Volume 10, Number 1 (Cumulative No.32) Part 14 March 25, 2013 ISSN:1097-8135

Life Science Journal

Acta Zhengzhou University Overseas Edition

Marsland Press
PO Box 180432
Richmond Hill, New York 11418, USA

Website:
http://www.lifesciencesite.com

Emails:
editor@sciencepub.net
lifesciencej@gmail.com

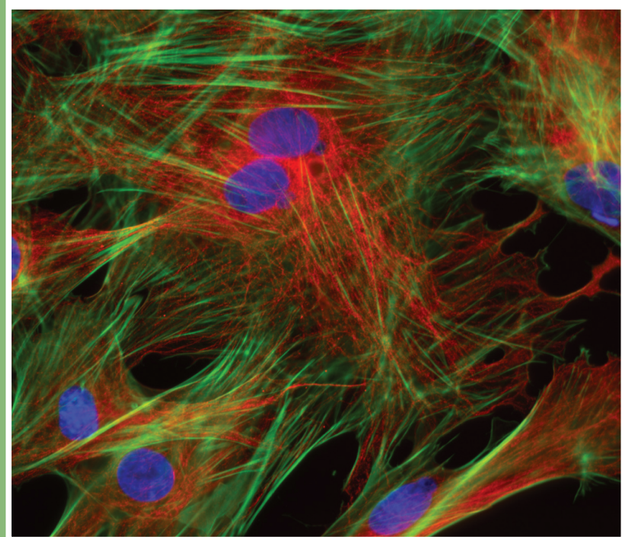
Phone: (347) 321-7172

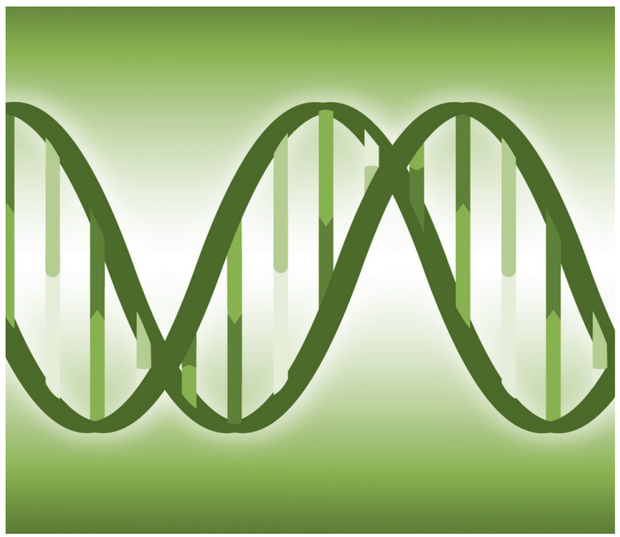
Cover design: CAO, Zhaolong
Photograph: YANG, Yizhong; YOUNG, Mary

Life Science Journal 2013 Volume 10, Number 1, Part 14 ISSN:1097-8135

Volume 10, Number 1, Part 14 March 25, 2013 ISSN:1097-8135

Life Science Journal





Copyright © 2013 Marsland Press / Zhengzhou University

ISSN 1097-8135

MARSLAND PRESS
Multidisciplinary Academic Journal Publisher

Website:
http://www.lifesciencesite.com

Emails:
editor@sciencepub.net
lifesciencej@gmail.com

Volume 10, Number 1, Part 14 March 25, 2013 ISSN:1097-8135

Life Science Journal

MARSLAND PRESS
Multidisciplinary Academic Journal Publisher

Website:
http://www.lifesciencesite.com

Emails:
editor@sciencepub.net
lifesciencej@gmail.com

Life Science Journal - Acta Zhengzhou University Oversea Version

ISSN: 1097-8135

Life Science Journal, the Acta Zhengzhou University Oversea Version, is an international journal with the purpose to enhance our natural and scientific knowledge dissemination in the world under the free publication principle. The journal is calling for papers from all who are associated with Zhengzhou University-home and abroad. Any valuable papers or reports that are related to life science - in their broadest sense - are welcome. Other academic articles that are less relevant but are of high quality will also be considered and published. Papers submitted could be reviews, objective descriptions, research reports, opinions/debates, news, letters, and other types of writings. Let's work together to disseminate our research results and our opinions.

***Editor-in-Chief*:** Shen, Changyu, Ph.D., Professor, Chinese Academy of Sciences

***Associate Editors-in-Chief*:** Ma, Hongbao; Cherng, Shen; Xin, Shijun

***Editorial Boards*:** Aghdam, Hashemi; An, Xiuli; Cao, Zhaolong; Chandra, Avinash; Chen, George; Dong, Ziming; Duan, uangcai; Edmondson, Jingjing; Fu, Qiang; Fu, Xianzhen; Gao, Danying; Han, Quancheng; Han, Zhijiang; Hosseini, Seyyed Mousa; Huang, Shuan-Yu; Kan, Quancheng; Lang, Jinghe; Li, Hui; Li, Xinhua; Li, Yan; Li, Yuhua; Li, Yusheng; Li, Zhen; Lindley, Mark; Liu, Hongmin; Liu, Hua; Liu, Xinjing; Ma, Changsheng; Ma, Margret; Ma, Yongsheng; Maio, Zhongrong; Ming, Gongguang; Mousavi, Amin; Nie, Wei; Qi, Yuanming; Qiao, Nancy X; Qin, Jie; Qin, Qin; Qin, Yuhua; Ouyang, Da; Sabyasachi Chatterjee; Seryasat, Omid Rahmani; Shang, Fude; Shi, Lifeng; Song, Chunpeng; Song, Yongping; Spurr, Lydia; Sun, Yan; Sun, Yingpu; Sun, Yiwu; Tan, Song; Venkatesh, Jaganathan; Wang, Jianliu; Wang, Lidong; Wang, Longde; Wang, Xiaoying; Wang, Yilong; Wang, Yongjun; Wang, Zhonggao; Wen, Jianguo; Wu, Ziying; Xu, Cunshuan; Xu, Yuming; Xue, Changgui; Yang, Qian; Young, Jenny; Zaki, Mona; Zhang, Guangxian; Zhang, Hong; Zhang, Jianying; Zhang, Kehao; Zhang, Liusuo; Zhang, Mingzhi; Zhang, Rui; Zhang, Shengjun; Zhang, Shuijun; Zhang, Suming; Zhang, Wei; Zhang, Xueguo; Zhang, Yanzhou; Zhang, Zhan; Zhang, Zhao; Zhang, Zhenxiang; Zhu, Huaijie.

Introductions to Authors

1. General Information: (1) Goals: As an international journal published both in print and on internet, **Life Science Journal** is dedicated to the dissemination of fundamental knowledge in all areas of nature and science. The main purpose of **Life Science Journal** is to enhance our knowledge spreading in the world under the free publication principle. It publishes full-length papers (original contributions), reviews, rapid communications, and any debates and opinions in all the fields of nature and science. **(2) What to Do**: The **Life Science Journal** provides a place for discussion of scientific news, research, theory, philosophy, profession and technology - that will drive scientific progress. Research reports and regular manuscripts that contain new and significant information of general interest are welcome. **(3) Who**: All people are welcome to submit manuscripts in life science fields. Papers of other fields are also considered. **(4) Copyright and Responsibility of Authors to their Articles:** When the manuscript(s) is submitted to the journal, the authors agree the following: All the authors have participated sufficiently in this work; The article is not published elsewhere; Authors are responsibility on the contents of the article; The journal and author(s) have same right for the copyright of the article and either of the journal or author(s) can use it by anyway without noting the other party. **(5) Publication Costs**: US$640 per article to defray costs of the publication will be paid by the authors when it is received. **(6) Advertisements**: The price will be US$400/page, and we normally accept one advertisement for one page. Other size of the advertisement please contact with our editorial office through email.

2. Manuscript Preparation - Each manuscript is suggested to include the following components but authors can do their own ways: **(1) Title**: including the complete article title; each author's full name; institution(s) with which each author is affiliated, with city, state/province, zip code, and country; and the name, complete mailing address, telephone number, facsimile number (if available), and at least one email address for author(s). **(2) Abstract**: including Background, Materials and Methods, Results, and Discussions. **(3) Key Words**. **(4) Introduction**. **(5) Materials and Methods**. **(6) Results**. **(7) Discussions**. (8) **Acknowledgments**. (**9) References**.

3. Manuscripts Submission: (1) Submission Methods: Submission through email (editor@sciencepub.net or lifesciencej@gmail.com) is encouraged. **(2) Software**: The Microsoft Word file. **(3) Font**: Normal, Times New Roman, 10 pt, single space. **(4) Page Setup**: Paper-Letter size; Margins-2.54 cm each side; Header/Footer-1.27 cm. **(5) Indent:** Type 8 spaces in the beginning of each new paragraph. **(6) Manuscript**: Don't use "Footnote" or "Header and Footer". **(7) Email:** At least one author's email must be put under title. **(8) Title:** Use Title Case in the title and subtitles, e.g. "**Molecular Study of Stem Cell". (9) Figures and Tables:** Use full word of figure and table, e.g. Figure 1**.** Annul Income of Different Groups**,** Table 1. Annual Increase of Investment**. (10) References:** Cite references by "last name, year", e.g. "(Smith, 2003)". References should include all the authors' last names and initials, title, journal, year, volume, issue, and pages etc. **Reference Examples**: **Journal Article**: Hacker J, Hentschel U, Dobrindt U. Prokaryotic chromosomes and disease. Science 2003;301(34):790-3. **Book**: Berkowitz BA, Katzung BG. Basic and clinical evaluation of new drugs. In: Katzung BG, ed. Basic and clinical pharmacology. Appleton & Lance Publisher. Norwalk, Connecticut, USA. 1995:60-9. **(11) Submission Address:** editor@sciencepub.net or lifesciencej@gmail.com, Marsland Press, PO Box 180432, Richmond Hill, New York 11418, USA, 347-321-7172.

© 2013 Marsland Press / Zhengzhou University

CONTENTS

280	**Identification and Some Probiotic Potential of Lactic Acid Bacteria Isolated From Egyptian Camels Milk** Eman Hamed and Aisha Elattar	**1952-1961**
281	**Evaluation of inactivated AIV vaccines in conjunction with antiviral drugs in chickens challenged with Egyptian H5N1 HPAIV** Ali, A. Salama; El-Bakry, M. Ismail; Fatma, M. Abdallah and Gemelat, K. Farag	**1962-1968**
282	**Oral exposure to zinc oxide nanoparticles induced oxidative damage, inflammation and genotoxicity in rat's lung** Howaida Nounou , Hala Attia , Manal Shalaby , Maha Arafah	**1969-1979**
283	**Calf Scours: Definition and causes** Nagwa S.Ata ,Sohad M. Dorgham , Eman A.Khairy and Mona S, Zaki	**1980-1983**
284	**Interference of preventive caries system with microshear bond strength of enamel surface bonded to etch &rinse or self etch adhesive system with nanofilled composite** Ola. M. Sakr and Mohammad Almohaimeed	**1984-1987**
285	**Expression of Galectin-3 in Thyroid Lesions; Immunohistochemical Analysis** Jaudah Al-Maghrabi, Sherine Salama, Adhari Al-Selmi and Mahmoud Al-Ahwal.	**1988-1992**
286	**Utility of 2-Thiohydantoin Derivatives in the Synthesis of Some Condensed Heterocyclic Compounds with Expected Biological Activity** A.Y. Hassan, M.M.Said; M.T. Sarg; H.S.Al-Zahabi and E.M.Hussein	**1993-2011**
287	**Human Papillomavirus and Cervical Cancer: Use of Molecular Diagnostic Techniques** Hammoudah S.A.F., Hannan M.A., Al Harbi A.E. and Al Harbi K.M.	**2012-2022**
288	**Water content controlled instead of suction controlled strength tests** Reza Ahmadi-Naghadeh, Nabi Kartal Toker, Mohammad Ahmadi-Adli	**2023-2030**
289	**Antihypertensive Prescribing Pattern and Blood Pressure Control among hypertensive patients over a Ten Year period in a Primary Care Setting in Malaysia** Chia Yook Chin Victoria L Keevil Ching Siew Mooi	**2031-2035**
290	**Designing and Validating Standards of Nursing Practice in Radiology Department of El-Manial University Hospital** Elham Youssef Elhanafy and Touto Abdel-Hamid Ismail	**2036-2047**
291	**Biochemical and histopathological effects of melamine on liver, spleen, heart and testes in male rats** Abdulbasit I. Al- Sieni , Haddad A. El Rabey and Abdullah A. Majami	**2048-2059**
292	**Unusual Abdominal Metastasis from Marjolin's Ulcer (Case Report and Review of Literature)** Munaser S. Alamoodi	**2060-2062**
293	**Effect of Breast-feeding and Formula- feeding on Antibody Response of Hepatitis B Vaccination**	**2063-2068**

Mohsenzadeh A, Ahmadipour SH, Firouzi M, Babaei Homa , Anbari KH

294 **Attenuation of specific CTL responses by highly efficient transduction of the recombinant adenovirus expressing His-tag-ICP47 fusion gene** **2069-2074**
Wang Peng , Zhang Zhenxiang , Kan Quancheng , Yu Zujiang , Li Ling , Pan Xue , He Hongjun , Feng Ting , Li Xiangnan , Jiang Li-li , Zhai Guangyu and Cui Guanglin

295 **Growth of the green alga *Chlorella vulgaris* as affected by different carbon sources** **2075-2081**
Battah M. G. El-Sayed, A.B. and El-Sayed, E.W

296 **Estimation of soil Fertility and Yield Productivity of Three Alfalfa (*Medicago sativa* L.) Cultivars Under Sahl El-Tina Saline Soils Conditions** **2082-2095**
Zeinab M. Abd El-Naby, Nabila, A. Mohamed. and Kh. A. Shaban

297 **Distinctive Features of the Professional Nursing Practice Environment As Perceived By Bachelor Nursing Students and Nurses at University of Dammam – Saudi Arabia** **2096-2106**
Dr. Sana A. Al-Mahmoud

298 **Serum Antibody Detection in Ecchinococcosis: Specificity of *Hydatidosis* enzyme-linked immunosorbent assay (ELISA) IgG** **2107-2110**
Metwally D M and Al-Olayan E M

299 **Screening of Some Antibiotics and Anabolic Steroids Residues in Broiler Fillet Marketed in El-Sharkia Governorate** **2111-2118**
Mohamed Abdallah Hussein and Samah Khalil

300 **The toxic effect of melamine on the kidney of male rats as revealed by biochemical and histopathological investigations**
Haddad A. El Rabey, Abdulbasit I. Al- Sieni and Abdullah A. Majami

301 **Evaluation of *Ginkgo biloba* as Alternative Medicine on Ova-Induced Eotaxin and Eosinophilia in Asthmatic Lung** **2131-2136**
Ghada Tabl and Abd El-Hamid Mohamed Elwy

302 **Effect of Breast Milk versus Therapeutic Honey (Apicare) on Cracked Nipples' healing** **2137-2147**
Rasha Mohamed Essa and Enas Mohamed Ebrahim

303 **Exploring the Environmental Knowledge of Urban and Rural Consumers and Its Impact on Green Purchase Behavior** **2148-2153**
Nalini Palaniswamy,Dr. Muruganandam Duraiswamy

304 **Sex Differences and the Ordering of Lengths of the Proximal phalanges in *Macaca Mulatta*** **2154-2159**
Xiaojin Zhao, Fengchan Wang, Xuan Zhao, Jie Song, Xiaojing Mao

305 **Nurses' Perceptions of Safety Climate and Barriers to Report Medication Errors** **2160-2168**
Ebtsam Aly Abou Hashish and Gehan Galal El-Bialy

Identification and Some Probiotic Potential of Lactic Acid Bacteria Isolated From Egyptian Camels Milk

Eman Hamed[1] and Aisha Elattar[*2]

[1]Department of Botany and Microbiology, Faculty of Science, Alexandria University
[2] Department of Dairy Sciences and Technology Faculty of Agriculture, Alexandria University. Egypt.
elattar2001@yahoo.com

Abstract: A study was carried out to investigate the probiotic potential of isolated lactic acid bacteria from camel's row milk collected from Arabian camels (*Camelus dromedaries*) in Egypt. Eleven gram positive, catalase negative isolates were identified using API 20STREP identification system for the identification of cocci isolates and API 50CHL for bacilli. Isolates were identified as *Enterococcus faecium* (seven isolates), *Enterococcus durans* (one isolate), *Aerococcus viridians* (one isolate), *Lactococcus lactis* (one isolate) and *Lactobacillus plantarum* (one isolate). The probiotic potential of these isolates was investigated using *in vitro* antagonistic tests against *Salmonella typhi* ATCC 14028, *E.coli* ATCC 25922 and *Vibrio fluvialis* using agar spot test. All of the isolates were proved to be effective against those pathogens. Isolate ES08 was able to inhibit the growth of indicator pathogens with an average inhibition zone of 3.3, 3.7 and 2.0 cm in diameter against *Salmonella typhi*, *E.coli* and *Vibrio fluvialis*, respectively. All of the isolates showed resistance to stomach pH (pH 3.0), tolerance against 0.3% bile salts concentration and none of the isolates caused blood hemolysis. Isolate ES08 was farther identified by sequencing their 16S rRNA encoding gene.

[Eman Hamed and Aisha Elattar. **Identification and Some Probiotic Potential of Lactic Acid Bacteria Isolated From Egyptian Camels Milk**. *Life Sci J* 2013;10(1):1952-1961] (ISSN:1097-8135). http://www.lifesciencesite.com. 280

Key Words: Camels milk, Lactic Acid Bacteria, Probiotic potential.

1. Introduction

According to the FAO statistics (FAO, 2004), there are about 19 million camels in the World, of which 15 millions are found in Africa and 4 millions in Asia. About 79% of the world's population is found in Africa, and all are one-humped. Camel populations are more concentrated in North East Africa. In Egypt, their numbers were previously estimated as 230,000 camels (GOVS, 2005). Nowadays, camel milk is considered as one of the main source of animal protein in some Egyptian provinces. It was reported that patients suffering from chronic hepatitis had improved liver functions after drinking camel milk. Camel milk is also succefully used for stabilization of juvenile diabetes (Yagil, 1987). This is confirmed by the presence of insulin-like protein in camel milk (Beg *et al.,* 1986). In different countries of Africa (Egypt, Sudan and Somalia) there is a common belief that among herdsmen of camels, especially those grazing on herbs, that men who drink such camel milk become strong, swift and virile (El Agamy, 2006). In pastoral societies, milk is traditionally consumed predominantly in the form of fermented milk. Fermentation is the only means of preserving milk under warm condition (Mohamed *et al.,* 1990; Farah, 1993; Kamoun, 1990). In many arid areas, camels play a central role as milk suppliers where they are either home-consumed or sold (Yagil, 1982; Kamoun, 1995; Lhoste, 2004).

Micro-organisms are important in dairy products. One of the most important groups of acid producing bacteria in the food industry is the Lactic Acid Bacteria (LAB) which are used in making starter culture for dairy products. The proper selection and balance for starter culture is critical for the manufacture of fermented products of desirable texture and flavour. The microbiological quality of milk and milk products is influenced by the initial flora of raw milk (Ritcher and Vadamuthu, 2001). When camel milk is left to stand, its acidity rapidly increases due to presence of LAB (Ohris and Joshi, 1961). It has also been recognized that LAB are capable of producing inhibitory substances other than organic acids (lactate and acetate) that are antagonistic toward other microorganisms (Daeschel, 1989). Certain LAB strain characterized by their ability to transform lactose and improves the digestibility of fermented dairy products (Weinberg *et al.,* 2007) as well as their preservation (Abdelbasset and Djamila, 2008). They also employed for improvement of the taste, texture and viscosity in the manufacture of dairy products (Soukoulis *et al.,* 2007). The ability of LAB to produce probiotics (Temmerman et al., 2002) and stimulation of the immune system (Kalliomäki *et al.,* 2001) render this group of microorganisms' essential importance dairy industry. Bacteria used as probiotic adjuncts are commonly delivered in a food system and, therefore, upon oral administration, they begin

their journey from the stomach to the lower intestinal tract. Therefore, probiotic bacteria should have the ability to resist the digestion process in the stomach and the intestinal tract. A large number of lactic acid bacteria have been classified as probiotics. According to the definition adopted by the World Health Organization, probiotics are live microorganisms that when administered in adequate amounts confer a health benefit to the host (Corsetti and Valmorri. 2011). Strains of the genera Lactobacillus, Bifidobacterium (Yateem *et al.,* 2008) and Enterococcus (Ogier and Serror (2008) are the most widely used and commonly studied probiotic bacteria. Today, there is a growing need for new strains of LAB that carry the probiotic traits mentioned above and with favorable health effects on human and animals. This can be obtained from other natural ecological niches which remain unexploited.

This study was undertaken to isolate and identify the lactic acid bacteria from row Camel's milk obtained from different locations in Egypt. The identification tests were applied using the phenotypic and genotypic methods. These isolates were investigated for bile salt and, acidic pH values resistant, hemolytic activities and bacteriocin production. Our goal is the selection of potential probiotic strains from camel's milk.

2. Materials and Methods

Sample collection

Bacterial strains were isolated from camel's milk samples collected from local lactating Arabian camels (*Camelus dromedaries*) in Egypt. A total of 21 camel milk samples were collected, five samples were collected from Mersa Matrouh, 14 samples from Wadi El Natrun area, and two samples from the farm of the Faculty of Veterinary Medicine - Menoufia University, Sadat city area. The samples were collected in sterile plastic containers and kept in ice box until delivery to the laboratory for the achievement of the isolation procedure.

Isolation of lactic acid bacteria

Each camel's milk sample was immediately cultured on MRS agar plates, and then serial dilutions were prepared from each sample. 1 ml of these dilutions was pour-plated in the MRS agar (de Man *et al.,* 1960). After incubation at 37°C for 48 hrs under anaerobic condition, individual different colonies were phenotypically selected.

The purity of the isolates was checked by streaking again to fresh agar plates, followed by macroscopic and microscopic examinations. The strains displaying the general characteristics of lactic acid bacteria were chosen from each plate for further studies. The strains of lactic acid bacteria were stored without appreciable loss of properties in skimmed milk with 20% glycerol at -20°C.

Identification of isolates

All isolates were microscopically examined for Gram stain reaction, cell morphology and cellular arrangement. Catalase activity was examined by adding drop of 3 percent hydrogen peroxide on a clean microscopic slide. A visible amount of bacterial growth was added with the inoculating loop. Both were mixed and observed for gas bubble production. Only Gram-positive and Catalase negative isolates were identified at species level.

API 20STREP (Biomérieux, Marcy-l'Étoil, France) was used for cocci isolates identification according to the manufacturer's instructions. Results reading were done after 24 and 48 hrs at 37°C, on the other hand bacilli isolates were evaluated according to the carbohydrates fermentation profiles using API 50CHL (Biomérieux, Marcy-l'Étoil, France). The tests were also done according to the manufacturer's instructions and the results were interpreted after incubation at 37°C for 24 and 48 hrs. Identification of the isolates was done by the interpretation of the fermentation profiles using the computerized database program API WEB software V 1.2.1.

Determining the antagonistic activity of isolated LAB using in vitro tests

The antagonistic activity of the isolated LAB bacteria against *Salmonella typhi* ATCC 14028, *E. coli* ATCC 25922 (obtained from the High Institute of Public Health, Egypt) and *Vibrio fluvialis* (obtained from the National Institute of Oceanography and Fisheries, Egypt), was determined using agar spot test (Jacobsen *et al*., 1999).

Prior to conducting the test, the potential LAB isolates were propagated in MRS broth medium and incubated at 37°C for 24 h. For the agar spot test, 4 µL of each bacterial isolate were spotted on the surface of MRS agar medium and incubated at 37°C for 24 hrs to allow colonies to develop. Overnight culture of each test pathogen was inoculated (1% v/v) in 15 ml of soft Nutrient agar (containing 0.7% agar) and poured onto the inoculated MRS agar plates (Yavuzdurmaz, 2007). After incubation at 37°C for 24 hrs, the antimicrobial activity of tested strains was determined by measuring the diameter of the inhibition (clear) zones surrounding the colonies.

Resistance to acidic pH

The resistance of LAB isolates to acidic pH was performed according to (Nawaz *et al*., 2011). Each bacterial isolate was inoculated using 1% (v/v) of an overnight LAB culture, in sterile MRS broth adjusted to pH 2, 3 and 4 then incubated at 37ºC for 6 hrs. The absorbance at 620 nm using spectrophotometer (Optima, Japan) was monitored at hourly intervals. Control samples without acidification were also prepared and similarly handled.

Bile salts tolerance

Isolates were tested for their ability to grow in presence of different bile salts concentrations. For this purpose 0.1% and 0.3% (w/v) bile concentrations were selected. An aliquots of 15 ml sterile MRS broth containing 0, 0.1% and 0.3% of bile salts was inoculated with an overnight LAB culture and incubated at 37ºC for 4 hrs. The absorbance at 620 nm was monitored using spectrophotometer (Optima, Japan) at hourly intervals (Yavuzdurmaz, 2007).

Blood hemolysis

The hemolytic activity of isolates was determined according to Guttmann and Ellar (2000) on blood agar base (Biolife, Milano. Italy) plates containing 5% v/vs of sheep blood. After incubating the plates at 37 °C for 24 hrs, β-hemolytic, no haemolysis or γ and α-haemolysis reactions were recorded by the observation of a clear zone around the colonies, the non-hemolysed area under and around the colonies and the greenish zone, respectively.

Genotypic Identification using 16S rDNA

The genomic DNA of the presumptive LAB strain was isolated using the DNA extraction and purification kit according to the manufacturer instructions (Fermentas, UK). DNA preparations were then analyzed by electrophoresis in 1% agarose gel.

The PCR reaction mixture contained 5 µL of template DNA, 2 µL of reverse primer (10 mM), 2 µL of forward primer (10 mM), 2 µL of dNTP (25 mM), 4 µL of $MgCL_2$ (25 mM), 5 µL of PCR buffer (10X) and 1µL Taq polymerase, Distilled water was added to obtain 50 µL final volume in the PCR tube. The primers used for PCR amplification of 16S rRNA gene were S-C-Act-235-a-S-20 F: 5´-CGCGGCCTATCACTTGTTG-3´ and S-C-Act-878-a-A-19 R: 5´-CCGTACTCCCCAGGCGGGG-3´ (Jaatinen *et al*., 2008), with expected product size of 400 bp. The cycling program was 95ºC for 5 min, 35 cycles at 95ºC for 30 sec, 50ºC for 30 sec and 72ºC for 2 min. At the end, the reaction was incubated at 72ºC for 10 min.

Gel electrophoresis was carried out by using 1% agarose gel prepared in TAE buffer (2.0 M Tris base, 1.0 M glacial acetic acid and 0.05 M EDTA at pH 8).

The DNA samples were loaded in the gel after mixing with the loading dye (a solution of 0.1% bromophenol blue, 50% glycerol, 0.1 M EDTA pH 8 and 1% SDS), and the voltage was then applied (90 v/cm) after soaking in TAE buffer. The DNA was visualized using a UV transilluminater (Bio-Rad, USA) after staining the gel with ethidium bromide (10 mg/ml) for 20 min.

Sequencing of PCR product was made by the sequencing facility offered by the U.S.B. American Company through SIGMA-Egypt.

3. Results and Discussion

Isolation of lactic acid bacteria

From the twenty one collected samples, a total of 60 isolates randomly picked, after the original characterization. They are all gram positive bacteria, moreover, a lot of catalase positive bacteria and yeast were observed. The presence of yeast in the tested samples is possibly a result of contamination from udder skin, as previously mentioned by Yavuzdurmaz, (2007). Only eleven isolates were stable after purification and sub-culture (Table1), consequently, they were applied in this study. They were Gram positive cocci or rods, catalase negative and non spore forming. Ten of them (91%) were found to be cocci with spherical morphology and appeared mostly as forming chains or groups therefore they tentatively referred to *lactococcus*. Only one isolate (9%) was bacilli mostly appeared as short rod, pairs or single cells and this could cautiously determined as derivatives of the genus *Lactobacillus*. Brasca *et al*. (2008) purified 92 isolates of lactic acid bacteria using frozen camel's milk. These isolates were classified as 55.43% and 44.56% of cocci and rods isolates, respectively. While Ashmaig *et al*. (2009) isolated 24 LAB from 12 samples of gariss (fermented camel's milk) in the Sudan. The isolates were classified into 66.6% rods and 33.3% cocci. Also, Khay *et al*. (2011) isolated a total of 450 cultures from 25 samples of dromadedary milk collected from Laâyoune region of Morocco. Out of these, 30 were determined to be lactic acid bacteria.

Physiological and biochemical identification of the isolated lactic acid bacteria

Based on phenotypic, biochemical characteristics and interpretation of the API database, 11 strains were satisfactorily identified, of which 10 (cocci) were identified using the enzymatic and carbohydrate fermentation profile API 20 STREP and one isolate (rod shaped) was identified using API 50 CHL. The biochemical profiles of the tested strains and the suggested identification are shown in Tables (2 and 3).

All isolates fermented lactose, trehalose and ribose. Only isolate (EW01) failed to ferment arabinose and mannitol. All the isolates did not ferment raffinose and did not utilize inulin or sorbitol. Only two isolates (EW02 and LS07) could not utilize esculine. All of the isolates were not able to hydrolysis hippuric acid.

Table 1. Morphological characterization of the bacterial isolates.

Isolate Number	Shape	Gram staining	Catalase reaction
EW01	Cocci	G +ve	-ve
EW02	Cocci	G +ve	-ve
EW03	Cocci	G +ve	-ve
AW04	Cocci	G +ve	-ve
ES05	Cocci	G +ve	-ve
ES06	Cocci	G +ve	-ve
LS07	Cocci	G +ve	-ve
ES08	Cocci	G +ve	-ve
ES09	Cocci	G +ve	-ve
ES10	Cocci	G +ve	-ve
LS11	Short rods	G +ve	-ve

Table 2. Analysis results of isolated LAB using API 20 STREP* and API 50 CHL**.

Strain	Identification	Confidence (%)
EW01*	*Enterococcus durans*	83.20
EW02*	*Enterococcus faecium*	85.40
EW03*	*Enterococcus faecium*	85.80
AW04*	*Aerococcus viridans*	95
ES05*	*Enterococcus faecium*	85.80
ES06*	*Enterococcus faecium*	85.80
LS07*	*Lactococcus lactis* subsp. *cremoris*	42.80
ES08*	*Enterococcus faecium*	85.80
ES09*	*Enterococcus faecium*	85.80
ES10*	*Enterococcus faecium*	85.80
LS11**	*Lactobacillus plantarum*	99.9

On the other hand, they could produce acetoin as they gave positive results in the Voges Proskauer test. All isolates did not show blood hemolysis. Based on API 20 STREP identification, 7 isolates which represent 63.6% of the experimental isolates were identified as *Enterococcus faecium*, one isolate (9%) as *Enterococcus durans*, one isolate (9%) as *Aerococcus viridans*, one isolate (9%) as *Lactococcus lactis* and one isolate (9%) as *Lactobacillus plantarum*. The presence of *E. faecium* and *E. durans* in raw camel's milk and cheese is common (Rodríguez *et al*., 1995; Freitas *et al*., 1999) and this observation was in agreement with our results. Enterococcal strains, mainly those of *E. faecium* are frequently present in various food systems and their technological and probiotic benefits are widely recognized (Giraffa, 1995). The isolates, which were identified as *E. faecium* produced acid from mannitol and arabinose (Durlu-Ozkaya *et al*., 2001). The confidence results were highly (83.2% – 99.9%) for all the isolates except for the isolate LS07 (42.8%) which was not clearly identified but it could be referred to *Lactococcus lactis* subsp. *cremoris* (Table.2).

Enterococci represented a large part of the bacterial microflora of this work as a total of 7 isolated strains were identified as *E. faecium*. This group of bacteria plays a major role in the ripening and aroma development in butter and many types of traditional cheeses (Centeno *et al*., 1996 and Malek *et al.,* 2012). In addition, Egyptian Ras and Domiatti cheese were made in the presence and absence of selected *E. faecium* strains. The cheese containing *E. faecium* exhibited higher levels of free fatty acids and amino acids. The organoleptic evaluation of the different cheeses revealed a preference to the *E. faecium* containing cheeses, which suggest a desirable role of this microorganism during the ripening of Egyptian cheeses (El Soda. 2002) In addition to these technological aspects, clinical research on enterococci emphasizes that the safety of dairy products containing enterococci should be carefully addressed before use (Giraffa, 2003). Contrary to other lactic acid bacteria, some strains of enterococci are not considered as "Generally Recognized As Safe" (GRAS) microorganisms (Cariolato *et al.,* 2008). And their detection in water is regarded as an indicator of fecal contamination. So, enterococci are generally considered as having an ambiguous status concerning their safety assessment procedure (Oigier and Serror. 2008).

On the other hand, *Lactobacillus* phenotypes were represented by only one out of the eleven isolates, this may be due to a possibility that camel's milk is not an adequate medium for their growth, their sensitivity to natural inhibitors which represent in the milk or to the lack of essential growth factors (Benkerroum *et al*., 2003). The experimental isolate (LS07) that fermented lactose, ribose, mannitol, trehalose and arabinose but not raffinose, inulin and sorbitol was identified as *Lactococcus lactis*. The isolation of *Lactococcus lactis* from camel's milk was also reported by Hardie (1986) and Khay *et al*. (2011). *Lactococcus lactis* subsp. *Lactis* and *Lactococcus lactis* subsp. *cremoris* are important in food technology (Garvie, 1984; Sandine, 1985; Salama *et al*., 1991) as the bacteriocin producing lactococcal strains have been used successfully in starter cultures for cheese to improve the safety and quality of the product (Maisnier-Patin *et al*., 1992; Delves-Broughton *et al*., 1996 and Ryan *et al*., 1996). The experimental isolate (AW04) that gave positive reactions for hydrolysis of esculin and acidification of lactose and trehalose was identified as *Aerococcus viridans* (MacFaddin, 1980).

Probiotic properties

Antagonestic activity

LAB have been shown to inhibit the growth of many enteric pathogens *in vitro* and have been used for treatment of a broad range of gastrointestinal disorders in both humans and animals (Rolfe, 2000). Therefore, experimental isolates were tested against the indicator microorganisms such as, *Salmonella typhi* ATCC 14028, *E. coli* ATCC 25922 and *Vibrio fluvialis* (Fig. 1.). The diameter of inhibition zones (Table 4) showed that all of the isolates have antibacterial effect on the indicator microorganisms. Isolate ES10 gave the largest inhibition zone against *E. coli* with a diameter of 3.8 cm followed by isolate ES08 which gave an inhibition zone with a diameter of 3.7 cm, while isolates ES05 and ES09 were less effective against *E. coli* and gave inhibition zones with almost the same diameter (3.1 cm). On the other hand, isolate ES08 was the most effective isolate against *Salmonella typhi* as it gave an inhibition zone with a diameter of 3.3 cm followed by LS07 (3 cm). Isolates LS11 and EW02 were the lowest effective with inhibition zones of 2.4 and 2.3 cm respectively. While, against *Vibrio fluvialis*, ES08 and ES10 were the most effective strains as they gave clear zones with a diameter of 2 cm and strains ES05 and ES09 were the lowest effective as they gave clear zones with 1 cm in diameter. The inhibitory action of LAB bacteria is mainly due to the accumulation of main primary metabolites such as lactic and acetic acids, ethanol and carbon dioxide. Additionally, LAB are also capable of producing antimicrobial compounds such as formic and benzoic acids, **hydrogen peroxide**, diacetyl, acetoin and bacteriocins. The production levels and the proportions among those compounds depend on the strain, medium compounds and physical parameters (Tannock, 2004).

Table 3. Biochemical identification of the ten cocci isolates using API 20 STREP identification system.

Strain	TIME	VP	HIP	ESC	PYRA	αGAL	βGUR 4	βGAL	PAL	LAP	ADH	RIP	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLYG	β-hem
EW03	4h	+	-	+	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
	24_h	+	-	+	+	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-
AW04	4h	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-
	24_h	+	-	+	+	-	-	-	-	-	+	+	+	+	-	+	+	-	-	-	-	-
EW01	4h	+	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
	24_h	+	-	+	+	-	-	-	-	+	+	+	-	-	-	+	+	-	-	-	-	-
EW02	4h	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	24_h	+	-	-	+	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-
ES05	4h	+	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
	24_h	+	-	+	+	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-
ES06	4h	+	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
	24_h	+	-	+	+	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-
LS07	4h	+	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
	24_h	+	-	-	+	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-
ES08	4h	+	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-
	24_h	+	-	+	+	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-
ES09	4h	+	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
	24_h	+	-	+	+	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-
ES10	4h	+	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
	24_h	+	-	+	+	-	-	-	-	+	+	+	+	+	-	+	+	-	-	-	-	-

+ = positive reaction, - = negative reaction, test was done under anaerobic conditions at 37ºC /4 and 24 hrs.

Table 4. Antagonistic activity of isolated LAB against *Salmonella typhi* ATCC 14028, *E. coli* ATCC 25922 and *Vibrio fluvialis* as determined by the agar spot test.

Isolate number	Inhibition zones diameter in cm		
	Salmonella typhi	***E. coli***	***Vibrio fluvialis***
EW01	2.5	3.5	1.2
EW02	2.3	3.4	1.4
EW03	2.5	3.5	1.6

AW04	2.7	3.4	1.7
ES05	2.5	3.1	1.0
ES06	2.9	3.6	1.4
LS07	3.0	3.3	1.8
ES08	3.3	3.7	2.0
ES09	2.5	3.1	1.0
ES10	2.8	3.8	2.0
LS11	2.4	3.4	1.8

LAB has shown to possess inhibitory activities mostly towards Gram positive pathogens and closely related bacteria due to the bactericidal effect of protease sensitive bacteriocins (Jack *et al*., 1995). LAB strains are mostly inactive against Gram-negative bacteria due to the resistance conferred by the outer membrane. However, inhibitory effects of nisin (Cutter and Siragusa, 1995), bacteriocin produced by *Lactobacillus paracasei* subsp. *paracasei* (Caridi, 2002), bacteriocin ST151BR produced by *Lactobacillus pentosus* ST151BR (Todorov and Dicks, 2004), thermophylin produced by *Streptococcus thermophillus* (Ivanova *et al*., 1998) and some enterocins produced by enterococcus sp. (Jennes *et al*., 1999) on Gram-negative bacteria through their synergetic effects with other antimicrobials has gained increased interest (Helander *et al*., 1997). LAB were also able to control the growth of Gram negative pathogens including food borne pathogens by the production of **organic acid**s and **hydrogen peroxide** (Lu and Walker, 2001 and Ito *et al*., 2003).

Resistance to low pH

One of the major selection criteria for probiotic strains is to be resistant to low pH (Chou and Weimer, 1999; Quwehand, *et al*., 1999). Since, they have to pass through the stressful conditions of stomach to reach the small intestine. Although in the stomach, pH can be as low as 1.0, however, *in vitro* assays pH 3.0 has been preferred, due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below (Prasad, *et al*. 1998). For selection of strains resistant to low pH, MRS broth with pH-adjusted to 4.0, 3.0 and 2.0 were used. Berrada *et al.* (1991) mentioned that the time from entrance to release from the stomach was reported to be 90 min and the bactericidal effect of the acid is evident at pH values below 2.5 (Maffei and Nobrega, 1975). After the examination, all the isolates survived in pH 3.0 were taken to the next step. Experiments were run twice. The growth was monitored by measuring the O.D at 620 nm.

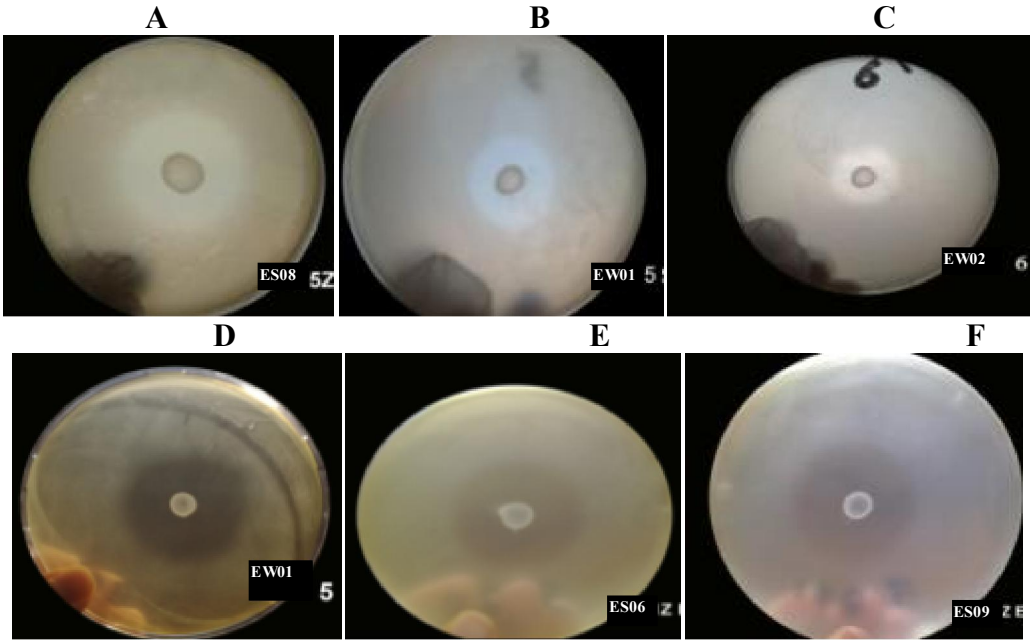


Fig. 1. Antagonistic effects of isolated LAB against *Salmonella typhi* (A, B), *Vibrio fluvialis* (C) and *E. coli* (D, E, F) using agar spot test.

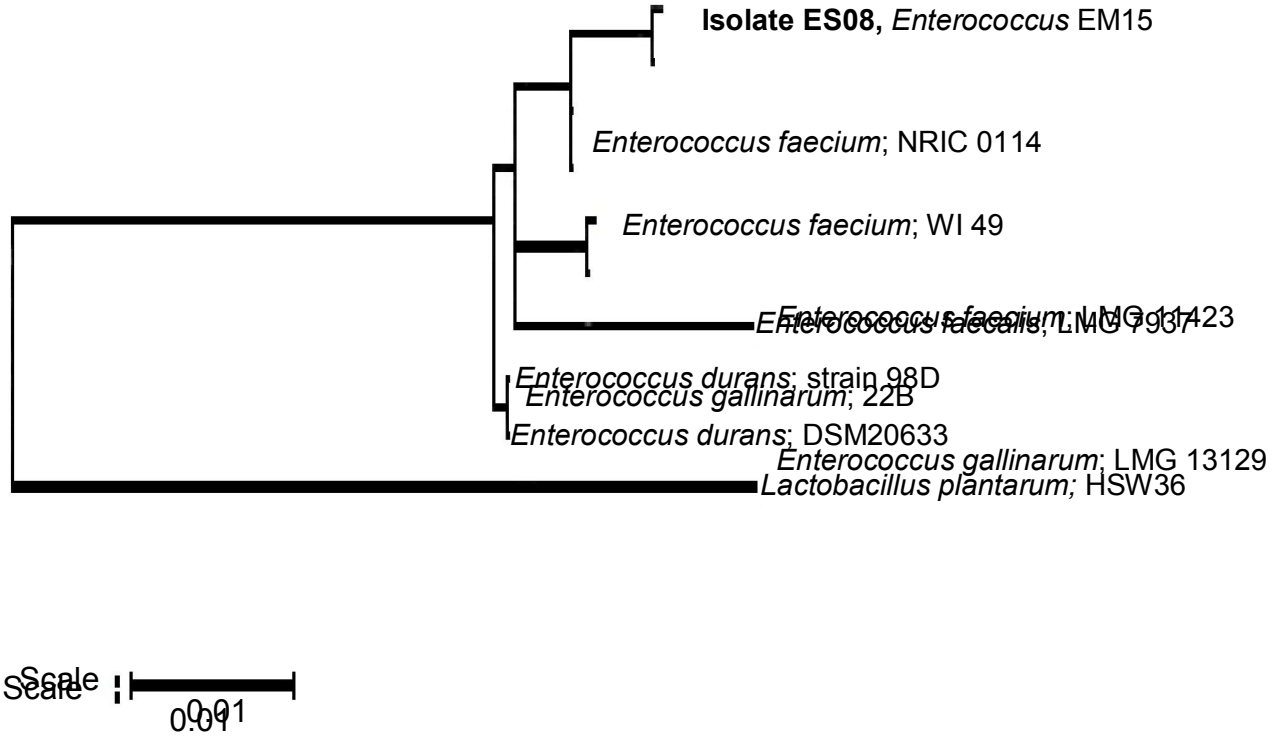


Fig. 2. Phylogenetic analysis of ES08 isolate based on partial sequence of 16s rRNA gene. The scale indicated substitution per site.

In this study, all the isolates classified as *E. faecium* 63.6% survived in pH 3.0 for 3 hours. This result is in accordance with the study of Strompfová and Lauková (2007) which found that *E. faecium* can survive at pH 3.0 for 3 h. Accordingly, it could be suggested that the *E. faecium* phenotypes isolated in this study could be used as probiotic.

Isolate LS11, followed by isolate EW01, seemed to be the most stable isolates as they could maintain themselves and increase the cell density at pH 3. Isolates 6, EW03, AW04, ES05, ES06, ES08 and ES10 showed a good stability at pH 3 for 3 hours. Isolates ES09 and LS07 were more sensitive to low pH than the other isolates.

Tolerance against bile salts

The isolated bacterial phenotypes were tested for their ability to grow in the presence of bile salts. Although the bile concentration of the human gastrointestinal tract varies, the mean intestinal bile concentration is believed to be 0.3% w/v and the staying time is suggested to be 4 hrs (Prasad, *et al*., 1998). The isolates showed a good stability at 0.1% concentration indicated by the increase in the optical density. At 0.3% concentration, none of the isolates showed a marked increase in the optical density; instead they could survive at this concentration for 4 hours. Similar results were observed by Ouled-Haddar *et al*. (2012) who reported an increase in cell viability by alginate microencapsulation. In addition, Strompfova *et al*. (2004) reported that *E. faecium* isolated from dogs can tolerate up to 1% bile for 24 hours.

Bile tolerance is an important characteristic of bacteria to survive in small intestine. Bile resistance of some strains is related to specific enzyme activity, bile salt hydrolase (BSH) which helps to hydrolyse conjugated bile, thus reducing its toxic effect (Du Toit *et al*., 1998). Hydrolyzation of bile salt by enzyme hydrolases (BSHs) had been explained by Tanaka *et al*. (2000), which can be found in *Lactobacillus* sp. (De *et al*., 1995) and *Enterococcus* sp. (Agus, 2003).

Molecular phylogeny of the selected isolate

Isolate ES08 was selected according to its probiotic properties. This isolate was farther identified by partial sequencing of the gene coding

for the 16S rRNA. The phylogenetic relationship of the experimental sequence and its close relatives was analyzed through the facilities of the Ribosomal Database Project (http://rdp.cme.msu.edu/) and summarized in a dendrogram (Fig.2). Therefore, the confirmation of the identified isolate was as *Enterococcus faecium.*

Conclusion

As a conclusion, the results obtained from this study demonstrated the potential probiotic ability of the isolated LAB species from camel's milk. In addition it is recommended that these species can be further studied according to selection criteria like stimulation of immunological system, antibiotic resistance and adhesion to epithelium tissue.

Corresponding author
Aisha Elattar
Department of Dairy Sciences and Technology Faculty of Agriculture, Alexandria University. Egypt.
elattar2001@yahoo.com

References

1. Abdelbasset, M. and Djamila, K. (2008). Antimicrobial activity of autochthonous lactic acid bacteria isolated from Algerian traditional fermented milk "Raïb". *Afr. J. Biotechnol*. 7, 2908-2914.
2. Agus, W. (2003). Investigation into the influence of a bacteriocin-producing *Enterococcus* strain on the intestinal microflora, PhD. Thesis, Graduate School, Karlsruhe University, Germany, p. 124.
3. Ashmaig, A., Hasan, A. and El Gaali, E. (2009). Identification of lactic acid bacteria isolated from traditional Sudanese fermented camel's milk (Gariss). *Afr. J. Microbiol. Res.* 3, 451-457.
4. Benkerroum, N., Boughdadi, A., Bennani, N. and Hidane, K. (2003). Microbiological quality assessment of Moroccan camel's milk and identification of predominating lactic acid bacteria. *World J. Microbiol. Biotechnol.* 19, 645-648.
5. Beg, O. U., Von Bahr-Linstrom, H., Zaidi, Z. H. and Jornvall, H. (1987). Characterization of a heterogenous camel milk whey non-casein pro-protein. *Fed. Eur. Biochem. Soc. Lett.* 216, 270-274.
6. Berrada, N., Lemeland, J. F. and Laroche, G. (1991). *Bifidobacterium* from fermented milks: Survival during gastric transit. *J. Dairy Sci*. 74, 409-413.
7. Brasca, M., Lodi, R. and Morandi, S. (2008). Metabolic Characteristics of Lactic Acid Bacteria from Camel Milk "5th IDF symposium on cheese ripening", 9 -13 March, Bern, Switzerland, p. 139.
8. Caridi A. (2002). Selection of *Escherichia coli* inhibiting strains of *Lactobacillus paracasei* subsp. *paracasei*. *J. Ind. Microbiol. Biotechnol*. 29, 303-308.
9. Cariolato, D., Andrighetto, C., Lombardi, A., 2008. Occurrence of virulence factors and antibiotic resistances in *Enterococcus faecalis* and *Enterococcus faecium* collected from dairy and human samples in North Italy. Food Control 19, 886–892.
10. Centeno, J. A., Menéndez, S. and Rodriguez-Otero, J. L. (1996). Main microbial flora present as natural starters in Cebreiro raw cow's-milk cheese (Northwest Spain). Int. Food Microbiol. 33,307-313.
11. Chou, L. S. and Weimer, B. (1999). Isolation and characterization of acid and bile tolerant isolates from strains of *Lactobacillus acidophilus*. J. Dairy Sci. 82, 23-31.
12. Corsetti. A. and Valmorri S. 2011. *Lactobacillus* spp.: *Lactobacillus plantarum. In* Encyclopedia of Dairy Sciences, Second Edition, 1, pp. 111-118
13. Cutter, C. N. and Siragusa, G. P. (1995). Population reductions of Gram-negative pathogens following treatments with nisin and chelators under various conditions. J. Food. Protect. 58, 977-983.
14. Daeschel, M.A., 1989. Antimicrobial substances from lactic acid bacteria for use as food preservatives. Food Technol., 43: 164-166.
15. De, S., Van, H. L., Vande, W. M., Christiaens, H. and Verstracte, W. (1995). Significance of bile salt hydrolytic activities of Lactobacilli. J. Appl. Bacteriol. 78, 292-301.
16. De Man, J. D., Rogosa, M. and Sharpe, M. E. (1960). A Medium for the cultivation of Lactobacilli. J. Appl. Bacteriol. 23, 130–135.
17. Delves-Broughton, J., Blackburn, P., Evans, R. J. and Hugenholtz, J. (1996). Application of the bacteriocin, nisin. Anton. Leeuw. Int. J. Gen. Mol. Microbiol. 69, 193-202
18. Du Toit, M., Franz, C., Schillinger, U., Warles, B. and Holzappfel, W. (1998). Characterization and selection of probiotic lactobacilli for a preliminary minipig-feeding trail and their effect on serum cholesterol level, faeces moisture contents. Int. Food Microbiol. 40, 93-104.
19. Durlu-Ozkaya, F., Xanthopoulos, V., Tunail, N. and Litopoulou-Tzanetaki, E. (2001). Technologically important properties of lactic acid bacteria isolated from Beyas cheese made from raw ewes' milk. J. Appl. Microbiol. 91, 861-870.
20. El-Sayed I. El-Agamy. 2006. Camel Milk. In Handbook of Milk of Non-Bovine Mammals. Edited by Young W. Park and George F.W. Haenlein. Blackwell Publishing. pag: 297-344
21. El Soda. M. 2002. Selection of *Enterococcus faecium* strains for Egyptian cheesemaking. Abstract in congree Enterococci in Foods Functional and Safety Aspects. 30-31 May 2002, Berlin. Germany.
22. FAO, (2004). FAO-STAT DATA, Food and Agricultural Organization, Statistical Databases at

http://earthtrends.wri.org/text/agriculture-food/variable-334.html
23. Farah, Z. (1993). Composition and characteristics of camel milk. J. Dairy Res. 60, 603-626.
24. Freitas, A. C., Pintado, A. E., Pintado, E. M. and Malcasa, F. X. (1999). Role of dominant microflora of Picante cheese on proteolysis and lipolysis. Int. Dairy J. 9, 593-603.
25. Garvie, E. I. (1984). Separation of species of the genus *Leuconostoc* and differentiation of the Leuconostocs from other lactic acid bacteria. In: Methods In Microbiology. Bergan, T. (ed.). Academic press, London, pp. 147-178.
26. Giraffa, G. (1995). Enterococcal bacteriocins: their potential as anti-*Listeria* factors in dairy technology. Food Microbiol. 12, 291–299.
27. Giraffa, G., 2003. Functionality of enterococci in dairy products. International Journal of Food Microbiology 88, 215–222.
28. GOVS (2005). General organization for veterinary services: Animal report of general organization for veterinary services, ministry of agriculture Egypt.
29. Guttmann, D. M., and Ellar, D. J. (2000). Phenotypic and genotypic comparisons of 23 strains from the *Bacillus cereus* complex for a selection of known and putative *B. thuringiensis* virulence factors. FEMS Microbiol Lett. 188, 7–13.
30. Hardie, J. M. (1986). Genus *Streptococcus*. In: Bergey's Manual of Systematic Bacteriology, Vol. 2. Sneath, P. H. A. (ed.), Williams and Wilkins, London, pp. 1043-1047.
31. Helander, I. M., Von Wright, A. and Mattila-Sandholm, T. M. (1997). Potential of lactic acid bacteria and novel antimicrobials against Gram-negative bacteria. Trends. Food. Sci. Technol. 8, 146-150.
32. Ito, A., Sato, Y., Kudo, S., Sato, S., Nakajima, H. and Toba, T. (2003). The screening of hydrogen peroxide-producing lactic acid bacteria and their application to inactivating psychrotrophic food-born pathogens. Curr. Microbiol. 47, 231-236.
33. Ivanova, I., Miteva, V., Stefanova, T. S., Pantev, A., Budakov, I., Danova, S., Moncheva, P., Nikolova, I., Dousset, X. and Bouvaval, P. (1998). Characterization of a bacteriocin produced by *Streptococcus thermophilus* 81. Int. J. Food. Microbiol. 42, 147-158.
34. Jack, R. W., Tagg, J. R. and Ray, B. (1995). Bacteriocins of gram-positive bacteria. Microbiol. Rev. 59, 171-200.
35. Jacobsen, C. N., Nielsen, V. R., Hayford, A. E., Moller, P. L. and Michaelsen, K. F. (1999). Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by *in vitro* techniques and evaluation of the colonization ability of five selected strains in humans. Appl. Environ. Microbiol. 65, 4949-4956.
36. Jennes, W., Dicks, L. M. T. and Verwoerd, D. J. (1999). Enterocin 012, a bacteriocin produced by *Enterococcus gallinarum* isolated from the intestinal tract of ostrich. J. Appl. Microbiol. 88, 349-357
37. Kalliomäki, M., Salminen, S., Arvilommi, H., Kero, P., Koskinen, P. and Isolauri, E. (2001). Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. The Lancet. 357, 1076-1079.
38. Kamoun, M. 1990. Cheese production from camel milk. Options Mediterrannees, Ser. Nr. 12, p. 119–124.
39. Kamoun M (1995). Le lait de dromadaire: production, aspects qualitatifs et aptitude à la transformation. Options méditerranéennes, Séries séminaires, 13: 81-103.
40. Khay, E., Idaomar, M., Castro, L. P., Bernárdez, P. F., Senhaji, N. S. and Abrini, J. (2011). Antimicrobial activities of the bacteriocin-like substances produced by lactic acid bacteria isolated from Moroccan dromedary milk. Afr. J. Biotechnol. 10, 10447-10455
41. Lhoste F (2004). Lait de chamelle pour l'Afrique. Atelier sur la filière cameline en Afrique, Niamey, 5-8 novembre 2003, Animal production and health, Publication FAO, Rome.
42. Lu, L. and Walker, W. A. (2001). Pathologic and physiologic interactions of bacteria with the gastrointestinal epithelium. Am. J. Clin. Nutr. 73, 1124S-1130S.
43. MacFaddin, J. F. (1980). Biochemical Tests for Identification of Medical Bacteria, 2nd ed. Williams and Wilkins, Baltimore
44. Maffei, H. V. and Nobrega, F. J. (1975). Gastric pH and microflora of normal and diarrhoeic infants. *Gut* 16, 719-726.
45. Maisnier-Patin, S., Deschamps, N., Tatini, S. R., Richard J (1992). Inhibition of *Listeria monocytogenes* in Camembert cheese made with nisinproducing starter. Le Lait. 72, 249-263.
46. Malek, R. El-Attar, A., Mohamed, M. Anwar, S. El-Soda, M. and Béal, C. 2012. Technological and safety properties display biodiversity among enterococci isolated from two Egyptian cheeses, "Ras" and "Domiati". International Journal of Food Microbiology 153. pp: 314–322.
47. Mohamed, M. A., Larsson-raznikiewicz, M. and Mohamud, M. U. (1990). Hard cheese making from camel milk. Milchwissenschaft. 45, 716–718.
48. Nawaz, M., Wang, J., Zhou, A., Ma, C., Wu, X. and Xu, J. (2011). Screening and characterization of new potentially probiotic lactobacilli from breast-fed healthy babies in Pakistan. Afr. J. Microbiol. Res. 5, pp. 1428-1436.
49. Ogier JC and Serror P (2008) Safety assessment of dairy microorganisms: The Enterococcus genus. International Journal of Food Microbiology 126: 291–301.
50. Ohris, S.P. and B.K. Joshi, 1961. Composition of camel milk. Ind.Veterinary j., 38: 514-516.

51. Ouled-Haddar, H., Idoui, T., Sifour, M., Guezira, M. and Bouthabet, M. (2012). Isolation, characterization and microencapsulation of probiotic *Lactobacillus curvatus* G7 from chicken crop. J. Sci. Technol. 2, 47- 53.
52. Prasad, J., Gill, H., Smart, J., and Gopal, P. K. (1998). Selection and Characterization of *Lactobacillus* and *Bifidobacterium* strains for use as probiotic. Int. Dairy J. 8, 993-1002.
53. Ritcher. R. L and R. Vadamuthu., 2001. Microbiology Examination of Food. 4th Ed. American Public Health Association, pp: 483-485.
54. Rodríguez, M. M. L., Tornadijo, M. E., Carballo, J. and Sarmiento, R. M. (1995). Microbiological study of León raw cow-milk cheese, a Spanish craft variety. J. Food. Protect. 58, 998-1006.
55. Rolfe, D. R. (2000). The role of probiotic cultures in the control of gastrointestinal health. J. Nutr. 130, 396S–402S
56. Ryan, M. P., Rea, M.C., Hill, C. and Ross, R. P. (1996). An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. Appl. Environ. Microbiol. 62, 612-619.
57. Quwehand, A. C., Kirjavainen, P. V., Shortt, C. and Salminen, S. (1999). Probiotics: Mechanisms and established effects. Int. Dairy J. 9, 43-52.
58. Salama, M., Sandine, W. and Giovannoni, S. (1991). Development and application of oligonucleotide probes for identification of *Lactococcus lactis* subsp. *cremoris*. Appl. Environ. Microbiol. 57, 1313–1318.
59. Sandine, W. E. (1985). The streptococci: milk products. In: Bacterial Starter Cultures for Food. Gilliland S. E. (ed.), CRC Press, Boca Raton, Florida. pp. 5-23.
60. Soukoulis, C., Panagiotidis, P., Koureli, R. and Tzia, C. (2007). Industrial yogurt manufacture: monitoring of fermentation process and improvement of final product quality. J. Dairy Sci. 90, 2641-2654.
61. Strompfova, V. and Laukova, A. (2007). *In vitro* study on bacteriocin production of Enterococci associated with chickens. Anaerobe. 13, 228-237.
62. Strompfova, V., Laukova, A. and Ouwehand, A. C. (2004). Selection of enterococci for potential canine probiotic additives. Vet. Microbiol. 100, 107-114.
63. Tannock, G. W. (2004). A special fondness for lactobacilli. Appl. Environ. Microbiol. 70, 3189-3194.
64. Tanaka, H., Hashiba, H., Kok, J. and Mierau, I. (2000). Bile salt hydrolase of *Bifidobacterium longum*: biochemical and genetic characterization. Appl. Environ. Microbiol. 66, 2502-2512.
65. Temmerman, R., Pot, B., Huys, G. and Swings, J. (2002). Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int. J. Food Microbiol. 81, 1-10.
66. Todorov, S. D. and Dicks, L. M. T. (2004). Effect of medium components on bacteriocin production by *Lactobacillus pentosus* ST151BR, a strain isolated from beer produced by the fermentation of maize, barley and soy flour. World. J. Microbiol. Biotechnol. 20, 643-650.
67. Weinberg, Z., Shatz, O., Chen, Y., Yosef, E., Nikbahat, M., Ben-Ghedalia, D. and Miron, J. (2007). Effect of lactic acid bacteria inoculants on *in vitro* digestibility of wheat and corn silages. J. Dairy Sci. 90, 4754-4762.
68. Yagil, R. (1982). Camels and camel milk Animal Production and Health Paper No. 26. FAO, Rome. 26, 14-19.
69. Yagil, R. (1987). Camel milk. A review. *Int. J. Anim. Sci.* 2, 81
70. Yateem, M.T. Balba, T. Al-Surrayai, B. Al-Mutairi and Al-Daher,R. 2008. Isolation of lactic acid bacteria with probiotic potential from camel milk. International Journal of Dairy Science. 3 (4): 194-199.
71. Yavuzdurmaz, H. (2007). Isolation, characterization, determination of probiotic properties of lactic acid bacteria from human milk. M.Sc. Thesis, The graduate School of Engineering and Sciences of Izmir Institute of Technology.

2/2/2013

Evaluation of inactivated AIV vaccines in conjunction with antiviral drugs in chickens challenged with Egyptian H5N1 HPAIV

Ali, A. Salama; El-Bakry, M. Ismail; Fatma, M. Abdallah and Gemelat, K. Farag

Virology Department, Faculty of Veterinary Medicine, Zagazig University, Egypt
mm.fatma @yahoo.com

Abstract: The goal of the presented research was to synthesis focuses on the effectiveness of vaccines and antiviral drugs in the prevention and treatment of avian influenza virus (AIV) in chickens. Antibody responses, and virus shedding were evaluated after challenge with Egyptian H5N1 HPAIV (A/chicken/faquos/amn/2/2011 (H5N1)). The results revealed that, the antibody titers in sera of the broiler chickens vaccinated with AI H5N1 vaccine alone or in combination with potent neuraminidase inhibitor antiviral drugs (NAI) were higher than antibody titers in sera of the broiler chickens vaccinated with AI H5N2 vaccine alone or in combination with NAI antiviral drugs against AIV with significant difference ($p< 0.05$). Furthermore, NAI antiviral drugs provided significant protection and reduction the duration and titer of virus shedding especially in vaccinated chickens. These investigations showed that NAI antiviral drugs used in conjunction with vaccination strategies in chicken farms reduced the risk of avian influenza virus.

[Ali, A. Salama; El-Bakry, M. Ismail; Fatma, M. Abdallah and Gemelat, K. Farag. **Evaluation of inactivated AIV vaccines in conjunction with antiviral drugs in chickens challenged with Egyptian H5N1 HPAIV.** *Life Sci J* 2013;10(1):1962-1968] (ISSN:1097-8135). http://www.lifesciencesite.com. 281

Key Words: HPAIV, H5N1, NAI, H5N2, broiler chickens, antiviral drugs, vaccines.

1. Introduction

Avian influenza (AI) is a contagious viral disease, worldwide in distribution caused by a single stranded, negative-sense RNA virus in the family Orthomyxoviridae, genus influenza virus A with the genome divided into eight gene segments**.** The surface is covered by two types of glycoprotein projections; rod shaped timers of haemagglutinin (HA) and mushroom shaped tetramers of neuraminidase (NA) **[1].** Influenza A virus is further categorized by serological reaction of the two surface glycoproteins into 16 different hemagglutinin (H1–16) and 9 different neuraminidase (N1–9) subtypes **[2].** Protection is primarily the result of humoral immune response against the hemagglutinin (HA), and secondarily against the neuraminidase **[3].** Avian influenza (AI) viruses vary in virulence either being of low or high pathogenicity **[4].** It affects the chickens of all ages with variable morbidity and mortality. With the HPAI viruses, morbidity and mortality rates are very high (50–89%) and can reach 100%in some flocks **[5].**

Vaccines have been used in AI control programs achieve one of three broad goals: (1) prevention, (2) management, or (3) eradication. The best protection is produced from the humoral response against the hemagglutinin (HA) protein **[6].** Vaccination has been shown to increase resistance to field challenge and reduce virus shedding levels in vaccinated birds and subsequently reduce transmission **[7].** However, vaccines have not been a universal solution in the control of AIV in the field **[3]** and as described previously, M2 ion channel inhibitors[amantadine and rimantadine] & neuraminidase inhibitors [oseltamivir (Tamiflu®) and zanamivir (Relenza®)] have comparable effectiveness in the prevention and treatment of influenza **[8].** Thus, in the present study, we investigated the protective efficacy of available inactivated oil emulsion whole-virus H5 (H5N1 & H5N2) influenza vaccines against Egyptian H5N1 HPAIV in conjunction with neuraminidase inhibitors (oseltamivir® & zanamivir®) as anti-influenza A virus drug therapies in chickens.

2. Material and methods

Chickens:

One hundred and eighty one day old commercial Hubbard chicks were purchased from (Dakahlia Poultry Company). The chicks were reared in isolation cabinets with continuous light exposure and were individually identified by means of a numbered wing tag. Chickens were fed with water and feed *ad libitum* daily with commercial compound suitable for their age.

Vaccines and vaccine administration:

a. The inactivated oil emulsion reassortant avian influenza vaccine (H5N1 subtype, Re-1 strain), China. The vaccine strain is (H5N1 subtype, Egy/PR8-1 strain).

b. Volvac® AI KV avian influenza killed virus. The inactivated oil emulsion LPAI H5N2 vaccine. The vaccine strain is H5N2, A/ chicken / Mexico / 322/94/CPA.

The vaccine dose of the two vaccines under study was 0.5 ml /bird, it were inoculated in the lower (dorsal) part of the neck by the subcutaneous route.

Potent neuraminidase inhibitor antiviral drugs:

a. Oseltamivir capsule (Tamiflu®) is 75 mg/capsule manufactured by the Nile Company for pharmaceutical and chemicals industries, Cairo. It was administrated orally as 10 mg/bird for three successive days.

b. Zanamivir powder (relenza®) for inhalation, each blister contains zanamivir 5 mg manufactured by the Glaxowellcome. It was administrated by inhalation as 10 mg/bird for three successive days.

Challenge avian influenza virus (AIV):

Avian influenza virus (A/chicken/faquos/amn /2/2011 (H5N1)) with accession number JQ627585 was kindly supplied by Virology Department, Faculty of Veterinary Medicine, Zagazig University.

Experimental design:

One hundred and eighty day-old broiler chicks were randomly divided into 18groups, each group containing 10 chicks. Six groups (I-VI) of chicks were vaccinated with H5N1 and other six groups (VII-XII) were vaccinated H5N2 AI vaccines at 7days-old via subcutaneous injection with dose 0.5 ml / chick. Groups (I- XII) were treated differentially either with oseltamivir or zanamivir (10 mg/bird for three successive days) at 24 and /or 48hrs post challenge. The groups of (XIII-XVI) were administered oseltamivir and zanamivir (10 mg/bird for three successive days) without vaccination at 24 and /or 48hrs post challenge. The chickens of group XVII&XVIII were left as control groups. All chickens were challenged intranasally with 0.1 ml viral suspension containing 10^6EID_{50}/ml of the challenge locally isolated AIV strain (A/chicken/faquos/amn/2/2011 (H5N1)) after three weeks post vaccination. Whereas chickens of (VI and XII) were vaccinated and remained unchallenged & untreated .Thereafter, the experimental chickens were observed daily over a period of two weeks & the clinical signs and the mortality rate were recorded. In order to monitor virus shedding after challenge, Oropharyngeal and cloacal swabs were collected at 3rd, 5th, 7th, and 9th day post challenge for analysis of viral shedding. Swab samples were subjected to RRT-PCR analyses and at the same time they were processed for virus titration in 10-day-old SPF - ECEs. To determine the level of specific antibodies against AIV by Hemagglutination inhibition (HI) test and Commercially available H5 avian influenza (AIV) antibody ELISA test kit, blood samples were taken at 7 days (pre vaccination), 14 & 21 day post vaccination, and 14 day post challenge.

Sampling:

Chickens were observed daily for clinical signs throughout the duration of the study. Following challenge, oropharyngeal and cloacal swabs were processed for attempted virus isolation in SPF-ECEs and were analyzed by RRT-PCR. After collection, oropharyngeal and cloacal samples were placed in 1 ml phosphate buffered saline (PBS). 100 µL from each sample were used for RNA extraction. RNA was analyzed by real-time RT-PCR. The remaining sample was mixed with an equal volume of PBS containing penicillin (2000 IU/ml), streptomycin (2 mg/ml), gentamicin (0.05 g/ml) and mycostatin (1000 IU/ml) for virus isolation attempts. Also blood samples were collected from wing vein and kept in a slope position at 4°C overnight. Sera were then separated by centrifugation at 3000 rpm for 10 minutes and stored at -20°C. Sera were inactivated at 56°C for 30 minutes before testing **[9].**

Reference AIV antiserum:

Anti-avian influenza hyper immune serum against H5N1 AIV was kindly provided by Virology Department, Veterinary Medicine, Zagazig University.

Washed Chicken red blood cells (RBCs):

Blood samples were collected from wing veins of 2 – 3 apparently healthy of 4-6 weeks old chickens. Blood was received in sterile tubes containing 4% sodium citrate solution, and was subjected to three successive washing cycles by centrifugation at 1200 rpm for 10 minutes using PBS. For haemagglutination inhibition test (HI) test, the RBCs were used as 1 % suspension in PBS **[9].**

Hemagglutination inhibition (HI) test:

Hemagglutination units (HAU) of the H5N1 AIV were determined before each test using twofold dilutions. Sera were serially diluted twofold in 50 µl PBS, and 4 HAU of H5N1 were used in 50 µl. The contents of each well were gently mixed with a micropipettor and the plates were incubated for 30 min at room temperature. Finally, 50 µl of a 1 % chicken erythrocyte suspension was added to each well. The highest serum dilution capable of preventing hemagglutination was scored as the HI titer. The test was applied to quantify AIV antibodies in chicken sera and the data were reported as $\log_2$ titer according to OIE Manual[10]**.**

Enzyme-Linked Immunosorbent Assay (ELISA):

Commercially available H5 avian influenza (AIV) antibody ELISA test kit (ProFLOK® PLUS, Synbiotics Corporation, San Diego, CA, USA) was used under the manufacturer's instructions. Optical density values were read at 450 nm using an ELISA reader (Behring EL311). The kits used for detection of antibodies to haemaggltinins (HA) of influenza A virus, H5 strain.

Specific pathogen free embryonated chicken eggs (SPF – ECEs):

Eleven day old SPF-ECEs were purchased from poultry farm at Qom Osheem- Al Fayoum, Egypt.

Avian influenza virus titration in (SPF – ECE):

Virus containing oropharyngeal and cloacal swab samples were titrated and expressed as the 50% egg infectious dose (EID_{50}) using SPF–ECEs as previously described **[11].** Briefly, 200µl of each dilution (ten fold serial dilution) of swab samples suspended in PBS was inoculated into five10-day-old SPF–ECEs and incubated for 5 days or until death of the embryo. The allantoic fluids were collected and subjected for the hemagglutination activity . Titration was applied to quantify AIV in swabs and the data were reported as $\log_{10}EID_{50}$/ml according to Reed & Muench[12]

Real-time reverse transcriptase polymerase chain reaction (RRT-PCR):

For quantitation, swab samples were run together with known amounts of control viral RNA. To prepare control RNA, the reference virus used in this study was titrated using SPF-ECEs as described above and RNA was extracted from serially diluted virus (10^4–10^8 EID_{50}/ml). Standard curves were generated with this control viral RNA.

Sequences of the primer and hydrolysis probe sets specific for the H5 gene **(Table 1)** has been previously described by Spackman *et al.* [13].

Table 1. primer and hydrolysis probe sequences used for TaqMan RRT-PCR*(Metabion Company).*

H5+ 1456	ACG TAT GAC TAT CCA CAA TAC TCA G
H5 - 1685	AGA CCA GCT ACC ATG ATT GC
H5+ 1637	FAM-TCA ACA GTG GCG AGT TCC CTA GCA-TAMRA

The probe was labeled at the 5′ end with the 6-carboxyfluorescein (FAM) reporter dye and at the 3′ end with the 6-carboxytetramethylrhodamine (TAMRA) quencher dye.

Extraction of the RNA using QIAamp viral RNA mini kit (QIAGEN, Valencia, Calif, USA):

Ribonucleic acid was extracted using the RNeasy mini kit (Qiagen Inc., Valencia, CA) from fluid containing swabs following the instructions of the manufacturer. Briefly, 500µL of swab fluid was mixed with 500 µL of the kit-supplied RLT Buffer and the entire sample was applied to the RNeasy spin column. The column was washed with buffers and then RNA was eluted in 50 µL of nuclease free water. 5µL per RRT-PCR reaction was used as a template.

One step RRT-PCR using TaqMan probe:

One-tube RRT-PCR was performed using the Qiagen one-step RRT-PCR kit in a 50µl reaction mixture containing 25 µL of the kit-supplied mix and 0.5 µL from 30 pmol of each primer, 0. 5 µL from H5 probe 50 pmol, 0. 5 µL from Access Quick RT-Enzyme and 18 µL nuclease free water and 5 µL of RNA that amplified using Stratagen PCR machine. The RT-PCR program consisted of 30 min at 50°C and 10 min at 95°C and a three-step cycling protocol was used as 95°C for 30 s, 50°C for 1min and 72°C for 30 Sec for 35 cycles. Fluorescence data were acquired at the end of each annealing step. The result of the avian influenza H5N1 one step real-time RT-PCR assay showed positive amplification signals with FAM dye for the original isolate and the first four dilutions from 10^{-4} to 10^{-8}. Since samples with threshold cycle (Ct) values lower than 35 were counted as indicative of the presence of virus.

Statistical Analysis:

The egg infective dose fifty ($\log_{10}EID_{50}$ /ml) of virus shed from cloaca and oropharynx in each group was determined for consecutive days postchallenge and compared between groups by ANOVA.The logarithm_2 mean titre ($\log_2$) of H5 HI and ELISA antibody responses to H5N1 HPAIV were compared within and between groups postvaccination and postchallenge by ANOVA.

3. Result

- **Serological analyses:**

The goal of the presented research was to evaluate immunogenicity of the commercially available inactivated influenza vaccines either alone or in combination with NAI antiviral drugs (oseltamivir and/or zanamivir) of (Gp I - XII) and the efficacy of NAI antiviral drugs (oseltamivir and/or zanamivir) alone without vaccination of (Gp XIII- XVI) against the highly pathogenic avian influenza virus (A/chicken/faquos/amn /2/2011(H5N1)) in broiler chickens. All pre vaccination sera were negative for H5 antibodies in the HI and ELISA test.

The mean HI titers were 7.1 $\log_2$ of H5N1 inactivated vaccine immunized chickens at 21days post vaccination were higher than the mean HI titers in sera of chickens at 14 days post vaccination of 3.6 $\log_2$. While, the mean HI titers were 6.5 $\log_2$ of H5N2 inactivated vaccine immunized chickens at 21days post vaccination were higher than the mean HI titers in sera of chickens at 14 days post vaccination of 3.1 $\log_2$ **(Table 2).** A marked increase

of H5-specific antibody HI titers (7.8 & 7.4 log_2) at 14 days post challenge were observed in surviving chickens of the (GP I & VII) immunized with H5N1 vaccine as well as H5N2 vaccine, respectively **.** Mean HI titers in sera were 7.9& 7.6 log_2 of chickens immunized with H5N1& H5N2 vaccines in combination with NAI drugs in (Gp II-V& GPVIII-XI) , respectively **.** At the end of the experimental trial, the uninfected vaccinated groups (GPVI&XII) showed a slight increase in HI titers (7.2& 6.0 to 7.4& 6.3), respectively when vaccinated with H5N1 &H5N2 vaccines

The data were analyzed by HI test **(Table 2)**, and reconfirmed by commercially available H5 avian influenza (AIV) antibody ELISA test kit.

The mean ELISA titers (1964) of H5N1 inactivated vaccine immunized chickens at 21days post vaccination were higher than the mean ELISA titers in sera of chickens at 14 days post vaccination (1633). While, the mean ELISA titers (1775) of H5N2 inactivated vaccine immunized chickens at 21days post vaccination were higher than the mean ELISA titers in sera of chickens at 14 days post vaccination of (1476) **.** A marked increase of H5-specific antibody ELISA titers (2522 & 2462) at 14 days post challenge was observed in surviving chickens of the (GP I & VII) immunized with H5N1 vaccine as well as H5N2 vaccine, respectively **.** Mean ELISA titers in sera of chickens immunized with H5N1& H5N2 vaccines in combination with NAI drugs increased slightly in (GpII-V& GPVIII- XI) was 2597& 2520 , respectively **(Table 2).** At the end of the experimental trial, the uninfected vaccinated groups (GPVI&XII) showed a slight increase in ELISA titers (1743& 1442 to 1939&1631), respectively when vaccinated with H5N1 &H5N2 vaccines.

- **Analyses of viral shedding**

During the period of 14 days, the challenged chickens were observed, oropharyngeal and cloacal swabs were collected at 3rd, 5th, 7th and 9th days post challenge to reveal possible virus shedding.

The highest value of mean $log_{10}EID_{50}$/ml of recovered AI challenge virus from all oropharyngeal swabs was frequently recorded at 3rd day post challenge. In (GPI-V) was $10^{5.2}$, $10^{4.6}$, $10^{5.0}$, $10^{4.3}$, and $10^{5.4}$ log_{10} EID_{50}/ml. whereas in (GPVII-XI), the mean log_{10} EID_{50}/ml was $10^{5.7}$, $10^{4.9}$, $10^{5.3}$, $10^{4.7}$, and $10^{5.6}$.The mean log_{10} EID_{50}/ml were $10^{5.7}$, $10^{5.9}$, $10^{5.3}$, and $10^{5.7}$ in groups XIII- XVI. During the entire period of observation, the all non vaccinated chickens (GPXVII) were died at the 2nd day post challenge and the AIV was reisolated in high titers $10^{6.2}$ and $10^{5.7}log_{10}$ EID_{50}, respectively via oropharyngeal and cloacal swabs . The AI challenge virus was recovered less frequently and the viral titers were observed rather low from all cloacal samples in the experimental study than oropharyngeal swabs as shown in **(Table 3).** Recovered AI challenge virus titers were considerably reduced at 5th day post challenge. The AI virus was also detected from the oropharyngeal and cloacal swabs of chickens at day 7 with low titer rather than at 5 th days. While no virus was detected from any chickens on day 9th post challenge **.** However, (GPVI, XII, and XVIII), all chickens survived and no symptoms of disease were observed. Also, Virus could no longer be isolated from the pooled oropharyngeal and cloacal swabs of these chickens.

Oropharyngeal and cloacal swab samples in which AIV was titrated in SPF-ECEs were subjected to TaqMan RRT-PCR. Since the mean threshold cycle (Ct) values were observed ranged between 24.0 - 35.0, all groups shed virus at a comparable level.The RRT-PCR was performed to quantify the titer and variations in AIV RNA levels over time in swabs of challenged chickens. High loads of viral RNA were frequently detected at 3rd day post challenge **(Table 3).** The mean C_t in (GPI-V) were 29.3, 31.6, 30.7, 31, and 30 whereas the mean C_t in (GPVII-XI) were 28.7, 29.8, 29, 29.5, and 29.01 In addition to, the mean C_t were 28.3, 27, 28.07, and 26.3 in groups XIII- XVI. The viral load continued to decrease at 5th, 7th day post challenge. However, AIV RNA levels were dropped at 9 th day post challenge **.** However, (GPVI, XII, and XVIII), AIV could not be detectable. Also, Viral RNA could not be detectable in all the pooled cloacal swabs as shown in **(Table 3).**

Table 2. Immune response of broiler chickens post vaccination with inactivated AIV vaccines and administration with NAI drugs using HI and ELISA.

Groups	14 day post vaccination		21day post vaccination		14 day post challenge	
	HI	ELISA	HI	ELISA	HI	ELISA
I	3.5± 0.28	1633± 37.52	7.0± 0.12	1964± 25.98	7.8± 0.17	2522± 11.54
II	3.5± 0.11	1566± 25.98	7.0± 0.12	1990± 11.54	7.9± 0.17	2577± 23.09
III	3.6± 0.12	1462± 23.09	7.1± 0.12	1943± 25.98	7.7± 0.17	2686± 23.09
IV	3.9± 0.11	1544± 11.54	7.4± 0.16	1934± 25.98	7.9± 0.17	2554± 17.32
V	3.4± 015	1484± 17.32	7.0± 0.12	1989± 11.54	7.8± 0.16	2574± 17.32
VI	3.0± 0.10	1366± 17.32	7.2± 0.16	1743± 25.98	7.4± 0.16	1939± 23.09
VII	3.4± 0.13	1476± 11.54	6.5± 015	1775± 11.54	7.4± 0.16	2462± 11.54
VIII	3.2± 0.11	1351± 23.09	6..7± 0.10	1739± 25.98	7.6± 0.16	2578± 23.09

IX	2.9± 0.16	1306± 11.54	6.5± 0.10	1668± 11.54	7.5± 0.16	2490± 17.32
X	3.0± 0.17	1390± 17.32	6.3± 015	1624± 25.98	7.6± 0.12	2512± 23.09
XI	3.0± 0.21	1310± 22.80	6.5± 0.10	1569± 17.32	7.4± 0.12	2500± 11.54
XII	3.0± 0.12	1105± 22.80	6.0± 015	1442± 17.32	6.3± 0.12	1631± 23.09
XIII	-	207± 1.15	-	272± 1.57	4.2± 0.05	708± 1.57
XIV	-	102± 1.15	-	256± 1.57	4.6± 0.12	939± 1.15
XV	-	302± 1.57	-	308± 1.15	3.7± 0.22	801± 1.57
XVI	-	207± 1.57	-	256± 1.57	4.5± 0.16	708± 1.15
XVII	-	117± 1.15	-	200± 1.15	4.0± 0.17	649± 1.57
XVIII	-	132± 1.57	-	184± 1.57	-	184± 1.15

Table 3. The efficacy of inactivated AIV vaccines and NAI drugs against challenging H5N1 HPAIV.

Titration of excreted H5N1HPAIVafter challenge in SPF-ECEs& RRT-PCR												
Groups	3 days post challenge			5 days post challenge			7 days post challenge			9 days post challenge		
	$log_{10}EID_{50}$		Ct[c]	$log_{10}EID_{50}$		Ct	$log_{10}EID_{50}$		Ct	$log_{10}EID_{50}$		Ct
	Op[a]	Cl[b]		Op	Cl		Op	Cl		Op	Cl	
I	5.2± 0.17	4.6± 0.15	29.3± 1.57	4.0± 0.21	2.5± 0.05	31.73± 0.31	2.0± 0.13	1.0± 0.18	33.34± 0.98	-	-	34.62± 2.87
II	4.6± 0.15	4.0± 0.18	31.6± 2.12	3.5± 0.11	2.8± 0.21	32.82± 0.18	1.8± 0.21	1.0± 0.07	34.15± 1.02	-	-	35.0± 1.89
III	5.0± 0.21	4.3± 0.21	30.7± 0.98	3.5± 0.11	2.9± 0.05	32.0± 0.31	1.8± 0.31	1.0± 0.13	33.18± 0.57	-	-	34.05± 2.87
IV	4.3± 0.15	3.9± 0.15	31.0± 1.64	3.2± 0.11	2.5± 0.07	32.1± 0.18	1.5± 0.13	1.0± 0.07	33.34± 1.47	-	-	34.62± 1.89
V	5.4± 0.21	4.0± 0.18	30.0± 2.12	4.0± 0.21	3.0± 0.21	32.01± 0.18	-	-	33.97± 1.02	-	-	34.06± 2.87
VI	-	-	-	-	-	-	-	-	-	-	-	-
VII	5.7± 0.31	4..4± 0.21	28.7± 2.12	4.3± 0.31	3.3± 0.11	30.21± 0.31	2.5± 0.05	1.0± 0.31	32.0± 0.98	-	-	33.7± 0.57
VIII	4.9± 0.07	4.4± 0.21	29.8± 1.57	4.0± 0.07	3.0± 0.11	32.85± 0.18	2.0± 0.21	1.0± 0.13	33.92± 1.02	-	-	34.62± 1.49
IX	5.3± 0.31	4.6± 0.18	29.0± 1.57	3.5± 0.18	2.9± 0.21	31.5± 0.18	-	-	32.44± 1.47	-	-	34.0± 0.57
X	4.7± 0.18	3.9± 0.18	29.5± 1.57	4.0± 0.31	3.0± 0.11	32.12± 0.18	2.6± 0.05	1.5± 0.13	33.89± 1.47	-	-	34.17± 0.57
XI	5.6± 0.07	4.4± 0.21	29.01± 1.57	4.0± 0.18	3.0± 0.21	31.44± 0.31	2.5± 0.31	1.5± 0.13	32.7± 0.98	-	-	33.54± 1.49
XII	-	-	-	-	-	-	-	-	-	-	-	-
XIII	5.7± 0.31	4.2± 0.21	29.3± 1.64	4.1± 0.07	3.2± 0.11	30.32± 0.31	3.0± 0.21	1.5± 0.13	31.35± 0.57	-	-	33.0± 0.84
XIV	5.9± 0.31	4.5± 0.21	27.0± 2.12	4.0± 0.13	2.9± 0.21	29.0± 0.18	2.5± 0.05	1.8± 0.18	30.0± 1.02	-	-	32.0± 0.57
XV	5.3± 0.18	4.0± 0.21	28.07± 1.64	3.9± 0.31	2.9± 0.05	30.45± 1.64	2.0± 0.07	1.0± 0.31	32.0± 0.98	-	-	33.0± 0.97
XVI	5.7± 0.31	4.3± 0.21	26.3± 2.12	4.0± 0.13	3.1± 0.11	28.0± 0.07	2.0± 0.31	1.0± 0.18	30.0± 0.57	-	-	32.0± 0.57
XVII	6.2± 0.18	5.7± 0.18	24.0± 1.64	4.5± 0.21	-	-	-	-	-	-	-	-
XVIII	-	-	-	-	-	-	-	-	-	-	-	-

[a] Op= oropharyngeal swab [b] Cl= cloacal swab [c] Ct= threshold cycle

4. Discussion

The objective of this study was to evaluate in a comparative setting the protective efficacy of available inactivated H5N1 and H5N2 AIV vaccines either alone or in combination with NAI antiviral drugs (oseltamivir® and/or zanamivir®) in chickens against H5N1 HPAIV.

Hemagglutination inhibition test and enzyme-linked immunosorbent assay were used to evaluate immunogenicity of the available inactivated avian influenza vaccines either alone or in combination with NAI antiviral drugs .The mean HI titer of inactivated vaccines in immunized chickens at 21day post vaccination were higher than the mean HI titer in sera of chickens at 14 day post vaccination. These data were analyzed by HI test, and reconfirmed by available H5 avian influenza (AIV) antibody ELISA test kit as shown in **(Table2).** Previous studies had indicated that the vaccinated chickens could be completely protected from highly pathogenic AIV challenge when the antibody titers to the challenge virus equaled or were greater than $4log_2$ at three weeks after vaccination **[14].** A mean HI titer in the sera of broiler chickens vaccinated with AI H5N1 vaccine alone or in combination with NAI antiviral drugs was significantly higher ($P < .05$) than

antibody titers in sera of the broiler chickens vaccinated with AI H5N2 vaccine alone or in combination with NAI antiviral drugs. As previously mentioned by Tian *et al.*[14]**,** Most consistent reduction in respiratory shedding was afforded when the vaccine was more similar to the challenge virus. Significant increase of antibody titers were observed in vaccinated and treated chickens two weeks after challenge. This is in accordance with the other Studies **[16]** and Could possibly represent a lack of replication of the challenge virus in the surviving birds.

The efficacy evaluations have been based on a challenge study performed few weeks post vaccination. Oropharyngeal and cloacal swabs for virus titration were taken at the peak of replication, i.e. 3 day post challenge. As **Swayne and Halvorson, 2003** were taken Oropharyngeal and cloacal swabs at the peak of virus shedding, day 3 post-inoculation, for virus isolation attempts in 10 days embryonating chicken eggs .

After challenge , protective efficacy was evaluated based on clinical observations and the magnitude of viral shedding. The challenge virus was highly pathogenic for the control group as causing 100 % mortalities within 48 hours .Challenge of other groups showed difference in immune response and protective efficacy of vaccines and drugs. Also our results were in agreement with Villegas & Swayne [17] who reported that all unvaccinated challenged birds died within 2 days, whereas 90% and 100% of chickens vaccinated with H5N1and H5N2 respectively were protected against morbidity and mortality.

The data obtained from this study show a significant increase of the survival rate in chickens, a significant reduction in sick /dead chickens, and a significant reduction in the number of chickens shedding the challenge virus , which results in an overall reduction of shedding leaves in vaccinated treated chickens **(data not shown)** is consistent with previous studies where chemotherapy may be useful in the treatment of a highly pathogenic influenza virus outbreak in humans or other animals when used in combination with vaccine**[19,20].**

The quantitation of virus shed from infected chickens was done by titration in ECE-SPFs and expressed as 50% embryo infective dose **[18].** RRT-PCR also has been successfully applied in the quantitation of AIV samples and is a reliable alternative to virus isolation in ECEs **[13].**

The AI challenge virus was recovered less frequently and the viral titers were observed rather low for all cloacal samples in the experimental study than oropharyngeal swabs. **Tian and his colleague,2005** recorded the viral titers shed from the trachea were higher than from the cloaca and is believed to be related to a shift in replication efficiency for the upper respiratory tract after infection with challenge virus **[14, 21].**

The AI challenge virus shedding peaked at day 3 post challenge, which might be related to reisolation of inoculum, and remained significantly lower ($P < .05$) in consecutive days postchallenge. while no virus was detected in chickens on day 9th post challenge by titration in ECE-SPFs as reported by Webster *et al.* [19].Also, High loads of viral RNA were frequently detected at 3rd day post challenge and the viral load continued to decrease at 5th, 7th day post challenge. However, AIV RNA levels were dropped at 9 th day post challenge.This is in accordance with other studies**[22,19]** that demonstrated, high titers of virus were detected in birds at 3 day post challenge, However, the titer of virus decreased significantly and the number of virus positive birds also decreased at 7 day post challenge. By contrast, Viral RNA could not be detectable in all the pooled cloacal swabs of any challenged chickens in this experiment.The failure of detection of viral RNA in cloacal swabs in comparison to SPF-ECEs titration might relate to inhibitory substances present in fecal specimens that reduce or block PCR amplification and most commercial RNA extraction kits have limited capacity to remove inhibitors from these clinical samples **[18].**

Conclusion

The serological response and protection percentage in vaccinated chickens were improved following administration of potent neuraminidase inhibitor antiviral drugs.Thus, chemotherapy may be useful in the treatment of a highly pathogenic influenza virus when used in combination with vaccine.

References

1. Swayne, D.E., Halvorson D.A. 2003.Influenza. In: Saif, Y.M., Barnes, H.J., Fadly, A.M., Glisson, J.R., McDougald ,L.R. Diseases of poultry. Ames, IA, Iowa State University Press, p. 135–160.
2. Easterday, B.C., Hinshaw, V.S., Halvorson, D.A., 1997. Infuenza. In: Calnek, B.W., Barnes, H.J., Beard, C.W.,McDougald, L.R., Saif, Y.M. (Eds.), Diseases of Poultry 10th Edition, Iowa State University Press, Ames,IA, pp. 583-605.
3. Swayne,D.E. 2009. Avian influenza vaccines and therapies for poultry. Comparative Immunology, Microbiology and Infectious Diseases 32 , 351–363.
4. Perdue, M.L., Suarez, D.L., Swayne, D.E., 2000. Avian Infuenza in the 1990s. Poultry and Avian Biol. Rev., 10(in press).

5. Capua, I. F., Mutinelli, M.A., Bozza, C., Terregino, G. Cattoli, C., 2000. Highly pathogenic avian influenza (H7N1) in ostriches (Struthiocamelus). Avian Pathol., 29, 643–646.
6. Swayne, D.E., Akey, B. 2005. Avian influenza control strategies in the United States of America. In: Schrijver R.S., Koch, G., editors. Avian influenza. Prevention and control. Dordrecht: Springer; p. 113–30.
7. Swayne, D.E., Beck, J.R., Garcia, M., Stone, H.D. 1999. Influence of virus strain and antigen mass on efficacy of H5avian influenza inactivated vaccines. Avian Pathol., 28,245–55.
8. WHO.2004. Guidelines on the Use of Vaccines and Antiviral during Influenza Pandemics. Geneva, World Health Organization, (http://www.who.int/csr/resources/publications/influenza/1129_01_A.pdf, accessed 17, November 2005).
9. OIE Manual, 2005. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter, 2.7.12 .
10. OIE Manual, 2008. Avian influenza .In, Manual of Diagnostic tests and Vaccines for terrestial animals, OIE (Ed) .6th Edn. OIE, Paris, France.
11. OIE Manual, 2009. Manual of diagnostic tests and vaccines for terrestrial animals purtz, section 2-1. Chapter 2-1.
12. .Reed, L.J. , Muench, H. 1938. A simple method of estimating fifty percent end point. Am .J. Hyg. 27, 493 – 497.
13. Spackman, E., Senne, D.A., Myers, T.J., Bulaga, L.L., Garber, L.P., Perdue, M.L., Suarez, D.L. 2002. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. Journal of Clinical Microbiology, 40(9), 3256-3260.
14. Tian ,G.,Zhang, S.,Li ,Y., Buc Zh, Liu, P., Zhoub ,J., Li Ch, Shi, J., Yua, K., Chen ,H . 2005. Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics. Virol, 341, 153 – 162.
15. Swayne,D. E., Michael, L., Perdue, Joan R. Beck, Maricarmen Garcia ,David L. Suarez.2000.Vaccines protect chickens against H5 highly pathogenic avian infuenza in the face of genetic changes infield viruses over multiple years.Veterinary Microbiology. 74 ,165-172.
16. Ellis, T.M., Leung, C.Y., Chow, M.K., Bissett, L.A., Wong, W., Guan, Y., Peiris, M. 2004. Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. Avian Pathol. 33, 405–412.
17. Villegas, P., Swayne, D.E. 1998. Titration of biological suspensions. A Laboratory Manual for the Isolation and Identification of Avian Pathogens.fourth ed. American Association of Avian Pathologists,Kennett Square, PA, 248–254.
18. Das, A., Spackman, E., Mary, J., Pantin-Jackwood, David ,L. ,Suarez,1. 2009. Removal of real-time reverse transcription polymerase chain reaction(RT-PCR) inhibitors associated with cloacal swab samples and tissues for improved diagnosis of Avian influenza virus by RT-PCR..J .Vet .Diagn .Invest 21,771–778 .
19. Webster,R. G., Webster, Y. Kawaoka , Bean, W. J. 1986 . Vaccination as a strategy to reduce the emergence of amantadine- and rimantadine-resistant strains of A/Chick/Pennsylvania/83 (H5N2) influenza virus. *Antimicrob. Chemother.* 18 (Supplement B), 157-164.
20. Lee, D.H., Lee, Y.N., Park, J.K., Yuk, S.S., Lee, J.W., Kim, J.I., Han, J.S., Lee, J.B., Park, S.Y., Choi, I.S., Song, C.S. 2011. Antiviral efficacy of oseltamivir against avian influenza virus in avian species. Avian Dis. 55(4):677-9.
21. Webster,R.G.,Guan,Y.,Peiris,M.,Chen,2006.H5N1 influenza contiuous to circulate and change. Microbe,1(12):559-565.
22. Lee, C.W., Suarez, D.L.2004. Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype avian influenza virus. J .Virol. Methods 119,151–8.

1/20/2013

Oral exposure to zinc oxide nanoparticles induced oxidative damage, inflammation and genotoxicity in rat's lung

Howaida Nounou [1, 2], Hala Attia [3, 4], Manal Shalaby [2, 5], Maha Arafah [6]

[1.] Department of Medical Biochemistry, Faculty of Medicine, Alexandria University, Alexandria 21111, Egypt. Email: drhan2008@yahoo.com
[2.] Department of Biochemistry, College of Science, King Saud University, Riyadh, 11421
[3.] Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh 11495, KSA.
[4.] Department of Biochemistry, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt.
[5.] Department of Biomedical technology, Institute of science biotechnology, City of scientific research and biotechnology. Alexandria 21111, Egypt. [6.] Department of Pathology, College of Medicine, King Saud University, Riyadh 11495

Abstract: This study aimed to investigate the toxicity of oral ZnONPs on the rat's lung. Rats were divided into four groups each of ten rats. Groups I and II were treated orally with 40 and 100 mg/kg ZnONPs for 24 hrs. Groups III and IV received daily 40 and 100 mg/kg ZnONPs orally for 1 week. Ten untreated rats were used as control. Oral administration of ZnONPs induced eosinophilia and lymphocytes infiltration in the lungs in the four tested groups that peaked at 100 mg/kg/day for 1 week. Lipid peroxidation was significantly higher, while levels of reduced glutathione and catalase activity were lower in all ZnONPs-treated groups. Nitrite concentrations increased significantly in rat's lung treated with 100 mg/kg for 24 hrs and in those treated with 40 and 100 mg/kg daily for 1 week. Levels of lung TNF-α were significantly higher after 24 hrs at high dose and after 1 week at both low and high doses. Interleukin-1β and pentraxin-3 levels were significantly increased at 1 week only at both low and high doses. There were lower levels of paraoxonase-1 and increased DNA damage in the four studied groups. Oral administration of ZnONPs induced lung injury possibly through oxidative stress, inflammatory response and DNA damage.

[Nounou H, Attia H, Shalaby M, Arafah M. **Oral exposure to zinc oxide nanoparticles induced oxidative damage, inflammation and genotoxicity in rat's lung**. *Life Sci J* 2013;10(1):1969-1979] (ISSN:1097-8135). http://www.lifesciencesite.com. 282

Keywords: ZnONPs; oxidative stress markers; inflammatory markers; DNA damage; lungs

1. Introduction

Nanoparticles (NPs) have unique and novel properties that lead to a diverse array of products with applications in diagnosis, drug delivery, food industry, paints, electronics, environmental cleanup, cosmetics and sunscreens **(Nohynek et al., 2007, Nel et al., 2009)**. Due to this expanding use of NPs and rapid growth in nanotechnology, the potential for human exposure has increased tremendously **(Roco, 2007).** It is necessary to assess the potential toxicity of engineered NPs to avoid their adverse effects on human health before widespread industrial application.

Zinc oxide nanoparticles (ZnONPs) are one of the most widely used engineered nanoparticles in consumer products. They are utilized in many commercial products including cosmetics, paints, textiles, and personal hygiene products. ZnONPs are highly effective in protection against ultraviolet A and B radiation; therefore, they are important ingredients of sunscreens and moisturizers **(Nohynek et al., 2007).** In addition, ZnONPs are being used in the food industry as additives and in packaging due to their antimicrobial properties **(Jin et al., 2009).** They also have potential use as fungicides in agriculture **(He et al., 2011)** and as anticancer in medical applications **(Rasmussen et al., 2010).** The increased production and use of ZnONPs enhances the probability of occupational and environmental exposure.

Experimental work done on cell cultures of colon cells compared the effects of ZnONPs to ZnO sold as a conventional powder. That experimental work found that the nanoparticles were twice as toxic to the cells as the larger particles **(Oberdörster et al., 2005).** The commonly proposed pathogenic mechanisms initiated by NPs are dominated by inflammation-driven effects; including fibrosis, oxidative stress, and DNA damage **(Nel et al., 2009, Lu et al., 2009).**

Most of toxicity studies of ZnONPs have mainly performed using cell lines. Exposure of human skin epithelial cells, for example, to ZnONPs produced severe cytotoxicity accompanied by oxidative stress and genotoxicity **(Kroll et al., 2009)**. While the in vitro assays have some limitations **(Sharma et al., 2009**), the in vivo systems involving; the complex cell–cell and cell–matrix interactions as

well as the diversity of cell types and hormonal effects are not present in cultured cells.

Few studies have been reported concerning the in vivo toxicity of ZnONPs although intratracheal instillation of ZnONPs in Sprague-Dawley rats induced cytotoxicity and severe inflammation after instillation **(Sayes et al., 2007, Cho et al., 2011).** Keeping in view the fact that ZnONPs can be ingested directly when used in food, food packaging, drug delivery and cosmetics, oral exposure and uptake through the gastrointestinal route needs to be considered. Workers involved in the synthesis of ZnONPs can be exposed by unintentional hand-to-mouth transfer of nanomaterials. When discharged accidentally into the environment, these nanoparticles may enter the human body through the food chain. Nanoparticles could be then translocated from the lumen of the intestinal tract and blood into different organs.

Previous experiments have revealed liver, kidney and spleen as the target organs for engineered nanoparticles after uptake by the gastrointestinal tract **(Wang et al., 2007).** However, a previous study had reported that, when 50 mg/kg and 300 mg/kg ZnO nanoparticle doses were administered orally by the gavage tube, elevated zinc levels were observed in the liver, lung, and kidney in 6–24 hours. This was explained by the ionization properties of ZnO nanoparticles following oral administration **(Baek et al., 2012).** Most studies on the toxicity of ZnONPs particles in mammals were focused on the pulmonary impact of inhaled ZnONPs nanoparticles or dermal exposure, but most available work has been undertaken on the impacts of oral exposure of these nanoparticles on the GIT and not on the lung. The present study was undertaken to investigate the oral toxicity of ZnONPs in lungs only and the mechanisms of toxicity involved. For this, we exposed rats to ZnONPs via the oral route and the toxic effects of nanoparticles on lung tissue were investigated. Oxidative tissue damage, inflammatory responses, genotoxic effects by comet assay, DNA fragmentation and organ damage by histopathology were investigated.

2. Material and Methods

Chemicals and reagents:

ZnONPs (<100 nm, surface area 15-25 m2/g, purity >99%, Cat. No. 544906) were obtained from Sigma-Aldrich (St Louis, Missouri, USA). Low melting point agarose (LMA), normal melting point agarose (NMA), ethidium bromide, thiobarbituric acid, Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid, DTNB), sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, were purchased from Sigma-Aldrich chemical Co. (St Louis, Missouri, USA). Kits for TNF-α and IL-1β were Quantikine Immunoassay kits (R&D Systems, Inc, Minnea- polis, USA), Pentraxin-3 kit was from CUSABIO BIOTECH Co., Ltd. (Wuhan, China) and paroxonase-1 kit was purchased from Life Science Co. (Florida, USA). All other chemicals used in this study were of high analytical grade.

Experimental animals and treatment:

The experimental procedures were carried out according to the National Institute of Health Guidelines for Animal Care and approved by the local Ethics Committee, IRB NO: 00007555. Adult male Wistar albino rats weighing 150-200 g were obtained from Animal Care Centre, College of Pharmacy, King Saud University, Riyadh, KSA. The animals were kept in a 12-hour light/dark cycle, at a temperature of 22°C ± 2°C with relative humidity 50% ± 20%. Animals were fed on standard food pellets and tap water ad libitum for the entire test period. The doses suggested in OECD TG 420 for investigating the oral toxicity of a new test chemicals are 5, 50, 300, 2000 and 5000 mg/kg. In case of ZnONPs and according to previous work **(Wang et al., 2007),** high doses showed extensive agglomeration which has suggested the need to study ZnONPs induced oral toxicity at low doses. While previous experiments had reported that, when 50 mg/kg and 300 mg/kg ZnO nanoparticle administered orally remarkable signs of toxicity had been observed in different organs including lungs **(Baek et al., 2012)** , we investigated toxicity of ZnONPs at concentrations 40 and 100 mg/kg. The rats were divided into 5 groups (10 rats in each group): control group, group I; low-dose one day group (40 mg/kg/24 hrs), group II; high-dose one day group (100 mg/kg/24 hrs), group III; low dose one week group (40 mg/kg/day for1 week), and group IV; high-dose one week group (100 mg/kg/day for 1 week). All animals were weighed at the beginning and end of each treatment. The ZnONPs suspension was given to rats by oral gavage tube according to body weight. The control groups received only the vehicle (distilled water) by oral gavage tube for 24 hrs and one week.

Particle characterization:

The morphology and size of ZnONPs was determined by transmission electron microscopy (TEM). The hydrodynamic size was determined using dynamic light scattering (DLS) in a Zetasizer Nano-ZS, Model ZEN3600 equipped with 4.0 mW, 633 nm laser (Malvern instruments Ltd., Malvern, UK).The surface zeta potential measurements were also performed with the Malvern Zetasizer Nano ZS. ZnONPs were suspended in Milli-Q water (filtered with 0.22 μm filter) at a concentration of 15 mg/ml and probe sonicated (Sonics & Material Inc., Newtown, CT, USA) at 30 W for 10 min (2.5 min on and 30 s off). For TEM a drop of ZnONPs solution (8

µg/ml in Milli-Q) was deposited on carbon-coated copper grids. The films on the TEM grids were allowed to dry prior to measurement **(Sharma et al., 2009).**

Preparation of ZnONPs:

ZnONPs were dissolved in distilled water in a concentration of 20 mg/ml for the dose of 40 mg/kg and in a concentration of 50 mg/ml for the dose of 100 mg/kg. The stock suspension was sonicated for 120 sec at a high energy level using a continuous mode to create a high grade of dispersion according to the protocol of **Bihari et al., (2008).**

Processing of lung tissue:

At specified time (24 hrs and one week); rats were anesthetized and sacrificed by decapitation. Lungs were isolated, immersed in normal saline and dissected into parts. One part of each lung was weighed and homogenized with phosphate buffered saline (PBS, pH 7.4) in an Ultra-Turrax homogenizer (IKA Labortechnik, Staufen, Germany). The homogenate was then centrifuged at 3,000 rpm for 10 min. at 4°C and supernatants were divided into aliquots and kept at -80° C for the subsequent assessment of oxidative stress and inflammatory markers, comet assay and DNA fragmentation. Small pieces of the other part of the lung were fixed in a 10% phosphate-buffered formalin solution thereafter; the organs were embedded in paraffin, stained with hematoxylin and eosin, and examined under light microscope.

Assessment of Oxidative Stress Markers

Assay of Lipid Peroxidation

Malondialdehyde (MDA, a product in the sequence of lipid peroxidation reactions) was quantified using thiobarbituric acid (TBA) assay as described by **Dubovskiy et al., (2008).** Briefly, 0.5 mL of 0.6 %TBA and 125 µL of 20% trichloroacetic acid (TCA) were mixed with 250 µL of lung homogenate. The mixture was heated for 30 min. in boiling water bath then cooled and centrifuged at 3000 rpm for 10 min. at 4°C. The absorbance of the developed pink colored chromogen was measured at 535 nm against reagent blank.

Assay of Reduced Glutathione (GSH)

GSH (the most important nonprotein sulphydryl antioxidant in the cell) was estimated using Ellman's reagent (5, 5'- dithiobis-2-nitrobenzoic acid; DTNB) according to the method described by **Moron et al., (1979)** with some modification. Briefly, a sample of lung homogenate was deproteinized by adding equal volume of 25% TCA and then centrifuged at 4°C at 3000 rpm for 10 minutes. 0.5 mL of supernatant was then added to 4.5 mL of Ellman's reagent and the produced yellow color was measured at 412 nm against reagent blank.

Assay of Catalase (CAT) Activity

CAT activity was estimated as the decomposition rate of hydrogen peroxide (H2O2) according to **Wang et al., (2001).** 500 µL 1% H2O2 in PBS was mixed with 5 µL of homogenate. The mixture was incubated at 28°C for 10 min and the rate of decomposition of H2O2 was measured at 240 nm.

Measurement of Nitrite (An Index of Nitric Oxide, NO) In Lung Tissues

Biologically produced NO is rapidly oxidized to nitrite and nitrate, thus, nitrite concentrations can reflect NO production, and may be measured colorimetrically using Griess reagent **(Kleinbongard et al., 2002).** 100 µL of tissue homogenate was added to 100 µL Griess reagent (1:1 mixture of 1% sulfanilamide in 2.5% orthophosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine in distilled water). After 10 min of color development at room temperature, absorbance was measured at 540 nm.

Assessment of Inflammatory Markers:

Levels of tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), pentraxin-3 (PTX-3) and paroxonase-1 (PON-1) in lung tissues were determined using ELISA technique according to the manufacturer's instructions.

Assessment of DNA damage:

Comet DNA Assay

The comet assay protocol was modified form previous report **(Hu et al., 2002)**. Lung was minced, suspended in 4mL, chilled homogenizing solution (pH 7.5) containing 0.075 M NaCl and 0.024 M Na2EDTA and then homogenized gently using a potter-Elvehjem homogenizer at 500 to 800 rpm in ice. To obtain nuclei, the homogenate was centrifuged at 700 X g for 10 min at 0°C and the precipitate was resuspended in chilled homogenizing buffer at 1 g organ weight/mL. 100 µL of lung homogenate was mixed with 600 µL of low-melting agarose (0.8% in PBS, Sigma, USA). 100 µL of this mixture was spread on slides pre-coated with 300 µL of 0.6% normal melting point agarose (NMP). After application of a third layer of 0.6 % NMP (300 µL), slides were immersed in ice-cold lysis buffer (0.045 M Tris-borate-EDTA (TBE) buffer, pH 8.4) for 1 hrs at 4°C. Slides were then removed from the lysing solution and placed for 20 min in a horizontal electrophoresis unit filled with an alkaline buffer (1 mM Na2 EDTA and 300 mM NaOH, pH 13) to allow the unwinding of DNA. After the unwinding of DNA, electrophoresis was carried out under standard conditions (25 V, 300 mA, distance between electrodes 30 cm) for 20 min. at room temperature in the same alkaline solution (pH 13). Electrophoresis at high pH results in structures resembling comets, as observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the number of DNA breaks. The slides were then neutralized by adding 0.4 M Tris–

HCl buffer (pH 7.5), stained with ethidium bromide (20µg/ml, Sigma USA) at 4°C, covered and stored in sealed boxes at 4°C until analysis. All preparation steps were performed under dimmed light to prevent additional DNA damage by UV. Images of 100 randomly selected cells were analyzed for each sample. DNA fragment migration patterns was evaluated with Leitz Orthoplan epifluorescence microscope (magnification 250×) equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. The microscope was connected through a camera to a computer-based image analysis system (Comet Assay IV software, Perspective Instruments). Comets were randomly captured at a constant depth of the gel, avoiding the edges of the gel, occasional dead cells, and superimposed comets. DNA damage was measured as tail length (TL = distance of DNA migration from the centre of the body of the nuclear core, tail intensity DNA (TI = % of DNA that migrated from the nuclear core to the tail) and tail moment. The tail moment (TMOM) was determined according to the formula:

TMOM =DNA in tail as a % of total DNA x tail length (TL).

Measurement of DNA fragmentation

The fragmented DNA was quantified using diphenylamine (DPA) reagent according to the method of **Burton (1956)** and modified by **Suenobu et al., (1999)**. Lung homogenate were lysed with 0.4 mL hypotonic lysis buffer (10 mmol/L Tris, 1 mmol/L EDTA, and 0.1% NP-40, pH 7.5) and centrifuged at 13000 g for 10 minutes to separate intact from fragmented chromatin. The supernatant containing fragmented DNA was placed in a separate Microfuge tube, and both pellet and supernatant were precipitated overnight at 4°C in 12.5% trichloroacetic acid. The precipitates were sedimented at 13000 g for 4 minutes. The DNA precipitates were hydrolyzed by heating to 90°C for 10 minutes in 5% trichloroacetic acid. For quantification of fragmented DNA, in brief, 0.16 mL of DPA reagent (0.15 g DPA, 0.15 mL H2SO4, and 0.05 mL acetaldehyde per 10 mL glacial acetic acid) was added to each tube, and the absorbance at 570 nm was measured after overnight color development. "Percent fragmentation" refers to the ratio of DNA in the supernatant ("fragmented") to the total DNA recovered in both supernatant and pellet ("fragmented plus intact").

Statistical analysis:

Results are expressed as mean ± SEM. Statistical comparisons between groups were performed using one way analysis of variance (ANOVA) followed by Tukey Krammer as post multiple test. Statistical analysis was performed using Graph pad Instat 3 software Inc, San Diego, CA, USA. Results were considered significant at $P<0.05$.

3. Results

Particle characterization:

The counted particles by TEM were 105 and no aggregation could be detected as shown in Figure

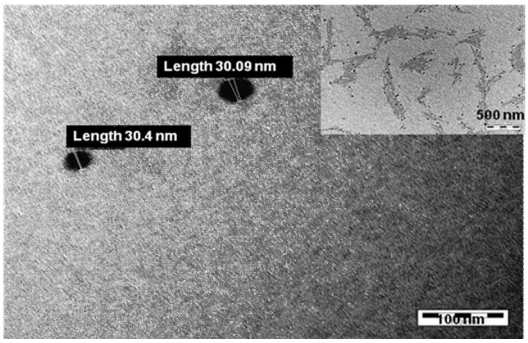


Figure 1, ZnONPs demonstrated a spheroid shape; the minimum size was 23 nm and the maximum was 34 nm. The mean size measured by the TEM was 30 nm with a standard error ± 1.12. The mean hydrodynamic diameter of the nanoparticle suspension in Milli-Q water as determined by the DLS measurement was 272 nm ± 8.75 (minimum diameter was 234 nm and the maximum was 386 nm). The surface zeta potential was – 41.2 ± 0.65 mV. Data are reported as mean ± SE.

Biochemical Measurements in Lung Tissues:

There was no difference between the results of the two control groups, so for simplicity the two control groups for 24 hrs and 1 week were combined in one control group.

Oxidative Stress Markers in Lung Tissues

A significant elevation of malondialdehyde (MDA; a marker of lipid peroxidation) in lung tissues was observed in all groups treated with ZnONPs at different doses (40 mg/kg and 100 mg/kg) and for different durations (24 hrs and 1 week) compared to the normal control group ($P < 0.01$, $P < 0.001$, respectively). As shown in Figure (2A), the level of MDA was significantly higher in the rats treated at 100 mg/ kg/day for1 week (group IV) compared to those treated at 40 and 100 mg for 24 hrs (groups I, II, $P < 0.001$) and to those treated at 40 mg/ kg/day for 1 week (group III, $P < 0.01$).

Reduced glutathione (GSH) levels were significantly lower in lung tissues in the four studied groups compared to the normal control group ($P < 0.05$, $P <0.001$), also there was a significant decrease in GSH levels in the rats treated daily with ZnONPs for 1 week at both low and high doses (40 and 100 mg/kg) (groups III, IV) compared with those treated for 24 hrs at a dose of 40 mg/kg (group I) ($P < 0.01$) Figure (2B).

Figure (2C) demonstrates that all the four studied groups, showed significant decrease in catalase (CAT) activity in lung homogenates compared to the normal control group ($P < 0.05$, $P < 0.01$, $P < 0.001$). As shown in Figure (2D), nitrite concentrations in lung tissues were increased in all groups, however the increase was highly significant in the rats treated at high dose (100mg/kg) for 24 hrs (group II), and in those treated at both low and high doses (40 and 100 mg/kg) for one week (groups III, IV) compared to the control group. The increase was time-dependent since nitrite concentrations in rats treated with ZnONPs 100mg/kg/day for 1 week (group IV) showed a significantly higher levels as compared to those treated with 40 and 100 mg/kg/24 hrs (groups I, II) while nitrite concentrations were significantly higher in rats treated with ZnONPs 40mg/kg/day for 1 week (group III) than group I treated with 40mg/kg/24 hrs.

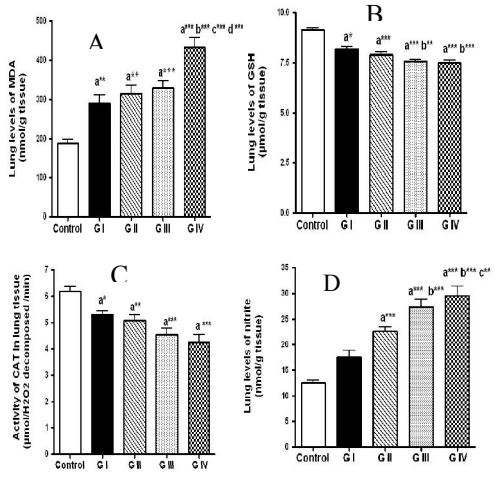


Figure 2, Levels of oxidative stress markers in lung tissues in the different studied groups. A: malondiladehyde (MDA); B: reduced glutathione (GSH); C: catalase activity (CAT) and D: nitrite (as index of NO). Data are expressed as mean ± SEM of 10 rats in each group. Control group, G I rats treated at 40 mg/kg/24 hrs and G II rats treated at 100 mg/kg/24 hrs, G III rats treated at 40 mg/kg/day for 1 week and G IV rats treated at 100 mg/kg /day for 1 week. a: significantly different from normal control rats; b: significantly different from group I (40 mg/kg/24 hrs); c: significantly different from group II (100 mg/kg/24 hrs); d: significantly different from group III (40 mg/kg/day for 1 week) *: P<0.05; **: P<0.01; ***: P<0.001

Pro-inflammatory and anti-inflammatory markers in lung tissues:

The concentrations of TNF-α, IL1-β and PTX-3 were significantly higher in ZnONPs- treated groups at 1 week either at the low dose (40 mg/kg/day for 1 week) or the high dose (100 mg/kg/day for 1 week) (groups III, IV) compared to the normal control group ($P < 0.001$) (Figure 3). TNF-α levels were significantly higher in the rats treated with ZnONPs at a high dose 100 mg/kg /24 hrs (group II) and in the groups treated at 40 and 100 mg/kg/day for 1 week (groups III, IV), compared to group I (40 mg/kg/24 hrs, $P < 0.001$) Figure (3A).

Oral treatment with ZnONPs produced significant increases in IL-1β and PTX-3 as two inflammatory biochemical parameters at 1 week in group III (40mg/kg/day for 1 week) and group IV (100 mg/kg/day for 1 week) compared to control whilst, at 24 hrs in group I (40 mg/kg) and group II (100 mg/kg) there were no significant changes compared to control (Figures 3B, 3C). IL-1 β and PTX-3 concentrations were elevated in group IV that was treated at high dose for one week (100 mg/kg) compared to the concentrations observed in groups treated for 24 hrs either at low (40 mg/ kg) or high (100 mg/ kg) doses (groups I, II).

Activity of PON-1 which is antioxidant and anti- inflammatory was significantly lower in all the four studied groups compared with normal control group ($P < 0.01$, $P < 0.001$). There was a significantly decreased activity in group II (100 mg/kg/24 hrs) as compared to group I (40 mg/kg/24 hrs). Groups that were treated daily for 1 week (groups III 40mg/kg and IV 100mg/kg) showed highly significant reduction in PON-1 activity compared to those treated for 24 hrs (groups I 40mg/kg and II 100mg/kg) ($P < 0.001$). There was no significant difference in PON-1 activity in group IV that was treated at high dose 100 mg/kg/day for 1 week compared with those treated at low dose group III 40 mg/kg/day for 1 week (Figure 3D).

Assessment of DNA Damage:

Comet DNA assay in lung tissues

Comet DNA assay as a marker for DNA fragmentation was significantly increased in the four studied groups compared to the normal control group and was maximum in ZnONPs treated with 100 mg/kg/day for 1 week (group IV). Treatment daily for one week at both low and high doses 40 and 100 mg/kg (groups III, IV) showed increased DNA damage as compared to treatment for 24 hrs (groups I, II) (Figure 4 and Table 1 a, b). These results were ascertained with the % DNA migrated (TI) which shows same pattern of changes. Tail moment showed similar results however non significant increase was observed with group I (40 mg/kg/24 hrs) compared with normal control.

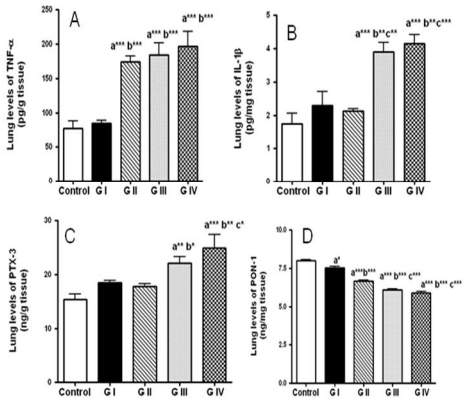


Figure 3, Levels of pro-inflammatory and anti-inflammatory markers in lung tissues A: Tumor necrosis factor- α (TNF-α); B: interleukin-1β (IL-1β); C: Pentraxin-3 (PTX-3) and D: Paroxonase-1(PON-1). Data are expressed as mean ± SEM of 10 rats in each group. Control group, G I rats treated at 40 mg/kg/24 hrs and GII rats treated at 100 mg/kg/24 hrs, G III rats treated at 40 mg/kg/day for 1 week and G IV rats treated at 100 mg/kg /day for 1 week. a: significantly different from normal control rats; b: significantly different from group I (40 mg/kg/24 hrs); c: significantly different from group II (100 mg/kg/24 hrs); *: P<0.05; **: P<0.01; ***: P<0.001.

Table 1 (a). Comet DNA assay in lung tissues

Group	Tailed DNA %	P value	Tail length (µm)	P value
Control	4.25 ± 0.31		2.51 ± 0.116	
G I	8.33 [a] ± 0.25	[a] *P<0.001*	3.91 [a] ± 0.12	[a]*P<0.001*
G II	11.17[a b] ± 0.33	[a,b]*P<0.001*	5.02[ab] ± 0.17	[a]*P<0.01* [b] *P<0.05*
G III	15.2 [a b c] ± 0.34	[a,b,c]*P<0.001*	7.95[abc]± 0.19	[a,b,c]*P<0.001*
G IV	20.33[abcd] ± 0.67	[a,b,c,d]*P<0.001*	11.37[abcd]± 0.38	[a,b,c,d]*P<0.001*

Table 1 (b). Comet DNA assay in lung tissues (Cont.)

Group	Tail intensity (TI)	P value	Tail moment	P value
Control	2.23 ± 0.085		5.36 ± 0.24	
G I	3.86 [a] ± 0.111	[a]*P<0.001*	15.31 ± 0.64	Ns
G II	4.63 [a] ± 0.2	[a]*P<0.001*	22.47 [a] ± 0.17	[a] *P<0. 01*
G III	6.42 [a b c] ± 0.2	[a,b,c]*P<0.001*	50.03[a,b,c] ± 1.46	[a,b,c] *P<0.001*
G IV	9.22[a b c d] ± 0.67	[a,b,c,d]*P<0.001*	108.2 [a b c d] ± 7.44	[a,b,c,d]*P<0.0 01*

Data are expressed as mean ± SEM; NS: not significant; a: significantly different from normal control rats. b: significantly different from group I (40 mg/kg/24 hrs); c: significantly different from group II (100 mg/kg/24 hrs); d: significantly different from group III (40 mg/kg/day for 1 week).

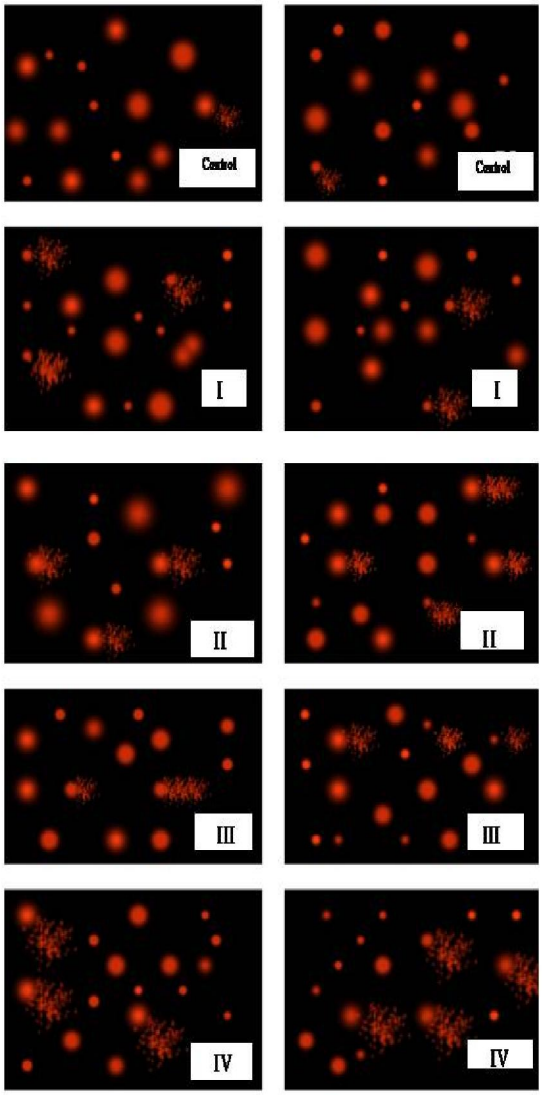


Figure 4, Comet DNA assay in lung tissues. Control group, G I rats treated at 40 mg/kg/24 hrs and GII rats treated at 100 mg/kg/24 hrs G III rats treated at 40 mg/kg/day for 1 week and G IV rats treated at 100 mg/kg/day for 1 week. Tail length as marker for DNA fragmentation has increased in the four studied groups compared to the normal control group and was maximum in G IV (100 mg/ kg/day for1 week).

DNA fragmentation in lung tissues and its correlations with PON-1

DNA fragmentation % was significantly higher (P< 0.001) in the four ZnONPs treated groups

compared to the normal control group as shown in Table 2 which confirm the DNA comet assay results. Data of at least six rats showed that, there were negative correlations between DNA fragmentation and PON-1 concentrations in all studied four groups which were significant in groups II, III and IV (G I, r = - 0.63, P=0.086; G II, r = - 0.751, P <0.05; G III, r = - 0.93, P <0.001 and G IV, r = - 0.882, P <0.001) (Figures 5, 6).

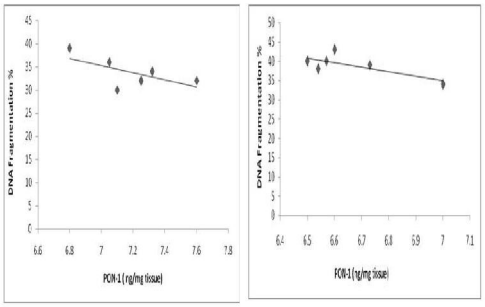


Figure 5, Negative correlation between DNA fragmentation and concentrations of PON-1 (ng/mg tissue) in lung in rats administered ZnONPs for 24 hrs (A): 40 mg/kg ZnONPs (G I, r = -0.638, P=0.086) and (B): 100 mg/kg (G II, r = - 0.751, P<0. 05).

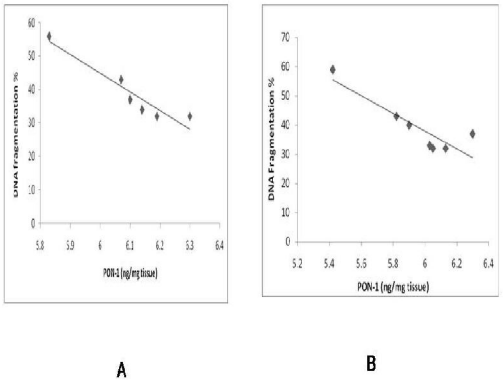


Figure 6, Significant negative correlation between DNA fragmentation and concentrations of PON-1 (ng/mg tissue) in lung tissues of rats administered ZnONPs daily for 1 week at (A): 40 mg/kg ZnONPs (G III, r = -0.93, P<0.01) and (B): 100 mg/kg (G IV, r = -0.882, P<0.01).

Table 2. DNA fragmentation in lung tissues.

Groups	DNA fragmentation (%)
Control	10.36 ±1.77
G I (40 mg/kg/24 hrs)	33.53 ^{a***} ± 1.34
G II (100 mg/kg/24 hrs)	38.65 ^{a***} ± 2.7
G III (40 mg/kg/day for 1 week)	36.9 ^{a***} ± 4.18
G IV(100 mg/kg/day for 1 week)	39.52 ^{a***} ± 3.633

Data are expressed as mean ± SEM of 10 rats in each group; a: significantly different from normal control rats ***: P<0.001.

Lung histopathology (H & E):

ZnONPs induced diverse pathological lung lesions when rats were treated with both low (40 mg/ kg) or high (100 mg/ kg) doses either for 24 hrs only or for 1 week. The representative pathological lesions could be classified as eosinophilic inflammation, lymphocytes and mast cells infiltration around arterioles, in the alveolar interstitium and hemorrhage. Number of eosinophils around arterioles and bronchioles and hemorrhage, were remarkably increased among all the studied groups and peaked at 1 week after oral treatment with ZnONPs 100mg/ kg (group IV). Furthermore, dense infiltration by lymphocytes and macrophages was observed after one week in groups III and IV (Figures 7, 8).

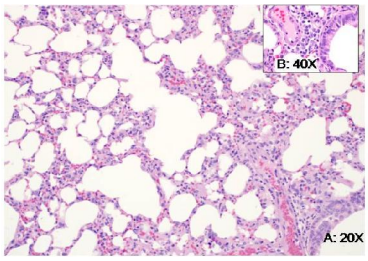


Figure 7, H& E stain of lung tissues in the control group. A few lymphocytes and other mononuclear cells in the interstitium and around the bronchioles in the control lung. H&E; A is 20X and B is 40X.

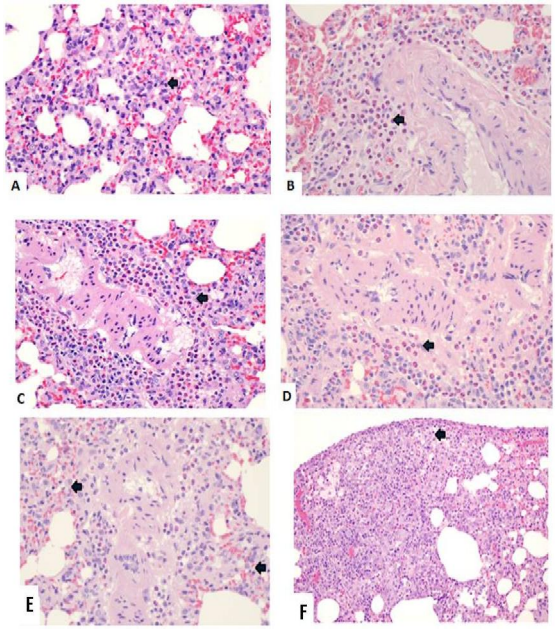


Figure 8, H& E stain of lung tissues in the studied groups. A: GI (40mg/kg/24 hrs): infiltration by mononuclear cell in the interstitium with hemorrhage. H&E 40X. B. G II (100 mg/kg/24 hrs): infiltration by

eosinophils and mast cells in the interstitium and around the arterioles associated with mild hemorrhage, H&E 40X. C: G III (40 mg/ kg/day for 1 week): infiltration by eosinophils and mononuclear cells in the interstitium and around arterioles with hemorrhage. H&E 40X. D: G IV (100 mg/kg/day for 1 week): Dense infiltration by eosinophils and other mononuclear cells around arterioles. H&E 40X. E& F: G IV (100 mg/ kg/day for 1 week): Dense infiltration by lymphocytes, eosinophils and other mononuclear cells in the interstitium (E) H&E. 40X and subpleural areas (F) with hemorrhage H&E 20X.

4. Discussions

The escalating uses of zinc oxide nanoparticles (ZnONPs) in coatings, paints, personal care products, food industry and medical applications increase the possibility of different organs exposure to ZnONPs. In the present study, oral administration of ZnONPs caused severe inflammatory lung injury, in groups treated with 40 and 100 mg/kg for either 24 hrs or one week, as observed with H&E staining showing eosinophilic inflammation, hemorrhage in addition to lymphocytes and mast cells infiltration. These results were similar to intratracheal instillation studies in which neutrophilic and eosinophilic inflammation was reported using ZnONPs (Sayes et al., 2007, Cho et al., 2011), indicating that pulmonary toxicity of ZnONPs is elicited not only by direct instillation or inhalation but extended to oral exposure.

One of the proposed mechanisms of ZnONPs-induced lung injury is the release of reactive oxygen species (ROS) with the subsequent oxidative stress. Oxidative damage of ZnONPs on lung tissues was indicated in our study by the higher levels of MDA; a product of lipid peroxidation, together with lower levels of GSH and CAT activity in lung tissues treated with ZnONPs compared to normal control. These effects were observed by both 40 and 100 mg/kg either at 24 hrs and one week. Decreased GSH levels with the duration of ZnONPs exposure (Figure 2B) indicated time-dependent elevation of oxidative stress in the lung. Significant increased levels of MDA in group IV (100 mg/kg/day for 1 week) compared to group III (40 mg/kg/day for 1 week) may reflect the dose dependent effect of ZnONPs toxicity (Figure 2A). Our results are consistent with previous studies suggesting that cytotoxcity of ZnONPs was mediated through ROS generation and oxidative stress (**Hackenberg et al., 2011, Huang et al., 2010).** Lipid peroxidation; the peroxidative breakdown of polyunsaturated fatty acids, leads to lung injury due to the effects on membrane function, inactivation of membrane-bound receptors and enzymes, and increased tissue permeability **(Rahman, 2005).** Several studies have suggested that GSH homeostasis may play a central role in the maintenance of the integrity of the lung airspace epithelial barrier. It has been shown that GSH depletion, as observed in our study by all groups treated with ZnONPs, sensitizes both alveolar and bronchial epithelial cells to the injurious effects of H2O2, resulting in an increased membrane permeability and activation of nuclear factor kB (NF-kB) leading to inflammation **(Rahman et al., 2001).** Under normal conditions, scavenging of H_2O_2 is catalyzed by catalase enzyme, which activity was significantly decreased in the present study by all the four studied groups treated with ZnONPs (Figure 2C).

Reactive nitrogen species, derivatives of nitric oxide (NO), have also been implicated in oxidative stress. In the present study, the levels of nitrite (a stable metabolite of NO) were markedly increased in the lung homogenate of rats treated with 100 mg/kg/24 hrs and in those treated with either 40 or 100 mg/kg/1 week (Figure 2D) reflecting increased NO production possibly through activation of NO synthase (NOS). Increased nitrite levels with the duration of treatment [levels in rats treated daily for 1 week at either low or high doses were significantly higher than levels in rats treated for 24 hrs] may indicate time-dependent effect of ZnONPs on lung injury. Neutrophils and macrophages are generally considered to be the most prodigious source of highly reactive oxidants in the lung **(Brigham, 1986).** It is postulated that ZnONPs may be capable of priming and/or activating neutrophils to generate ROS and to stimulate NOS to produce more NO in lung tissues.

Second possible mechanism of the toxic effect of ZnONPs on lung is the inflammatory response. Levels of lung TNF-α were significantly increased after 24 hrs at high dose (group II) and after one week of administration of ZnONPs at both low and high doses (groups III, IV). Oral treatment with ZnONPs produced significant increases in IL-1β at 1 week (groups III, IV) whilst at 24 hrs (groups I, II) there were no significant changes compared to control (Figure 3B). Collectively, these data may suggest the role of TNF-α in acute oral toxicity, while IL-1β was involved in long term inflammatory response, this may be because TNF-α represents an early phase mediator of inflammation **(Pereda et al., 2006).** It was revealed that nanoparticles are more potent in instigating the proinflammatory cytokine production by macrophages compared to bulk size ZnO **(Roy et al., 2011),** indicating that their small size may help in evading the macrophage response. Pulmonary inflammation in the current study may be also attributed to ZnONPs-induced ROS which has been implicated to enhance gene expression of pro-inflammatory mediators **(Kamata et al., 2005).** Likewise, lipid peroxidation products have also been shown to act as a signal for activation of transcription

factors and gene expression, leading to an inflammatory response **(Uchida et al., 1999)**. Direct or indirect oxidant stress to the airway epithelium and alveolar macrophages may also generate cytokines, such as TNF-α, which in turn can activate airway epithelial cells to induce proinflammatory genes **(Rahman, 2005).**

Pentraxin-3 (PTX-3) is a glycoprotein belonging to the pentraxin family of acute-phase proteins. It was originally described as a gene inducible by TNF-α in human fibroblasts and, soon after, it was also identified as being induced by IL-1β in endothelial cells **(Breviario et al., 1992).** Increased PTX-3 levels were found in the lung tissue in multiple models and their levels were strikingly correlated with the severity of lung injury **(Okutani et al., 2007).** This finding supports our results where PTX-3 levels in the lung tissues were significantly elevated compared to normal control only after administration of ZnONPs for one week either at 40 or 100 mg/kg, while non significant increase was observed with 24 hrs treatment at both low and high dose (Figure 3C). In our study severe inflammation and hemorrhage were clearly recorded in groups III and IV. Increased PTX-3 in groups III and IV could be explained by either the direct effect of cumulative doses of ZnONPs that may induce PTX-3 gene expression in the rat's lung or by the consequence of elevated IL-1β and TNF-α, such explanation is potentiated by cell culture studies in which treating lung cells with TNF-α or IL-1β induced a significant increase in PTX-3 gene expression and protein production **(Souza et al., 2002, Han et al., 2005).**

Paraoxonase-1(PON-1) is an antioxidant that has been suggested to play an important role in metabolizing and detoxifying lipid peroxides **(Harangi et al., 2004).** The results of our study showed lower activities of PON-1 in all the four studied groups compared to normal control (Figure 3D). As reported by **Feingold et al., (1998**) and **Kumon et al., (2003),** PON-1 was down-regulated by the administration of TNF-α and IL-1β, therefore, we suggest that the decrease in lung PON-1 activity in our study may be secondary to inflammatory response in ZnONPs-treated groups. We also can conclude that the reduction of PON-1 activity, may contribute to oxidative damage of ZnONPs in the lung tissues.

Although the genotoxic potential of ZnONPs has been reported **(Sharma et al., 2009, Wang et al., 2007, Hackenberg et al., 2011, Yang et al., 2009, Lin et al., 2009)**, however, there is a lack of information on oral ZnONPs-induced DNA damage. Our results showed DNA damage in all groups treated orally with ZnONPs compared to normal control as demonstrated by comet assay (Figure 4 and Tables 1a and 1b) and DNA fragmentation (Table 2). The longer DNA tail lengths represent higher percentages of DNA strand break. In our study, tail length and tail moment were significantly increased in the four studied groups compared to the normal control in both dose and time-dependent manner (Tables 1a and 1b). DNA damage may occur due to direct interaction of ZnONPs with the DNA after its nuclear uptake, or by intracellular ROS generation induced by ZnONPs **(Hackenberg et al., 2011).** Products of lipid peroxidation can interact with DNA causing genetic damage and disturbance of cell signaling **(Harangi et al., 2004)**. The PON-1, via hydrolyzing lipid peroxides, inhibits the development of further oxidative stress and thus, prevents the oxidative damage of DNA (Harangi et al., 2004). Accordingly, in the current study, the significantly reduced levels of PON-1 in ZnONPs-treated rats are postulated to play a crucial role in ZnONPs-induced DNA damage. This finding was further confirmed by the negative correlations found in our study between DNA fragmentation and PON-1 activity in all studied groups with significant correlation detected in groups II (100mg/kg/24 hrs) , III (40mg/kg/day for 1 week) and IV (100mg/kg/day for 1 week) (Figures 5 and 6). Our results are similar to those carried out on cell lines that reported oxidative DNA damage after exposure to ZnONPs **(Sharma et al., 2009, Hackenberg et al., 2011, Lin et al., 2009).** Ongoing DNA damage in groups III (40mg/kg/day for 1 week) and IV (100mg/kg/day for 1 week) could be attributed to the persisting presence of intracellular ZnONPs, which may even increase with time because of the release from endocytotic vesicles or continued cellular uptake. Effects of ZnONPs on the oxidative stress, antioxidants, selective inflammatory markers and DNA damage are summarized in the illustrated **figure 9.**

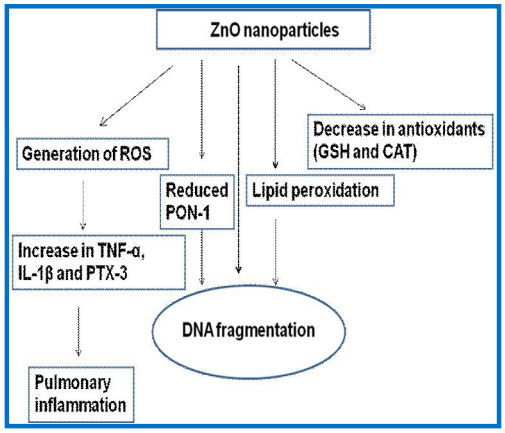


Figure 9, effects of ZnONPs on oxidative stress, antioxidant system, mediators of inflammation and DNA damage as suggested by the results of the present study.

Most studies focus on the toxic effect of ZnONPs on lung when administered through inhalation. Our result highlighted that the oral administration of ZnONPs also poses a unique and substantial hazard to the lungs via a multitude of mechanisms. ZnONPs induced oxidative damage as evidenced by the elevation of lipid peroxidation and the decline of the antioxidants; GSH, CAT and PON-1. In addition, they resulted in inflammatory response through release of TNF-α, IL-1β and PTX-3 as well as gentoxicity through DNA damage. These results emphasize the need for caution during the use and disposal of such manufactured nanomaterials to prevent unintended environmental impacts.

Acknowledgements:

The authors extend their appreciation to the deanship of Scientific Research at King Saud University for funding the work through the research group project number RGP-VPP-079.

References

1. Baek M, Hae-Eun Chung J Y, Jung-A L, Tae-Hyun K, Jae-Min O, Won-Jae L, et al. Pharmacokinetics, tissue distribution, and excretion of zinc oxide nanoparticles. I. J. Nanomed 2012; 7: 3081-97.
2. Bihari P, Vippola M, Schultes S, Praetner M, Khandoga AG, Reichel CA et al. Optimized dispersion of nanoparticles for biological in vitro and in vivo studies. Part Fibre Toxicol 2008: 5; 14.1.
3. Breviario F, d'Aniello EM, Golay J, Peri G, Bottazzi B, Bairoch A, et al. Interleukin-1- inducible genes in endothelial cells. Cloning of a new gene related to C-reactive protein and serum amyloid P component. J Biol Chem 1992; 267: 22190 –7.
4. Brigham KL. Role of free radicals in lung injury. Chest 1986; 89: 859–63.
5. Burton K.A. Study of the Conditions and Mechanism of the Diphenylamine Reaction
for the Colorimetric Estimation of Deoxyribonucleic Acid. Biochem J 1956; 62: 315–23.
6. Cho WS, Duffin R, Howie SAM., Scotton CJ, Wallace AH, MacNee W, et al. Progressive severe lung injury by zinc oxide nanoparticles; the role of Zn2+ dissolution inside lysosomes. Particle and Fibre Toxicology 2011; 8: 27- 35.
7. Dubovskiy IM, Martemyanov BB, Vorontsova YL, Rantala MJ, Gryzanova EV, Glupov VV. Effect of bacterial infection on antioxidant activity and lipid peroxidation in the midgut of Gaalleria mellonella L.larva (Lepidoptera, Pyralide). Comparative Biochemistry and Physiology, Part C 2008; 148: 1-5.
8. Feingold KR, Memon RA, Moser AH, Grunfeld C. Paraoxonase activity in the serum and hepatic mRNA levels decrease during the acute phase response. Atherosclerosis 1998; 139: 307–15.
9. Hackenberg S, Zimmermann FZ, Scherzed A, Friehs G, Froelich K, Ginzkey C. Repetitive Exposure to Zinc Oxide Nanoparticles Induces DNA Damage in Human Nasal Mucosa Mini Organ Cultures. Environmental and Molecular Mutagenesis 2011; 52: 582- 9.
10. Han B, Mura M, Andrade C, Okutani D, Lodyga M, Dos Santos CC, et al. TNF-alpha-induced long pentraxin PTX3 expression in human lung epithelial cells via c-Jun Nterminal kinase. J Immunol 2005; 175: 8303–11.
11. Harangi M, Seres I, Varga Z, Emri G, Szilva´ ssy Z, Paragh G, et al. Atorvastatin effect on high-density lipoprotein-associated paraoxonase activity and oxidative DNA damage. Eur J Clin Pharmacol 2004; 60: 685–91.
12. He L, Liu Y, Mustapha A, Lin M. Antifungal activity of zinc oxide nanoparticles against Botrytis cinerea and Penicillium expansum. Microbiol. Res 2011; 166: 207-15.
13. Huang CC, Aronstam RS, Chen DR, Huang YW. Oxidative stress, calcium homeostasis, and altered gene expression in human lung epithelial cells exposed to ZnO nanoparticles. Toxicol in Vitro 2010; 24: 45-55.
14. Hu ML, Chuang CH, Sio HM, Yeh SL. Simple cryoprotection and cell dissociation techniques for application of the comet assay to fresh and frozen rat tissues. Free Radic. Res 2002; 36: 203–209.
15. Jin T, Sun D, Su JY, Zhang H, Sue HJ. Antimicrobial efficacy of zinc oxide quantum dots against Listeria monocytogenes, Salmonella enteritidis, and Escherichia coli. J. Food Sci 2009; 74: M46–M52.
16. Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNF alpha induced death and sustained JNK activiation by inhibiting MAP kinase phosphatases. Cell 2005; 120: 649–61.
17. Kleinbongard P, Rasaf T, Dejam A, Kerber S, Kelm M. Griess method for nitrite measurement of aqueous and protein containing sample. Meth Enzymol 2002; 359: 158-68.
18. Kroll A, Pillukat MH, Hahn D, Schnekenburger J. Current in vitro methods in nanoparticle risk assessment: limitations and challenges. Eur J Pharm Biopharm 2009; 72: 370–7.
19. Kumon Y, Suehiro T, Ikeda Y, Hashimoto K. Human paraoxonase- 1 gene expression by HepG2 cells is downregulated by interleukin-1β and tumor necrosis factor α, but is upregulated by interleukin-6. Life Sci 2003; 73: 2807–15.
20. Lin W, Xu Y, Huang CC, Ma Y, Shannon KB, Chen DR, et al. Toxicity of nano- and micro-sized ZnO particles in human lung epithelial cells. J Nanopart Res 2009; 11: 25-39.8.
21. Lu S, Duffin R, Poland C, Daly P, Murphy F, Drost E, et al. Efficacy of simple short-term in vitro assays for predicting the potential of metal oxide

nanoparticles to cause pulmonary inflammation. Environ Health Perspect 2009; 117: 241–7.
22. Moron MS, Depierre J, Mannervik B. Levels of glutathione, glutathione reductase and glutathione – S- transferase activities in rat lung and liver. Biochem. Biophys. Acta 1979; 582: 67-78.
23. Nel AE, Madler L, Velegol D, Xia T, Hoek EM., Somasundaran P, et al. Understanding biophysicochemical interactions at the nano-bio interface. Nat Mater 2009; 8: 543–57.
24. Nohynek GJ, Lademann J, Ribaud C, Roberts MS. Grey goo on the skin? Nanotechnology, cosmetic and sunscreen safety. Crit Rev Toxicol 2007; 37: 251–77.
25. Oberdörster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K, et al Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol 2005; 2: 8-14.
26. Okutani D, Han B, Mura M, Waddell TK, Keshavjee S, Liu M. High-volume ventilation induces pentraxin 3 expression in multiple acute lung injury models in rats. Am J Physiol Lung Cell Mol Physiol 2007; 292: L144–L153.
27. Pereda J, Sabater L, Aparisi L, Escobar J, Sandoval J, Viña J. Interaction between cytokines and oxidative stress in acute pancreatitis. Current medicinal chemistry 2006; 13: 2775-87.
28. Rahman I, Mulier B, Gilmour PS, Watchorn T, Donaldson K, Jeffery PK. Oxidant-mediated lung epithelial cell tolerance: the role of intracellular glutathione and nuclear factor-kB. Biochem Pharmacol 2001; 62: 787–94.
29. Rahman I. The role of oxidative stress in the pathogenesis of COPD: implications for therapy. Treat Respir Med 2005; 4: 175–200.
30. Rahman I. Oxidative stress in pathogenesis of chronic obstructive pulmonary disease: cellular and molecular mechanisms. Cell Biochem Biophys 2005; 43: 167–188.
31. Rasmussen JW, Martinez E, Louka P, Wingett DG. Zinc oxide nanoparticles for selective destruction of tumor cells and potential for drug delivery applications. Expert Opin. Drug Deliv 2010; 7: 1063–77.
32. Roco, M.C. The National Nanotechnology Initiative: Past, Present and Future. 2nd ed; Lyshevski S, Iafrate G, Eds.; Taylor and Francis Press: Boca Raton and London, CRC,; 2007, pp. 1-42.
33. Roy R, Tripathi A, Das M, Dwivedi PD. Cytotoxicity and uptake of zinc oxide nanoparticles leading to enhanced inflammatory cytokines levels in murine macrophages: comparison with bulk zinc oxide. J Biomed Nanotechnol 2011; 7: 110-1.
34. Sayes CM, Reed KL, Warheit DB. Assessing toxicity of fine and nanoparticles: comparing in vitro measurements to in vivo pulmonary toxicity profiles. Toxicol Sci 2007; 97: 163-80.
35. Sharma V, Shukla RK, Saxena N, Parmar D, Das M, Dhawan A. DNA damaging potential of zinc oxide nanoparticles in human epidermal cells. Toxicol Lett 2009: 185, 211-8.
36. Souza DG, Soares AC, Pinho V, Torloni H, Reis LF, Teixeira MM., et al. Increased mortality and inflammation in tumor necrosis factor-stimulated gene-14 transgenic mice after ischemia and reperfusion injury. Am J Pathol 2002; 160: 1755–65.
37. Suenobu N, Shichiri M, Iwashina M, Marumo F, Hirata Y. Natriuretic Peptides and Nitric Oxide Induce Endothelial Apoptosis via a cGMP–Dependent Mechanism. Arterioscler Thromb Vasc Biol 1999; 19: 140-6.
38. Wang B, Feng W, Wang M, Wang T, Gu T, Zhu M, et al. Acute toxicological impact of nano- and submicro-scaled zinc oxide powder on healthy adult mice. J. Nanopart. Res 2007; 10: 263–76.
39. Wang J, Zhou G, Chen C, Yu H, Wang T, Ma Y, et al. Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. Toxicol. Lett 2007; 168: 176–85.
40. Wang Y, Oberley LW, Murhhammer DW. Evidence of oxidative stress following the viral infection of two Lepidopteran insect cell lines. Free Rad Biol Med 2001; 31: 1448-55.
41. Uchida K, Shiraishi M, Naito Y, Torii N, Nakamura Y, Osawa T. Activation of stress signaling pathways by the end product of lipid peroxidation. J Biol Chem 1999; 274: 2234–42.
42. Yang H, Liu C, Yang D, Zhang H; Xi Z. Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: The role of particle size, shape and composition. J Appl Toxicol 2009; 29: 69–78.

1/20/2013

Calf Scours: Definition and causes

[1]Nagwa S.Ata , [1]Sohad M. Dorgham , [1]Eman A.Khairy and [2]Mona S, Zaki

[1]Dept.Microbiology and Immunology, National Research Centre,Giza,Egypt
[2]Dept.Hydrobiology, National Research Centre, Giza, Egypt.
Dr_mona_zaki@yahoo.co.uk

Abstract: Calf scours is a broad, descriptive term referring to diarrhea in calves. It is not a specific disease with a specific cause, but it is actually a clinical sign of a disease complex with many possible causes. The present literature is review of the causes, symptoms, diagnosis, treatment, control and vaccination of calf scours. Scours occur within the first several days of life are the most important cause of calf sickness and death. Almost no herd goes through a calving season without some scours. In severe outbreaks, the effects of scours in an individual herd can be overwhelming. Morbidity may occur in 70 % of calves born and mortality may occur in 50 %. The present study was concluded that the causes of calf scours divided to noninfectious scours (nutritional) and infectious scours (Viral, bacterial and protozoal agents).
[Nagwa S. Ata, Sohad M. Dorgham , Eman A. Khairy and Mona S, Zaki. **Calf Scours: Definition and causes.** *Life Sci J* 2013;10(1):1980-1983] (ISSN:1097-8135). http://www.lifesciencesite.com. 283

Key words: Scour; calf; noninfectious and infectious scours

1. Introduction

One of the major economic losses problems is scour in young calves. This problem usually occurs when the calves are less than one month of age. The primary harm full effect from scours is loss of water and electrolytes (body salts) in the diarrhea. This loss of water and salts creates dehydration and alteration of the acid-base balance of the bodily fluids. Inflammation of the intestinal lining impaired the calf's ability to digest nutrients, creating weight loss and the potential for hypoglycemia (low blood sugar). If untreated, these changes can be severe enough to result in death. In addition, certain bacteria (certain strains of *Salmonella* and *Clostridium perfringens*) can release toxins that cause harm to multiple vital organs in the calf.

Many factors influence the occurrence of diarrheal disease.

Factors that predispose calves to scours include: Dystocia, poor health of dam, less mothering ability of dam, etc. These factors may be difficult to control. When they occur, they lower the calf's ability to resist infectious diseases, and extra care of the calf is required to decrease the risk of scours. When the calf's resistance was lowered, exposure and invasion by infectious agents could occurred which played an important role in producing diarrhea.

Causes of Scours

1) - Noninfectious Scours (Nutritional Scours)

Calves do best under consistent circumstances. Sudden changes, especially to the feeding program, Overfeeding, switching milk replacer brands or changing from a high to a low quality milk replacer formulation could adversely affect digestion. Ingredient differences, taste, nutrient and product density (how much fits in an 8 oz cup) can affect a calf's willingness to drink as well as its performance. Changes like these should be evaluated and made gradually. When waste milk is fed, nutrient quality and quantity vary depending upon the condition and health status of cows contributing to the waste milk supply. Calves may scour in response to these changes. Pasteurization does not affect this characteristic of waste milk **Walter Baumgartner. 2012.**

2) - Infectious Scours

A- Viral Scours

1-Rota virus Scours.

A reo-like virus can cause scours in calves within 24 hours of birth. However, when the infection- is first introduced into the herd, it can affect calves up to 30 days of age or older. Infected calves are severely depressed. There may be a drooling of saliva and profuse watery diarrhea. The feces will vary in color from yellow to green. Calves lose their appetite and the death rate may be as high as 50 percent, depending on the secondary bacteria present **Haschek *et al*., 2006.**

Diagnosis of *rota* virus

The diagnosis depends on an accurate history, clinical signs, and proper specimen collection and submission to a laboratory. The reo-like virus infection alone causes no diagnostic gross lesions in the intestine, but there is an increased volume of fluid in both the small and large intestine.

2-Corona virus Scours.

Scours caused by corona virus occurs in calves that are over 5 days of age. When the infection first starts in a herd, calves up to 6 weeks of age may scour. These calves are not as depressed as those infected with rotavirus. Initially, the fecal material may have the same appearance as that caused by rotavirus. As the calf continues to scour for several hours, however, the fecal material may contain clear mucus that resembles the white of an egg. Diarrhea may continue for several days. Mortality from corona virus scours ranges from 1 to 25 percent **Heinrichs and Radostits .2001**.

Control of *Corona* virus

Scours of *corona* virus is the same as that for rotavirus scours. Many herds have been found to be infected with both the *rota*- and *corona* viruses. A vaccine that is specific for the *rota*- and *corona* viruses is available. It can be administered in one of two ways:

Orally to the calf soon after birth; or as a vaccination to the pregnant cow. The first year that a vaccination program is started in the beef cow herd, the cow receives two vaccinations-the first at 6 to 12 weeks before calving, and the second as close to calving as possible. The next year, the cows are given a booster vaccination just before calving. In herds where the calving period extends over more than 6 to 8 weeks, cows that have not calved at the end of a 6-week period should receive a second booster vaccination. Following this procedure insures that the calf receives a high level of rota- and corona virus antibodies in the colostrum. However, the calf must receive adequate colostrum, preferably within the first 4 hours after birth as the antibodies cannot be absorbed later than 24 hours after birth. This cow vaccination program fits well into a beef cow herd health program and helps prevent virus build-up in the herd.

Diagnosis of *Rota*- and *Corona virus* Scours. Accurate diagnosis of viral scours can be made only by laboratory tests.

3-Bovine Virus Diarrhea.

The virus of bovine virus diarrhea can cause diarrhea and death in young calves. Diarrhea begins 2 to 3 days after exposure and may persist for quite a long time. Ulcers on the tongue, lips, and in the mouth are the usual lesions that can be found in the live calf. These lesions are similar to those found in yearlings and adult animals affected with bovine virus diarrhea. Diagnosis is by history, lesions, and diagnostic laboratory assistance.

Treatment and control

Similar to that used for other viral scours. Bovine virus diarrhea is controlled by vaccinating all replacement heifers 1 to 2 months before breeding.

B) - Bacterial Scours

1- *Escherichia coli (Colibacillosis).*

Escherichia coli (E. coli) have been incriminated as a major cause of scours **Yamamoto and Nakazawa, 1997**. Many times this is the only organism identified following routine bacteriologic culturing. Many different serotypes of *E. coli* have been identified; some cause scours while others do not.

E. coli is always present in the intestinal tract and is usually the agent that causes a secondary infection following viral agents or other intestinal irritants. *E. coli* scours is characterized by diarrhea and progressive dehydration. Death may occur in a few hours before diarrhea develops. The color and consistency of the feces are of little value in making a diagnosis of any type of diarrhea **Khan *et al*., 2002; Chattopadhyay *et al*., 2003; Wani *et al*., 2004.**

The course varies from 2 to 4 days, and severity depends on age of the calf when scours starts and on the particular serotype of *E. coli*.

Upon postmortem examination, lesions are nonspecific. However, the small intestine may be filled with fluid and the large intestine may contain yellowish feces.

Diagnosis of *Colibacillosis*

It depends on an accurate history, clinical signs, and culture of internal organs for bacteria and serotyping of the organism. The location at which the culture from the intestine was taken is also important.

Control of *E. coli* scours

It can be difficult in a severe herd outbreak. All calves should receive colostrum as soon after birth as possible. This helps the calf resist *E. coli* infection. Early isolation and treatment of scours help to prevent new cases. There are new *E. coli* cow vaccines now on the market. These vaccines contain K99 antigen which should give immunity to many types of *E. coli*. The vaccine is administered 6 weeks and 3 weeks prior to calving. The new *E. coli* vaccine is also available in combination with the rota- and corona virus vaccine. This vaccination builds high antibody levels in the colostrum, but the calf must get colostrum in the first few hours of life for the vaccine to be effective.

2- Salmonella.

There are more than 1000 types of *salmonella*, all of which are potential disease producers. In Egypt, a varying prevalence of *Salmonella* infections in calves was recorded with predominance of *Salmonella enteric* serovar Typhimurium (ST) and *Salmonella enterica* serovar Enteritidis (SE) **Seleim *et al* 2004; Younis *et al* 2009; Moussa *et al* 2010**and **2012.**

Calves are usually affected at 6 days of age or older. This age corresponds very closely to the age of the Corona virus infection. The source of

salmonella infection in a herd can be from cattle, birds, cats, rodents, the water supply, or a human carrier **Islam *et al.*, 2004**.

Clinical signs associated with *salmonella* infection include diarrhea, blood and fibrin in the feces, depression, and elevated temperature.

The disease is more severe in young or debilitated calves. Finding a membrane like coating in the intestine on necropsy is strong presumptive evidence that *salmonella* might be involved.

3- *Clostridium perfringens*

Usually a harmless member of the normal microflora, under certain conditions it can multiply rapidly and secrete toxins and degradative enzymes that are associated with serious enteric disease **Songer.1996**.

There are 6 types of *Clostridium perfringens* that can produce toxins, of which types B, C, and D appeared to be the most important in calves **McDonel.1980** .The disease has a sudden onset. Affected calves became listless, display uneasiness, and strain or kick at their abdomen. Bloody diarrhea may occur or not. It is usually associated with a change in weather, a change in feed of the cows, or management practices that cause the calf to not nurse for a longer period of time than usual. The hungry calf may over-consume milk which established a media in the gut that was conducive to the growth and production of toxins by the clostridial organisms. In many cases, calves died without clinical signs being observed. Postmortem lesions may be a hemorrhagic intestinal tract; thus, the common name, "purple gut." In the small intestine, there may be large hemorrhagic or bloody, purplish areas where the tissue looked dead. This was usually attributed to type C. Types B and D produced diarrhea without the usual postmortem lesions. Diagnosis of these toxins was done by finding the toxin in the small intestine by laboratory methods. This toxin breaked down rather rapidly so the contents of the intestinal tract must be collected very soon after death and preserved by freezing. Finding lesions of hemorrhagic enteritis at postmortem in a calf that has died suddenly was basis for a tentative diagnosis **Trotz-Williams *et al.*, 2005**.

Vaccination programs

This disease controlled by vaccinating the cows with *Clostridium perfringens* toxoid 60 and 30 days before calving. A single booster dose of toxoid should be given annually thereafter before calving. If this problem diagnosed in calves from non immunized cows, antitoxin can be given to the calf. Administration of antitoxin and oral antibiotics were the only effective treatment.

C) - Protozoal scour

1-*Coccidiosis.*

It caused by one-celled parasites that invade the intestinal tract of animals. There were many species of coccidia. Two, *Eimeria zurnii* and *Eimeria bovis*, were usually associated with clinical infections in cattle. Coccidiosis has been observed in calves 3 weeks of age and older, usually following stress, poor sanitation, overcrowding or sudden changes of feed. It often occured in calves 7 to 14 days after they moved from the calving lots onto pasture. Clinical coccidiosis was diagnosed by finding significant numbers of parasites in the feces. The results of the fecal examination must be related to the clinical signs and intestinal lesions. Occasionally, clinical coccidiosis would be present with bleeding and very few parasites in the fecal material. Laboratory examination of sections of the intestine may be required for diagnosis. A typical sign of coccidiosis in young calves was diarrhea with fecal material smeared over the rump as far around as the tail will reach. This may contain blood or not. Death may occur during the acute period or later from secondary complications **Rob Costello .2005 .**

Sulfonamides have been the treatment of choice for coccidiosis for many years. If treatment was given before signs appeared, the disease can largely be prevented. Amprolium has been cleared for use in calves as a preventative; this should be supplied at the rate of 5 mg/kg of body weight for a period of 21 days to cover the time period during which this disease anticipated. Good feeding practices, management, and sanitation were the control methods of choice.

2- *Cryptosporidium.*

Cryptosporidium is a protozoan parasite that is much smaller than coccidia. It has the ability to adhere to the cells that line the small intestine and to damage the microvilli. Several reports from researchers and diagnosticians have associated cryptosporidium with outbreaks of calf scours **Björkmann *et al.*,2003**. As a rule, cryptosporidium is detected in combination with corona virus, rotavirus, and/or *E. coli*. Calves infected by cryptosporidium have ranged from 1 to 3 weeks in age **Steiner *et al.*, 1997**.

References

1. **Chattopadhyay, U.K.; Gupta, S. and Dutta, S. (2003).** Search for Shiga toxin producing *Escherichia coli* (STEC) including O157:H7 strains in and around Kolkata. Indian J. Med. Microbiol. **21**: 17-20.
2. **Khan, A.; Yamasaki, S.; Sato, T.; Ramamurthy, T.; Pal, A.; Datta, S.;**

Chowdhury, N.R.; Das, S.C.; Sikdar, A.; Tsukamoto, T.; Bhattacharya, S.K.; Takeda, Y. and Nair, G.B. (2002). Prevalence and genetic profiling of virulence determinants of non-O157 Shiga toxin-producing *Escherichia coli* isolated from cattle, beef and humans, Calcutta, India. Emerg. Infect. Dis. **8**: 54-62.

3. **Wani, S.A.; Bhatt, M.A.; Samanta, I.; Nishikawa, Y. and Buchh, A.S. (2004).** *Escherichia coli* O116 associated with an outbreak of calf diarrhoea. Vet Rec. **154:** 506-508.
4. **Yamamoto,T. and Nakazawa M.(1997).** Detection and sequences of the enteroaggregative *Escherichia coli* heat stable enterotoxin 1 gene in ETEC strains isolated from piglets and calves with diarrhea. J. Clin Microbiol. **35:**223-227.
5. **Moussa, I. M., Ashgan M. H., Mahmoud M.H., Mohamed K. F. H., Al- Doss, A. A., 2010.** Rapid detection of *Salmonella* species in new borne calves by polymerase chain reaction. International Journal of Genentics and Molecular Biology 2:62-66.
6. **Moussa, I. M., Ashgan M. H., Mahmoud M. H., Al-Doss A. A., 2012.** Rapid detection and characterization of *Salmonella enterica* serovars by multiplex polymerase chain reaction assay. African Journal of Biotechnology 11:3452-3458.
7. **Seleim, R. S., sahar R. M., Novert M. H., Gobran R. A., 2004.** *Salmonella* infection in calves: virulence proteins and its immunogenic properties. J Vet online. (http://www.priory.com/vet/salmonella.htm).
8. **Younis, E. E., Ahmed A. M., El-Khodery S. A., Osman S. A., El-Naker Y. F., 2009.** Molecular screening and risk factors of enterotoxigenic *Escherichia coli* and *Salmonella* spp. in diarrheic neonatal calves in Egypt. Res Vet Sci 87:373-379.
9. **Islam, M., Morgan J., Doyle M. P., Phatak S. C., Millner P., Jiang X., 2004**. Persistence of *Salmonella enterica* serovar typhimurium on lettuce and parsley and in soils on which they were grown in fields treated with contaminated manure composts or irrigation water. Foodborne Pathog Dis 1:27-35.
10. **Trotz-Williams LA, Jarvei BD, Martin SW, Leslie KE, Peregrine AS.** Prevalence of *Cryptosporidium parvum* infection in southern Ontario and its association with diarrhea in neonatal dairy calves. Can Vet J. 2005; 46:349–51**.**
11. **McDonel, J. L. 1980.** *Clostridium perfringens* toxins (type A, B, C, D, E). Pharmacol. Ther. **10:**617–655.
12. **Songer JG (1996)** Clostridial enteric diseases of domestic animals. Clin Microbiol Rev 9: 216–234.
13. **Steiner, L., Busato, A., Burnens, A., Gaillard, C. (1997):** Häufigkeiten und Ursachen von Kälberkrankheiten in Mutterkuhbetrieben. II. Mikrobiologische und parasitologische Diagnosen bei Kälbern mit Durchfall. Dtsch. Tierärztl. Wschr. 104, 169-173.
14. **Björkmann, C., Svensson, C., Christensson, B., Verdier de, K. (2003):** *Cryptosporidium parvum* and *Giardia intestinalis* in Calf Diarrhoea in Sweden. Acta Vet. Scand. 44, 145-152.
15. **Haschek, B., Klein, D., Benetka, V., Herrera, C., Sommerfeld-Stur, I., Vilcek, Š., Moestl, K., Baumgartner, W. (2006):** Detection of bovine torovirus in neonatal calf diarrhoea in Lower Austria and Styria (Austria). J. Vet. Med. B 53, 160-165.
16. **Heinrichs, A. J., Radostits, O. M. (2001)**: Health and Production Management of Dairy Calves andReplacement Heifers. *In*: Radostits, O. M. (Ed.): Herd Health. Food Animal Production Medicine. 3rd Ed., Saunders Company, USA. pp. 333-395.
17. **Walter Baumgartner 2012.** Diarrhoea in calves and young cattle. Lucrări Ştiinţifice - Seria Zootehnie, vol. 57.
18. **Rob Costello2005.** Calf Scours Causative Agents of Calf hood Diarrhea. Merrick's Inc., 2005

1/20/2013

Interference of preventive caries system with microshear bond strength of enamel surface bonded to etch &rinse or self etch adhesive system with nanofilled composite

Ola. M. Sakr[1] and Mohammad Almohaimeed [2]

[1]Departments of Operative Dentistry, College of Dentistry ,Qassim University (Saudi Arabia) and Misr University for Science and technology (Egypt)
[2] Department of Orthodontics and Pediatric Dentistry, College of Dentistry , Qassim University, Saudi Arabia
olasakr2004@yahoo.com

Abstract: Purpose: To evaluate the microshear bond strength of self etch and etch &rinse adhesives of nanofilled composite on enamel substrates after fluoride application. **Materials and Methods**: Forty Enamel samples were obtained from human premolars and randomly divided into 2 groups (n = 10) according to enamel substrates treatment first group samples are painted with fluoride varnish for 20 min and stored in artificial saliva for 24 hours , then divided into 2 subdivisions : first subdivision is treated with etch & rinse adhesive then. 2nd subdivision samples are treated with self etch adhesive. The second group divided into 2 subdivision : first subdivision are treated with etch & rinse adhesive. 2nd subdivision samples are treated with self etch adhesive. All prepared samples ,Prior to adhesive curing, a hollow cylinder (2.0 mm height/0.75mm internal diameter) was placed on the treated surfaces. A nanofilled resin composite was then inserted into the tube and cured. After artificial saliva storage for 24 hrs, the tube was removed and microshear bond strength was determined in a universal testing machine at a crosshead speed of 0.5 mm/min. **Results:** The mean and standard deviation values of micro-shear bond strength were 15.9 ± 4 MPa and 9.7 ± 3.8 MPa for normal and fluoridated enamel, respectively. Normal enamel showed statistically significantly higher mean micro-shear bond strength than fluoridated enamel. **Conclusions**: The microshear bond strength decrease in cases of recently fluoridated enamel.

[Ola. M. Sakr and Mohammed Almohaimeed. **Interference of preventive caries system with microshear bond strength of enamel surface bonded to etch &rinse or self etch adhesive system with nanofilled composite.** *Life Sci J* 2013;10(1):1984-1987] (ISSN:1097-8135). http://www.lifesciencesite.com. 284

Key Words: preventive therapy, microshear bond strength**,** adhesion , nanofilled composite.

1. Introduction

Improvements in dental adhesive technology have extensively influenced modern restorative dentistry. Nowadays, extension for prevention' proposed by GV Black[1] in 1917 is no longer has any explain, also it has been replaced by the concept of 'minimal invasive dentistry'.[2] This modern approach focuses on the achievement of a more conservative cavity design. The subsequent restorative procedure relies on the bonding effectiveness of adhesive materials such as resin composites, which do not require the removal of sound dental structure for additional mechanical retention. Although these restorations tend to fulfill the main requirements of a more conservative and aesthetic treatment, their clinical longevity is still a topical issue, mainly due to the degradation of the adhesive interface over time.[3]

Initial enamel caries lesions are usually not treated operatively to avoid the sacrifice of sound hard tissues [4].Thus, preventive action at an early stage is important to prevent caries development. The maintenance of oral hygiene in conjunction with dietary advice , fluoride therapy and may prudent use of pit-and-fissure sealants has been shown to be a reliable preventive strategy in these populations [5].

In order to obtain long-term clinical success, the integrity of composite -enamel bond are important and the criteria for successful prevention of leakage of bacteria and oral fluids that initiate caries [6,7].

Recently, the microshear bond test has been developed as an alternative to the microtensile test [8]. The microshear bond test was further developed by Shimada and his group, who have researched the shear strengths of enamel and resin-based adhesives [9].

The objective of the present study was to compare the resin-enamel bond strength of etch &rinse and self etch adhesives of fluoridated and normal enamel. The null hypothesis was that there was no difference between the self-etching materials and etch-and-rinse adhesive in their bond strengths

2.Materials and Methods

I- Preparation of the Samples

A total of 40 caries-free permanent premolars, which were extracted for orthodontic reasons, were used in this study. Individual tooth surfaces were hand scaled to remove any remaining soft tissue. All teeth were stored in distilled water at -20°C. Crowns were separated from the roots 2 to 3 mm apical to the cementoenamel junction using a diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) under water

irrigation at a low speed. With their labial surfaces exposed, the crowns were then embedded in self-curing acrylic resin (Meliodent, Heraeus Kulzer, Dormagen, Germany) into Teflon molds. The convex enamel surfaces on the outermost buccal surfaces were reduced up to 0.5 mm by gently polishing on a 600-grit silicone carbide paper under running water to prepare a flat enamel surface. All enamel surfaces were examined under a stereomicroscope (Leica, MZ 12, Leica AG, CH-9435 Heerbrugg, Switzerland), and any specimens with cracks or hypoplastic defects were excluded.

II- Sample classification:

The total 40 Specimens were randomly divided into two groups.

Group I : 20 specimens are painted with fluoride gel for 30 minutes , rinsed and stored in artificial saliva for 24 hours Group II : 20 specimens are not painted with fluoride gel.

Each group is subdivided into 2 subdivisions

1st subdivision is treated with etch &rinse adhesive

2nd subdivision is treated with self etch adhesive

III- Application of Bonding Agents and Preparation of Resin Composite specimens:

Enamel surfaces were cleaned with water spray for 5 seconds and dried with oil and water-free compressed air for 3 seconds. Details of bonding adhesives and composite are provided in Table 1 . Prior to application of the bonding resin on each specimen, hollow cylinders 2.0 mm in height were cut from micro-bore tygon tubing (Norton Performance Plastic; OH, USA) with an internal diameter of 0.75mm and were placed on the treated surfaces. .

Each adhesive system was applied according to the manufacturer's instructions as follows:

Etch & rinse adhesives :

The enamel surface was etched (using Scotchbond) for 15 s with 37% phosphoric acid, and rinsed with water spray for 15 s. Excess water was removed with cotton pellet or mini sponge leaving the enamel moist. Bond (Adper Single bond 2)was applied with a disposable brush, 2 to 3 consecutive coats for 15 s with gentle agitation using a fully saturated applicator . Gently air thin for five seconds in evaporative solvents . light cured for 10 s using a halogen light source (Visulux curing unit, Vivadent; Schaan, Liechtenstein). The output of the light curing unit was regularly checked (500 mW/mm2).

Self Etch adhesives :

Adper Easy one : The adhesive was applied to the enamel surface for 20 s, blown with mild air for 5 s and light cured for 10 s. A nanofilled restorative composite (Filtek Z350 Enamel Shade A2, 3 M , USA) was carefully inserted into the tubing lumens and irradiated for 40 s according to the manufacturer's instructions. The specimens were then stored in artificial saliva at 37°C for 24 hrs. After removal from artificial saliva, the tygon tubing around composite cylinders was removed by gently cutting the tube into two hemi cylinders using a feather-edge blade.

IV- Microshear bond strength testing:

These tests were performed using NEXYGEN from Lloyd Instruments. Each acrylic embedded molar tooth with its own bonded composite micro-cylinders was secured with tightening screws to the lower fixed compartment of a materials testing machine (Model LRX-plus; Lloyd Instruments Ltd., Fareham, UK) with a loadcell of 5 kN and data were recorded using computer software (Nexygen-MT Lloyd Instruments). A loop prepared from an orthodontic wire (0.014" in diameter) was wrapped around the bonded micro-cylinder assembly as close as possible to the base of the microcylinder and aligned with the loading axis of the upper movable compartment of the testing machine.

A shearing load with tensile mode of force was applied via materials testing machine at a crosshead speed of 0.5 mm/min. The relatively slow crosshead speed was selected in order to produce a shearing force that resulted in debonding of the microcylinder along the substrate-adhesive interface. The load required to debonding was recorded in Newton.

Micro-Shear bond strength calculation;

- The load at failure was divided by bonding area to express the bond strength in MPa :

$$\tau = P/ \pi r^2$$

where ; τ =bond strength (in MPa), **P** =load at failure (in N), π =3.14

r = radius of micro-cylinder (in mm)

A total of 40 bond strength values were recorded for two adhesives.

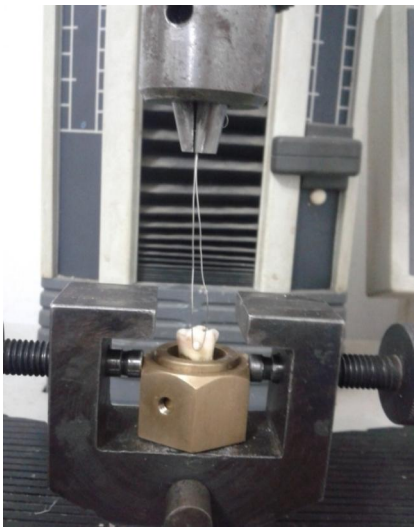


Fig.(1): lloyd universal testing machine with microshear bond strength sample.

Table (1) Composition, lot number, and manufacture of the tested materials.

Material	composition	Lot number	Manufacture
Topex DuraShield	5% sodium fluoride varnish	#0411131105	Sultan Health Care
Scotchbond etchant gel	35 % phosphoric acid	N 110268	3M ESPE
Adper Single bond 2	(10% colloidal nanofiller) BisGMA, HEMA, dimethacrylates, ethanol, water, a novel photoinitiator system and a methacrylate functional copolymer of polyacrylic and polyitaconic acids	N353034	3M ESPE
Adper Easy Bond	2-hydroxyethyl methacryate (HEMA) Bis-GMA Methacrylated phosphoric esters 1,6 hexanediol dimethacrylateMethacrylate functionalized Polyalkenoic acid (Vitrebond™ Copolymer) Finely dispersed bonded silica filler with 7 nm primary particle size Ethanol Water Initiators based on camphorquinone Stabilizers	434163	3M ESPE
Filtek Z350 XT	(20 nm silica filler 4-11 nm zirconia filler) as 72.5% by w filler bis-GMA, UDMA,TEGDMA , PEGDMA and bis-EMA resins	N339145	3M ESPE

V-Statistical Analysis:

Data were presented as mean and standard deviation (SD) values. Regression model using two-way Analysis of Variance (ANOVA) was used in testing significance for the effect of adhesive system, enamel condition and their interactions on mean micro-shear bond strength.

The significance level was set at $P \leq 0.05$. Statistical analysis was performed with IBM®SPSS® Statistics Version 20.

3.Results

Two-way ANOVA results

The results showed that enamel condition and the interaction between the two variables had a statistically significant effect on mean micro-shear bond strength.

Table (2): Regression model results for the effect of different variables on mean micro-shear bond strength

Source of variation	Type III Sum of Squares	df	Mean Square	*F*-value	*P-value*
Adhesive system	23.5	1	23.5	2.3	0.154
Enamel condition	134.3	1	134.3	13.4	0.004*
Adhesive system x Enamel condition	77.9	1	77.9	7.8	0.018*

df: degrees of freedom = (n-1), *: Significant at P ≤ 0.05

Effect of enamel condition:

The mean and standard deviation values of micro-shear bond strength were 15.9 ± 4 MPa and 9.7 ± 3.8 MPa for normal and fluoridated enamel, respectively. Normal enamel showed statistically significantly higher mean micro-shear bond strength than fluoridated enamel.

Table (3): Comparison between micro-shear bond strength of the two enamel conditions regardless of adhesive system

Normal		Fluoridated		*P*-value
Mean	±SD	Mean	±SD	
15.9	±4	9.7	±3.8	<0.001*

*: Significant at P ≤ 0.05

Effect of different interactions:

The statistically significantly highest mean micro-shear bond strength was found with (Self-etch & normal enamel). The statistically significantly lowest mean micro-shear bond strength was found with (etch & rinse & fluoridated enamel) and (Self-etch & fluoridated enamel) with no statistically significant difference between the two groups. From the interactions table, we can also conclude the following:

- **Detailed comparisons between adhesive systems:**
  - With normal enamel; self-etch showed statistically significantly higher mean micro-shear bond strength than etch & rinse.
  - With fluoridated enamel; there was no statistically significant difference between the two adhesive systems.
- **Detailed comparisons between enamel conditions:**
  - With total etch; normal enamel showed statistically significantly higher mean micro-shear bond strength than fluoridated enamel.
  - With self-etch; normal enamel showed statistically significantly higher mean micro-shear bond strength than fluoridated enamel.

Table (4): Comparison between micro-shear bond strength of different variables' interactions

Adhesive system	Enamel condition	Mean	SD	*P*-value
Total etch	Normal	12.3 [b]	1	0.018*
	Fluoridated	10.8 [c]	4.1	
Self-etch	Normal	19.5 [a]	0.8	
	Fluoridated	8.7 [c]	3.7	

*: Significant at P ≤ 0.05, Different letters are statistically significantly different

4.Discussion :

The null hypothesis that there are no differences in the bond strength of the enamel substrates and different resin adhesive materials was rejected. In this study, differences were noted between the Microshear of normal enamel and fluoridated enamel to resin adhesive. The fluoride application of 5% NaF varnish decrease microshear bond strength of enamel to different types of adhesives.

The use of a varnish as a vehicle for topical fluoride application was chosen in this study due to its prolonged period of contact with the enamel surface to allow greater uptake of fluoride ions into the enamel and making it more resistant to demineralization [(10)].

Shimada and his group who have researched the shear bond strengths of enamel and resin-based adhesives. They also who developed microshear bond strength [(9)] . The microshear bond test is considered more useful for testing bond strengths to enamel, as the microtensile method is not easy to use on this substrate and there is a high probability that the enamel will be pulled off the dentin when a tensile stress is applied to such small specimens. Also the microshear bond test is less demanding in terms of specimen production, and bond test areas can be much better controlled by the use of known diameter microbore tubing.

Little is known about the microshear bond strength of resin composite to fluoridated enamel. In current study normal enamel showed statistically significantly higher mean micro-shear bond strength than fluoridated enamel, in accordance with other studies have indicated that topical fluoride application fills the interprismatic spaces occupied by $Ca_5(PO)_3$ and CaF_2 and reduces the bonding capacity of adhesives.[(11,12)] On the other hand, studies have shown that shear bond strength is not significantly different in groups with and without fluoride pretreatment [(13-15)] . In these studies, researchers saw globular structures only on the prism cores of ground enamel surfaces etched with H3PO4 containing higher fluoride concentrations; they did not observe adverse effects on the bond strength of bonding resin to etched enamel.

5. Conclusion:

It can be concluded that the microshear bond strength was significantly influenced by fluoride application of 5% NaF varnish . The microshear bond strength decrease in cases of recently fluoridated enamel .

References:

1. Black GV. (1917):A work in operative dentistry in two volumes. Chicago: Medico-Dental Publishing.
2. Degrange M, Roulet JF. (1997):Minimally invasive restorations with bonding. Chicago: Quintessence Publishing.
3. De Munck J, Van Landuyt K, Peumans M.: (2005):A critical review of the durability of adhesion to tooth tissue: methods and results. J Dent Res;84:118–132.
4. Kidd EAM, Van Amerongen JP. (2003):The role of operative treatment. In: Fejerskov O, Kidd E (eds). Dental caries. The disease and its clinical management. Ames, Iowa, USA: Blackwell Munksgaard,245-250.
5. Seppa L, Leppanen T, Hausen H. (1995):Fluoride varnish versus acidulated phosphate fluoride gel: a 3-year clinical trial. Caries Res;29:327-330.
6. Simonsen RJ. Pit and fissure sealant(2002): review of the literature. Pediatr Dent;24:393-414.
7. Tandon S, Mathew TA. (1997):Effect of acid-etching on fluoride-treated caries-like lesions on enamel: a SEM study. ASDC J Dent Child;64:344-348.
8. McDonough WG, Antonucci JM, He J, Shimada Y, Chiang MY, Schumacher GE, Schultheisz CR. (2002):A microshear test to measure bond strengths of dentin-polymer interfaces. Biomaterials;23:3603-3608.
9. Shimada Y, Kikushima D, Tagami J. (2002):Microshear bond strength of resin bonding systems to cervical enamel. Am J Dent;15:373-377.
10. Beltran-Aguilar ED, Goldstein JW, Lockwood SA. (2000):Fluoride varnishes. A review of their clinical use, cariostatic mechanism, efficacy and safety. J Am Dent Assoc;131:589-596.
11. Lee H, Stoffey D, Orlowski J, Swartz ML, Ocumpaugh D, Neville K. (1972): Sealing of developmental pits and fissures. Effects of fluoride on dhesion of rigid and flexible sealers. J Dent Res;51:151-152.
12. Low T, von Fraunhofer JA, Winter GB. (1975):The bonding of a polymeric fissure sealant to topical fluoride-treated teeth. J Oral Rehabil :2;303-307
13. Garcia-Godoy F.(1993):Shear bond strength of a resin composite to enamel treated with an APF gel. Pediatr Dent;15:272-274.
14. Kidd EAM, Van Amerongen JP. (2003):The role of operative treatment. In: Fejerskov O, Kidd E (eds). Dental caries. The disease and its clinical management. Ames, Iowa, USA: Blackwell Munksgaard, 245-250.
15. Kimura T, Dunn WJ, Taloumis LJ.(2004):Effect of fluoride varnish on the in vitro bond strength of orthodontic brackets using a self-etching primer system. J Orthod Dentofacial Orthop;125:351-356.

2/20/2013

Expression of Galectin-3 in Thyroid Lesions; Immunohistochemical Analysis

Jaudah Al-Maghrabi[1], Sherine Salama[1], Adhari Al-Selmi[1] and Mahmoud Al-Ahwal[2].

Department of Pathology[1], Department of Medicine[2] King Abdulaziz University, Faculty of Medicine, Jeddah, Saudi Arabia
jalmaghrabi@hotmail.com

Abstract: Objectives: Evaluation of a single thyroid nodule is considered as one of the diagnostic challenges for pathologists. Recently Galectin-3 has been found to be a promising immunohistochemical marker for papillary carcinoma. The objective of this study is to test the value of Galectin-3 expression in differentiating PTC from other thyroid lesions.**Method:** In this study we evaluated a total of 74 cases of thyroid lesions which share a common clinical presentation as solitary thyroid nodule; 38 cases of papillary thyroid carcinoma, 5 cases of follicular thyroid carcinoma, 16 cases of thyroid adenoma, and 15 cases of hyperplasic thyroid nodules. The cases were immunohistochemically stained for Galectin-3 using the conventional biotin – avidin immunoenzymatic technique.
Results: Positive staining for Galectin-3 was detected in 35 cases out of thirty-eight (92%) of PTC (27 cases show moderate or strong diffuse staining). Of the three negative cases 2 were follicular variant of papillary carcinoma, and one case was conventional type. Weak or focal staining was detected in 2 out of 5 cases of FTC (40%), 5 out of 16 cases of follicular adenoma (31%), and 3 out of 15 of hyperplastic nodules (20%). None of the non papillary lesions show moderate or strong staining.**Conclusion**: Diffuse and strong immunohistochemical staining for Galectin-3 carries high significance in the diagnosis of PTC and differentiating benign from malignant tumors. However, we recommend its use with caution in diagnosing unconventional variants of PTC.
[Jaudah Al-Maghrabi, Sherine Salama, Adhari Al-Selm and Mahmoud Al-Ahwal. **Expression of Galectin-3 in Thyroid Lesions; Immunohistochemical Analysis.** *Life Sci J* 2013;10(1):1988-1992] (ISSN:1097-8135).
http://www.lifesciencesite.com. 285

Key words*:* Thyroid lesions, Galactin-3 expression, IHC, differential diagnosis.

1. Introduction

The burden of thyroid disease in the general population is enormous. As many as 50% of people in the community have microscopic nodules [1] whereas palpable nodules are encountered in 4% of the population of the United States between the ages of 30-60 years [2]. One of the challenging areas in surgical pathology is the differential diagnosis of encapsulated, follicular-patterned tumors with less-than-typical nuclei and equivocal signs of invasiveness. This necessitates the discrimination between; dominant nodule of nodular hyperplasia, follicular adenoma, minimally invasive follicular carcinoma, and the follicular variant of papillary carcinoma. Although the current diagnostic 'gold standard' for most thyroid lesions, is pathological evaluation using routine hematoxylin and eosin (H&E) stains by expert pathologists, yet, the diagnostic agreement among pathologists remains suboptimal. Malignancy of the thyroid gland is a common health problem worldwide [3-5]. In the Saudi Society, thyroid cancer is the second most common malignancy in females in all age groups (9.4%), being only preceded by breast cancer, as reported in Saudi Cancer Registry, 2004. PTC is the most common type of thyroid malignancy [6]. The histopathological diagnosis of PTC depends mainly on the appreciation of the characteristic nuclear features rather than the presence of true papillary fronds [7-9]. Recent studies pointed to some immunohistochemical markers questioning their diagnostic and prognostic utility in different thyroid tumors. Among these promising markers is Galectin-3. Galectin-3 is a unique member of an ancient lectin family [10]. Galectin-3 is also expressed in a variety of normal tissue and tumors [11] Malignant transformation of thyroid cells has been found to be accompanied with intense nuclear localization of galectin-3 [12]. Galectin-3 expression recently emerged as a potential diagnostic and/or prognostic marker of some cancers [13]. Although galectin-3 is not a universal and unambiguous marker of thyroid cancers, it could be a helpful parameter in diagnosis of these tumors as well as possible potential therapeutic target [14-17]. However the data is not very clear yet regarding the reliability of using Galectin-3 for confirmation of the diagnosis of PTC. In this study we test the value of Galectin-3 expression using immunohistochemistry in differentiating PTC from other thyroid lesions.

2. Material and Method:

Seventy four cases of different thyroid tumors were retrieved from the archival files of pathology department at king Abdulaziz University Hospital (KAUH). We selected 38 cases diagnosed as papillary thyroid carcinoma (PTC); 18 cases were

conventional type, 9 cases were follicular variants (FVPTC), 6 cases were encapsulated type, one case was tall cell variant, and 4 cases were microcarcinomas. Seven cases showed multifocal tumor. Four cases of PTC were associated with lymph node metastasis and one of the involved lymph nodes from each case was also stained with Galectin-3 to study the staining pattern in metastatic tumor as well. The study also included 5 cases diagnosed as minimally invasive follicular thyroid carcinoma (MIFTC), 16 cases of follicular adenoma, and 15 cases of hyperplastic nodules. The cases were reviewed by two pathologists, and stained with Galectin-3, using the conventional biotin – avidin immunoenzymatic technique as follows: Five-micrometer sections from selected tumor blocks are mounted on 3-aminopropyltriethoxysilane coated (Sigma, St. Louis, MO) slides, Sections are deparaffinized in xylene, rehydrated in graded alcohols, and rinsed in 0.05 M Tris-buffered saline (TBS), sections then boiled in 10 mM citrate buffer for antigen retrieval, at pH 6.0, blocking of endogenous peroxidase with aqueous 0.3% H_2O_2 for 15 min. Sections are then incubated for one hour with the monoclonal antibody; Galectin-3 (Novacastra, UK, clone 9C4). The dilution used was 1:100 as per the company instruction. The antigen-antibody immunoreactions are visualized using 3, 3'diaminobenzidine. All immunoreactions are carried out at room temperature. Negative control sections are made by exclusion of the primary antibody. Positive control sections are obtained from sections of normal prostatic tissue. Gal-3 gives nuclear and/or cytoplasmic staining. Tumor cells were considered positive only when the appropriate staining pattern was noted. Positive histological reaction for different antibodies used is visualized as follows; A semi quantitative scoring system is used to score immunohistochemical positivity; The extent of immunoreactivity was categorized as negative (0); less than 5% f the cells show positive staining; equivocal (+/-), focal or weak staining (1+); 5% to 10%, moderate (2+); 11% to 50%, strong and diffuse (3+); greater than 50% positivity of tumor cells.

3. Results:

The study investigated 74 cases of different thyroid lesions; 38 cases of papillary thyroid carcinoma (PTC), 5 cases of minimally invasive follicular thyroid carcinoma (FTC), 16 cases of thyroid adenoma, and 15 cases of hyperplasic nodule. All share a common clinical presentation as single thyroid nodule. The thirty-eight cases of PTC were excised from 33 females and 5 males (female to male ratio 6.6/1). The age ranged from 17 to 70 years with the mean age of 38 years. Only 3 cases out of 38 were negative for Galectin-3 immunostaining. The positive cases represented 92% of the total number of PTC (table 1 and figure 1). Of the three negative cases 2 were FVPTC, and one case was conventional type. As for the staining intensity; 13 cases stained strongly and diffuse (+3), 14 cases revealed moderate staining (+2), 7 cases stained weakly or focal (+1), while another one case revealed equivocal pattern of staining (+/-). The localization of staining was mainly cytoplasmic, both nuclear and cytoplasmic staining was encountered in (+3) cases. Neither The staining localization nor its intensity showed relation to specific type of the tumor. The study included 5 cases of minimally invasive FTC; three cases were females and 2 cases were males. The age ranged from 21 to 43 with mean age of 33 years. Three cases were negative for the Galectin-3 immunostaining, while two cases were positive (+1). The included 16 cases of adenoma were excised from 13 females and three male patients (female to male ratio was 4.3/1), the mean age was 34 years. Negativity was demonstrated in 11 cases (73 %). The 5 positive cases either weak (+) or equivocal (+/-), whereas in the hyperplasic nodules; 12 cases were negative (80%), three cases were weakly positive (+1). The female to male ratio in hyperplastic nodules' cases was 6.5/1, while the mean age was 33 years. The results showed significant Galactin-3 expression differences between the benign and malignant lesions in which the tendency of positive expression is mainly more towards the malignant cases as comparing to benign lesions (**$p<0.0001$**).

Table 1: Intensity of Galectin-3 positivity in different thyroid lesions:

	0	**+/-**	**+1**	**+2**	**+3**	**Total**
PTC	3	1	7	14	13	38
FTC	3	0	2	0	0	5
Adenoma	11	2	3	0	0	16
Hyperplastic	12	0	3	0	0	15
Total	29	3	15	14	13	**74**

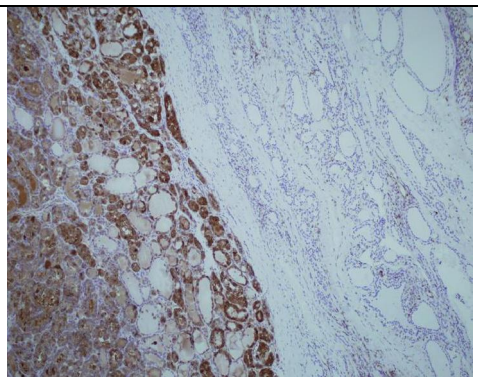


Fig. 1A) Gal-3 positivity in encapsulated FPTC against surrounding negatively stained normal thyroid tissue.

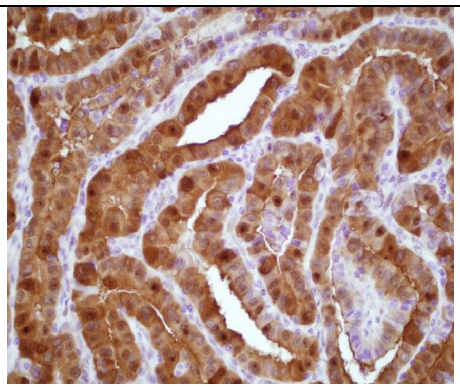


Fig. 1B) Pattern of Gal-3 staining (nuclear and cytoplasmic (+3) in PTC.

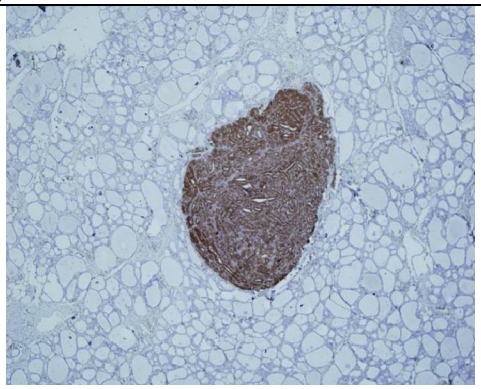


Fig. 1C) Gal-3 highlighting incidentally found papillay microcarcinoma background of normal thyroid with negative staining.

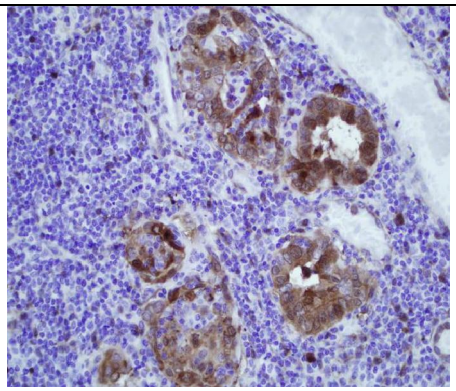


Fig. 1D) Gal-3 highlighting metastatic PTC in lymph node

4. Discussion:

Saxen et al [18] tested the reducibility of WHO classification of thyroid tumors on 696 cases and found only 58% agreement among five Nordic pathologists. A more recent study by Hirokawa et al. [19] who compared the diagnoses of 21 follicular nodules by four American and four Japanese pathologists; the agreement of benign versus malignant was encountered in only 62% of the nodules. Fassina et al.,[20], review of 200 thyroid tumors revealed good agreement for papillary and anaplastic thyroid carcinomas, moderate for medullary and poor for follicular thyroid carcinomas. In more recent review of 41 follicular carcinomas by five experienced French thyroid pathologists, the agreement for malignancy varied from 5% among all five pathologists to 56% between two pathologists. It is clear from these studies and others [21] that there is interobserver variation in the diagnosis of thyroid neoplasms.

Among the recent ancillary techniques emerged, Galectin-3 has been found to be a promising marker with high specificity for papillary thyroid carcinoma (PTC). In the study of **Smenov, et al** who examined fourty cases of thyroid nodules, positive immunohistochemistry staining for Galectin-3 was found to be a highly sensitive oncomarker for PTC: sensitivity of 100%, specificity of 71%, diagnostic value of 85% [22]. This study was in agreement with ours which demonstrated positivity for galectin-3 in 90% cases of PTC including microcarcinoma and were positive in metastatic carcinoma in the lymph nodes in all the four cases with lymph nodes metastasis. This was also in concordance with the study of **Cvejic et al,** [23] who reported immunopositivity in 80.9% of papillary microcarcinomas.

Our study revealed different intensity of immunostaining among PTC cases ranging from (+1 to +3), and showed predominant cytoplasmic localization with or without nuclear staining. The strong diffuse (3+) positivity was seen only in papillary carcinoma and none of the other lesions show this strong intense staining pattern.

Our results were also in keeping with that of **Cvejic et al,** which revealed that localization of galectin-3 was mainly cytoplasmic, and the intensity of staining was variable, ranging from strong to weak. The

immunonegative cases in their study were of non-classical types lacking the papillary architecture. They didn't relate galectin-3 positivity to rate of growth or aggressiveness of the tumor, rather suggested its role in tumor biology [23]. Our study also demonstrated that two out of the three negative cases of PTC were of FVPTC, an observation that could be a feature of Galectin-3 specificity to tumors with papillary architecture.

In another study by **Cvejic et al** [24] conducted on wider scale of papillary thyroid cases (202 cases), they studied Galectin-3 expression in different histological patterns of PTC, cases with lymph node metastasis and cases with extrathyroid invasion. Their results revealed sensitivity of galectin-3 immunostaining for conventional histology in 98% (100 of 102) for classical PTC, 85.2% (46 of 54) for follicular variant, and 50% (23 of 46) for follicular/solid variant of PTC. They also demonstrated that Galectin-3 immunohistochemical expression itself is not an indicator of lymph node metastasis or extrathyroid invasion of PTC. They declared as well as other investigators [25-27] that Galectin-3 as an excellent marker for classical PTC yet recommended its use with caution in diagnosing unconventional variants of PTC because of the possibility of false-negative results. On the other hand, **Mehrotra et al** [28] found that galectin-3 immunopositivity is not only in PTC, but also in a large proportion of follicular adenomas (72%) and multinodular goitres (57%) which is in contrast to our study and other studies [29].

Few studies investigated Galectin-3 expression in follicular thyroid carcinoma (FTC), positivity was encountered in minimally invasive FTC [30], as well as in fully invasive FTC in a study of 260 cases [31] which also correlated the galectin-3 positivity to the degree of capsular or vascular invasion. They concluded that Galectin-3 expression level significantly increased with the degree of vascular or capsular invasion ($p<0.0001$). However, its diagnostic value for follicular carcinoma was not high because the sensitivity and specificity were 68.7% and 57.5%, respectively. In our study three cases out of five of FTC were negative for galectin-3 immunostaining and the two positive cases only show weak (+1) staining pattern.

In conclusion our results as well as those of the vast majority of researchers confirmed that galectin-3 is an excellent marker for supporting the diagnosis of papillary thyroid carcinoma and for distinguishing malignant from benign or hyperplasic lesions when the morphologic criteria are equivocal. Strong (3+) positivity for galectin-3 is almost characteristic for papillary carcinoma. Although the number of FVPTC is small, the data demonstrate that negative staining for galectin-3 does not exclude papillary carcinoma in those cases and the presence of strong diffuse staining (3+) is very suggestive of papillary carcinoma. Galectin-3 expression is also successful in highlighting metastasis in lymph nodes, yet its role in FTC is controversial. Further studies using combination of galectin-3 and other immunohistochemical markers such as HBME-1, CITED-1, CK-19 and fibronectin would be useful to establish a panel for the diagnosis thyroid neoplasms with higher sensitivity and specificity.

Corresponding author
Jaudah Al-Maghrabi
Department of Pathology[1]
King Abdulaziz University, Faculty of Medicine, Jeddah, Saudi Arabia
jalmaghrabi@hotmail.com

References

1. Wang C, Crapo LM: The epidemiology of thyroid disease and implications for screening. *Endocrinology and metabolism clinics of North America* 1997, 26(1):189-218.
2. Mazzaferri EL: Management of a solitary thyroid nodule. *N Engl J Med* 1993, 328(8):553-55.9
3. Al-Jaradi M, Sallam A, Jabr H, Borda A, Decaussin-Petrucci M, Berger N: Prevalence of differentiated thyroid cancer in 810 cases of surgically treated goiter in Yemen. *Ann Saudi Med* 2005, 25(5):394-397.
4. Abdeluakhab M, Mziuad O, Gavrailov M: [Thyroid cancer--its prevalence, carcinogenic factors, classifications of the cancer, types, variants amd prognostic factors]. *Khirurgiia* 1995, 48(2):32-38.
5. Maruchi N, Furihata R, Makiuchi M: Population surveys on the prevalence of thyroid cancer in a non-endemic region, Nagano, Japan. *Int J Cancer* 1971, 7(3):575-583.
6. Schlumberger MJ: Papillary and follicular thyroid carcinoma. *N Engl J Med* 1998, 338(5):297-306.
7. Naganuma H, Murayama H, Ohtani N, Takaya K, Mori Y, Sakai N, Kakudo K: Optically clear nuclei in papillary carcinoma of the thyroid: demonstration of one of the fixation artifacts and its practical usefulness. *Pathol Int* 2000, 50(2):113-118.
8. Pedio G, Hedinger C, Zobeli L: Ground-glass nuclei in papillary carcinoma of the thyroid. *Acta Cytol*.728:(6)25 ,1981
9. Gray A, Doniach I: Morphology of the nuclei of papillary carcinoma of the thyroid. *Br J Cancer* 1969, 23(1):49-51.
10. Hirabayashi J, Kasai K: The family of metazoan metal-independent beta-galactoside-binding

lectins: structure, function and molecular evolution. *Glycobiology* 1993, 3(4):297-304.
11. Dumic J, Dabelic S, Flogel M: Galectin-3: an open-ended story. *Biochim Biophys Acta* 2006, 1760(4):616-635.
12. Paron I, Scaloni A, Pines A, Bachi A, Liu FT, Puppin C, Pandolfi M, Ledda L, Di Loreto C, Damante G *et al*: Nuclear localization of Galectin-3 in transformed thyroid cells: a role in transcriptional regulation. *Biochemical and biophysical research communications* 2003, 302(3):545-553.
13. Danguy A, Camby I, Kiss R: Galectins and cancer. *Biochim Biophys Acta* 2002, 1572(2-3):285-293.
14. van den Brule F, Califice S, Castronovo V: Expression of galectins in cancer: a critical review. *Glycoconjugate journal* 2004, 19(7-9):537-542.
15. Johnson KD, Glinskii OV, Mossine VV, Turk JR, Mawhinney TP, Anthony DC, Henry CJ, Huxley VH, Glinsky GV, Pienta KJ *et al*: Galectin-3 as a potential therapeutic target in tumors arising from malignant endothelia. *Neoplasia* 2007, 9(8):662-670.
16. Sawangareetrakul P, Srisomsap C, Chokchaichamnankit D, Svasti J :Galectin-3 expression in human papillary thyroid carcinoma. *Cancer genomics & proteomics* 2008, 5(2):117-122.
17. Nangia-Makker P, Nakahara S, Hogan V, Raz A: Galectin-3 in apoptosis, a novel therapeutic target. *Journal of bioenergetics and biomembranes* 2 ,007 -79:(1)39 .84
18. Saxen E, Franssila K, Bjarnason O, Normann T, Ringertz N: Observer variation in histologic classification of thyroid cancer. *Acta pathologica et microbiologica Scandinavica Section A, Pathology* 1978, 86A(6):483-486.
19. Hirokawa M, Carney JA, Goellner JR, DeLellis RA, Heffess CS, Katoh R, Tsujimoto M, Kakudo K: Observer variation of encapsulated follicular lesions of the thyroid gland. *Am J Surg Pathol* 2002, 26(11):1508-1514.
20. Fassina AS, Montesco MC, Ninfo V, Denti P, Masarotto G :Histological evaluation of thyroid carcinomas: reproducibility of the "WHO" classification. *Tumori* 1993, 79(5):314-320.
21. Franc B: Observer variation of lesions of the thyroid. *Am J Surg Pathol* 2003, 27(8):1177-1179.
22. Semenov D, Pozharisskii KM, Boriskova ME, Pankova PA, Mukhina MS, Feshchenko NS: [Galectin-3 in diagnosis of thyroid cancer]. *Voprosy onkologii* 2008, 54(3):321-323.
23. Cvejic D, Savin S, Petrovic I, Paunovic I, Tatic S, Krgovic K, Havelka M: Galectin-3 expression in papillary microcarcinoma of the thyroid. *Histopathology* 2005, 47(2):209-214.
24. Cvejic DS, Savin SB, Petrovic IM, Paunovic IR, Tatic SB, Havelka MJ: Galectin-3 expression in papillary thyroid carcinoma: relation to histomorphologic growth pattern, lymph node metastasis ,extrathyroid invasion, and tumor size. *Head & neck* 2005, 27(12):1049-1055.
25. Turkoz HK, Oksuz H, Yurdakul Z, Ozcan D: Galectin-3 expression in tumor progression and metastasis of papillary thyroid carcinoma. *Endocrine pathology* 2008, 19(2):92-96.
26. Torregrossa L, Faviana P, Camacci T, Materazzi G, Berti P, Minuto M, Elisei R, Vitti P, Miccoli P, Basolo F: Galectin-3 is highly expressed in nonencapsulated papillary thyroid carcinoma but weakly expressed in encapsulated type; comparison with Hector Battifora mesothelial cell 1 immunoreactivity. *Hum Pathol* 2007, 38(10):1482-1488.
27. Prasad ML, Pellegata NS, Huang Y, Nagaraja HN, de la Chapelle A, Kloos RT: Galectin-3, fibronectin-1, CITED-1, HBME1 and cytokeratin-19 immunohistochemistry is useful for the differential diagnosis of thyroid tumors. *Mod Pathol* 2005, 18(1):48-57.
28. Mehrotra P, Okpokam A, Bouhaidar R, Johnson SJ, Wilson JA, Davies BR, Lennard TW: Galectin-3 does not reliably distinguish benign from malignant thyroid neoplasms. *Histopathology* 2004, 45(5):493-500.
29. Kawachi K, Matsushita Y, Yonezawa S, Nakano S, Shirao K, Natsugoe S, Sueyoshi K, Aikou T, Sato E: Galectin-3 expression in various thyroid neoplasms and its possible role in metastasis formation. *Hum Pathol* 2000, 31(4):428-433.
30. Saggiorato E, Cappia S, De Giuli P, Mussa A, Pancani G, Caraci P, Angeli A, Orlandi F: Galectin-3 as a presurgical immunocytodiagnostic marker of minimally invasive follicular thyroid carcinoma. *The Journal of clinical endocrinology and metabolism* 200.5158-5152:(11)86 ,1
31. Ito Y, Yoshida H, Tomoda C, Miya A, Kobayashi K, Matsuzuka F, Yasuoka H, Kakudo K, Inohara H, Kuma K *et al*: Galectin-3 expression in follicular tumours: an immunohistochemical study of its use as a marker of follicular carcinoma . *Pathology* 2005, 37(4):296-298.

2/2/2013

Utility of 2-Thiohydantoin Derivatives in the Synthesis of Some Condensed Heterocyclic Compounds with Expected Biological Activity

A.Y. Hassan[1], M.M.Said[2]; M.T. Sarg[3]; H.S.Al-Zahabi[4] and E.M.Hussein[3]

[1]Department of Chemistry, Faculty of Science (Girls), Al-Azhar University, Nasr City, Cairo; Egypt
[2]Department of Organic Chemistry, Faculty of Pharmacy, Cairo University, Cairo; Egypt
[3]Department of Organic Chemistry, Faculty of Pharmacy (Girls), Al-Azhar University, Nasr City, Cairo; Egypt
[4]Department of Pharmaceutical Chemistry, Faculty of Pharmacy (Girls), Al-Azhar University, Nasr City, Cairo. Egypt
m.t.sarg@hotmail.com

Abstract: On the pharmaceutical account of the reported anticancer activity of imidazole and condensed imidazole, new imidazo [2,1-b][1,3,5]thiadiazines **3a-d ,** pyrrolo[1,2-e]imidazole **5**, imidazo[2,1-b][1,3]thiazines **6a,b, 6\a**, imidazo[2,1-b][1,3]benzothiazines **7a,b, 9a,b** imidazo[2,1-b][1,3]thiazoles **11a,b, 12a,b, 18a,b, 19a,b, 20a,b, 24**; thieno[3\,2\,:4,5]pyrimido[1,2-a]imidazoles **14a,b, 15a,b,** pyrido[3',2':4,5]thieno[3,2-d]imidazo[1,2-a]pyrimidines **17a,b ,** imidazole derivatives **8a,b**, **10a,b, 21, 22**, **23** and imidazo[2\,1\:2,3]thiazolo[4,5-c]pyrazole **25** were synthesized through different chemical reactions. Structures of all synthesized compounds were supported by spectral and elemental analyses. The selected compounds by NCI were evaluated for their *in vitro* antitumor activity against 60 human tumour cell lines.

[A.Y. Hassan, M.M.Said; M.T. Sarg; H.S.Al-Zahabi and E.M.Hussein. **Utility of 2-Thiohydantoin Derivatives in the Synthesis of Some Condensed Heterocyclic Compounds with Expected Biological Activity.** *Life Sci J* 2013;10(1):1993-] (ISSN:1097-8135). http://www.lifesciencesite.com. 286

Keywords: imidazo[2,1-b][1,3,5]thiadiazine; imidazo[2,1-b][1,3]thiazine, imidazo [2,1-b] thiazole; imidazole derivatives; anticancer .

1. Introduction:

2-Thiohydantoin derivatives represent an important class of biologically active molecules having broad pharmacological activities, anticancer [1], anticonvulsant [2], antidiabetic [3], antimicrobial [4], antiarrythmic [6], hypolipidemic [6] and hypotensive [7] activities, as well as herbicidal and fungicidal [8] applications. Furthermore, many thiohydantions are responsible for inhibition of fatty acid hydrolase [9], glycogen phosphorylase [10], amylase [11] and serine protease [12] enzymes.

The aforementioned biological activities together with the industrial importance of these compounds stimulated our interest for the synthesis of several new condensed heterocyclic compounds containing thiohydantoin moiety condensed with each of thiadiazine, thiazine, benzothiazine, pyrimidine, thiazole and pyrrole.

The new condensed heterocyclic derivatives possessing latent functional substituents appear promising to fulfill the objectives of our biological activity studies and the desired chemical transformations. However, 2-thiohydantoin and 5-arylidene-2-thiohydantoin seemed to be excellent candidates for these synthesis.

2.Discussion:

1,3,5-Thiadiazine derivatives belong to a promising class of heterocyclic compounds [13] with a broad spectrum of biological activities. In particular, some functionalized thiadiazines have been found to exhibit antibacterial [14,15], fungicial, herbicidal [16,17] and insecticidal [18] activities. Fused derivatives of 1,3,5-thiadiazine are most suitably synthesized by the "Mannich condensation" of mercaptoazoles with formaline and primary amines [15,19-23]. The reaction of 5-arylidene-2-thiohydantoins **2a,b** with formaline and primary aromatic amine was readily accomplished in boiling DMF without any catalyst [24] to yield the novel imidazo[2,1-b][1,3,5]thiadiazine derivatives **3a-d .**

It should be noted that the aminomethylation of thiohydantoin derivatives **2a,b** has been repeatedly studied earlier [25,26]. It has been shown with a number of examples that the aminomethylation of 2-thiohydantoins exclusively involves the N(3) atom [25]. Based on these findings, we assumed that the reaction intermediate is the aminomethylthiohydantoin **2\,** whose structure permits subsequent cyclocondensation with formaline along the only possible pathway leading to imidazo[2,1-b][1,3,5] thiadiazines **3a-d**. It is worth noting that, documented examples of the synthesis of compounds containing the imidazo[2,1-b][1,3,5]thiadiazine fragment are few [17,23,27-32]. The structures of compounds **3a-d** were confirmed by spectroscopic data and elemental analyses. For instance, the IR spectra of compounds **3a-d** were devoid of absorption bands at 3400-3100 cm^{-1} characteristic for NH fragment, instead they showed

bands characteristic to stretching vibrations of the conjugated amide C=O group and the C=N fragments at 1739-1720 and 1658-1596 cm^{-1}. ^{1}H-NMR spectra of compounds **3a & 3c** showed, apart from the characteristic signals of the aromatic protons, singlets attributed to the methine protons at δ 6.74, 6.76 ppm, two peaks for the methylene protons of the 1,3,5-thiadiazine rings at δ 5.44, 5.96 and 4.90, 5.42 ppm, respectively. The mass spectrum of compound **3a** revealed the molecular ion peak at m/z 380 (0.02) and the base peak at m/z 61(100).

2-Thiohydantoin **1** was converted with N,N-dimethylformamide / dimethylacetal (DMF/DMA) into the corresponding 5-[(N,N-dimethylamino)methylene]-2-thioxoimidazolidine-4-one **4** by fusion, in 57% yield. The IR spectrum of compound **4** exhibited bands in the region 3398, 3300, 1680 and 1398 cm^{-1} corresponding to (two NH), (C=O) and (C=S) groups respectively. Furthermore, treatment of compound **4** with malononitrile by refluxing in ethanol and in the presence of a catalytic amount of piperidine, yielded 5-amino-2,3-dihydro-1-oxo-3-thioxo-1H-pyrrolo[1,2-e]imidazole-6-carbonitrile **5**. The structure of the latter product was established on the basis of its elemental analysis and spectral data. The IR spectrum of **5** showed absorption bands at 3305, 3117, 2207, 1694 cm^{-1} due to NH_2, NH, CN and amide carbonyl functions respectively. The mass spectrum of compound **5** revealed the molecular ion peak at m/z 192 (M^+, 5.99) and the base peak at 56(100). **(Scheme 1)**.

Since, the thiazine moiety of imidazo[2,1-b][1,3]thiazines is an important pharmacophoric group for the benzodiazepine receptor binding activity [23,33]. Bicyclic compounds of imidazo[2,1-b][1,3]thiazines **6-9** were synthesized and screened for their anticancer activities. This was accomplished through the reaction of 5-arylidene-2-thiohydantoin **2a,b**, with 1,3-dichloropropane in acetone in the presence of triethylamine [34]. Earlier, it was found by our research group [35,36], that during the reaction of 5-arylidene-2-thiohydantoins with 1,2-dibromoethane two isomeric products were obtained. In an analogous reaction, upon reacting 1,3-dichloropropane with 5-(4-chlorobenzylidne)-2-thiohydantoin **2a**, the two isomeric products **6a, 6\a** were obtained in a 1:4 ratio. On the contrary, when 5-(4-methoxybenzylidene)-2-thiohydantoin **2b** was reacted with 1,3-dichloropropane, only one product **6b** was obtained in a yield 65%. The structures of the products were supported by ^{1}H-NMR, IR spectroscopy and elemental analysis. The ^{1}H-NMR spectrum of **6a** showed resonance signals at δ 2.20-2.38, 3.10-3.20 and 3.70-3.80 ppm attributed to three CH_2 functions of the thiazine ring and a singlet at δ 6.89 ppm due to olefinic proton.

Cyclocondensation of o-chlorobenzaldehyde and 5-arylidene-2-thiohydantoins **2a,b** proceeds by fusion of equimolar amounts of these compounds affording 2-(4-substitutedbenzylidene)-5-hydroxy-2H-imidazo[2,1-b][1,3]benzothiazin-3-(5H)-ones **7a,b**. The structures of **7a,b** were supported by ^{1}H-NMR and IR spectroscopy and elemental analyses.

The mass spectrum of compound **7a** showed peaks at m/z 344 due to M+2 (0.04), molecular ion peak at 342 (0.06) in addition to the base peak at 139 (100). The ^{1}H-NMR spectrum of **7b** revealed characteristic signals at δ 3.20-3.40 ppm corresponding to methoxy protons and C5 proton [37]. The IR spectra of compounds **7a,b** showed broad bands due to OH group at 3494-3394cm^{-1}.

Acylation of 5-arylidene-2-thiohydantoin **2a,b** with different substituted benzoyl chloride in dry toluene and in presence of piperidene as catalyst afforded 4-(4-chlorobenzylidene)-1-(4-substitutedphenylcarbonyl)-2-thioxo-5-oxo-2,3,4,5-tetrahydro-1H-imidazoles **8a,b** and 2-(4-substitutedbenzylidene)-8-chloro-2H-benzo[e]imidazo[2,1-b][1,3]thiazine-3,5-diones **9a,b**. Actually the heating of 4-nitro or 4-bromobenzoyl chloride with the thiones **2a,b** under experimental conditions, we assumed to yield N-acyl derivatives **8a,b** which were isolated. However, further trials to cyclize **8a,b** to yield fused systems all failed under the same experimental conditions. While, upon the reaction of 2,4-dichlorobenzoyl chloride with thiones **2a,b** under experimental conditions, the intermediate N-acylated derivative **8** could not be isolated and it was capable of intramolecular cyclization to provide compounds **9a,b**.

The structures of the N-acyl derivatives **8a,b** have been established on the basis of their elemental analyses and spectral data. The IR spectra of compounds **8a,b** showed characteristic absorption bands at 3433-3305, 1712 and 1650 cm^{-1} attributed to NH and two C=O groups. The IR spectra of compounds **9a,b** lacked the absorption bands at 3400-3100 cm^{-1} characteristic of the NH group and showed absorption bands at 1712- 1652 cm^{-1} corresponding to carbonyl groups of imidazole and thiazine rings. The mass spectrum of **9a** revealed a peak at m/z 376 corresponding to (M+2, 8.47) and the base peak at m/z 50(100). While the mass spectrum of **9b** showed the molecular ion peak at m/z 370 (M^+, 6.01) and the base peak at 52(100). **(Scheme 2).**

As a part of our aim towards the development of simple new procedures for the synthesis of fused heterocyclic compounds, the reaction of 5-arylidene-2-thiohydantoins **2a,b** with monohalogenated

compounds such as ethyl iodide, chloro acetonitrile and methyl bromoacetate was accomplished under the influence of alkali metal alkoxides to give ethyl mercapto derivatives **10a,b**. However, further cyclization of alkylthio derivatives via the Thorpe reaction yielded 3-amino-6-(4-substitutedbenzylidene)imidazo[2,1-b][1,3]thiazol-5-(6H)-ones **11a,b** and 6-(4-substitutedbenzylidene)imidazo[2,1-b]thiazole-3,5(2H,6H)diones **12a,b** was observed in the case of electron acceptor substituents in the thioalkyl group (R=CN, $COOCH_3$) under the influence of alkali metal alkoxides. It should be noted that the non cyclic alkyl derivatives of compounds **11a,b** and **12a,b** intermediates were not isolated in such reaction . The structures of compounds **(10-12) a,b** were supported by elemental analyses and spectral data. The IR spectra of compounds **10a,b** showed the presence of absorption bands at 3303-3131 and 1711-1700 cm^{-1} could be attributed to NH and carbonyl groups respectively, while the spectra of compounds **11a,b** revealed the disappearance of bands due to cyano group and the presence of absorption bands at 3224-3130 and 1724-1697 cm^{-1} due to amino and carbonyl groups respectively. However, the IR spectra of compounds **12a,b** showed absorption bands at 1742-1735 cm^{-1} due to two carbonyl groups of imidazole and thiazole rings.

The ^{1}H-NMR spectrum of **10a** showed a triplet at δ1.37ppm, a quartet at δ 3.22 ppm assigned to ethyl group protons, in addition to a deuterium oxide exchangeable singlet at δ 11.81 ppm assigned to the NH function. The ^{1}H-NMR spectrum of **11a** revealed no signals of –S-CH_2– protons but revealed signals for thiazole and NH_2 protons at δ 6.40 and 10.61 ppm respectively. Also, the structure of compound **11b** was supported by its mass spectrum, which showed molecular ion peak at m/z 273 (7.19) and the base peak m/z 53(100), which is in agreement with its molecular formula ($C_{13}H_{11}N_3O_2S$). The ^{1}H-NMR spectrum of **12a** showed a signal for –S-CH_2– protons of the thiazole ring at δ 4.49 ppm and the disappearance of signals due to the two NH functions of the starting compounds, while the mass spectrum of compound **12b** showed the molecular ion peak at m/z 274(0.05) and the base peak at 156(100). **(Scheme 3).**

Compounds containing a fused pyrimidine ring represent a broad class of compounds which have received considerable attention over the past years due to their wide range of biological activity. With the development of clinically useful anticancer, antihypertensive agents, antiviral, antibacterial, antiallergic, antimalarial, analgesic and anti-inflammatory drugs [38, 39], there has been recently remarkable interest in the synthesis of annulated pyrimidines.

Reaction of o-aminoester (ethyl-2-amino-4,5-dimethylthiophene-3-carboxylate) **13** [40] or o-aminonitrile (3-amino-4,6-diphenylthieno[2,3-b]pyridine-2-carbonitrile) **16** [41] with 2-ethylthiohydantoin derivatives **10a,b** in glacial acetic acid or by fusion leading to condensed systems **(14, 15 &17)a,b**. The postulated mechanism for the reaction of the o-aminoester with **10a,b** involves an initial nucleophilic addition of the amino group of the o-aminoester to the electron deficient imidazole C2 cation to form the intermediates **14'** by elimination of ethyl mercaptane which carry out subsequent nucleophilic attack of the nitrogen atom of imidazole moiety on the sp^2 carbon of the carboxylate group followed by elimination of an ethanol moiety to give two products **14a,b** and **15a,b.**

The structure of the new compounds **(14, 15 & 17)a,b** were established on the basis of elemental analyses and spectral data. The IR spectra of compounds **14a,b** and **15a,b** showed absorption bands at 3410-3208, 3380-3213cm^{-1} corresponding to NH functions, respectively. In addition to bands at 1710-1666, 1720-1710 cm^{-1} for imidazole carbonyl group and 1666-1662, 1658-1656 cm^{-1} for thiazine carbonyl group, respectively. The mass spectrum of compound **14a** showed peaks at m/z 359 due to (M+2, 20.02), m/z 357 attributed to (M^+, 8.3) and the base peak at m/z 49 (100). ^{1}H-NMR spectrum of compound **15b** showed a deuterium oxide exchangeable singlet at δ 4.98 ppm assigned to the NH_2 protons. In addition to two doublets at δ 7.73, 8.15 ppm corresponding to aromatic protons. Furthermore, the electron impact mass spectrum of compound **15a** showed a peak corresponding to M+2 at m/z 359(6.7) and the molecular ion peak at m/z 357(9.72) and the base peak at m/z 59 (100) which are in agreement with its molecular formula $C_{17}H_{12}ClN_3O_2S$. Moreover, the spectra of compounds **17a,b** showed absorption band at 3417-3209 cm^{-1} characteristic to amino group and another absorption bands at 1712-1710 cm^{-1} assigned for carbonyl group respectively. While, the ^{1}H-NMR spectra of the compounds **18a,b** apart from the characteristic signals attributed to the aromatic protons, they revealed deuterium oxide exchangeable singlets attributed to the amino protons at δ 8.14 ppm and another singlets due to pyridine C3 proton at δ 7.41 and 7.88 ppm, respectively. **(Scheme 4).**

The work was extended to shed more light on the activity and synthetic potential of the (NH_2) group in each of compounds **11a & 11b.** Thus, compounds **11a,b** were reacted with phenyl isothiocyanate to yield the corresponding 1-[6(4-substitutedbenzylidene)-5,6-dihydro-5-

oxoimidazo[2,1-b][1,3]thiazol-3-yl]-3-phenylthioureas **18a,b**. The IR and ^{1}H-NMR spectral data of **18a,b** were found to be in agreement with the assigned structure. The IR spectra of compounds **18a,b** showed bands at 3467-3193 cm^{-1} characteristic to two NH groups. In addition to four bands corresponding to N-C=S functions at 1597-1590, 1258-1253, 1180-1096 and 1027-1014 cm^{-1}, respectively.

The electron impact mass spectrum of compound **18a** showed peaks at m/z 414(1.25), 412(1.1) due to M+2 and M$^+$, respectively. In addition to the base peak at 60 (100). However, ^{1}H-NMR spectrum of **18b** showed a deuterium oxide exchangeable singlet at δ 10.57 ppm attributed to two NH protons, in addition to other signals corresponding to other protons. Compounds **18a,b** were further reacted with both phenacyl bromide and chloroacetic acid to yield compounds **19a,b** and **20a,b** respectively. The reaction with phenacyl bromide was applied in ethanol as solvent and in the presence of a catalytic amount of triethylamine. While the reaction with chloroacetic acid was performed in acetic acid as solvent and by using sodium acetate as a catalyst. Both reactions were accomplished via dehydrobromination or dehydrochlorination followed by cyclization through water elimination. IR spectral analyses for compounds **19a** and **20a** revealed the disappearance of bands corresponding to NH and C=S functions. ^{1}H-NMR spectrum of compound **19b** showed two singlets at δ 7.49 and 7.52 ppm corresponding to thiazole and imidazothiazole protons, respectively. In addition to signals corresponding to aromatic protons. Also, ^{1}H-NMR spectrum of compound **20b** showed two singlets at δ 5.07 and 9.38 ppm attributed to S-CH_2 protons and thiazole CH proton, respectively. **(Scheme 5).**

The synthesis of new compounds outlined in **(Scheme 6)** was accomplished by hydrazinolysis of 6-(4-chlorobenzylidene)imidazo[2,1-b][1,3]thiazole-3,5-(2H,6H) dione **12a** by boiling with hydrazine hydrate in ethanol to furnish 2-[4-(4-chlorobenzylidene)-4,5-dihydro-5-oxo-1H-imidazol-2-ylthio]acetohydrazide **21.** This reaction has proceeded via the rupture of the endocyclic C-N bond. Compound **21** was further reacted with phenyl isothiocyanate in ethanol to give the thiosemicarbazide derivative **22**. The latter compound was cyclized with ethyl bromoacetate in the presence of anhydrous sodium acetate to yield the corresponding 2-[4-(4-chlorobenzylidene)-4,5-dihydro-5-oxo-1H-imidazol-2-ylthio]-N-[4-oxo-3-phenyl-thiazolidin-2-ylidne)acetohydrazide **23.**

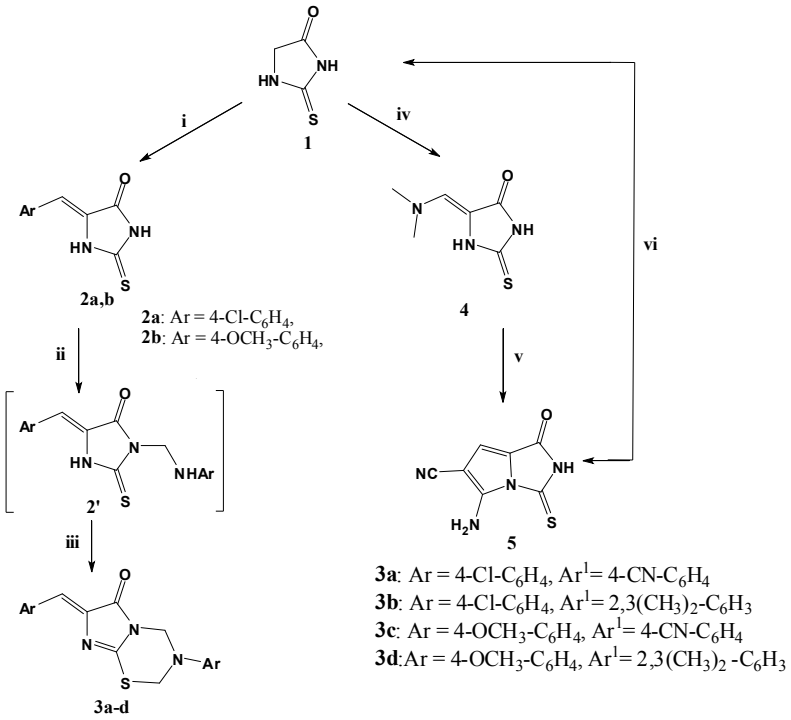
The IR spectrum of compound **21** displayed bands corresponding to imidazole and acidhydrazide NH functions at 3300, 3186 cm^{-1}, while that of compound **22** showed additional bands due to thioamidic functions at 1593, 1203, 1097 and 1033 cm^{-1}. However, the IR spectrum of compound **23** lacked the thioamidic absorption bands and displayed additional bands at 1658 cm^{-1} due to thiazolidin-C_4–carbonyl group. The ^{1}H-NMR spectrum of compound **21** revealed the presence of three deuterium oxide exchangeable singlets at δ 7.68, 7.70 and 10.44 ppm attributed to NH_2, acid hydrazide-NH and imidazole NH, respectively. In addition to a singlet at δ 4.20 ppm due to S-CH_2–CO protons. While the ^{1}H-NMR spectrum of compound **22** displayed signals at δ 7.30-7.35 ppm attributed to three NH protons and aromatic protons, in addition to the deuterium oxide exchangeable singlet at δ 11.02 ppm due to imidazole –NH. Besides to a singlet at δ 4.50 ppm corresponding to S-CH_2–CO- protons.

Moreover, the ^{1}H-NMR spectrum of **23** showed two deuterium oxide exchangeable singlets at δ 7.32 and 10.42 ppm attributed to CO-NH and imidazole NH, respectively. In addition to two singlets at δ 2.08 and 3.70 ppm corresponding to exocyclic –S-CH_2 and thiazolidin-S-$\underline{CH_2}$ protons, respectively.

Moreover, compound **12a** was condensed with 4-chlorobenzaldehyde in presence of anhydrous sodium acetate in glacial acetic acid to give the corresponding 2,6-bis(4-chlorobenzylidene)imidazo[2,1-b][1,3]thiazol-3,5-(2H, 6H)dione **24** which was further treated with hydrazine hydrate to afford 6-(4-chlorobenzylidene)-3-(4-chlorophenyl)-3,3a-dihydro-2H-imidazo[2',1':2,3][1,3]thiazolo[4,5-c]pyrazol-7(6H)-one **25** through cyclocondensation reaction.

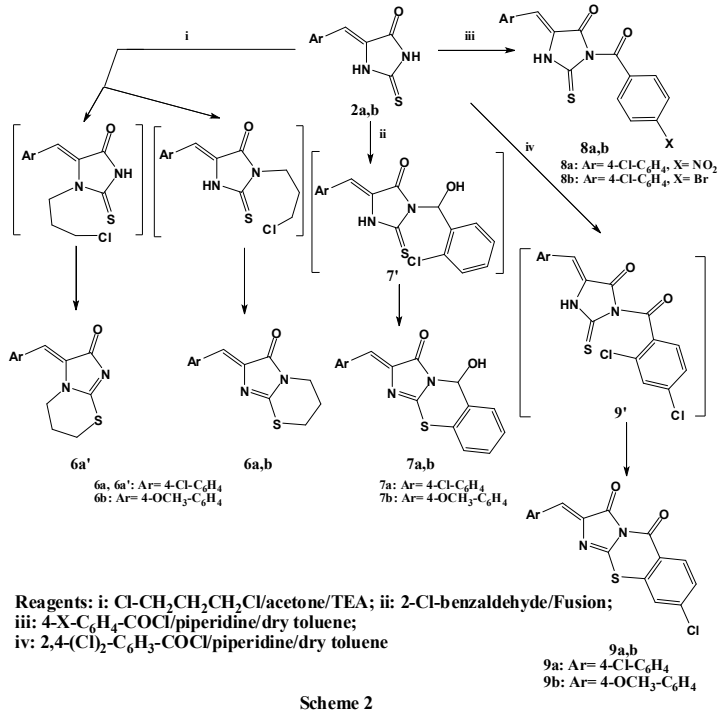
The electron impact mass spectrum of compound **24** revealed peaks at m/z 402(0.8) due to M+2 and the molecular ion peak at m/z 400(0.29), in addition to the base peak at m/z 90(100).

However, the IR spectrum of compound **25** showed absorption bands at 3421, 3224 cm^{-1} attributed to NH function. Moreover, the electron impact mass spectrum of compound **25** showed a peak at m/z 418(0.37) corresponding to (M+4) that complies with the molecular formula of the compound $C_{19}H_{12}Cl_2N_4OS$ and a base peak at m/z 57 (100).



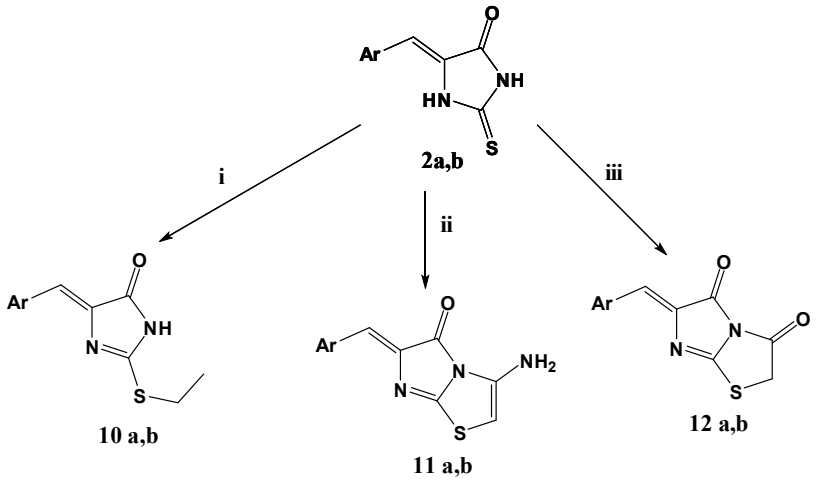
Reagents: i: Ar-CHO/CH_3COOH/CH_3COONa; ii: Ar_1NH_2/HCHO/DMF; iii: excess HCHO; v: DMF/DMA/Fusion; v: $CH_2(CN)_2$/Piperidine/CH_3CH_2OH; vi: DMF/DMA/$CH_2(CN)_2$/Piperidine/CH_3CH_2OH.

Scheme 1



Reagents: i: Cl-$CH_2CH_2CH_2Cl$/acetone/TEA; ii: 2-Cl-benzaldehyde/Fusion; iii: 4-X-C_6H_4-COCl/piperidine/dry toluene; iv: 2,4-$(Cl)_2$-C_6H_3-COCl/piperidine/dry toluene

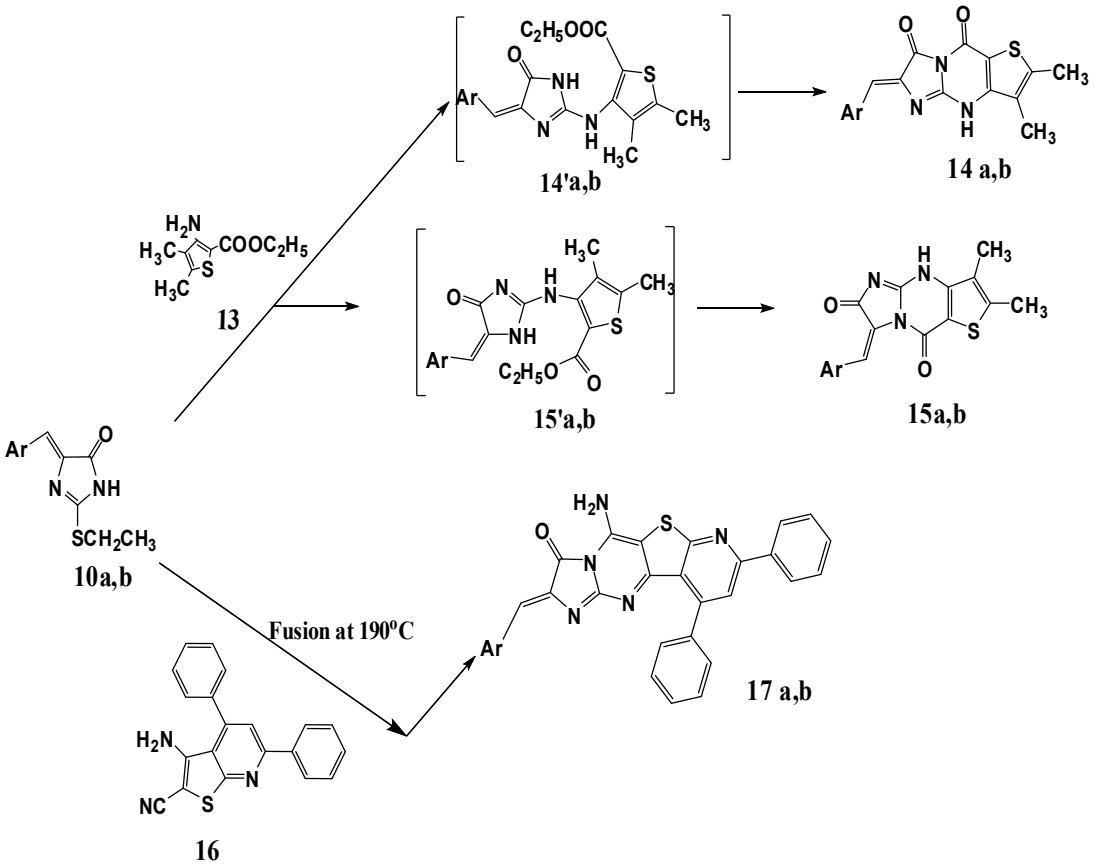
Scheme 2



a: Ar= 4- Cl-C_6H_4; b: Ar= 4-OCH_3-C_6H_4

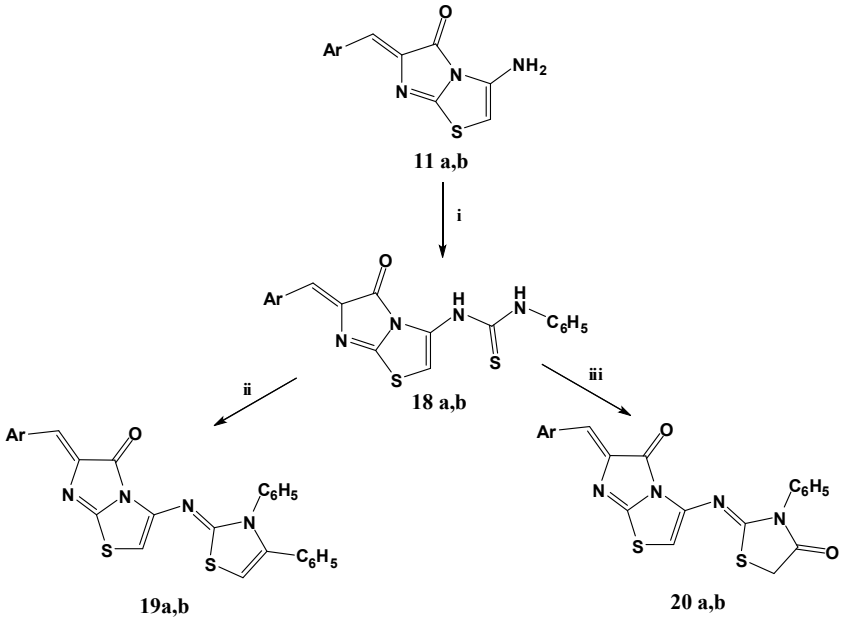
Reagents: i: CH_3CH_2I/ Na^+ $O^-C_2H_5$; ii: $ClCH_2CN$/ Na OC_2H_5 iii: $BrCH_2COOCH_3$/ $Na^+O^-C_2H_5$

Scheme 3



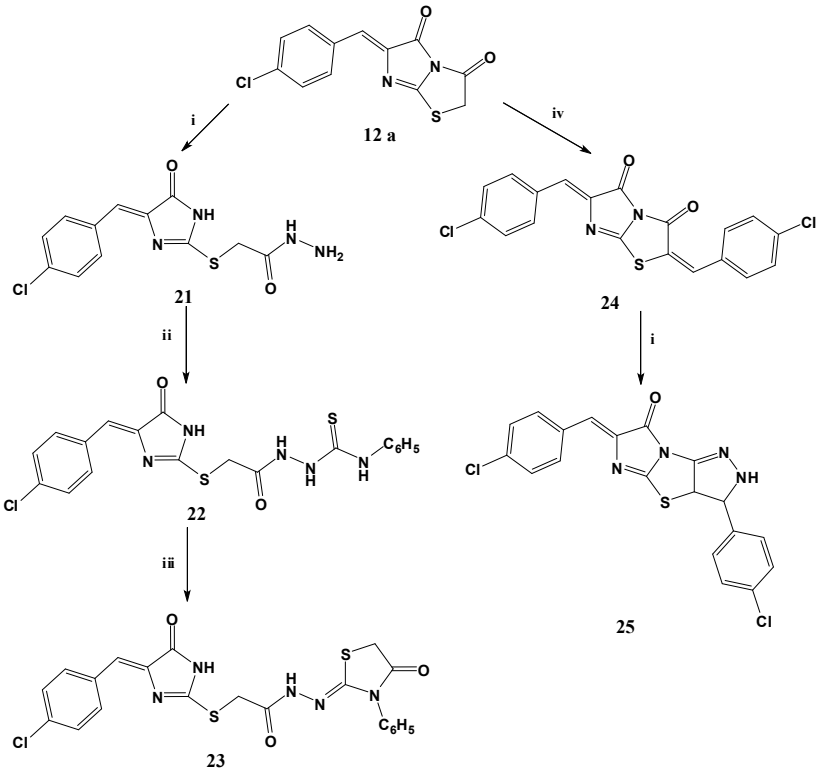
a : Ar = 4-Cl-C_6H_4 , b : Ar = 4-OCH_3-C_6H_4

Scheme 4



Reagents: i: C_6H_5NCS/ Benzene; ii: $C_6H_5COCH_2Br$/ TEA/ C_2H_5OH; iii: $ClCH_2COOH$/ CH_3COO Na/ CH_3COOH

Scheme 5



Reagents: i: NH_2NH_2/C_2H_5OH; ii: C_6H_5NCS/ pyridine; iii: $BrCH_2COOC_2H_5$/ C_2H_5OH;
iv: 4-Cl-C_6H_4-CHO/ CH_3COOH/ CH_3COO Na

Scheme 6

3.Experimental:

All melting points were measured on Electro thermal LA 9000 SERIS, Digital Melting point Apparatus and are uncorrected. IR spectra (KBr) were recorded on FT-IR 5300 spectrophotometer and Perkin Elmer spectrum RXIFT-IR system (ν, cm^{-1}). ^{1}H-NMR spectra were recorded in (DMSO-d_6) at 300 MHz on a Varian Gemini NMR spectrometer (δ, ppm) using TMS as an internal standard. Mass spectra were recorded on GC Ms-QP 1000 EX mass spectrometer at 70 eV. Microanalytical data were performed in Micro analytical Research Center, Cairo University. Thin layer chromatography was performed on pre-coated (0.25mm) silica gel GF_{254} plates (E. Merck, Germany). Compounds were detected with 254 nm UV lamp.

*General procedure for the synthesis of compounds **3a-d:***

To an equimolar mixture of compound **2a,b** (2.9 mmol) and the appropriate aromatic amine (2.9 mmol) in DMF (5 ml) an excess amount of 37% formaline solution (3ml) was added. The reaction mixture was refluxed for 30 min with vigorous stirring and then stirred at 20°C for 22h. Compounds **(3a-d)** were obtained as crystals which were filtered off and recrystallized from the suitable solvent.

*7-(4-Chlorobenzylidene)-3-(4-cyanophenyl)-3,4-dihydro-2H-imidazo[2,1-b][1,3,5] thiadiazin-6(7H)-one; **3a**.*

Red powder; recrystallized from EtOH; m.p. 215-217°C, yield 80%. **IR** (KBr, cm^{-1}): 3070 (CH–Ar); 2869 (CH-aliph.); 2210 (C≡N); 1735 (C=O); 1654 (C=N). **MS**: m/z(%): 381(M+1,0.01); 380($M^{+\cdot}$,0.02); 365(0.03); 358(0.02); 347(0.03); 345(0.03); 320(0.07); 318(0.03); 308(0.09); 307(0.09); 287(0.1); 285(0.08); 269(0.1); 268(0.13); 267(0.13); 266(0.14) ;265(0.11); 258(0.12); 256(0.12); 241(0.28); 240(0.27); 239(0.33); 238(0.18); 236(0.11); 229(0.24); 228(0.23); 227(0.21); 216(0.18); 215(0.27); 214(0.27); 213(0.26); 206(0.13); 204(0.43); 203(0.49); 201(0.41); 192(0.16); 190(0.27); 175(0.3); 167(0.23); 157(0.3); 155(0.68); 153(0.78); 152(0.98); 150(0.82); 148(0.31); 130(3.1); 129(3.32); 128(1.75); 120(0.98); 119(3.03); 114(0.96); 103(4.70); 102(5.98); 101(3.55); 100(1.53); 91(2.96); 89(3.35); 88(4.24); 87(9.06); 86(7.14); 78(2.19); 77(4.62); 76(5.66); 64(11.28); 63(13.52); 61(100); 53(71.84).**Anal. Form** : $C_{19}H_{13}ClN_4OS$. **Calcd.** (%): C, 59.92; H, 3.44; N, 14.71 **Found:** (%) C, 59.98; H, 3.47; N, 14.83.

*7-(4-Chlorobenzylidene)-3-(2,3-dimethylphenyl)-3,4-dihydro-2H-imidazo[2,1-b] [1,3,5]thiadiazin-6(7H)-one; **3b***

Yellow powder; recrystallized from EtOH; m.p. 213-215; yield 87%. **IR** (KBr, cm^{-1}): 3074 (CH–Ar); 2931, 2869 (CH-aliph.); 1739 (C=O); 1658 (C=N). **Anal. form**: $C_{20}H_{18}ClN_3OS$ **Calcd.** (%): C, 62.57; H, 4.73; N, 10.95. **Found** (%): C, 62.63; H, 4.78; N, 11.04.

*3-(4-Cyanophenyl)-7-(4-methoxybenzylidene)-3,4-dihydro-2H-imidazo[2,1-b][1,3,5] thiadiazin-6(7H)-one; **3c.***

Red powder; recrystallized from EtOH; m.p. 201-203; yield, 85%. **IR** (KBr, cm^{-1}): 3043 (CH–Ar); 2927, 2854 (CH–aliph.); 2218(C≡N); 1728 (C=O); 1596 (C=N); 1249, 1018 (C–O–C). **^{1}H-NMR** (DMSO-d_6-δppm): 3.87 (s, 3H, OCH_3); 5.44 (s, 2H, N–CH_2–N); 5.96 (s, 2H, S–CH_2–N); 6.74 (s, 1H, =CH); 6.99–7.01 (m, 4H, 4–OCH_3–C_6H_4); 7.38 (d, 2H, J= 8.9 Hz, 4–CN–C_6H_4–$C_{2,6}$–H); 7.47(d, 2H, J= 8.9 Hz, 4–CN-C_6H_4–$C_{3,5}$–H). **Anal. form**: $C_{20}H_{16}N_4O_2S$ **Calcd.** (%): C, 63.81; H, 4.28; N, 14.88. **Found** (%): C, 63.89; H, 4.32; N, 15.02.

*7-(4-Methoxybenzylidene)-3,4-dihydro-3-(2,3-dimethylphenyl)-2H-imidazo[2,1-b] [1,3,5]thiadiazin-6(7H)-one; **3d***

Yellow powder; recrystallized from EtOH; m.p. 223-225°C; yield 90%. **IR** (KBr, cm^{-1}): 3080 (CH–Ar); 2926, 2858 (CH–aliph.); 1720 (C=O); 1642 (C=N); 1290, 1000 (C–O–C). **^{1}H-NMR** (DMSO-d_6–δppm): 2.89 (s, 3H, phenyl–C_3-CH_3); 2.96 (s, 3H, phenyl–C_2–CH_3); 3.87 (s, 3H, OCH_3); 4.90 (s, 2H, N–CH_2–N); 5.42 (s,2H, S–CH_2-N); 6.76 (s, 1H, =CH); 6.94-6.96 (m, 3H, 2,3-$(CH_3)_2C_6H_4$–$C_{4,5,6,}$–H); 7.38 (d, 2H, J= 8.7Hz, 4-OCH_3-C_6H_4–$C_{3,5}$–H); 7.43 (d, 2H, J = 8.7Hz, 4–OCH_3–C_6H_4–$C_{2,6}$–H). **MS**: m/z(%): 379($M^{\cdot+}$, 50.31); 351(45.71); 345(46.75); 334(41.09); 308(41.33); 301(48.32); 285(74.72); 273(77.06); 248(88.82); 231 (67.14); 203(49.76); 173(66.78); 170(55.77); 131(47.17); 128(51.84); 108(44.43); 85(53.32); 69(44.97); 63(46.78); 54(40.24); 51(60.85); 50(100). **Anal. form**: $C_{21}H_{21}N_3O_2S$ **Calcd.** (%): N, 11.08. **Found** (%): N, 11.21.

*Synthesis of 5-[N,N-dimethylaminomethylene]-2-thioxoimidazolidin-4-one; **4** and 5-amino-2,3-dihyro-1-oxo-3-thioxo-1H-pyrrolo[1,2-e]imidazole-6-carbonitrile; **5.***

Method A:

Step 1:

An equimolar mixture of compound **1** (10 mmol) and DMF/DMA (1.06g, 1.48 mL, 10 mmol) was fused at 180°C for 6h. Then the solid product was collected, washed with EtOH and recrystallized from DMF to yield black crystals of compound **4** in 80% yield.

Step 2:

A mixture of compound **4** (10 mmol) and malononitrile (0.66g, 10 mmol) was refluxed in EtOH (20 mL) in the presence of pipridine (0.5 mL) as catalyst for 12h. The solvent was then evaporated

under reduced pressure and the solid product was collected and recrystallized from EtOH. Yield, 60%.

Method B:

A one pot reaction was carried out by refluxing an equimolar mixture of compound **1** and malononitrile (0.66g, 10 mmol) in (30 mL) of DMF/DMA (2:1) and in presence of a catalytic amount of piperidine. Reflux was allowed for 12hrs. and then the reaction was cooled, the solid product was collected and recrystallized from EtOH. Yield, 40%.

5-[N,N-Dimethylaminomethylene]-2-thioxoimidazolidin-4-one; ***4***.

Black powders; recrystallized from DMF; m.p.>360°C [42]; yield 80%. **IR** (KBr, cm^{-1}): 3398, 3300 (NH); 2928 (CH–aliph.); 1680 (C=O); 1398 (C=S).

5-Amino-2,3-dihydro-1-oxo-3-thioxo-1H-pyrrolo[1,2-e] imidazole-6-carbonitrile; ***5***.

Brownish black, crystals; recrystallized from EtOH; m.p. 358-360°C; yield 60%. **IR** (KBr, cm^{-1}): 3305, 3117 (NH, NH_2); 2207 (C≡N); 1694 (C=O); 1583 (C=N); 1359 (C=S). **MS:** m/z(%): 192(M^{+}, 5.99); 150(14.68); 149(10.88); 139(9.25); 128(10.09); 98(10.2); 91(10.24); 85(11.98); 84(17.81), 78(15.83); 77(14.93); 71 (20.97); 69(23.76), 68(35.14); 65(18.09); 57(44.44); 56 (100); 55(89.49); 53(32); 50(50.92). **Anal.form**: $C_7H_4N_4OS$ **Calcd**. (%): C, 43.74; H, 2.10; N, 29.15. **Found** (%): C, 43.75; H, 2.13; N, 29.23.

General procedure for the synthesis of compounds ***6a,b*** *and* ***6a'****:*

Compound **2a,b** (10 mmol) was refluxed with 1,3-dichloropropane (1.12 g, 0.94 mL, 10 mmol) in dry acetone (20 mL) as solvent and in presence of few drops of triethylamine (2 drops). Reflux was carried out for 50hrs., then the reaction mixture was cooled and the solid precipitate was collected and recrystallized from EtOH.

2-(4-Chlorobenzylidene)-6,7-dihydro-2H-imidazo[2,1-b][1,3]thiazin-3(5H)-one; ***6a***.

Yellow crystals; recrystallized by EtOH; m.p. 200-201 [43]; yield 77%. **IR**(KBr, cm^{-1}): 3058 (CH-Ar); 2916, 2828 (CH-aliph.); 1710 (C=O); 1632 (C=N). **^{1}H-NMR** (DMSO-d_6-δppm): 2.20–2.38 (m, 2H, perhydrothiazine–C_3–H); 3.10–3.20 (m, 2H, Perhydrothiazine-C_4–H); 3.70–3.80 (m, 2H, perhydrothiazine-C_2–H); 6.89 (s, 1H, =CH); 7.36 (d, 2H, J= 8.4 Hz, 4-Cl–C_6H_4–$C_{2,6}$–H); 8.04 (d, 2H, J = 8.4Hz, 4–Cl–C_6H_4–$C_{3,5}$–H). **MS:** m/z (%): 280(M+2 ,3.57); 278(M^{+},12.5); 237(10.71); 178(7.74); 152(10.12); 150(100); 123(39.29); 115(7.14); 114(22.02); 99(20.83); 87(26.79); 85(22.02); 76(4.17); 75(18.45); 73(15.48); 63(67.86); 62(79.76). **Anal. form**: $C_{13}H_{11}ClN_2OS$ **Calcd.** (%): C, 56.01; H, 3.98; N, 10.05. **Found** (%): C, 56.04; H, 4.02; N, 10.13.

2-(4-Methoxybenzylidene)-6,7-dihydro-2H-imidazo[2,1-b][1,3]thiazin-3(5H)-one; ***6b***

Yellowish orange crystals; recrystallized from EtOH; m.p. 243-245°C [43]; yield 65%. **IR**(KBr, cm^{-1}): 3020 (CH-Ar); 2927 (CH–aliph); 1701 (C=O); 1589 (C=N); 1245, 1018 (C–O–C). H). **Anal. Form**: $C_{14}H_{14}N_2O_2S$ **Calcd**. (%): C, 61.29; H, 5.14. **Found** (%): C, 61.38; H, 5.14.

3-(4-Chlorobenzylidene)-3,5,6,7-tetrahydro-2H-imidazo[2,1-b][1,3]thiazin-2-one; ***6'a***

Yellow crystals; insoluble in EtOH; m.p. 240-242 °C; yield 12%. **IR**(KBr, cm^{-1}): 3080 (CH–Ar); 2920 (CH–aliph.); 1710 (C=O); 1632 (C=N). **MS:** m/z (%): 280(M+2,1.62); 278(M^{+},2.6); 276(2.6) 271(2.6); 243(3.9); 219(2.6); 216(2.92); 214(3.57); 210(4.55); 199(4.87); 190(3.57); 175(2.6); 161(4.55); 154(3.57); 153(3.25); 150(4.55); 145(3.57); 140(3.9); 134(3.9); 120(5.19); 110(6.49); 104(6.17); 102(3.57); 94(4.22); 93(5.84); 89(7.47); 87(5.52); 83(5.52); 79(9.42); 77(15.58); 75(11.04); 70(30.19); 69(34.42); 66(24.35); 52(100). **Anal. form:** $C_{13}H_{11}ClN_2OS$ **Calcd**. (%): C, 56.01; H, 3.98; N, 10.05 **Found**(%): C, 56.05; H, 3.99; N, 10.12.

Synthesis of 2-(4-substitutedbenzylidene)-5-hydroxy-2H-imidazo[2,1-b][1,3] benzothiazin-3(5H)-ones; ***7a,b****:*

An equimolar mixture of compound **2a,b** (2 mmol) and o-chlorobenzaldehyde (0.28 g, 0.22 mL, 2 mmol) was fused at 180°C for 15-30hrs. The solid product was collected, washed with EtOH and recrystalized from EtOH.

2-(4-Chlorobenzylidene)-5-hydroxy-2H-imidazo[2,1-b][1,3]benzothiazin-3(5H)-one; ***7a.***

White crystals; recrystallized from EtOH; m.p. 158-160°C; yield 97%**.** **IR** (KBr, cm^{-1}): 3494, 3394 (OH broad band); 3075 (CH–Ar); 2900, 2869 (CH–aliph.); 1681 (C=O); 1604 (C=N). **MS:** m/z (%): 344**(**M+2, 0.04); 342(M^{+},0.06**)**; 333(14.56); 331(0.57); 317(16.5); 316(5.5); 315(0.05); 313(4.2); 311(0.02); 305(2.36); 293(3.85); 291(0.19); 287(14.31); 274(7.69); 265(10.4); 258(14.32) 251(2.27); 237(6.51); 216(2.18); 204(15.1); 202(5.02); 201(22.66); 190(14.85); 189(3.84); 177(6.42); 163(4.25); 159(4.64); 156(47.94); 155(9.99); 141(30.05); 139(100); 138(3.09); 113(11.7); 112(9.35); 111(6.98); 99(3.55); 89(4.9); 87(15.38); 76(33.48); 66(52.86); 63(5.74); 62(6.02); 55(3.97); 51(56.39). **Anal.form**: $C_{17}H_{11}ClN_2O_2S$ **Calcd.** (%): C, 59.56; H, 3.23; N, 8.17. **Found** (%): C, 59.62; H, 3.21; N, 8.29.

5-Hydroxy-2(4-methoxybenzylidene)-2H-imidazo[2,1-b][1,3]-benzothiazin-3(5H)-one; ***7b***

White crystals; recrystallized from EtOH; m.p. 142-144°C; yield 92%. **IR** (KBr, cm^{-1}): 3475, 3402 (OH broad band); 3060 (CH–Ar); 2890 (CH–aliph.); 1688 (C=O); 1590 (C=N); 1250, 1044 (C-O-C). **^{1}H-NMR** (DMSO-d_6-δppm): 3.20–3.40 (m, 4H, OCH_3, CH-OH); 7.34 (s, 1H, =CH); 7.35–7.43 (m, 4H, C_6H_4–H); 7.44–7.53 (m, 2H, 4-OCH_3-C_6H_4–$C_{3,5}$–H); 7.77 (d, 2H, J = 7.8Hz, 4–OCH_3–C_6H_4–$C_{2,6}$–H); 13.38 (s, 1H, OH, D_2O exchangeable). **Anal.form**: $C_{18}H_{14}N_2O_3S$ **Calcd**. (%): C, 63.89; H, 4.17; N, 8.28 **Found** (%): C, 63.92; H, 4.18; N, 8.36.

*General procedure for the synthesis of compounds **8a,b** and **9a,b**:*

Compound **2a, b** (8 mmol) was refluxed with the appropriate benzoyl chloride (12 mmol) in 15 mL of anhydrous toluene for 48 h. The reaction mixture was then cooled and the obtained product was collected and recrystallized from the suitable solvent.

*4-(4-Chlorobenzylidene)-1-(4-nitrophenylcarbonyl)-2-thioxo-5-oxo-2,3,4,5-tetrahydro -1H-imidazole; **8a.***

Greenish grey crystals; recrystallized from EtOH; m.p. 205-207; yield 90%. **IR** (KBr, cm^{-1}): 3363, 3305 (NH); 3075 (CH-Ar); 2939 (CH–aliph.); 1712, 1650 (two C=O); 1589, 1353 (NO_2); 1450, 1261, 1172, 1022 (I, II, III, IV bands of N–C=S). **Anal form**: $C_{17}H_{10}ClN_3O_4S$ **Calcd**. (%): C, 52.65; H, 2.60; N, 10.84. **Found** (%): C, 52.66; H, 2.58; N, 10.91.

*1-(4-Bromophenylcarbonyl)-4-(4-chlorobenzylidene)-2-thioxo-5-oxo-2,3,4,5-tetrahydro-1H-imidazole; **8b***

Yellow powder; recrystallized from ethyl acetate; m.p. 235-237°C; yield 76%. **IR** (KBr, cm^{-1}): 3433, 3363 (NH); 3136 (CH-Ar); 2927 (CH–aliph.); 1712 (C=O); 1647 (C=N); 1589, 1261, 1190, 1099 (I, II, III, IV bands of N–C=S). **Anal form**: $C_{17}H_{10}ClBrN_2O_2S$ **Calcd**. (%): C, 48.42; H, 2.39; N, 6.64. **Found** (%): C, 48.47; H, 2.41; N, 6.71.

*8-Chloro-2-(4-chlorobenzylidene)-2H-benzo[e]imidazo[2,1-b][1,3]thiazine-3,5-dione; **9a***

Yellowish orange crystals; recrystallized from EtOH; m.p. 234-235°C; yield 85%**. IR** (KBr, cm^{-1}): 3126, 3048 (CH-Ar), 1712, 1652 (two C=O); 1590 (C=N). **MS:** m/z (%): 376(M+2, 8.47); 374(M^+, 1.83); 360(5.06); 325(7.2); 323(5.47); 307(5.72) 295(7.83) 294(12.08); 271(8.23); 266(7.83); 264(6.92); 244(5.76); 241(6.61); 217(5.73); 215(6.87); 201(10.92); 187(10.89); 177(10.77); 171(8.55); 159(7.17); 158(11.41); 152(12.65); 151(14.74); 140(15.32); 137(5.92); 131(9.84); 123(6.6); 122(8.59); 114(7.34); 113(6.41); 92(18.23); 91(17.03); 79(8.85); 77(98.86); 76 (25.28); 75(23); 74(32.87); 73(32.64); 65(44.77); 63(55.53); 62(61.34); 61(34.09); 55(34.08); 54(25.1); 52(56.58); 51(44.8); 50(100); 49(85.63). **Anal. form**: $C_{17}H_8Cl_2N_2O_2S$ **Calcd.**: C, 54.42; H, 2.15. **Found** (%): C, 54.62; H, 2.11.

*8-Chloro-2-(4-methoxybenzylidene)-2H-benzo[e]imidazo[2,1-b][1,3]thiazine-3,5-dione; **9b***

Yellowish brown powder; recrystallized from acetone; m.p. 155-157°C; yield 56%. **IR** (KBr, cm^{-1}): 3010 (CH-Ar.); 1693 (C=O); 1589 (C=N); 1257, 1045 (C–O–C). **MS**: m/z (%): 370(M^+, 6.01); 327(4.77); 192(5.44); 191(4.69); 177(4.67); 176(18.03); 175(12.24); 174(28.18); 173(42.29); 147(4.22); 145(4.83); 138(5.21); 135(5.44); 133(5.67); 132(6.87); 111(4.79); 110(8.4); 100(5.45); 98(9.18); 96(5.69); 85(8.43); 83(11.18); 78(5.33); 76(8.69); 75(14.12); 74(17); 73(27.56); 72(20.05); 66(8.04); 65(21.37); 64(19.06); 63(34.65); 62(66.16); 61(20.55); 55(40.87); 54(37.25); 52(100); 51(98.24). **Anal. form :** $C_{18}H_{11}ClN_2O_3S$ **Calcd.** (%): C, 58.30; H, 2.99; N, 7.55 **Found** (%)**:** C, 58.32; H, 3.04; N, 7.63.

*Synthesis of 4-(4-substitutedbenzylidene)-2-ethylthio-1H-imidazol-5(4H)–ones **10a,b**:*

A solution of compound **2a,b** (10 mmol) in absolute ethanol (50 mL) confining sodium ethoxide [prepared from 0.23 g, 10 mmol atom sodium] were treated with ethyl iodide (1.56 g, 0.8 mL, 10 mmol). The reaction mixture was stirred for 3h. and left overnight at room temperature, the solid obtained was filtered off, washed with water and recrystallized from EtOH to give the target compounds.

*4-(4-Chlorobenzylidene)-2-ethylthio-1H-imidazol-5(4H)–one; **10a***

Yellow crystals; recrystallized from EtOH; m.p. 218-220°C; yield 85%. **IR:** (KBr, cm^{-1}): 3131 (NH); 3050 (CH-Ar); 2882, 2820 (CH-aliph.); 1711 (C=O); 1643 (C=N). **^{1}H-NMR** (DMSO-d_6-δppm): 1.37 (t, 3H, J=7.2Hz, SCH_2CH_3); 3.22 (q, 2H, J=7.2Hz, SCH_2CH_3); 6.61 (s, 1H, =CH); 7.44 (d, 2H, J= 8.5Hz, 4-Cl–C_6H_4–$C_{2,6}$-H); 8.12 (d, 2H, J= 8.5Hz, 4-Cl–C_6H_4–$C_{3,5}$–H); 11.81 (s, 1H, imidazole NH, D_2O exchangeable). **Anal. Form:** $C_{12}H_{11}ClN_2OS$ **Calcd.** (%): C, 54.03; H, 4.16; N, 10.50. **Found** (%): C, 54.09; H, 4.15 ; N, 10.61 .

*2-Ethylthio-4-(4-methoxy benzylidene)-1H-imidazol-5(4H)-one; **10b***

Yellow crystals; recrystallized from EtOH; m.p. 163-165°C [44]; yield 93%. **IR:** (KBr, cm^{-1}): 3303, 3143 (NH); 3068 (CH-Ar); 2907,2802 (CH-aliph.); 1700 (C=O); 1605 (C=N) ; 1256,1031 (C–O–C) .

*General procedure for the synthesis of compounds **11a,b** & **12a,b**:*

Compound **2a,b** (5 mmol) was dissolved in 10 mL 8.5% Na ethoxide, this solution was added to (2.25 g, 1.88 mL, 30 mmol) of chloroacetonitrile or (4.59 g, 2.284 mL, 30 mmol) methyl bromoacetate and refluxed for 12h. The reaction mixture was then poured onto crushed ice to yield crystalline precipitate

which was filtered off, washed with a large amount of water and recrystalized from the appropriate solvent.

3-Amino-6-(4-chlorobenzylidene)imidazo[2,1-b]thiazol-5(6H)-one; ***11a.***

Yellowish grey powder; recrystallized from EtOH; m.p. 232-234°C; yield 80%. **IR**(KBr, cm^{-1}): 3224 (NH_2); 1724 (C=O); 1646 (C=N); 1589 (C=C). **^{1}H-NMR** (DMSO-d_6-δppm): 6.40 (s, 1H, thiazole-CH); 6.87 (s,1H, =CH); 7.63 (d, 2H, J = 8.5 Hz, 4-Cl–C_6H_4–$C_{2,6}$–H); 7.76 (d, 2H, J=8.5 Hz, 4-Cl-C_6H_4–$C_{3,5}$–H); 10.61 (s, 2H, NH_2, D_2O exchangeable). **Anal.form**: $C_{12}H_8ClN_3OS$ **Calcd**. (%): C, 51.90; H, 2.90; N, 15.13**.** **Found**(%): C,51.88; H, 2.93; N, 15.21.

3-Amino-6-(4-methoxybenzylidene)imidazo[2,1-b]thiazol-5(6H)-one; ***11b.***

Orange powder; recrystallized from ethyl acetate; m.p. 205-207°C; yield 70%. **IR**(KBr, cm^{-1}): 3130 (NH_2); 3064 (CH-Ar); 1697 (C=O); 1595 (C=N); 1516 (C=C); 1260, 1030 (C–O–C)**. MS**: m/z (%): 273(M^+,7.19); 271(6.51); 257(6.11); 242(8.28); 239(12.21); 229(8.96); 228(8.14); 219(8.41); 215(7.46); 213(6.24); 211(7.19); 208(7.33); 207(6.51); 205(6.24); 204(8.41); 194(6.65); 191(7.33); 190(9.36); 188(7.46); 186(9.77); 180(6.11); 178(8.41); 176(13.57); 173(18.32); 172(16.55); 171(11.94); 149(13.16); 137(10.72); 133(15.2); 131(30.94); 130(26.32); 117(26.32); 116(21.3); 104(16.01); 101(18.32); 87(28.9); 78(30.12); 76(28.49); 72(45.05); 64(24.05); 62(45.86); 61(54.14); 56(69.47); 55(73.54); 53(100). **Anal.form:** $C_{13}H_{11}N_3O_2S$ **Calcd.** (%)**:** C, 57.13; H, 4.06; N, 15.37 **Found**(%): C, 57.17; H, 4.11; N, 15.42.

6-(4-Chlorobenzylidene)imidazo[2,1-b]thiazole-3,5-(2H, 6H)-dione; ***12a.***

Yellowish white powder; recrystallized from EtOH; m.p. 129-130°C [45]; yield 92%. **IR** (KBr, cm^{-1}): 3055 (CH-Ar); 2986, 2940 (CH-aliph.); 1742 (two C=O); 1640 (C=N). **^{1}H-NMR** (DMSO-d_6-δppm): 4.49 (s, 2H, thiazole–CH_2); 6.96 (s, 1H, =CH); 7.48 (d, 2H, J=8.8Hz, 4-Cl-C_6H_4–$C_{2,6}$–H); 8.19 (d, 2H, J = 8.8Hz, 4-Cl-C_6H_4–$C_{3,5}$–H)**.** **Anal. Form:** $C_{12}H_7ClN_2O_2S$ **Calcd.** (%): C, 51.71; H, 2.53; N, 10.05 **Found** (%). C, 51.73; H, 2.57; N, 10.18.

6-(4-Methoxybenzylidene)imidazo[2,1-b]thiazole-3,5(2H, 6H)-dione; ***12b.***

Yellow crystals; recrystallized from EtOH; m.p. 116-118°C; yield 80%. **IR** (KBr, cm^{-1}): 3078 (CH-Ar); 2981, 2927 (CH-aliph.); 1735 (C=O); 1693 (C=N); 1242, 1029 (C–O–C). **MS**: m/z (%): 274(M^+,0.05); 273(0.02); 260(0.02); 258(0.01); 243(0.02); 239(0.03); 227(0.05); 225(0.02); 213(0.02); 211(0.02); 205(0.04); 200(0.05); 195(0.02); 192(0.13); 182(0.03); 180(0.05); 171(0.01); 167(0.11); 156(100); 147(2.09); 146(1.7); 145(0.38); 140(0.13); 116(3.38); 105(0.9); 103(12.15); 93(0.8); 89(67.79); 86(1.88); 78(3.08); 76(1.77); 64(11.8); 59(13.54); 55(4.11). **Anal. form**: $C_{13}H_{10}N_2O_3S$ **Calcd.**(%): C, 56.92; H, 3.67; N, 10.21. **Found** (%): C, 57.01; H, 3.72; N, 10.29.

General procedure for the synthesis of compounds ***14a,b*** *&* ***15a,b****:*

Equimolar amounts of compound **10a,b** (5 mmol) and compound **13** [40] (0.99 g, 5 mmol) were refluxed in glacial acetic acid (6 mL) for 12h. The reaction mixture was allowed to cool and the obtained precipitate was filtered off to yield compounds **14a,b.** While the filtrate was poured onto crushed ice, filtered, washed with water to give compounds **15 a,b.**

2-(4-Chlorobenzylidene)-6,7-dimethylthieno[3',2':4,5]pyrimido[1,2-a]imidazole-3,5(2H, 9H)-dione*;* ***14a.***

Orange powder; recrystallized from EtOH; m.p.>300°C; yield 35%. **IR**(KBr, cm^{-1}): 3410, 3208 (NH); 3050 (CH–Ar); 1710 (C=O imidazole); 1662 (C=O thiazine); 1598 (C=N). **MS:** m/z(%): 359(M+2, 20.02); 357($M^{\cdot+}$, 8.3); 344(33.31); 335(33); 324(31.21); 322(36.8); 286(40.94); 247(22.4); 221(19.88); 216(34.46); 209(26.92); 179(20.82); 166(30.91); 160(26.04); 154(22.02); 153(33.18); 152(21.54); 151(21.71); 150(36.45); 148(96.88); 139(63.79); 128(44.88); 118(23.12); 112(52.38); 102(31.57); 100(57.36); 89(25.24); 86(62.82); 83(20.83); 79(28.39); 75(54.13); 74(60.99); 72(43.13); 70(49.97); 59(91.15); 54(72.73); 52(73.41); 49(100%); 48(42.16). **Anal.form**: $C_{17}H_{12}ClN_3O_2S$ **Calcd.** (%):N, 11.74; S, 8.96**.** **Found**(%): N, 11.83; S, 9.04.

6,7-Dimethyl-2-(4-methoxybenzylidene)thieno[3',2':4,5] pyrimido[1,2-a]imidazole-3,5(2H, 9H)-dione*;* ***14b.***

Yellowish brown powder; recrystallized form EtOH; m.p.> 300°C; yield 33%**. IR** (KBr, cm^{-1}): 3375, 3298 (NH); 3080 (CH–Ar); 2947 (CH–aliph.); 1666(C=O); 1593 (C=N); 1249, 1022 (C–O–C). **Anal.form**: $C_{18}H_{15}N_3O_3S$ **Calcd.**(%): N, 11.89; S, 9.07. **Found** (%): N, 12.04; S, 9.14.

3-(4-Chlorobenzylidene)-6,7-dimethylthieno[3',2':4,5]pyrimido [1,2-a] imidazole-2,5(3H, 9H)-dione*;* ***15a***

Greyish brown powder; recrystallized from EtOH; m.p. 278-280°C; yield 45%**.** **IR**(KBr, cm^{-1}): 3220 (NH); 3100 (CH–Ar); 2974 (CH-aliph.); 1720 (C=O imidazole); 1656 (C=O thiazine); 1560 (C=N). **MS:** m/z(%): 359(M+2, 6.7); 357($M^{\cdot+}$, 9.72); 344(9.29); 328(8.42); 321(5.18); 319(5.83); 304(9.07); 301(2.81); 299(5.4); 275(4.75); 273(4.1); 272(3.89); 270(4.75); 263(5.83); 261(2.81); 257(5.18); 255(3.02); 247(5.62); 245(5.62); 242(7.34); 240(6.7); 237(8.64); 236(5.62); 235(7.99);

230(10.37); 222(10.58); 218(3.46); 216(4.1); 204(9.5); 198(3.67); 197(13.39); 196(9.29); 193(8.42); 189(4.75); 187(4.32); 180(6.91); 176(3.46); 174(9.72); 169(10.8); 153(12.31); 144(5.62); 130(8.42); 127(11.88); 121(5.18); 115(15.77); 112(6.48); 110(7.34); 101(10.8); 90(11.02); 84(12.74); 77(4.97); 76(13.17); 75(12.1); 72(12.53); 70(19.22); 67(17.93); 63(20.73); 60(22.46); 59(100); 58(80.99); 57(37.37); 53(23.97); 50(29.59).**Anal.Form:** $C_{17}H_{12}ClN_3O_2S$ **Calcd**.(%): C, 57.06; H, 3.36; N, 11.74; S, 8.96. **Found**(%): C,57.11; H, 3.42; N, 11.81; S, 9.04.

6,7-Dimethyl-3-(4-methoxybenzylidene)thieno[3',2':4,5]pyrimido[1,2-a]imidazole-2,5(3H, 9H)-dione*; **15b.***

Pale brownish powder; recrystallized from EtOH; m.p. 268-270°C; yield 47%**. IR**(KBr, cm^{-1}): 3380, 3213 (NH); 3082 (CH–Ar); 2931 (CH–aliph.); 1710, 1658 (C=O); 1596 (C=N); 1245, 1014 (C–O–C)*.* **^{1}H-NMR** (DMSO-d_6–δppm): 2.28 (s, 3H, thienyl - C_3–H); 2.32 (s, 3H, thienyl–C_2–H); 3.82 (s, 1H, OCH_3); 4.98 (s, 1H, NH_2, D_2O exchangeable); 6.72 (s, 1H, =CH); 7.73 (d, 2H, J= 8.7Hz, 4–OCH_3–C_6H_4–$C_{3,5}$–H); 8.15 (d, 2H, J=8.7Hz, 4-OCH_3–C_6H_4–$C_{2,6}$–H). **Anal.form**: $C_{18}H_{15}N_3O_3S$ **Calcd.**(%): C, 61.18; H, 4.28; N, 11.89; S, 9.05. **Found** (%): C, 61.24; H, 4.23; N, 12.02; S, 9.12.

*Synthesis of 5-amino-8,10-diphenyl-2-(4-substitutedbenzylidene)pyrido[3',2':4,5] thieno[3,2-d]imidazo[1,2-a]pyrimidin-3(2H)–ones; **17 a,b***

An equimolar mixture of compound **10 a,b** (5 mmol) and compound **16** (1.63 g , 5 mmol) [41] was fused at 190°C for 6h. The formed precipitate was triturated with EtOH to yield compounds **17a,b** .

*5-Amino-2-(4-chlorobenzylidene)-8,10-diphenylpyrido[3',2':4,5]thieno[3,2-d] imidazo[1,2-a]pyrimidin-3(2H)–one ; **17a***

Black crystals; recrystallized from dioxane; m.p. 280-282°C; yield 60%. **IR**(KBr, cm^{-1}): 3359, 3301 (NH_2); 3070 (CH–Ar); 2927, 2858 (CH-aliph.); 1710 (C=O) ; 1608 (C=N)**. ^{1}H-NMR**(DMSO-d_6-δppm): 7.38 (s, 1H, =CH); 7.41 (s, 1H, pyridine-C_3-H); 7.56-7.72 (m, 10H, two C_6H_5-H); 7.76-7.80 (m, 2H, 4-Cl-C_6H_4–$C_{2,6}$–H); 8.14 (s, 2H, NH_2, D_2O exchangeable); 8.36-8.42 (m, 2H, 4-Cl- C_6H_4–$C_{3,5}$-H). **Anal. form**: $C_{30}H_{18}ClN_5OS$ **Calcd.** (%): C, 67.73; H, 3.41; N, 13.16; S, 6.03. **Found** (%): C, 67.75; H, 3.45; N, 13.27; S, 6.09.

*5-Amino-8,10-diphenyl-2-(4-methoxybenzylidene)pyrido[3',2':4,5]thieno[3,2-d] imidazo[1,2-a]pyrimidin-3(2H)–one ; **17b.***

Black crystals; recrystallized from dioxane; m.p. 265-267°C; yield 54%. **IR**(KBr, cm^{-1}): 3417, 3209 (NH_2); 2900 (CH–aliph.); 1712 (C=O); 1643, 1616 (C=N); 1245, 1041 (C-O-C). **^{1}H-NMR** (DMSO-d_6-δppm): 3.79 (s, 3H, OCH_3); 6.78 (s, 1H, =CH); 6.93 (d, 2H, J=8.4 Hz, 4-OCH_3- C_6H_4 – $C_{3,5}$ – H); 7.02-7.14 (m, 4H, two C_6H_4- $C_{3,5}$-H); 7.57-7.70 (m, 2H, two C_6H_4- C_4-H); 7.75-7.87 (m, 4H, two C_6H_4-$C_{2,6}$-H); 7.88 (s, 1H, pyridine-C_3-H); 8.14 (s, 2H, NH_2, D_2O exchangeable); 8.38 (d, 2H, J=8.4Hz, 4-OCH_3-C_6H_4 - $C_{2,6}$-H). **Anal. form**: $C_{31}H_{21}N_5O_2S$ **Calcd.** (%): C, 70.57; H, 4.01; N, 13.27; S, 6.08. **Found** (%): C, 70.61; H, 4.03; N, 13.35; S, 6.13.

*Synthesis of 1-[6-(4-substitutedbenzylidene)-5,6-dihydro-5-oxoimidazo[2,1-b] [1,3]thiazol-3-yl]-3-phenylthioureas; **18a,b**:*

1 mmol of compound **12a,b** was refluxed with phenyl isothiocyanate (0.14 g, 0.12 mL,1 mmol) in dry benzene (10 mL) for 12h. The reaction mixture was allowed to cool and the obtained product was collected, filtered off and recrystallized from the appropriate solvent.

*1-[6-(4-Chlorobenzylidene)-5,6-dihydro-5-oxoimidazo[2,1-b][1,3]thiazol-3-yl]-3-phenylthiourea; **18a.***

Brown powder; recrystallized from EtOH; m.p. 250-251°C; yield 75%. **IR** (KBr, cm^{-1}): 3467, 3236 (Two NH); 3050 (CH–Ar); 2854 (CH-aliph.); 1732 (C=O); 1647 (C=N); 1590, 1253, 1180, 1014 (I, II, III, IV bands of N–C=S). **MS:** m/z(%): 414(M+2, 1.25); 412(M^+, 1.1); 402(1.58); 400(1.29); 383(1.05); 381(1.25); 377(1.25); 375(2.06); 370(2.2); 341(1.63); 339(2.25); 260(2.3); 258(2.68); 215(4.07); 213(3.93); 175(4.98); 161(4.12); 160(6.61); 159(4.17); 152(15.09); 146(5.6); 136(5.41); 131(5.94); 116(10.2); 103(14.46); 101(1.15); 88(14.37); 80(11.06); 78(10.87); 62(26.68); 60(100); 59(35.97). **Anal form**: $C_{19}H_{13}ClN_4OS_2$ **Calcd**. (%): C, 55.27; H, 3.17; N, 13.57, S, 15.53. **Found** (%): C, 55.32; H, 3.19; N, 13.68; S, 15.59.

*1-[6-(4-Methoxybenzylidene)-5,6-dihydro-5-oxoimidazo[2,1-b]thiazol-3-yl]-3-phenyl-thiourea; **18b.***

Brown crystals; recrystalized from ethyl acetate; m.p. 249-251°C; yield 77%. **IR** (KBr, cm^{-1}): 3273, 3193 (NH); 3118 (CH-Ar), 2839 (CH-aliph.); 1719 (C=O); 1649 (C=N); 1597, 1258, 1096, 1027 (I,II,III,IV bands of N–C=S and C–O–C). **^{1}H-NMR** (DMSO-d_6-δppm): 3.86 (s, 3H, OCH_3); 6.70 (s, 1H, =CH); 6.90-7.02 (m, 2H, C_6H_5- $C_{3,5}$–H); 7.30 (t, 1H, J = 7.8Hz, C_6H_4–C_4–H); 7.45 (s, 1H, CH-thiazole); 7.55 (d, 2H, J = 7.8Hz, C_6H_4–$C_{2,6}$-H); 7.74 (d, 2H, J= 9.3Hz , 4-OCH_3-C_6H_4–$C_{3,5}$–H); 7.83 (d, 2H, J= 9.3 Hz, 4-OCH_3–C_6H_4–$C_{2,6}$-H); 10.57 (s, 2H, two NH, D_2O exchangeable). **Anal form**: $C_{20}H_{16}N_4O_2S_2$ **Calcd.** (%): C, 58.80; H, 3.95; N, 13.72; S, 15.68. **Found** (%): C, 58.81; H, 3.98; N, 13.79; S, 15.72.

*Synthesis of 6-(4-substitutedbenzylidene)3-(3,4-diphenylthiazol-2-(3H) ylideneamino) imidazo[2,1-b][1,3]thiazol-5(6H)-ones; **19a,b**:*

An equimolar mixture of compound **18a,b** (10 mmol) and phenacylbromide (1.99 g, 10 mmol.) in absolute EtOH (20 mL) and catalytic amount of triethylamine (1 mL) were refluxed for 6h. The reaction mixture was allowed to cool and the solid separated after neutralization with 10% HCl was recrystallized from acetone.

6-(4-Chlorobenzylidene)3-(3,4-diphenylthiazol-2-(3H)ylideneamino)imidazo[2,1-b] [1,3]thiazol-5(6H)-one; ***19a***

Brownish orange powder; recrystallized from acetone; m.p. 117-119°C; yield 50%**. IR**(KBr, cm^{-1}): 2820 (CH–aliph.); 1731 (C=O); 1639 (C=N). **MS**: m/z(%): 514(M+2, 0.01); 512(M^{+}, 0.01); 503(0.02); 500(0.23); 487(0.02); 485(0.03); 476(0.02); 474(0.09); 469(0.03); 467(0.02); 455(0.02); 452(0.09); 446(0.05); 439(0.17); 429(0.07); 427(0.08); 415(0.04); 413(0.12); 395(1.05); 369(6.88); 340(1.91); 313(0.62); 279(0.91); 263(2.89); 248(2.52); 238(11.11); 222(37.76); 207(1.01); 191(1.33); 167(2.21); 154(6.31); 152(17.07); 117(5.58); 105(19.22); 86(100). **Anal form:** $C_{27}H_{17}N_4OS_2$ **Calcd.** (%): N, 10.92; S, 12.50. **Found** (%): N, 11.09; S, 12.62.

6-(4-Methoxybenzylidene)-3-(3,4-diphenylthiazol-2(3H)-ylideneamino)imidazo[2,1-b] [1,3]thiazol-5(6H)-one; ***19b.***

Brownish red powder; recrystallized from acetone; m.p. 103-105°C; yield 55%. **IR**(KBr, cm^{-1}): 3090 (CH–Ar); 2923, 2852 (CH-aliph.); 1704 (C=O); 1643, 1596 (C=N); 1249, 1018 (C-O-C). **^{1}H-NMR** (DMSO-d_6–δppm): 3.61 (s, 3H, OCH_3); 5.15 (s, 1H, =CH); 7.49 (s, 1H, thiazole–C_5-H); 7.52 (s, 1H, imidazothiazole-C_2–H); 7.63 (d, 2H, J=7.6 Hz, 4-OCH_3-C_6H_4–$C_{3,5}$-H,); 7.67-7.74 (m, 10H, two C_6H_5); 8.05 (d, 2H, J=7.6 Hz, 4-OCH_3-C_6H_4–$C_{2,6}$–H**). Anal. form:** $C_{28}H_{20}N_4O_2S_2$ **Calcd.**(%): C, 66.12; H, 3.96; N, 11.02; S, 12.61. **Found**(%): C, 66.09; H, 3.98; N, 11.09; S, 12.64.

Synthesis of 6-(4-substitutedbenzylidene)-3-(4-oxo-3-phenylthiazolidin-2-ylidene amino)imidazo[2,1-b][1,3]thiazol-5(6H)-one; ***20a,b.***

A mixture of compound **18a,b** (5 mmol), chloroacetic acid (0.47 g, 5 mmol) and sodium acetate (0.25 g, 3 mmol) was refluxed in glacial acetic acid (20 mL) for 6h. The reaction mixture was then cooled, and poured on crushed ice. The yellow coloured solid separated was filtered; dried and recrystallized from the suitable solvent.

6-(4-Chlorobenzylidene)-3-(4-oxo-3-phenylthiazolidin-2-ylideneamino)imidazo[2,1-b][1,3]thiazol-5(6H)-one; ***20a.***

Yellowish powder; recrystallized from ethyl acetate ; m.p.>300°C; yield 70%. **IR**(KBr, cm^{-1}): 3078 (CH-Ar); 2920 (CH-aliph.); 1710 (C=O); 1608 (C=N). **Anal. form**: $C_{21}H_{13}N_4O_2S_2$ **Calcd**. (%): C, 55.69; H, 2.89; N, 12.37; S, 14.16 **Found** (%): C, 55.73; H, 2.93; N, 12.45; S, 14.21.

6-(4-Methoxybenzylidene)-3-(4-oxo-3-phenylthiazolidin-2-ylideneamino)imidazo [2,1-b][1,3]thiazol-5(6H)-one; ***20b.***

Pale yellowish powder; recrystallized from EtOH; m.p. 199-201°C; yield 70%. **IR** (KBr, cm^{-1}): 3025 (CH-Ar); 1700 (C=O); 1601 (C=N); 1251, 1074 (C-O-C). **^{1}H-NMR** (DMSO-d_6-δppm): 3.77 (s, 3H, OCH_3); 5.07 (s, 2H, S-CH_2); 7.67-7.69 (m, 10H, Ar-H & = CH); 9.38 (s, 1H, CH-thiazole). **MS:** m/z(%): 435(M- CH_3,1.52); 309(2.07); 290(2.2); 289(3.54); 280(3.54); 278(3.57); 276(3.14); 268(3.62); 267(3.11); 262(6.78); 249(4.43); 236(6.6); 219(4.09); 204(4.02); 197(6.07);185(3.65); 178(6.32); 174(5.81); 167(5.86); 159(3.77); 157(6.83); 152(5.89); 151(9.72); 150(15.69); 149(10.77); 141(9.84); 137(9.99); 136(10.86); 135(11.6); 125(9.16); 122(13.19); 109(18.43); 105(40.84); 97(10.77); 95(14.27); 91(16.35); 83(37.14); 81(52.34); 79(42.42); 76(42.52); 69(39.63); 68(32.92); 57(98.57); 55(100); 50(35.07). **Anal form**: $C_{22}H_{16}N_4O_3S_2$ **Calcd.** (%): N, 12.49; S, 14.30 **Found** (%): N, 12.63; S, 14.19.

Synthesis of 2-[4-(4-chlorobenzylidene)-4,5-dihydro-5-oxo-1H-imidazol-2-yl thio]acetohydrazide; ***21.***

Compound **12a** (2.78 g, 10 mmol) was refluxed in absolute EtOH (20 mL) in presence of hydrazine hydrate 99% (0.32 g, 0.31 mL, 10 mmol) for 6 h. The reaction mixture was cooled and the solid precipitate obtained was collected, dried and recrystallized from THF.

Yellow crystals; m.p.> 300°C; yield 65%. **IR**(KBr,cm^{-1}): 3300, 3186 (NH_2,NH); 3000 (CH-Ar); 2920 (CH-aliph.); 1700, 1671 (two C=O); 1500 (C=N). **^{1}H-NMR** (DMSO-d_6–δppm): 4.20 (s, 2H,S–CH_2-CO); 6.25 (s, 1H, =CH); 7.43 (d, 2H, J = 8.8Hz ,4–Cl–C_6H_4–$C_{2,6}$-H,); 7.63 (d, 2H, J=8.8Hz, 4–Cl-C_6H_4–$C_{3,5}$–H); 7.68 (s, 2H, NH_2, D_2O exchangeable); 7.70 (s, 1H, NH-NH_2, D_2O exchangeable); 10.44 (s, 1H, imidazole-NH; D_2O exchangeable). **Anal. Form:** $C_{12}H_{11}ClN_4O_2S$ **Calcd.** (%): C, 46.38; H, 3.57; N, 18.03. **Found** (%): C, 46.42; H, 3.59; N, 18.14.

Synthesis of 1-[2-(4-(4-Chlorobenzylidene)-4,5-dihydro-5-oxo-1H-imidazol-2-yl)thioacetyl]-4-phenylthiosemicarbazide; ***22.***

Compound **21** (3.1 g, 10 mmol) in pyridine (10 mL) was added to (1.35 g, 1.2 mL, 10 mmol) of phenyl isothiocyanate and heated under reflux for 8h. The resulted solution was allowed to cool at room temperature and light yellow crystals were collected, filtered off and recrystallized from EtOH.

Yellow crystals; m.p. 54-56°C; yield 95%. **IR**(KBr, cm^{-1}): 3210, 3119 (NH); 3045 (CH–Ar); 2988 (CH-aliph.); 1720,1692 (two C=O); 1593, 1203, 1097, 1033 (I, II, III, IV bands of N-C=S);

1544 (C=N). **^{1}H-NMR** (DMSO-d_6-δppm): 4.50 (s, 2H, S-CH_2); 6.14 (s, 1H, =CH); 7.30-7.35 (m, 8H, 3NH & C_6H_5); 7.40-7.80 (m, 4H, 4-Cl-C_6H_4); 11.02 (s, 1H, imidazole-NH, D_2O exchangeable). **Anal.form:** $C_{19}H_{16}ClN_5O_2S_2$ **Calcd**.(%): C, 51.17; H, 3.62; N, 15.70; S, 14.38. **Found.** (%): C, 51.22; H, 3.65; N, 15.79; S, 14.42.

Synthesis of 2-[4-(4-chlorobenzylidene)-4,5-dihydro-5-oxo-1H-imidazol-2-yl thio]-N-(4-oxo-3-phenylthiazolidin-2-ylidene)acetohydrazide; ***23.***

To a solution of compound **22** (4.45 g, 10 mmol) in EtOH (20 mL), an equimolar amount of ethyl bromoacetate (1.67 g, 1.1 mL, 10 mmol) was added and the reaction was refluxed for 8h. The reaction mixture was then cooled, poured onto crushed ice and the resulted precipitate was filtered, dried and recrystallized from acetone.

Pale yellow crystals; m.p. 142-144°C; yield 80%. **IR**(KBr, cm^1): 3282 (NH); 3020 (CH–Ar.); 2927 (CH–aliph.); 1712, 1658 (C=O); 1600 (C=N). **^{1}H-NMR**(DMSO-d_6-δppm): 2.08 (s, 2H, S-CH_2-CO); 3.70 (s, 2H, thiazolidine-CH_2); 7.03 (s, 1H, =CH); 7.06-7.08 (m, 5H, C_6H_5); 7.28 (d, 2H , J= 8.1Hz, 4–Cl–C_6H_4–$C_{2,6}$–H); 7.32 (s, 1H, NH, N-NH-CO, D_2O exchangeable); 7.44 (d, 2H, J = 8.1Hz, 4-Cl-C_6H_4-$C_{3,5}$–H); 10.42 (s, 1H, imidazole NH, D_2O exchangeable). **Anal.form:** $C_{21}H_{16}ClN_5O_3S_2$ **Calcd.**:(%): N, 14.41; S, 13.20. **Found**(%):N, 14.49; S, 13.26**.**

Synthesis of 2,6-bis (4-chlorobenzylidene)imidazo[2,1-b][1,3]thiazole–3,5-(2H, 6H)dione; ***24***

An equimolar mixture of compound **12a** (2.78 g, 10 mmol) and 4-chlorobenzaldehyde (1.4 g, 10 mmol) in glacial acetic acid (10 mL) was refluxed for 8 h. in presence of anhydrous sodium acetate (1.64 g, 20 mmol). The reaction mixture was allowed to cool and solid precipitate was filtered, dried and recrystallized from EtOH.

Brown powder; m.p. 220-222°C; yield 80%. **IR**(KBr, cm^{-1}): 3074 (CH–Ar.); 2927 (CH–aliph.); 1650 (C=O); 1566 (C=N). **MS:** m/z: 402(M+2,0.8); 400(M^+, 0.29); 392(0.61); 390(0.4); 386(0.36); 384(0.51); 382(0.36); 372(0.29); 370(0.69); 367(0.4); 365(0.58); 359(0.58); 349(0.83); 337(0.87); 325(1.01); 310(2.17); 308(0.98); 303(0.61); 295(0.61); 294(1.16); 289(1.08); 279(0.87); 277(0.9); 268(0.87); 266(0.98); 255(1.23); 250(1.3); 238(1.73); 195(2.1); 183(1.92); 181(2.6); 174(1.63); 172(2.39); 160(5.2); 159(6.11); 155(3.83); 143(4.26); 140(8.85); 133(9.07); 132(14.56); 131(17.28); 130(14.56); 126(11.82); 124(11.42); 118(20.46); 117(24.79); 106(13.05); 104(31.59); 91(5.75); 90(100); 89(90.64); 83(31.88); 80(50.34); 78(83.95); 77(75.82); 75(21.97); 65(46.98); 64(93.86); 60(57.14); 54(52.37). **Anal. form**: $C_{19}H_{10}Cl_2N_2O_2S$ **Calcd**. (%): C, 56.87; H, 2.51; N, 6.98. **Found** (%): C, 57.08; H, 2.49; N, 7.14.

Synthesis of 2-(4-chlorobenzylidene)-5-(4-chlorophenyl)-5,6-dihydro-4aH-imidazo[2',1':2,3][1,3]thiazolo[4,5-c]pyrazol-1(2H)-one*;* ***25***

Compound **24** (4 g, 10 mmol) and hydrazine hydrate (0.64 g, 0.62 mL, 20 mmol) were refluxed in absolute alcohol (20 mL) and in presence of a catalytic amount of glacial acetic acid (2 mL) for 8h. The reaction was cooled then poured onto crushed ice. The product obtained was filtered, washed with water, dried and recrystallized from methanol.

White crystals; m.p. 102-104°C; yield 88%. **IR**(KBr, cm^{-1}): 3421, 3224 (NH); 3050 (CH–Ar); 1660 (C=O); 1612 (C=N)**. MS:** m/z(%): 418(M+4,0.37); 403(0.52); 394(0.65); 365(0.55); 363(0.60); 362(0.49); 352(0.56); 307(0.58); 280(0.66); 279(0.95); 269(0.64); 268(1.64); 257(0.89); 256(1.4); 234(1.08); 226(1.61); 214(2.49); 211(1.24); 207(1.34); 203(1.4); 196(0.85); 195(1.86); 187(2.33); 184(2.33); 183(1.16); 180(1.06); 178(2.22); 177(1.29); 174(1.36); 173(1.84); 171(1.62); 170(3.04); 167(6.3); 154(7.08); 149(23.11); 141(4.31); 140(3.06); 139(6.46); 138(4.88); 136(3.84); 133(3.51); 129(3.94); 128(3.84); 127(10.31); 125(8.37); 123(4.05); 122(4.03); 119(4.99); 118(3.91); 113(7.17); 111(14); 105(6.28); 101(3.08); 97(17.51); 91(6.19); 85(10.67); 83(12.63); 82(36.86); 79(34.32); 76(12.91); 71(52.02); 69(44.53); 67(18.9); 63(12.69); 59(18.99); 58(16.1); 57(100); 54(89.92); 50(31.99); 46(23.34). **Anal. Form**: $C_{19}H_{12}Cl_2N_4OS$ **Calcd.** (%): N, 13.49. **Found**(%): N, 13.61.

Anticancer screening:

Developmental Therapeutic Program (DTP), Division of Cancer Treatment and Diagnosis (DCTD), National Cancer Institute (NCI), Bethesda, Maryland, USA has adopted an in-vitro model consisting of 60 human tumor cell lines for primary anticancer screening. Nineteen of the newly synthesized compounds were selected by the NCI for screening in a two stage process, beginning with evaluation of all compounds against 60 human tumor cell lines in a one dose (10 μmol) screening panel. The output from the single 60 cell panel screen is reported as a mean graph and is available for analysis by the COMPARE program. Compounds which inhibit growth by more than 50% in a threshold number of cell lines was determined by comparison with historical NCl 60 cell and in-vivo data (COMPARE program), were selected for 5-dose assay [46,47]. However, one compound **11b** of which was selected for 5-dose assay. The one dose screening results of the selected compounds are presented in tables **1-5**.

Table (1): The mean growth percent, delta values and the percent growth inhibition against some subpanel cell lines of the selected compounds of scheme 1.

Comp. No.	NSC-number	Mean growth percent	Delta	Panel	Subpanel cell lines (Growth inhibition percent)
3a	767500	94.70	20.81	Leukemia	MOLT-4(24.11), RPMI-8226 (25.77).
				Non-Small Cell Lung Cancer	HOP-92(26.11), NCI-H522 (19.38).
				Colon Cancer	HCT-116 (13.23).
				CNS Cancer	SNB-19 (11.38), SNB-75(20.95).
				Melanoma	SK-MEL-5(23.83), UACC-62 (13.63).
				Renal Cancer	786-0(14.70), SN12C(10.41),UO-31 (19.71).
				Prostate Cancer	PC-3(22).
				Breast Cancer	MCF7(10.51), MDA-MB-231/ATCC(21.01), BT-549(12.91).
3c	767501	98.82	24.80	Leukemia	MOLT-4(11.93), RPMI-8226 (17.52), SR(22.94).
				Non-Small Cell Lung Cancer	NCI-H522 (25.98).
				CNS Cancer	SNB-75 (23.54).
				Melanoma	LOX IMVI (11.61), UACC-62 (12.56).
				Breast Cancer	MCF7 (10.40).

Table (2): The mean growth percent, delta values and the percent growth inhibition against some subpanel cell lines of the selected compounds of scheme 2.

Comp. No.	NSC-number	Mean growth percent	Delta	Panel	Subpanel cell lines (Growth inhibition percent)
6a	767504	97.97	21.52	Leukemia	K-562(13.58), MOLT-4 (13.54).
				Non-Small Cell Lung Cancer	NCI-H522(23.55)
				Ovarian Cancer	OVCAR-4(10.09).
				Renal Cancer	CAKI-1(20.04), UO-31(13.33).
				Prostate Cancer	PC-3(10.67).
6b	767505	96.25	16.11	Leukemia	CCRF–CEM(17.52), MOLT-4(11.46), RPMI-8226(15.50), SR(19.86).
				Non-Small Cell Lung Cancer	A549/ATCC(11.07), NCI-H522 (18.24).
				Colon Cancer	HCT-15(11.01), HT29(12.34).
				Renal Cancer	786-0(13.13). A498(12.81), CAKI-1 (16.25), TK-10 (11.89), UO-31 (12).
9a	767502	100.51	24.72	Leukemia	HL-60(TB)(10.90). MOLT-4(10.91), SR(24.21)
				Non-Small Cell Lung Cancer	A549/ATCC (13.42), NCI-H522 (21.09)
				Renal Cancer	CAKI-1 (21.48)
9b	767503	98.18	33.16	Leukemia	SR(11.56)
				Non-Small Cell Lung Cancer	HOP-92(17.38), NCI-H226 (17.03), NCI-H522(12.51)
				CNS Cancer	SNB-75(23.25)
				Renal Cancer	CAKI-1(26.17),UO-31 (34.98).
				Prostate Cancer	PC-3 (14.51).

Table (3): The mean growth percent, delta values and the percent growth inhibition against some subpanel cell lines of the selected compounds of scheme 3.

Comp. No.	NSC-number	Mean growth percent	Delta	Panel	Subpanel cell lines (Growth inhibition percent)	Lethality
11a	767512	69.98	83.51	Leukemia	HL-60(TB) (60.08), K-562(59.93), MOLT-4 (91.88), RPMI-8226(60.08)	CCRF-CEM (13.53), SR(2.29)
				Non-Small Cell Lung Cancer	A549/ATCC (25.79), HOP-62(16.50), HOP-92 (30.45), NCI-H226(19.75), NCI-H23(27.41), NCI-H460(37.85), NCI-H522(66 .20)	
				Colon Cancer	HCT-116(33.93), HCT-15(33.25), HT29(37.38), KM12 (10.84), SW-620 (28.70).	
				CNS Cancer	SNB-19(13), SNB-75(12.64).	
				Melanoma	LOX IMVI (49.40), MALME-3M (35.11), M14 (23.22), MDA-MB-435(31.97), SK-MEL-5 (27.38).	
				Ovarian Cancer	IGROV1(20.79), OVCAR-3(28.38), OVCAR-4(15.87), OVCAR-8(27.70), NCI/ADR-RES(23.95), SK-OV-3 (10.68).	

				Renal Cancer	786-0(18.61), A498(34.44), ACHN(11.67), CAKI-1 (61.18), RXF393 (34.31), SN12C(23.15), TK-10(27.20), UO-31(41.79).	
				Prostate Cancer	PC-3(19.37), DU-145(22.09)	
				Breast Cancer	MCF7(32.78), MDA-MB-231/ ATCC(16.82), BT-549(24.68), T-47D(21.04), MDA-MB-468 (35.50).	
11b	767513	14.10	68.60	Leukemia	K-562(94.71).	CCRF-CEM (40.21), HL-60(TB) (39.98). MOLT-4 (15.92), RPMI-8226 (28.76), SR (21.30).
				Non-Small Cell Lung Cancer	A549/ATCC (67.23), HOP-62(59.77), NCI-H226(48.39), NCI-H23(76.38), NCI-H322M(53.36), NCI-H460(82.75).	HOP-92 (8.02), NCI-H522 (54.50).
				Colon Cancer	Colo 205 (87.49), HCC-2998(59.08), HCT-15 (79.26), HT29 (90.88), KM12(53.11), SW-620(78.06).	HCT-116(6.91).
				CNS Cancer	SF-268(62.65), SF-539(73.30), SNB-19(46.23), SNB-75(74.79).	
				Melanoma	M14(75.16), MDA-MB- 435(90.41), SK-MEL-28 (56.26), SK-MEL-5 (95.35), UACC-62 (77.02).	LOXIMVI (47.54), MALME-3M (15.30).
				Ovarian Cancer	IGROV1(90.19), OVCAR-3(75.76), OVCAR-4(78.50), OVCAR-5(40.11), OVCAR-8(64.26), NCI/ADR-RES (57.74), SK-OV-3(54.37).	
				Renal Cancer	786-0(66.83), A498(94.33), ACHN (67.04), SN12C (79.57), TK-10 (80.46).	CAKI-1(32.99), RXF 393 (21.24), UO-31(4.09).
				Prostate Cancer	PC-3(79.38), DU-145 (73.53).	
				Breast Cancer	MCF7(86.60), MDA-MB-231/ ATCC (57.53), HS 578T (49.64), BT-549 (88.25), T-47D (90.25).	MDA-MB-468 (31.93).
12a	767510	99.24	29.66	Leukemia	CCRF-CEM (12.99),HL-60(TB) (24.48), K-562 (14.06), MOLT-4 (18.86), RPMI-8226(15.92), SR(30.42).	
				Non-Small Cell Lung Cancer	HOP-92(14.81), NCI-H522(25.69).	
				Melanoma	MALME-3M (15.06).	
				Renal Cancer	CAKI-1 (12.07), UO-31(11.33).	
12b	767511	98.21	27.44	Leukemia	CCRF-CEM (10.73), HL-60(TB) (27.68), K-562 (14.07), MOLT-4 (20.86), SR(29.23).	
				Non-Small Cell Lung Cancer	HOP-92(13.69), NCI-H522 (24.00)	
				Ovarian Cancer	OVCAR-4 (10.29).	
				Renal Cancer	CAKI-1(14.14), UO-31(21.73).	

Table (4): The mean growth percent, delta values and the percent growth inhibition against some subpanel cell lines of the selected compounds of scheme 4.

Comp. No.	NSC-number	Mean growth percent	Delta	Panel	Subpanel cell lines (Growth inhibition percent)
14a	767507	100.66	18.94	Leukemia	SR(13.45).
				Non-Small Cell Lung Cancer	NCI-H522(11.94).
				CNS Cancer	SNB-75 (10.43).
				Ovarian Cancer	OVCAR-5(18.28).
14b	767508	98.03	17.75	Leukemia	CCRF-CEM(10.13), HL-60 (TB) (10.65), MOLT-4(12.67), SR (19.72).

				Non-Small Cell Lung Cancer	NCI-H522(16.22).
				Ovarian Cancer	OVCAR-4(10.52).
				Renal Cancer	CAKI-1(16.26), UO-31(11.97).
15a	767506	94.31	28.74	Leukemia	CCRF-CEM (25.81), K-562 (11.74), MOLT-4(31.87), SR (32.31).
				Non-Small Cell Lung Cancer	A549/ATCC (15.24), HOP-92(34.43).
				Lung Cancer	NCI-H23(13.07), NCI H522 (25.21).
				Colon Cancer	HCT-15(18.58).
				CNS Cancer	SF-268(15.82), SNB-19(16.13).
				Renal Cancer	786-0(19.59), A498(12.47), RXF 393 (12.19), SN12C (10.11), UO-31(22.81).
				Prostate Cancer	PC-3(16.61).
				Breast Cancer	BT-549(17.72).
15b	767509	89.26	35.31	Leukemia	CCRF-CEM (33.12), HL-60 (TB) (28.19), K-562(18.38), MOLT-4(37.66), RPMI-8226 (17.14), SR (46.05).
				Non-Small Cell Lung Cancer	A549/ATCC(20.30), NCI-H460 (11.68), NCI-H522(25.56).
				Colon Cancer	HCT-15(26.79), HT29(11.85).
				CNS Cancer	SF-268(11.52), SNB-19 (11.60).
				Melanoma	MDA-MB-435(13.17), UACC-62(19.56).
				Ovarian Cancer	OVCAR-4(18.21), OVCAR-8 (22.25), NCI/ADR-RES(20.70).
				Renal Cancer	786-0 (11.87), CAKI-1(25.76), SN12C(15.96), UO-31(27.04).
				Breast Cancer	BT-549(14.86), MDA-MB-468(16.89).
				Breast Cancer	MDA-MB-231/ATCC(11.31).

Table (5): The mean growth percent, delta values and the percent growth inhibition against some subpanel cell lines of the selected compounds of schemes 5 and 6.

Comp. No.	NSC-number	Mean growth percent	Delta	Panel	Subpanel cell lines (Growth inhibition percent)
19a	767514	102.82	23.52	Leukemia	MOLT-4(13.12), SR(14.06).
				Non-Small Cell Lung Cancer	HOP-92(20.37), NCI-H522 (12.76).
				CNS Cancer	SNB-75(10.85).
				Renal Cancer	UO-31(20.70).
				Prostate Cancer	PC-3(10.93).
19b	767515	101.11	23.27	Leukemia	MOLT-4(10.06), SR (13.91).
				Non-Small Cell Lung Cancer	NCI-H522 (12).
				CNS Cancer	SNB-75 (12.03).
				Renal Cancer	UO-31(22.16).
20a	767519	99.74	20.83	Leukemia	HL-60(TB)(20.74), MOLT-4 (17.33), SR(20.71).
				CNS Cancer	SNB-75(15.70).
				Renal Cancer	CAKI-1(19.18), UO-31(21.09).
20b	767520	99.52	18.96	Leukemia	K-562(10.30), MOLT-4(11.22), SR (19.44).
				CNS Cancer	SNB-75 (11.91).
				Renal Cancer	CAKI-1(16.17), UO-31(19.03).
				Breast Cancer	T-47D (11.18).
25	767516	74.10	60.67	Leukemia	CCRF-CEM(85.07), HL-60 (TB) (51.62), K-562(86.57), MOLT-4 (67.30), SR (73.34).
				Non-Small Cell Lung Cancer	A549/ATCC(52.32), HOP-62 (36.16), HOP-92 (17.06), NCI-H23(14.46), NCI-H460 (43.99), NCI-H522(56.20).
				Colon Cancer	COLO 205 (29.44), HCT-116 (52.74), HT29(77.18), KM12(15.93), SW-620(42.23).
				CNS Cancer	SF-268(29.75).
				Melanoma	LOX IMVI(79.38), M14(31.42), MDA-MR-435 (23.41), UACC-62(17.39).
				Ovarian Cancer	IGROV1(58.26), OVCAR-5 (22.16), OVCAR-8(21.24), SK-OV-3(15.90).
				Renal Cancer	786-0(58), A498(26.60), ACHN (13.84), CAKI-1 (22.42), RXF 393(23.55), SN12C (15.14). TK-10(14.28), UO-31(47.88).
				Breast Cancer	MCF7 (23.19), MDA-MB-231 /ATCC(63.62), MDA-MB-468 (10.87).

The imidazothiadiazine analogues **3a** and **3c** showed moderate anticancer activity against some tumor cell lines namely, Leukemia, Non-Small Cell Lung Cancer, Melanoma and CNS cancer. Moreover, compounds containing imidazo[1,3]thiazine ring such as compounds **6a** and **6b** exhibited a slight increase in the growth inhibition activity against all cell lines. While, compounds **9a** and **9b** having benzo[e] imidazo[2,1-b]thiazine backbone showed a significant growth inhibition activity against Leukemia SR, Non-Small Cell Lung Cancer NCI-H522 and Renal Cancer CAKI-1 cell lines.

Furthermore, the one dose screening results as presented in table **(3)** of the 3-aminoimidazo[2,1-b]thiazole derivatives **11a** and **11b** revealed that these compounds exhibited promising growth inhibition activity against most cancer cell lines, even that compound **11b** was selected for further evaluation in the 5-dose screening assay. However, replacement of the chloro function in compound **11a** by methoxy moiety in compound **11b** resulted in enormous increase in the anticancer activity against almost all cell lines as it exerted a high lethal effect against Leukemia CCRF-CEM, HL-60(TB), MOLT-4, RPMI-8226 and SR cell lines by 40.21%, 39.98%, 15.92%, 21.3%, respectively. It also exhibited lethal effects towards Non-Small Cell Lung Cancer HOP-92 (8.02%) and NCI-H522 (54.5%) cell lines as well as Melanoma cell lines LOXIMVI (47.54%) and MALME-3M (15.3%). The cytotoxic effect of compound **11b** has extended also to Colon Cancer HCT-116 (6.91%), Renal Cancer CAKI-1 (32.99%), RXF393 (21.24%), UO-31 (4.09%) and Breast Cancer MDA-MB-468 (31.93%). Moreover, it also showed significant growth inhibition activity against various cell lines such as Leukemia K-562(94.71%), Non-Small Cell Lung Cancer NCI-H23 (76.38%) and NCI-H460 (82.75%), Colon Cancer Colo 205 by 87.49%, HCT-15 by 79.26%, HT 29 by 90.88% and SW-620 by 78.06% as well as Melanoma cell lines M14 (75.16%), MDA-MB-435(90.41%), SK-MEL-5 (95.35%) and UACC-62(77.02%). Furthermore, Ovarian Cancer cell lines were inhibited severely by the target compound showing growth inhibition activity by 90.19% and 78.5% against IGROV1 and OVCAR-4, respectively, Renal Cancer A498 (94.33%), SN12C(79.57%) and TK-10 (80.46%) cell lines. It also exerted potent activity against Breast Cancer MCF-7 (86.6%), BT-549 (88.25%) and T-47D (90.25%) cell lines. Replacement of 3-amino group in compound **11** by the 3-oxo function in compound **12** resulted in severe decrease in anticancer activity against all cell lines. The imidazo [2,1-b] pyrimidine compounds fused to thiophene ring **14a,b & 15a,b** showed moderate inhibitory activity against many cell lines.

The one dose screening results of compounds **19a, 19b, 20a, 20b** and **25** revealed that attachment of thiazole ring to 3-amino group of compound **11** strongly diminished the anticancer activity. Moreover, fusion of a pyrazole ring to the thiazole moiety as in compound **25** resulted in marked increase in the growth inhibitory activity against most of the cell lines especially, Leukemia CCRF-CEM (85.07%), K-562 (86.57%), Colon Cancer HT29 (77.18%) and Melanoma LOX IMVI (79.38%) cell lines as it showed much higher activity than compounds **19a** and **19b** as well as compounds **20a** and **20b** but it is still lower in activity than compound **11a** with free 3-amino thiazole moiety.

Corresponding author
M.T. Sarg
Department of Organic Chemistry, Faculty of Pharmacy (Girls), Al-Azhar University, Nasr City, Cairo; Egypt
m.t.sarg@hotmail.com

References

1) Shih, R.U., Wu, J., Liu, Y., Liang, Y. C., Lin, S. Y., Sheu, M. T., Lee, W. S., 2004.. Biochem.Pharmacol. 67, 67-75.
2) Takashi, A., Matsuoka, H., Yamada, K., Uda, Y., 2005. Food Chem. Toxicol. 43, 521-528.
3) Al-Obaid, A. A., El-Subagh, H. I., Khodair, A. I. , Elmazar, M. M., 1996. Anticancer Drugs. 7, 873-880.
4) Chui, W. K., Wong, T. H. ,Thenomozhiyal, J. C. 2004.J. Med. Chem. 47, 1527-1535.
5) Poitout, L., Thurieau, C., Brault, V. 2001 Chem. Abstr. 134, 163050.
6) Szymanska, E., Kiec-Kononowicz, K., Bialecka, A. , Kasprowicz, A., 2002. Il Farmaco. 57, 39-44.
7) Gulati, D., Chauhan, P. M. S., Pratap, R., Bhakuni, D. S. 1994. Ind. J. Chem., 33B, 10-16.
8) Havera, H. J., Strycker, W. G. 1977. U.S. Patent 3994904: Chem. Abstr. 86, 106586m.
9) Tompkin, J. E. 1986. J. Med. Chem. 29, 855-859.
10) Gadwood, R. C., Kamdar, B. V., Dubray, L. A. C., Wolfe, M. L., Smith, M. P., Watt, W., Mizsak, S. A., Groppi, V. E. 1993. J. Med. Chem. 36, 1480-1487.
11) Mizuno, T., Kino, T., Ito, T., Miyata, T. 2000. Synth. Commun. 30, 1675-1688.
12) Mappes, C. J., pommer, E. H., Rentzea, C., Zeeh, B. 1980. U.S. Patent 4198423. 93, 71784.
13) Muccioli, G. C., Fazio, N., Scriba, G. K. E., Poppitz, W., Cannata, F., Poupaert, J. H., Wouters, J., Lambert, D. M. 2006. J. Med. Chem. 49, 417-425.
14) Agasimundin, Y. S., Mumper, M. W., Hasmane, R. S. 1998. Bioorg. Med. Chem. 6, 911-923.
15) Augstein, J., Green, S. M., Monro, A. M., Potter, G. W. H., Worthing, C. R., Wrigley, T. I. 1965. J. Med. Chem. 8, 446-456.
16) Gyemant, G., Kandra, L., Nagy, V., Somsak, L.2003. Biochem. Biophys. Res. Commun. 312, 334-339.

17) Kandra. L., Remenyik, J., Batta, G., Somsak, L., Gyemant, G., Park, K. H. 2005. Carbohydr. Res. 340, 1311-1317.
18) Kuang, R., Epp, J. B., Ruan, S., Chang, L. S., Venkataraman, R., Tu, J., He, S., Truong, T. M., Groutas, W. C. 2000.
19) Bioorg. Med. Chem. 8, 1005-1016. He, S., Kuang, R., Venkataraman, R., Tu, J., Truong, T. M., Chan, H. T., Groutas, W. C. 2000. Bioorg. Med. Chem. 8, 1713-1717.
20) Smalley, R. K. 1996. Six-Membered Rings with Two or More Heteroatoms and Fused Carbocyclic Derivatives. Comprehensive Heterocyclic Chemistry II. Vol.6. Ed. A. J. Boulton, Elsevier, Oxford, UK.
21) Wang, Z., Shi, H., Shi, H. 2001. Synth. Commun. 31, 2841. Wang, Z., You, T., Shi, H., Shi, H. 1996. Molecules. 1, 89.
22) Wang, Z., You, T., Shi, H., Shi, H. 1997. Gaodeng Xuexiao Huaxue Xuebao. 18(4), 550. Chem. Abstr. 1997, 127, 95265t.
23) Shi, H., Shi, H., Wang, Z., Huaxue,Y. 2000., 20 (3), 344. Chem. Abstr. 133, 120280c.
24) Ochoa, C., Perez, E., Perez, R., Suarez, M., Ochoa, E., Rodriguez, H., Barrio, A. G., Muelas, S., Nogal, J. J., Martinez, R. A. 1999. Arzneim. Forsch. 49, 746.
25) Song, Q., Zhang, Zh., Li, Zh., Mi, G., Ding, Y., 1997. Zhongguo Yaowu Huaxue Zazhi. 7, 93. 1999 Chem. Abstr. 130, 25042f. Ilhan, E., Capan, G., Ergenc, N., Uzun, M., Kiraz, M., Kaya, D., 1995. Farmaco. 50, 787.
26) Chande, M. S., Dravid, R. N., Shetgiri, N. P. 1988. Proc. Indian Acad. Sci. Chem. 100, 53.
27) Ertan, M., Bilgin, A. A., Palaska, E., Yulug, N. 1992. Arzeim. Forsch. 42, 160.
28) Ertan, M., Ayyildiz, H. G., Yulug, N. 1991. Arzeim. Forsch. 41, 1182. Yadav Lal Dhar, S., Vaish, A., Sharma, S. 1994. 42, 811.
29) Liu, S., Qian, X., Chen, J., Song, G., 2000. Monatsh. Chem. 131, 935.
30) Jpn. Pat. 0859643. 1996. Chem. Abstr. 125, 10860u. Jpn. Pat. 03215478. 1992. Chem. Abstr. 116, 59409g.
31) Shi, H., Wang, Zh., Shi, H. 1999. Synth. Commun. 29, 2027.
32) Hozein, Z. A., Abd El-Wareth, A. O. S., El-Sherief, H. A. H., Mahmoud, A. M., 1997. Z. Naturforsch., Tiel B. 52, 1401.
33) Shi, H., Shi, H., Wang, Zh. 2001. J. Heterocycl. Chem. 38, 929.
34) Shi, H., Wang, Zh., Shi, H. 1997. Chimia. 51.
35) Hozein, Z. A. 2000. J. Chem. Res. (S). 99.
36) Kiec-Kononowicz, K., Karolak-Wojciechowska, J., Muller, C. E., Schumacher, B., Pekala, E., Szymanska, E., 2001.Imidazo-thiazine, -diazinone and diazepinone derivatives. Synthesis, structure and benzodiazepine receptor binding. Eur. J. Med. Chem. 36, 407-419.
37) Aly, Y., El-Barbary, A., El-Shehawy, A. 2004. Phosphorus, Sulfur, Silicon, Relat. Elem. 179, 185.
38) Frolov, K. A., Dotsenko, V. V., Krivokolysko, S. G., Litvinov, V. P. 2005. Russian Chemical Bulletin, International Edition. 54, 2226-2228.
39) Griffiths, D., Hull, R., Seden, T. P. 1980. J. Chem. Soc., Perkin Trans.1, 1240.
40) Saczewski, F., Gdaniec, M. 1992. J. Chem. Soc., Perkin Trans.1, 47.
41) Hesek, D., Rybar, A., Alfoldi, J., Bella, J., Patoprsty, V. 1996. Collect. Czech. Chem. Commun. 61, 1642.
42) Vovk, M. V., Dorokhov, V. I., Samarai, L. I., 1993. Dokl. Akad. Nauk Ukr. 11, 140.
43) Fr. Pat. 2512450, 1983. Chem. Abstr. 99, 70775.
44) Johnson, T. B., Nicolet, B. H. 1911. J. Am. Chem. Soc. 33, 1973.
45) Broom, N. J. P., Harrington, F. P. 2003. 6-Substitutedmethylene penems and intermediates. US Patent 6600035 (B1).
46) Kiec-Kononowicz, K., Karolak-Wojciechowska, J., Muller, C. E.,Geis, U., Ksiazek, W., Szymanska, E., 1999. J. Heterocycl. Chem. 36, 257-263.
47) Kiec-Kononowicz, K., Karolak-Wojciechowska, J., 1992. Phosphorus, Sulfur, Silicon. 73, 235-248.
48) El Ashry, E. S. H., Rashed, N., Awad, L. F., Ramadan, E., Abdel-Maggeed, S. M., Rezki, N. 2007. Nucleosides, Nucleotides and Nucleic Acids. 26, 423-435.
49) Shvedov, V.I., Sycheva, T. P., Sakovich, T. V. 1979. Khim. Geterotsikl. Soedin. 10, 1331.
50) Shaifullah-Chowdhury, A.Z.M., Shibata, Y. 2001 J. Heterocyclic Chem., 38, 743.
51) Gursoy, A., Iyikosker, T., Terzioglu, N., Otuk, G. 2005 Turkish J. Pharm. Sci. 2(1), 1-10.
52) Gewald, K., Schinke, E., Boettcher, H., 1966. Chem.Ber., 99, 94.
53) (a) Attia, Abdel M., Elgemeie,Galal H. 2003 Synthetic Communications 33(13),2243-2255.
(b) Wagner,G., Leistner,S.,Vieweg,H., Krasselt,U., Prantz,J. 1993 Pharmazie 48(7),514-518.
54) Golovko, T.v., Parshin, V.A., Asnina, V.V., kuleshova, E.F., Anisimova, O.S., Bogdanova, G.A.m Granik, V.G. 1990 Khimiko Farmatsevticheskii Zhurnal, 24(4), 32-35.
55) Geis, U., Kiec-Kononowicz, K., MuellermChrista, E. 1996 Scientia Pharmaceutica ,64(3/4), 383-390.
56) Shirai, H., Yashiro, T. 1967 Yakugaku Zasshi, 87(2), 142-147.
57) Kiec-Kononowicz, K., Karolak-Wojcieckowska, J., Robak, J. 1997 Arch. der. Pharmazie, 330(4), 85-90.
58) Weislow, O.W., Kiser, R., Fine, D., Badr, J., Shoemaker, R.H., Boyed, M.R., 1989. J. National Cancer Inst., 81, 577.
59) Monks,A., Scdiero, D.A., Skehan, P., Shoemaker, R.H., Pall,K.D., Vistica, D.T., Hose, C., Langley, J., Cronice,P., Vaigro-Wolf, M., Gray-Goodrich, M., Campbell, H., Mayo, M.R., 1991 . J. National Cancer Inst., 83, 757.

2/20/2013

Human Papillomavirus and Cervical Cancer: Use of Molecular Diagnostic Techniques

Hammoudah S.A.F.[1,2,3], Hannan M.A.[1,3], Al Harbi A.E. [1,3] and Al Harbi K.M. [1,3]

[1]Centre of Genetics and Inherited Diseases, Taibah University, Saudi Arabia
[2]Faculty of Medicine, Tanta University, Egypt
[3]Faculty of Medicine, Taibah University, Saudi Arabia
saharfathi2011@yahoo.com

Abstract :Human papillomavirus (HPV) infection is associated with a number of diseases that vary from self-limited warts to life threatening types of cancers(head and neck, ano-genital, oropharengeal and cervical cancers). Cervical cancer is considered the third common cancer among women and the fourth cause of increased mortality. The etiology of cervical cancer has been attributed to Human Papilloma Virus (HPV) in >99% of cases. While the genotypes of HPV linked to cervical cancer may vary in different parts of the world, almost 70% of cases around the world have been found to be due to two types of HPV namely HPV 16 and HPV 18. Molecular techniques using target specific HPV DNA amplification by PCR is an essential step for genotyping HPV as HPV DNA quantity in most samples is very low. There are three main techniques used for HPV detection and genotyping: Target amplification [amplify a specific DNA sequence from a targeted gene e.g. polymerase chain reaction (PCR)], signal amplification (increase the DNA-proportional signal to detectable levels using branched DNA or hybrid capture technology) and probe amplification (amplify the probe e.g. ligase chain reaction). Other traditional, non-amplified based molecular techniques which include Southern blot hybridization, in situ hybridization (ISH), and dot blot are hardly used nowadays. The accurate detection of HPV genotypes has played a pivotal role in both molecular and epidemiological studies. Determining the prevalence and genotypes of HPV infection in women is mandatory for the development of vaccines targeting the oncogenic types of HPV. To achieve these goals, the HPV DNA test needed to be designed with the highest analytical sensitivity and specificity.

[Hammoudah S. A. F.. Hannan M. A., Al Harbi A. E. and Al Harbi K.M. **Human Papillomavirus and Cervical Cancer: Use of Molecular Diagnostic Techniques.** *Life Sci J* 2013;10(1):2012-2022] (ISSN:1097-8135). http://www.lifesciencesite.com. 287

Key words: Human papillomavirus, prevalence , genotyping, molecular diagnosis

1- Introduction

In developing countries, the leading cause of cancer mortality among women is believed to be cervical cancer. [(1)] Worldwide, cervical cancer is considered the third common cancer among women and the fourth cause of increased mortality. [(2)] Studies on the etiology of cervical cancer showed Human Papilloma Virus (HPV) to be associated with >99% of this cancer. [(3)] While the types of HPV linked to this disease may vary in different parts of the world, almost 70% of the tumor samples around the world have been shown to contain two types of HPV namely HPV 16 and HPV 18. [(4)]

The establishment of a causal relationship of HPV with cervical carcinoma led to the development of anti-HPV vaccines as a method of controlling HPV infection and hence, reducing the incidence of cervical cancer. In such a preventive strategy, the current world-wide vaccine formulation has targeted two most prevalent types of HPV (i.e HPV16 and HPV18) . [(5)] It is believed that by protecting women against infection with these two types of HPV, most cervical cancers would be prevented. Type specific anti-HPV vaccines is now a major strategy for preventing cervical cancers in women across the world. [(2,5)]

The accurate detection of HPV genotypes has played a pivotal role in both molecular and epidemiological studies. Determining the prevalence and genotypes of HPV infection in women is mandatory for the development of vaccines targeting the specific oncogenic types of HPV. To achieve these goals, the HPV DNA test needed to be designed with the highest analytical sensitivity and specificity. [(6,7)]

Molecular techniques using target specific HPV DNA amplification by PCR is an essential step for genotyping HPV as HPV DNA quantity in most samples is very low. [(5)] For genotyping HPV, while different methods are available, the INNO-LiPA assay is very useful in identifying oncogenic HPV-positive women. This assay is reliable, relatively cost-effective and easy to perform. [(7)]This review discusses the usefulness of different molecular techniques for detecting HPV genotypes.

History of HPV:

An outstanding work done by Jablonska and Gerard Orth at the Pasteur Institute in 1978 had led to the discovery of HPV-5 in skin cancer. [(8)] A documented hypothesis published in 1976 by Harald zur Hausen stated that the human papilloma virus plays an important role in the cause of cervical cancer. [(9)]

Human papillomavirus (HPV):

HPV is a double stranded DNA virus (dsDNA)-which infects human by infecting keratinocytes in

the mucus membrane or in the skin. [10] In persistent HPV infection, HPV viral sequences are always integrated into the cellular DNA of most HPV-induced cancers. [11]

HPV is replicated only in the basal cells of stratified epithelium, so HPV infection is restricted to such cells. The slow infectious process allows for the development of antibodies which play the most important neutralizing role while the virions still inhabit the basement membrane and cell surfaces. [12]

The genome of HPV, which is ds-DNA, contain Open Reading Frame (ORF) genes coding for eight types of proteins responsible for viral replication. These ORF gene products have been classified into 6 early stage (E1, E2, E3, E4, E5, E6) and 2 late stage (L1, L2) proteins . Once the HPV infects the host cells, E1 and E2 HPV proteins are expressed. When HPV dsDNA incorporats itself into the host DNA, the E2 HPV protein function is interrupted. Normally E2 suppresses the function of E6 and E7. The disruption of E2 function consequently leads to the activation of E6/E7. Persistent expression of oncoproteins E6 and E7 result in malignant transformation of infected cells. [2,13] E6 inactivates p53 while E7 inactivates pRb. The inactivation of these two very important tumor suppressor genes (p53 and pRb) might be the main etiological cause for cancer development. [14]

HPV genotypes and diseases:

It has been reported that, there are more than 200 genotypes of HPV. Fortunately most of these genotypes do not cause symptoms in people. Some types can cause warts and very few genotypes were related to cancer mainly cervical cancer or anogenital cancer. High-risk HPV genotypes include type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 which might lead to the development of various types of cervical, vulvar, and/or anal neoplasia. [15]

WHO/ICO Information Centre on HPV and Cervical Cancer (HPV Information Centre) had reported that, more than 99% of cervical cancers are associated with HPV infection. [16] There is a wide variation in cancer incidence, type and mortality among different ethnic groups, which might be due to a difference in socio-economic status, availability of health care, pathogen exposure and carcinogen exposure. Worldwide differences in cancer epidemiology might be also due to an interaction of genetic factors, environmental changes and various tumor biology. [17,18]

Although HPV genotypes directly linked to the pathogenesis of cervical cancer are somewhat variable worldwide, there are other diseases than cervical cancer that have been attributed to HPV. [13,14] For example, high risk HPV is also linked to the pathogenesis of anogenital and oropharengial cancers which might affect both males and females. International Agency for Research on Cancer (IARC) reported that although cervical cancer almost exclusively caused by HPV but other sexually transmitted (STD) diseases might be caused by HPV as well. [19]Non-cervical HPV-related cancers is reported to be linked to the risk HPV genotypes (HPV16 and HPV18) which is detected in 5.2% and 3.7% of the world population respectively. [20]

Infections by low risk HPV genotypes were found to be associated with benign genital warts (condyloma acuminata) in women and the penis, scrotum or anus in men. These low risk genotypes were also associated with juvenile respiratory papillomatosis or recurrent respiratory papillomatosis (JRP-RRP). [21]

HPV type 16, among other types of HPV has been reported to be associated with HPV-positive oropharyngeal cancer (OSCC). [11]

Due to large number of new cervical cancer cases caused by HPV globally every year, HPV is considered as an important transmittable cause of cancer. [22]

It is believed that, the early detection of high risk HPV genotypes and the use of vaccines against them will reduce both cervical cancer and other HPV triggered diseases.

2-Transmission

Genital infections

Sexual activity is the main cause for HPV transmission. Approximately 40 identified HPV genotypes infect the genital tract. The risk of infection is reported to be markedly increased in women having more than one partner. Condoms could not be considered as a protection from HPV infection as the areas around the genitals are not covered. [23,24] Male circumcision is reported to have protective effect against HPV infection and cervical cancer incidence in female partner. [25]

Prenatal transmission:

Juvenile-onset of recurrent respiratory papillomatosis (JORRP) can be caused by prenatal transmission of HPV types 6 and 1. JORRP represent a relatively rare disease in the United States. [26]

3-Epidemiology and prevalence of HPV globally:

Most cases of genital HPV infections do not cause any obvious symptoms and the immune system is able to get rid of the infection in a few months. Immunity is believed to be type specific especially for cutaneous HPVs. This immunological control and clearance may fail in some infected individuals. Persistent infection with high-risk HPV types might be responsible for the pathogenesis of cervical cancer or other types of cancer. [27]

High-risk HPV types 16 and 18 were reported to be the main cause of about two-thirds of cervical cancer cases. [28] Type 16 accounts for the vast majority of HPV-induced vaginal/vulvar cancers, anal cancers, penile cancers, and head and neck cancers. [29]

Lee et al., [30] investigated the prevalence and the genotypes of HPV among Korean women in a total sample of 60.775 (aged 18-79 yr, median 44). They reported that HPV positive rate was 34.2% of which 87.7% were found to be due to single type infections, while multiple HPV types were reported in 12.3%. As regard the genotype, HPV-16 was found to be the most prevalent, followed by HPV-52, HPV-58 and HPV-18 respectively.

In a meta-analysis review published by Shi et al.[31] collecting evidence for the load of cervical cancer in China, it was reported that HPV infection incidence in China was less than 5/100.000 which is considered to be low compared to other countries. These findings are intriguing and need to be interpreted cautiously as the registries examined were only from high socioeconomic status regions without a consideration of the low-socioeconomic rural areas which might have higher incidence of HPV.

Rivera et al.,[32] investigated the prevalence and genotype distribution of HPV in 929 apparently healthy women from Mexico City using Pap smear cytological examination and multiplex PCR for HPV DNA detection. HPV infection was detected in 9.1%. High risk genotypes (16-18) were detected in 99% of those cases. Only 15% of the HPV positive cases showed abnormal cervical cytology. Their study highlights the importance of HPV screening and genotyping in healthy women. Also, it showed increased frequency of high risk HPV genotypes among Mexican women. These results warrant further investigations as the high frequency of high risk HPV genotypes (16-18) among healthy women is unexpectedly high (99%).

In USA more than 24 million persons are infected with HPV, and annually reported new cases is about 4 millions. [33]

Raza et al., [34] reported that in Karachi, Pakistan, the prevalence of HPV among general population was 2.8%, and the most common genotypes were HPV16 and 18. Young women showed no evidence of higher prevalence.

In Iran, the prevalence of HPV has been reported to be 5% and, seven different HPV genotypes have been detected, six of them being high risk genotypes (16, 18, 31, 33, 51 and 56) beside HPV-66 which was "probably carcinogenic." [35] In Algeria, the prevalence and genotypes of HPV in cervical cancer cases and control women were similar to that reported in Europe unlike the one in sub-Saharan Africa, where HPV 16 is less prevalent. [36]

A population-based cohort study of HPV in Chilean women to evaluate the prevalence of HPV for five years had shown that, high risk-HPV prevalence increased by 43%. Incidence was the highest in women < 20 years of age. [37]

In Denmark, a large population-based study was done to determine the overall and age-specific HPV prevalence, and HPV type distribution in women. The HPV prevalence was found to be 26.4% with a peak in women with an age range of 20-24 years. HPV 16 was the most prevalent type followed by HPV 18. [38]

Cervical HPV DNA prevalence among general women population in Guatemala was reported to be 38.1% while 67.3% in sex worker women. The most prevalent genotypes were HPVs 51 66 and 16. [39]

The published data regarding the prevalence of HPV genotypes in invasive cervical cancers in Italy from 1996 to 2008 was reviewed by Rossi *et al.,* [40] They performed pooled and multivariate analysis and showed that, the proportion of HPV 16 and/or 18 decreased with age, although it increased in cancers diagnosed in more recent years. Their results support the theory that, HPV 16/18 vaccination has beneficial effect on early onset cervical cancers.

A systematic review was conducted for the U.S. Preventive Services Task Force to compare liquid based cytology versus HPV detection and genotyping for primary screening of cervical cancer in 141 566 participants. [41] It concluded that, primary HPV screening detected more cases of CIN3 or cancer in women older than 30 years. The limitation of this study was that, short –term trial data that have been included might cause ascertainment unfairness.

Tricco et al., [42] systematically reviewed the prevalence of oncogenic cervical HPV infection among Canadian females in order to establish most advantageous vaccination strategies. The data collected from all investigated studies support the previous published data regarding those high risk genotypes HPV16 and 18 as the most prevalent genotypes. Also they reported that, vaccination might enhance cancer protection for female under age of 20 years.

Martín et al., [43] investigated the prevalence and genotype of HPV infection among 2.461women aged15-60 years living in Madrid (Spain) with normal and abnormal cytology. They reported HPV-16 as the most frequent type, followed by HPV 53 and HPV 31, but HPV 18 showed low prevalence. The commercially available vaccination targeted HPV16 and 18. These vaccines could decrease cervical cancer by 50%. HPV genotypes that are not covered by the available vaccines were reported to be frequent in women from Spain. So, before introducing HPV vaccination to any population detailed studies on the most prevalent genotype distribution should be considered to target the relevant HPV genotype.

The possible etiological role of persistent infection with HPV in high-grade cervical neoplasia was systemically reviewed by Koshiol et al., [44] in more than 22.500 women. They concluded that, persistence infection with HPV was constantly and highly associated with high grade cervical

neoplasia. The duration of persistency, HPV test interval and sampling procedure should be standardized before validation of the concept that HPV persistency is associated with high grade cervical neoplasia.

To evaluate the prevalence and genotypes of HPV DNA in women with normal cytology de Sanjosé et al., [45] systematically reviewed all published literature. They reported that, in all studied reviews globally 291 million women were found to be carriers of HPV DNA , of them 32% were carriers of HPV16 or HPV18, or both. These detected HPV types were similar to those described in pre-neoplastic and neoplastic cervical lesions. It should be mentioned that, the role of HPV16 and HPV18 is significantly lower in women with normal cervical cytology.

HPV prevalence and genotyping in Saudi Arabia:

Al-Muammar et al., [46] studied the prevalence of HPV16/18 in 120 Saudi and non-Saudi women living in Riyadh, during routine gynecological examination. They reported a high prevalence of HPV-16/18 among examined women. The progression rate of cervical intraepithelial neoplasia in those cases was reported to be low in a follow-up period of 4 years.

Sait and Gazzaz in 2011 conducted a study to detect HPV in cervical biopsy samples from 45 women Saudi women diagnosed with cervical cancer or cervical dysplasia, attending department of Obstetrics and Gynecology, Faculty of Medicine, King Abdulaziz University. They stated that, the most common genotype detected was HPV 16 .However, when they included cases with mixed infection, HPV 18 was found to be the second most common. [47]

Sait et al., [48] published a review using the Pub Med database between January 2000 till June 2011, including all the publications concerned with cervical cancer and cervical dysplasia in Saudi Arabia. They concluded that the distribution of HPV subtype in cervical cancer in Saudi Arabia still has not been extensively studied. Their data showed that, the absence of proper screening programs deprive women for their right of early detection of cytological changes. They stated that, cervical cancer could be prevented by proper HPV screening and genotyping. [48]

A WHO report on Human Papillomavirus and related cancer in Saudi Arabia states that: [15] **1-** No data are available from cancer registry about the incidence of cervical cancer, or age standardized incidence rate by histological types among Saudi women.

2- There is no data available on the genotype-specific HPV prevalence among Saudi women with and without cervical lesions.

3- There is no data available regarding the predictable reports of cervical cancer screening in Saudi Arabia, by age and study.

Screening and genotyping of HPV in a large number of native Saudi women with or without cervical cancer is now considered a high priority. This will provide data regarding the incidence and genotype of HPV among Saudi women, which is not available till now. A service platform based on these results is required to provide the clinical and genetic data for population screening service which is urgently needed in Saudi Arabia. Data on the HPV prevalence and genotypes will be helpful for the Ministry Of Health (MOH) before vaccination against HPV is introduced at the population level.

4-HPV infection, cervical cancer and vaccine development:

HPV infections in young females are almost temporary, with little or no long-term significance. Roughly after one year of infection, about two thirds of such infections will disappear and about 90% in two years. [18] Only 50–60% of women infected with HPV express HPV serum antibodies after natural infection. [49]

The approximate annual record of worldwide new cases of cervical cancer is about 500, 000 of which almost 80% have been diagnosed in developing countries. HPV is reported to be the main cause of cervical cancer. Standard gynecological screening combined with HPV DNA testing is believed to be of great value in preventing or reducing neoplastic transformation by discovery and treatment of precancerous lesions. [21]

The accurate detection of HPV genotypes has played a pivotal role in both molecular and epidemiological studies. Defining the prevalence and genotypes of HPV infection in women is mandatory for the development of vaccines targeting the oncogenic types of HPV. To achieve these goals, the HPV DNA test needed to be designed with the highest analytical sensitivity and specificity. [6] A relatively inexpensive but a reliable method of genotyping like INNO-LiPA can be routinely used for molecular epidemiological studies aimed at classifying the HPV positive women as well as for specific vaccine development. [7]

5- The current consideration for vaccines:

The current world-wide vaccine formulation has targeted two most prevalent types (i.e HPV16 and HPV18) associated with cervical cancer. It is believed that by protecting women against infection with these two types of HPV, most cervical cancers would be prevented. [5]

In April 2009, WHO published a paper on HPV vaccines. [50] The following quotation summarizes its recommendations: **"WHO recognizes the importance of cervical cancer and other HPV-related diseases as worldwide public-health trouble and recommends that regular HPV**

vaccination must be included in nationalized immunization programs. "

Types and use of HPV vaccines :

HPV vaccines besides being cost effective, is expected to decrease the incidence of cervical cancer, particularly in low-resource settings. In fact, model studies suggest that combining HPV vaccination and organized screening programs may have the highest impact on the disease control worldwide. (51)

HPV vaccines might be categorized into two groups, prophylactic vaccines which has been developed since 2006 and therapeutic which need to be developed in the near future. The prophylactic HPV vaccines include two types which were commercially available to avoid infection by some HPV types: quadrivalent vaccine (Gardasil, marketed by Merck against HPV 16, 18, 6, 11) and bivalent (Cervarix, marketed by GlaxoSmithKline against HPV 16 and 18). These two types of vaccines protect against preliminary infection with high risk genotypes 16 and 18. Gardasil is reported to be effective also towards low risk genotypes 6 and 11, which is the main causative agent for genital warts. (52)

These commercially available HPV vaccines were designed to be prophylactic (i.e. to prevent infection and consequent disease). Some reports stated that, sexually active women who have been infected with high risk HPV genotypes will get no or little benefit from these vaccines, but those who still are not sexually active will be protected by such vaccinations. (53)

In many countries, the vaccines are recommended to be given to females only. Only USA and UK have supported male HPV vaccination. No therapeutic effects of the vaccines on already HPV infected people have been reported. In 2010 >60% of teens in the US had gotten vaccination against meningitis and DPT and only about 49% have receive HPV vaccinations. (54)

After receiving the vaccine women should continue to undergo cervical screening, such as Pap smear testing. Recommendations for cervical cancer screening have not changed for females receiving HPV vaccine. Combination of both vaccination and continued Pap smear scanning might decrease the risk of cervical cancer development. Regarding children under 15 years of age, no efficacy trials have been performed. There is no strong practical clinical trial that can determine the duration of vaccine efficacy; however Cervarix effectiveness is proven for 6.4-7.4 years while Gardasil is proven for 5 years. (55) Protection after vaccination lasts for almost 5 years. Data are not yet available on the safety and efficacy of HPV vaccines in Africa. (20)

Data published from a meta-analysis by Lu et al. (56) evaluating the efficacy and safety of prophylactic vaccines targeting HPV infection and cervical cancer among women, concluded that prophylactic HPV vaccines were found to be very effective in preventing long standing HPV infection, safe in young female (less than 30 years of age). These vaccines were reported to be simply tolerated. It should be emphasized that, these vaccines prevent infection and cervical disease in a type specific manner. Further studies are needed to evaluate the safety, tolerability of vaccine related side effects and effectiveness of HPV vaccines in older age groups**.**

Earlier systemic reviews addressing evaluation of the efficacy HPV vaccine in preventing persistent cervical HPV infection in women by La Torre et al., (57) and Rambout et al., (58) had documented the protective role of the vaccine in all studies they had reviewed. They stated that the HPV vaccine efficacy in markedly evident in young women aged 15-25 years who had not been infected with HPV yet. We think that in order to evaluate the protective role of HPV in reduction in cervical cancer mortality, long-term follow-up is recommended.

6- Early Diagnosis and cervical cancer prevention:

Initial screening using a Papanicolaou (Pap) test or liquid-based cytology is used to check patients for premalignant or malignant lesions that require further evaluation. Early detection and appropriate treatment of any detected premalignant lesion is essential to prevent cervical cancer development. Pap-smear screening is used to detect any abnormal cytological changes. If any abnormal cytological changes are detected, colposcopy and biopsy of the suspected lesion are to be done. (59)

Cytological examination of cervical smears is mandatory to detect any abnormal squamous cell growth which termed as squamous intraepithelial lesions (SIL), graded low to high according to the degree of cervical epithelium affection and the presence of abnormal cells. Histopathological examination of cervical biopsies can detect and define cervical intraepithelial neoplasia (CIN), a term which means abnormal cells in the cervix that are graded from 1 to 3 according to the thickness of the cervical epithelium. In CIN 3, neoplastic cells invade more than 2/3s of the cervical epithelium. Similar grading exists for vaginal and vulvar lesions. In long standing HPV infection, the virus DNA integrated into the human DNA leads to moderate or severe carcinogenesis classified according to severity into: cervical intra-epithelial neoplasia (CIN 2, CIN 3 or adenocarcinoma in situ (AIS). (60)

Value of molecular testing in diagnosis of cervical cancer:

In developing countries, visual inspection and cytological detection of cervical cancer have been successful in screening large population at risk and provide adequate medical care. However, the necessity was felt for developing more refined and

accurate diagnostic tools. In developed countries, the use of HPV DNA testing have been useful in decreasing mortality rate due to cervical cancer up to 50%. Cuzick et al., [61] stated that, HPV DNA testing is the most beneficial choice for primary screening, but women who are HPV positive follow-up should include cytological examination plus HPV DNA testing.

In a meta-analysis reviewing the results of more than 25 non-randomized studies evaluating the diagnostic accuracy of HPV DNA testing in primary cervical screening Koliopoulos et al., [62] stated that HPV DNA testing is more sensitive but less specific than cytology in diagnosis of CIN2. A combination of cytological examination and HPV DNA has the high sensitivity but decreased specificity. As regard the prognostic value of HPV DNA testing versus cytology, there is no difference in cancer mortality or invasiveness in either cytologically defined or HPV DNA defined cases.

In an earlier study by Zielinski et al., [63] evaluating the use of HPV DNA testing for monitoring women treated for high grade cervical neoplasia, recommended the use of combination of both cytology and HPV DNA testing for better results.

Several studies have shown HPV screening and genotyping to be more sensitive than cytological testing for primary cervical screening. A meta-analysis and systematic review including all randomized controlled trials conducted from 2005 to 2010 was published in 2012 by Murphy et al., [64] who compared the value of HPV testing versus conventional cytological examination in primary cervical screening. They concluded that, HPV testing as the primary screening test for women 30 or 35 years of age and older is the most accurate and reliable. Optimal screening strategies for younger women need to be further evaluated.

A similar conclusion was drawn in a recent study conducted by Ogilive et al., [65] comparing primary cervical cancer screening with HPV DNA testing to liquid –based cytology. They stated that HPV DNA testing had increased cervical intraepithelial neoplasia detection when compared to liquid based cytology.

The usefulness, specificity, sensitivity and cost effectiveness of HPV DNA testing and genotyping compared to visual inspection methods for cervical cancer screening had been evaluated by Shi et al., [31] in rural China. They concluded that, if the test is to be done once-lifetime, maximum effectiveness had been achieved for women between 35-50 years of age .

7- Various Molecular techniques for detecting HPV:

Since, HPV cannot be cultured in-vitro and serological assays lack sensitivity and specificity, so the detection is entirely based on molecular methods. HPV infection is diagnosed indirectly by detection of its DNA in the cells obtained from a particular anatomic site. [66]

There are three main techniques used for HPV detection and genotyping: Target amplification [amplify a specific DNA sequence from a targeted gene e.g. polymerase chain reaction (PCR)], signal amplification (increase the DNA-proportional signal to detectable levels using branched DNA or hybrid capture technology) and probe amplification (amplify the probe e.g. ligase chain reaction). Other traditional, non-amplified based molecular techniques which include Southern blot hybridization, in situ hybridization (ISH), and dot blot are hardly used nowadays. [33,67]

A-Target Amplification Techniques

Polymerase Chain Reaction

Detection and amplification of HPV DNA is done mainly using polymerase chain reaction (PCR). In order to recognize and amplify a single genotype of HPV type specific PCR is usually done through targeting type-specific DNA sequence. Several repeats of PCR is necessary to determine the exact genotype existing in the sample. [68]

Reverse Line Blot and Linear Array

27 different HPV genotypes could be detected using the reverse line blot assay (Alameda, CA) developed by Roche Molecular Systems. This assay use L1 consensus primer-based PCR with PGMY09/11 primers. It is based on reverse line blot hybridization. The probes are membrane fixed [69] The Linear Array Test is CE-Marked for in vitro diagnostic use in Europe.

The reverse line blot hybridization linear array is the principle of another commercially available INNO-LiPA HPV Genotyping (Innogenetics, Ghent, Belgium), which could indentify 24 low- and high-risk HPV genotypes. This kit also is CE-Marked. The INNO- LiPA test amplifies HPV DNA using SPF10 primers located at the L1 region. The probes are membrane strips in sequence-specific lines and visualized with unaided eye as purple/brown bands. [70]

Amplicor HPV

The Amplicor HPV test developed by Roche Molecular Systems is a PCR-based test. 13 high-risk HPV types could be detected using this assay. PCR is used for amplification of the target DNA which will be followed by nucleic acid hybridization. Amplicor HPV only detects HPV but could not identify HPV specific genotype. [71]

PapilloCheck

PapilloCheck (Greiner Bio-One, Monroe, NC) is a commercial DNA-array based test used for HPV genotyping. Twenty-four genotypes of HPV could be detected using this technique. It depends on the amplification of E1 gene and DNA hybridization to HPV oligoprobes. [72] A laser scanner is needed to

detect the excitation from fluorescently labeled probes which bind to the HPV primers fixed on the PapilloCheck DNA chip containing the immobilized HPV probes.

Multiplex HPV Genotyping Kit

Multiplex Genotyping is a relatively new commercially available test (Multimetrix, Heidelberg, Germany). Multiplex HPV Genotyping kit is PCR-based fluorescent bead array. It could detect up to 24 HPV types with high and low risk genotypes. [73]

Although the Multiplex HPV Genotyping Kit is available for research only , in the near future it might be available for routine diagnostic use due to its high sensitivity. [74]

Real-Time PCR

Real-Time qPCR is a technique that combines the use of fluorescent probe and PCR primers. It is characterized by accurate quantification of HPV in the sample. RT-qPCR is considered a sensitive technique for HPV- DNA identification. [75]

GenoID assay is a commercially available kit using RT-PCR. It can identify 14 high risk and 5 low-risk in about three hours. This kit is also available for three types of RT-PCR platforms: Applied Biosystems 7900 HT, Corbett Rotor-Gene 6600 and Roche LightCycler 2.

B-Signal Amplification Techniques

Hybrid Capture Assay

In this assay fluorescent or chemiluminescent signal is amplified to assist detection, rather than the target DNA amplification. This assay is FDA approved. It is only useful for HPV detection not for genotyping. The principle is that specimens containing HPV DNA are hybridized with a HPV-specific RNA probe, this will produce a DNA:RNA hybrid molecule. A microplate-well coated with the first antibody targeting the DNA-RNA hybrids. Alkaline phosphatase-conjugated secondary antibody targeting the first antigen-antibody molecule then a signal is detected after the addition of a chemi- luminescent substrate. Signal amplification is due to several alkaline phosphatase molecules which have been conjugated to each antibody, and multiple conjugated antibodies can bind each captured hybrid. [76]

CareHPV

CareHPV is based on Hybrid Capture Assay (HC2) principle and is considered a HC2 spin-off (developed by Qiagen). It is the most common type of tests used for cervical screening in under developed countries. It can detect HPV but not genotypes. It is less expensive and almost about 90% accurate. [77]

Cervista HPV HR and Cervista HPV 16/18

This is the only test for HPV screening and genotyping which is FDA approved since 2009. The Cervista HPV HR which detect 14 high risk HPV genotypes and Cervista HPV16/18 which detect only high risk genotypes 16 and 18 are commercially available supplied by Third Wave Technologies, now Hologic, Bedford, MA.

Cervista test is based on a unique signal amplification technique called the Invader technology which uses 2 simultaneous isothermal reactions. Simply, the target DNA is denatured, a DNA probe including a sequence-specific region binds to the HPV DNA molecule. Then a specific Cervista test probe called the Invader which associates also with the target sequence, resulting in a 1-base overlapping. A specific Cervista test enzymes cleave the probe, releasing a 5' oligo flap. Multiple flaps are generated from each target DNA molecule. That flap then associates with a florescence resonance energy transfer (FRET) probe, resulting in cleavage at the indicated site. The fluorophore, is released from the quencher, causing a fluorescent signal which is directly proportional to the amount of target DNA. This results in a detectable fluorescent signal. [78]

The genome of HPV (ds-DNA molecule) is composed of eight types of Open Reading Frame (ORF) proteins responsible for replication, of them E6 and E7 has important role in cell proliferation and closely linked with HPV-associated carcinogenesis. [12-13] It could be of diagnostic interest if we were be able to measure the expression of HPV E6 and E7 genes, as over-expression of these genes will denote ongoing HPV-related carcinogensis. This could be achieved using the reverse-transcriptase (RT)-CR to detect HPV mRNA. Using RT-PCR for sensitive quantification of gene expression (protein products) of these genes (E6-E7) might give an indication on HPV virulence. Monitoring the levels of expression of these genes might be useful for screening and monitoring of cancer progression. [79] The only limitation is that RT-PCR still needs to be optimized for use in large scale HPV screening to ensure clinical sensitivity and specificity. [80]

Clinical efficacy of any HPV screening method should depend on the usefulness of the test to predict cervical cancer, not only HPV virus detection.. This is very important especially for young women, since virus detection has no clinical value because HPV infections in young females are almost temporary, with little or no long-term significance. HPV in these women mostly self- resolves and never develops into cancer. [81]

Before approval of any appropriate technique for use in clinical laboratory many aspects should be clarified such as the cost of the test, population to whom this test is directed, specificity and sensitivity

of the test. In underdeveloped countries, although our goal is early and accurate detection of cervical cancer, we might have to compromise for specificity and sensitivity in order to get rapid accurate , less expensive and easy to use test. Searching for molecular technique that is sensitive and specific for early and accurate diagnosis of cervical cancer must be a priority for researchers working in this field.

Corresponding author
Hammoudah SAF
-Center of Genetics and Inherited Diseases(CGID), Taibah University. Al Madinah AL Moonwarrah, Saudi Arabia. 009664243248
-Clinical and Chemical Pathology Department, Tanta University, Faculty of Medicine, Tanta, Egypt.
Saharfathi2011@yahoo.com
00201001709456,00201000030773, 009664243248

References:

1- Ferlay J, Bray F, Pisani P , and Parkin D M , "Globocan 2002 cancer incidence. Mortality and prevalence worldwide," IARC Cancer Base no 5, version 2.0, IARC Press, Lyon, France, 2004. http://globocan.iarc.fr/DataSource_and_methods.asp
2- Chao A, Huang HJ, and Lai CH: Human Papillomavirus Research on the Prevention, Diagnosis, and Prognosis of Cervical Cancer in Taiwan. Chang Gung Med J 2012;35:297-308.
3- World Health Organization : Cervical cancer, human papillomavirus (HPV), and HPV vaccines. Key points for policy-makers and health professionals. Geneva, World Health Organization, December 2008. http://www.who.int/reproductivehealth/publications/policy/en/index.html
4- Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R, Clifford GM: Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. International journal of cancer. Journal international du cancer, 2007, 121:621–632. http://www.ncbi.nlm.nih.gov/pubmed/17405118
5- Harper DM, Franco EL, Wheeler CM, Moscicki AB, Romanowski B, Roteli-Martins CM, Jenkins D, Schuind A, Costa Clemens SA, Dubin G; HPV Vaccine Study group : Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. Lancet. 2006;367(9518):1247–1255. http://www.ncbi.nlm.nih.gov/pubmed/16631880
6- Castle PE, Porras C, Quint WG, Rodriguez AC, Schiffman M, Gravitt PE, González P, Katki HA, Silva S, Freer E, Van Doorn LJ, Jiménez S, Herrero R, Hildesheim A; CVT Group: comparison of two PCR-based human papillomavirus genotyping methods [published online ahead of print August 20, 2008] J Clin Microbiol. 2008;46(10):3437–3445.
7- Iftner T, Germ L, Swoyer R, Kjaer SK, Breugelmans JG, Munk C, Stubenrauch F, Antonello J, Bryan JT, Taddeo FJ. Study comparing real-time multiplex human papillomavirus (HPV) PCR assays with INNO-LiPA HPV genotyping extra assay. J Clin Microbiol. 2009 Jul;47(7):2106-13.
8- Orth G, Hablonska S, Favre M, Croissant O, Jarzabek-Chorzelska M, and Rzesa G : Characterization of two types of human papillomaviruses in lesions of epidermodysplasia verruciformis. Proc Natl Acad Sci U S A. 1978 March; 75(3): 1537–1541. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC411508/
9- zur Hausen H, Meinhof W, Scheiber W, Bornkamm GW. Attempts to detect virus-secific DNA in human tumors. Nucleic acid hybridizations with complementary RNA of human wart virus. Int J Cancer 1974;13:650-6. www.benthamscience.com/open/toccj/articles/V005/1TOCCJ.pdf
10- Insinga RP, Dasbach EJ, Elbasha EH : Epidemiologic natural history and clinical management of Human Papillomavirus (HPV) Disease: a critical and systematic review of the literature in the development of an HPV dynamic transmission model. BMC Infect Dis. 2009; 9: 119. Published online 2009 July 29.
11-D'Souza G, Kreimer AR, Viscidi R: "Case-control study of human papillomavirus and oropharyngeal cancer". N. Engl. J. Med. 2007; 356 (19): 1944–56. www.ncbi.nlm.nih.gov/pubmed/17494927
12- Anil C and Gillison ML (March 4, 2010). "Human Papillomavirus and Head and Neck Cancer". In Andrew F. Olshan. Epidemiology, Pathogenesis, and Prevention of Head and Neck Cancer (1st ed.). New York: Springer en.wikipedia.org/wiki/HPV-positive_oropharyngeal_cance
13-Altinbas S, and Tapisiz O: Human papillomavirus vaccines, and protection from cervical cancer. Saudi Med J 2012;33(12);1270-1277. smj.psmmc.med.sa/ojs/index.php/smj/article/viewFile/467.
14- Boccardo E, Lepique A, and Villa LL: The role of inflammation in HPV carcinogenesis. Carcinogenesis vol.31 no.11 pp.1905–1912, 2010 doi:10.1093/carcin/bgq176 Advance Access publication September 5, 2010. http://carcin.oxfordjournals.org/content/31/11/1905.full.pdf
15-Insinga PR, Liaw KL, JohnsonLG, Madeleine MM: A Systematic Review of the Prevalence and Attribution of Human Papillomavirus Types Among Cervical, Vaginal and Vulvar Pre-cancers and Cancers in the United States . Cancer Epidemiol Biomarkers Prev. Author manuscript; available in PMC 2009 July 1. Published in final edited form as: Cancer Epidemiol Biomarkers Prev. 2008 July; 17 (7) : 1611–1622.
16-WHO/ICO Information Centre on HPV and Cervical Cancer (HPV Information Centre). Human Papilloma virus and Related Cancers in Saudi Arabia. Summary Report 2010. http://apps.who.int/hpvcentre/statistics/dynamic/ico/country-pdf/SAU.pdf?CFID=4005139&CFTOKEN=50211239
17- DennyL : Cervical cancer : prevention and treatment. Discov Med. 2012 Aug;14(75):125-31. http://www.ncbi.nlm.nih.gov/pubmed /22935209
18- Wallace TA, Martin DM and Ambs S:Interactions among genes, tumor biology and the environment in cancer health disparities: examining the evidence on

a national and global scale. Carcinogenesis vol.32 no.8 pp.1107–1121, 2011.

19- Human Papillomavirus and Oral Cancer: The International Agency for Research on Cancer Multicenter Study. JNCI J Natl Cancer Inst (2003) 95 (23): 1772-1783. jnci.oxfordjournals.org/content/95/23/1772.full

20- Chaturvedj AK: Beyond cervical cancer: burden of other HPV-related cancers among men and women: J Adolesc Health. 2010 Apr;46(4 Suppl):S20-6. www.ncbi.nlm.nih.gov/pubmed/20307840

21- Cutts FT , Franceschi S, Goldie S, Castellsague X, de Sanjose S, Garnett G, Edmunds, P Claeys WJ, Goldenthal KL, Harper DM, and Markowitz L: Human papillomavirus and HPV vaccines: a review Bull World Health Organ. 2007 September; 85(9): 719–726.

22- Parkin DM (2006). "The global health burden of infection-associated cancers in the year 2002". Int. J. Cancer 118 (12): 3030–44. http://www.ncbi.nlm.nih.gov/pubmed/16404738

23- Montalvo MT, Lobato I, Villanueva H, Borquez C, Navarrete D, Abarca J, Calaf GM.: Prevalence of human papillomavirus in university young women. Oncol Lett. 2011 Jul;2(4):701-706. www.ncbi.nlm.nih.gov/pubmed/22848252

24-Boccalini S, Tiscione E, Bechini A, Levi M, Mencacci M, Petrucci F, Bani Assad G, Santini MG, Bonanni P.: Sexual behavior, use of contraceptive methods and risk factors for HPV infections of students living in central Italy: implications for vaccination strategies. J Prev Med Hyg. 2012 Mar;53(1):24-9. www.ncbi.nlm.nih.gov/pubmed/22803316

25-Castellsague X, Bosch FX, Munoz N, Meijer CJ, Shah KV, De Sanjose S, et al. Male circumcision, penile human papillomavirus infection, and cervical cancer in female partners. N Engl J Med. 2002;346:1105–12.

26- Venkatesan NN, Pine HS, Underbrink MP.: Recurrent respiratory papillomatosis. Otolaryngol Clin North Am. 2012 Jun;45(3):671-94, viii-ix. http://www.ncbi.nlm.nih.gov/pubmed/22588043

27- Patel S, and Chiplunkar S : Host immunresponses to cervical cancer.Curr Opin Obstet Gynecol. 2009 Feb;21(1):54-9. www.ncbi.nlm.nih.gov/pubmed/19125004

28- Moyer VA : U.S. Preventive Services Task Force Screening for cervical cancer: U.S. Preventive Services Task Force recommendation statement. Ann Intern Med. 2012 Jun 19;156(12):880-91, W312. http://www.ncbi.nlm.nih.gov/pubmed/22711102

29- Lu B, Hagensee ME, Lee JH, et al. (February 2010). "Epidemiologic factors associated with seropositivity to human papillomavirus type 16 and 18 virus-like particles and risk of subsequent infection in men". Cancer Epidemiol. Biomarkers Prev. 19 (2): 511–6. doi:10.1158/1055-9965.EPI-09-0790. http://cebp.aacrjournals.org/content/early/2010/01/18/1055-9965.EPI-09-0790

30-Lee EH, Um TH, Chi HS, Hong YJ, and Cha YJ: Prevalence and Distribution of Human Papillomavirus Infection in Korean Women as Determined by Restriction Fragment Mass Polymorphism Assay. J Korean Med Sci 2012; 27: 1091-1097 http://dx.doi.org/10.3346/jkms.2012.27.9.1091

31-Shi JF, Canfell K, Lew JB, Qiao YL:The burden of cervical cancer in China: synthesis of the evidence. Int J Cancer. 2012 Feb 1;130(3):641-52.

32- Rivera MGL, Flores MOM, VillalbaMagdaleno GDA, and Monroy VS: Prevalence of Human Papillomavirus in Women from Mexico City. Infectious Diseases in Obstetrics and Gynecology, Volume 2012, Article ID 384758.

33- Arney A and Bennett K: Molecular Diagnostics of Human Papillomavirus. Labmedicine,2010; 41 (9); 523-530.

34- Raza SR, Franceschi S, Pallardy S, Malik FR, Avan BI, Zafar A, Ali SH, Pervez S, Serajuddaula S, Snijders PJF, van Kemenade FJ, Meijer C J L M , Shershah S,[3] and CliffordGM: Human papillomavirus infection in women with and without cervical cancer in Karachi, Pakistan. Br J Cancer. 2010 May 25; 102(11): 1657–1660. Published online 2010 April 20.

35- Eghbali SS, Amirinejad R, Obeidi N, Mosadeghzadeh S, Vahdat K, Azizi F, Pazoki R, Sanjdideh Z, Amiri Z, Nabipour I, and Zandi K: Oncogenic human papillomavirus genital infection in southern Iranian women: population-based study versus clinic-based data. Virol J. 2012; 9: 194. Published online 2012 September 12.

36-Hammouda D, Muñoz N, Herrero R, Arslan A, Bouhadef A, Oublil M, Djedeat B, Fontanière B, Snijders P, Meijer C, Franceschi S.: Cervical carcinoma in Algiers, Algeria: human papillomavirus and lifestyle risk factors. Int J Cancer. 2005 Jan 20;113(3):483-9.

37-Ferreccio C, Van De Wyngard V, Olcay F, Domínguez MA, Puschel K, Corvalán AH, Franceschi S, Snijders PJ.: High-risk HPV infection after five years in a population-based cohort of Chilean women. fect Agent Cancer. 2011 Nov 16;6(1):21.

38-Kjaer SK, Breugelmans G, Munk C, Junge J, Watson M, Iftner T.: Population-based prevalence, type- and age-specific distribution of HPV in women before introduction of an HPV-vaccination program in Denmark. Int J Cancer. 2008 Oct 15;123(8):1864-70.

39- Vallès X, Murga GB, Hernández G, Sabidó M, Chuy A, Lloveras B, Alameda F, de San José S, Bosch FX, Pedroza I, Castellsagué X, Casabona J.: High prevalence of human papillomavirus infection in the female population of Guatemala. Int J Cancer. 2009 Sep 1;125(5):1161-7.

40- Rossi PG, Sideri M, Carozzi FM, Vocaturo A, Buonaguro FM, Tornesello ML, Burroni E, Mariani L, Boveri S, Zaffina LM and Chini F: the HPV Prevalence Italian Working Group: HPV type distribution in invasive cervical cancers in Italy: pooled analysis of three large studies. Infectious Agents and Cancer 2012, 7:26http://www.infectagentscancer.com/content/7/1/26

41-Whitlock EP, Vesco KK, Eder M, Lin JS, Senger CA, Burda BU.:Liquid-based cytology and human papilloma virus testing to screen for cervical cancer: a systematic review for the U.S. Preventive Services Task Force. Ann Intern Med. 2011 Nov 15;155(10):687-97, W214-5.

42-Tricco AC, Ng CH, Gilca V, Anonychuk A, Pham B, Berliner S.: Canadian oncogenic human papillomavirus cervical infection prevalence:

systematic review and meta-analysis. BMC Infect Dis. 2011 Sep 5;11:235.
43- Martín P, Kilany L , García D, López-García AM, Martín-Azaña AJ, Abraira V , Bellas C**:** Human papillomavirus genotype distribution in Madrid and correlation with cytological data. *BMC Infectious Diseases* 2011, 11:316
44-Koshiol J, Lindsay L, Pimenta JM, Poole C, Jenkins D, Smith JS: Persistent human papillomavirus infection and cervical neoplasia: a systematic review and meta-analysis. Am J Epidemiol. 2008 Jul 15;168(2):123-37.
45-de Sanjosé S, Diaz M, Castellsagué X, Clifford G, Bruni L, Muñoz N, Bosch FX.: Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. Lancet Infect Dis. 2007 Jul;7(7):453-9.
46-Al-Muammar T, Al-Ahdal MN, Hassan A, Kessie G, Dela Cruz DM, Mohamed GE: Human papilloma virus-16/18 cervical infection among women attending a family medical clinic in Riyadh. Ann Saudi Med. 2007 Jan-Feb;27(1):1-5.
47-Sait KH, Gazzaz FS :Molecular tests to detect human papillomavirus infection in patients with cervical dysplasia and invasive cervical cancer in Saudi Arabia. Pathology and Laboratory Medicine International 2011:3 25–32.
48- Sait KH, Bentley J, Anfinan N, Power. P:Cervical Cancer Prevention in Saudi Arabia: It is Time to Call for Action! The Open Women's Health Journal, 2012, 6, 1-5 (http://creativecommons.org/licenses/by-nc/3.0/)
49-Carter JJ, Koutsky LA, Hughes JP, Lee SK, Kuypers J, Kiviat N, et al. Comparison of human papillomavirus types 16, 18, and 6 capsid antibody responses following incident infection. J Infect Dis. 2000;181:1911–9.
50-Human papillomavirus (HPV) vaccine background paper. Geneva, World Health Organization, September 2008. http://www.who.int/immunization/documents/HPVBGpaper_final_03_04_2009.pdf
51- Goldie S. A public-health approach to cervical cancer control: considerations of screening and vaccination strategies. International Journal of Gynaecology and Obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics, 2006, 94 (Suppl. 1):S95–S105. http://www.ncbi.nlm.nih.gov/pubmed/17276172
52-Lowy DR, Schiller JT (2006). "Prophylactic human papillomavirus vaccines". J. Clin. Invest. 116 (5): 1167–73. doi:10.1172/JCI28607.http://www.jci.org/articles/view/JCI28607
53-Human papillomavirus vaccines. WHO position paper" Wkly. Epidemiol. Rec. 84 (15): 118–31. April 2009. www.ncbi.nlm.nih.gov/pubmed/19360985
54-Markowitz LE, Dunne EF, Saraiya M, Lawson HW, Chesson H, Unger ER (March 2007). Cervical Cancer Screening Among Vaccinated Females. "Quadrivalent Human Papillomavirus Vaccine: Recommendations of the Advisory Committee on Immunization Practices (ACIP)". MMWR Recomm Rep 56 (RR–2): 17. www.ncbi.nlm.nih.gov/pubmed/17380109
55-Harper D : "Current prophylactic HPV vaccines and gynecologic premalignancies" (pdf). Current opinion in obstetrics & gynecology; 2009; 21 (6): 457–464. http://sverigesradio.se/diverse/appdata/isidor/files/4278/12375.pdf
56-Lu B, Kumar A, Castellsagué X, Giuliano AR: Efficacy and safety of prophylactic vaccines against cervical HPV infection and diseases among women: a systematic review & meta-analysis. BMC Infect Dis. 2011 Jan 12;11:13.
57-La Torre G, de Waure C, Chiaradia G, Mannocci A, Ricciardi WHPV vaccine efficacy in preventing persistent cervical HPV infection: a systematic review and meta-analysis. Vaccine. 2007 Dec 5;25(50):8352-8. Epub 2007 Sep 29.
58-Rambout L, Hopkins L, Hutton B, Fergusson D.:Prophylactic vaccination against human papillomavirus infection and disease in women: a systematic review of randomized controlled trials. CMAJ. 2007 Aug 28;177(5):469-79.
59-Hormuzd A. Katki, Walter K. Kinney, Barbara Fetterman, Thomas Lorey, Nancy E. Poitras, Li Cheung, Franklin Demuth, Mark Schiffman, Sholom Wacholder, Philip E. Castle: Cervical Cancer Risk for 330,000 Women Undergoing Concurrent HPV Testing and Cervical Cytology in Routine Clinical Practice at a Large Managed Care Organization Lancet Oncol. Author manuscript; available in PMC 2012 February 6. Published in final edited form as: Lancet Oncol. 2011 July; 12 (7): 663–672. Published online 2011 June 16.
60-Ostor AG. Natural history of cervical intraepithelial neoplasia: a critical review. Int J Gynecol Pathol. 1993;12:186–92.
61-**C**uzick J, Arbyn M, Sankaranarayanan R, Tsu V, Ronco G, Mayrand MH, Dillner J, Meijer CJ:Overview of human papillomavirus-based and other novel options for cervical cancer screening in developed and developing countries. Vaccine. 2008 Aug 19;26 Suppl 10:K29-41.
62-Koliopoulos G, Arbyn M, Martin-Hirsch P, Kyrgiou M, Prendiville W, Paraskevaidis E: Diagnostic accuracy of human papillomavirus testing in primary cervical screening: a systematic review and meta-analysis of non-randomized studies. Gynecol Oncol. 2007 Jan; 104(1):232-46.
63-Zielinski GD, Bais AG, Helmerhorst TJ, Verheijen RH, de Schipper FA, Snijders PJ, Voorhorst FJ, van Kemenade FJ, Rozendaal L, Meijer CJ.: HPV testing and monitoring of women after treatment of CIN 3: review of the literature and meta-analysis. Obstet Gynecol Surv. 2004 Jul;59(7):543-53.
64-Murphy J, Kennedy EB, Dunn S, McLachlin CM, Fung Kee Fung M, Gzik D, Shier M, Paszat LHPV testing in primary cervical screening: a systematic review and meta-analysis. J Obstet Gynaecol Can. 2012 May;34(5):443-52.
65-Ogilvie GS, Krajden M, van Niekerk DJ, Martin RE, Ehlen TG, Ceballos K, Smith LW, Kan L, Cook DA, Peacock S, Stuart GC, Franco EL, Coldman AJ: Primary cervical cancer screening with HPV testing compared with liquid-based cytology: results of round 1 of a randomised controlled trial - the HPV FOCAL Study. Br J Cancer. 2012 Dec ;107(12):1917-24.
66- Molijn A, Kleter B, Quint W, van Doorn LJ.: Molecular diagnosis of human papillomavirus (HPV) infections. J Clin Virol. 2005;32(suppl 1):S43–S51.

http://www.journalofclinicalvirology.com/article/S1386-6532%2804%2900362-2/abstract
67-Malloy C, Sherris, J, Herdman, C. HPV DNA Testing: Technical and Programmatic Issues for Cervical Cancer Prevention in Low-Resource Settings. 2000. Available at: www.path.org/publications/details.php at March 16, 2010.
68- Chin-Hong PV, Klausner JD. New diagnostic tests for HPV in the developed and the developing world. MLO Med Lab Obs. 2008;40:48,50,52–53. http://labmed.ascpjournals.org/content/41/9/523.full.pdf
69-Gravitt PE, Peyton CL, Apple RJ, Wheeler CM: Genotyping of 27 human papillomavirus types by using L1 consensus PCR products by a single-hybridization, reverse line blot detection method. J Clin Microbiol. 1998 Oct; 36(10):3020-7.
70- INNO-LiPA HPV Genotyping Extra [package insert]. Ghent, Belgium: Innogenetics; 2007. http://www.bio-protech.com.tw/databank/DataSheet/PathoDiag/Innogenetics/user%27s%20manual/81064_HPVamp_110704.pdf
71- Sandri MT, Lentati P, Benini E, Dell'Orto P, Zorzino L, Carozzi FM, Maisonneuve P, Passerini R, Salvatici M, Casadio C, Boveri S, Sideri M :Comparison of the Digene HC2 assay and the Roche AMPLICOR human papillomavirus (HPV) test for detection of high-risk HPVgenotypes in cervical samples. J Clin Microbiol. 2006;44:2141–2146. http://www.ncbi.nlm.nih.gov/pubmed/16757611
72- Dalstein V, Merlin S, Bali C, Saunier M, Dachez R, Ronsin C. :Analytical evaluation of the PapilloCheck test, a new commercial DNA chip for detection and genotyping of human papillomavirus. J Virol Methods. 2009; 156: 77–83. http://www.sciencedirect.com/science/article/pii/S0166093408004114
73- Rautava J, Willberg J, Louvanto K, Wideman L, Syrjänen K, et al. (2012) Prevalence, Genotype Distribution and Persistence of Human Papillomavirus in Oral Mucosa of Women: A Six-Year Follow-Up Study. PLoS ONE 7(8): e42171.
74-. Schmitt M, Bravo IG, Snijders PJ, et al. Bead-based multiplex genotyping of human papillomaviruses. J Clin Microbiol. 2006;44:504–512.
75-Villa LL, Denny, L. Chapter 7: Methods for detection of HPV infection and its clinical utility. International Journal of Gynecology & Obstetrics. 2006;94(suppl 1):S71–S80. http://screening.iarc.fr/doc/HPV%20supplement%20-%20chapter%2007.pdf
76- Clavel C, Masure M, Putaud I, Thomas K, Bory JP, Gabriel R, Quereux C, Birembaut P. :Hybrid capture II, a new sensitive test for human papillomavirus detection. Comparison with hybrid capture I and PCR results in cervical lesions. J Clin Pathol. 1998;51:737–740. http://www.ncbi.nlm.nih.gov/pubmed /10023335
77-Qiao YL, Sellors JW, Eder PS, Bao YP, Lim JM, Zhao FH, Weigl B, Zhang WH, Peck RB, Li L, Chen F, Pan QJ, Lorincz AT. :A new HPV-DNA test for cervical-cancer screening in developing regions: A cross-sectional study of clinical accuracy in rural China. Lancet Oncol. 2008;9:929–936. http://www.ncbi.nlm.nih.gov/pubmed/18805733
78-Invader. 2009. Available at: www.twt.com/ invader. html. Accessed December 18, 2012.
79-Cuzick J, Mayrand MH, Ronco G, et al. Chapter 10: New dimensions in cervical cancer screening. Vaccine. 2006; 24(suppl 3):S3/90–97.
80-Lie AK and Kristensen G: Human papillomavirus E6/E7 mRNA testing as a predictive marker for cervical carcinoma. Expert Rev Mol Diagn. 2008;8:405–415.
81- Wiley D and Masongsong E: Human papillomavirus: The burden of infection Obstet Gynecol Surv. 2006;61(suppl 1):S3–S1.

2/3/3013

Water content controlled instead of suction controlled strength tests

Reza Ahmadi-Naghadeh[1], Nabi Kartal Toker[2], Mohammad Ahmadi-Adli[3]

[1.2.3.] Department of Civil Engineering, METU, Ankara, Turkey
e176494@metu.edu.tr

Abstract: Most soils that concern geotechnical engineering are in the state of partial water saturation. Current practice tries to predict engineering properties of cohesionless soils using data from tests on saturated specimens, regardless of the saturation in the field. Due to complexity of test setups and high technical requirements, unsaturated soil tests are not among the common equipment of soil mechanics laboratory. One of these problems is the existence of suction, which is a function of water content and affects the strength behavior of unsaturated soils. Procedures to keep the water content of the partially saturated specimens constant and homogeneous in conventional soil tests are not well-established. The exception to this is unsaturated test setups, which are costly, complicated and found only in research institutions, hence prohibiting the industry from keeping up with the developments in this field. This study explores simple modifications to conventional methodologies of triaxial and direct shear tests, with the ultimate aim of preventing temporal and spatial variability of specimen water content throughout test duration. For different modifications, specimens of each test are dissected at the end of the test, and water content profiles of the specimens are obtained.
[R Ahmadi-Naghadeh, N. K. Toker, M. Ahmadi-Adli. **Water content controlled instead of suction controlled strength tests**. *Life Sci J* 2013;10(1):2023-2030] (ISSN:1097-8135). http://www.lifesciencesite.com. 288

Keywords: Unsaturated soil; soil suction; water content; triaxial test; direct shear test

1. Introduction

In most geotechnical problems, engineers encounter soils in partially saturated condition. And yet, unsaturated soil mechanics is a relatively new branch that has developed significantly during the past few decades. This is due to the complexity of the unsaturated soil behavior and complication of experimental setups required to study this behavior.

Unsaturated soil tests do not see widespread use in soil mechanics laboratories due to a few difficulties. One of these difficulties is the need for measurement and/or control of suction, which is the unsaturated counterpart to pore water pressure in classical (saturated) soil mechanics. Matric suction is one of the fundamental state variables that control the shear strength behavior of unsaturated soil specimens, which in turn is dependent on the initial water content and the method of specimen preparation (Vanapalli et al., 1996).

Geotechnical engineers resort to laboratory strength tests to measure the response of saturated soils to varying stress conditions. The most common strength tests are triaxial test, which is primarily employed for testing of saturated undisturbed specimens, and direct shear test, which is more commonly used for (dry or saturated) reconstituted specimens. Strength tests are also classified according to the control of drainage and pre-shear stress: consolidated drained (CD) consolidated undrained (CU) and unconsolidated undrained (UU). For unsaturated soils, tests can be run with controlled suction (Cunningham et al., 2003; Jotisankasa et al., 2007 &Teng1 and Ou, 2011) or constant water content (Bishop et al., 1960; Bishop and Donald, 1961; Rahardjo et al., 2004). In a constant water content (CW) test, air phase is drained, and the water phase is undrained. This test simulates the field condition at lower degrees of saturation, with continuous air phase, where excess pore-air pressure can dissipate instantaneously, and excess pore-water pressure dissipates with time. However, in such tests, uniformity of water content throughout the specimen may be questionable.

This study explores simple modifications to conventional testing methodologies with the ultimate aim of preventing temporal and spatial variability of specimen water content throughout strength tests. For this purpose, two series of laboratory tests (triaxial and direct shear) were done on sandy specimens under various water content and stress conditions. The only test results discussed here are water contents at sample preparation and end of test.

2. Equipment and Material Overview

2.1 Triaxial Setup

In this study, a GEOCOMP fully automated triaxial setup is employed to carry out the triaxial tests. The triaxial system consists of a load frame, a pressure volume actuator (PVA) for controlling cell pressure, a computer for test control and data acquisition. A second PVA for pore pressure and volume control and measurement is also a part of the setup, but this component is not used in unsaturated tests. Each PVA utilizes a high speed, precision

micro stepper motor to regulate pressure and volume in the cell or specimen. The built-in microprocessor controls the micro stepper motor, which drives a piston in and out of a sealed cylinder. A pressure transducer on the end of the cylinder provides the feedback for control of pressure. Movement of the motor is used to compute volume change. The PVAs are capable of maintaining the desired pressure to within ±0.35 kPa (0.05 psi) while monitoring volume changes to within ±0.001 cc or ±1 mm^3 (Geocomp, 2010).

A water trap (sealed container of air and water with two inlets); and another container with an atmospheric air-water interface are added to the setup (Figure 1) in the later stages of this work, as described in section 3.

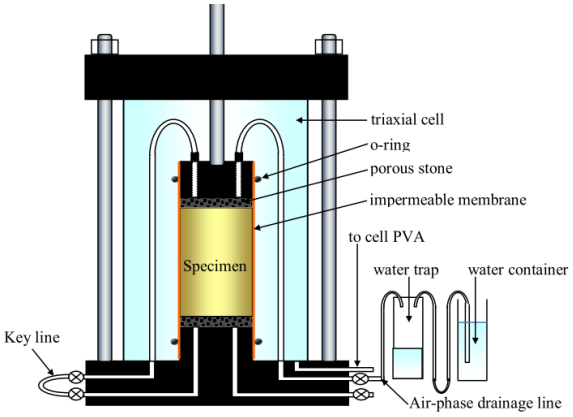


Figure 1. Schematic diagram of the triaxial cell with additions

number of individuals of most important species, Ns is the number of individuals of least important species and E is the evenness index.

2.2 Direct Shear Setup

For direct shear tests (DST) a VJ automated DST machine used. This setup uses a 60 mm circular shear box. Its shearing motor rate is adjustable in 0.05mm/min increments. Added to the equipment are impermeable plexiglas discs with the same dimensions as the porous stones, and a wet towel.

2.3 Soil Properties

The soil used in this study is a sandy soil from Izmir area (shortened as Iz Sand), with no plastic fines. Table 1 and Figure 2 present its index properties, grain size distribution (GSD) and soil water characteristic curve (SWCC). The SWCC lays out three distinct drainage modes of soils: (i) saturated regime at suctions lower than the air entry value (about 7 kPa for this soil), (ii) bulk drainage or funicular regime where small variations of suction cause significant changes in water content (i.e. the steep portion of the curve between 8-22% water contents) and (iii) residual or pendular regime where all water is trapped around the particle-particle contacts.

Table 1. Material properties

Index properties	Gs	2.68
	USCS	SM
GSD indexes	D10 (mm)	0.11
	D30 (mm)	0.26
	D50 (mm)	0.49
	D60 (mm)	0.6
Atterberg limits	LL %	N.P.
	PL %	N.P.
	PI %	N.P.

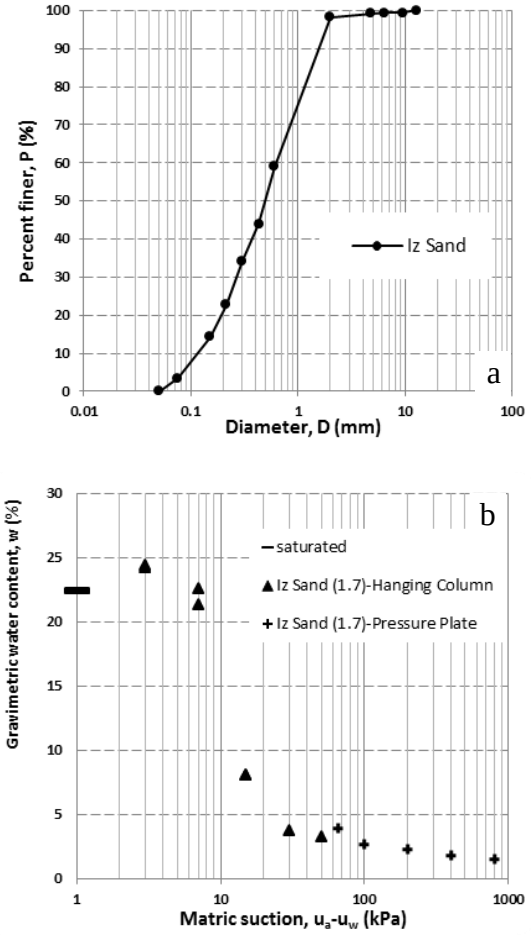


Figure 2. a) Particle size distribution, b) Soil Water Characteristic Curve of this soil at dry density of 1.7 g/cm3

3. Triaxial Tests

Triaxial specimens with 5 cm diameter and 10 cm height are prepared by the under-compaction method (Ladd, 1978) on the triaxial pedestal by controlling the density and water content. The

preparation equipment consists of a conventional split mold and custom made hammer as shown in Figure 3. This hammer includes a steel rod for tamping and an adjustable collar for precise control of layer height.

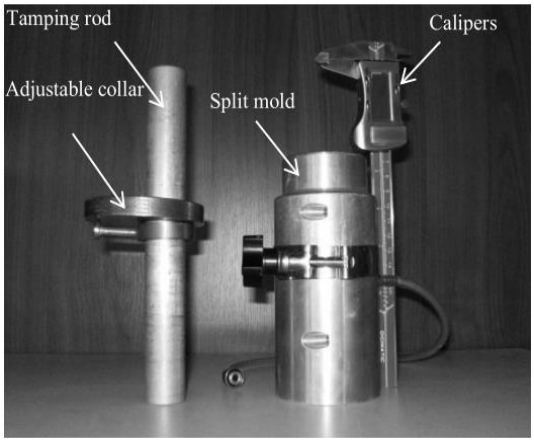


Figure 3. Equipment used for compaction

Specimens are arranged in five layers. Initially, the entire sample was mixed as a single batch, from which the soil was taken as small portions for each layer. However, this meant the lowermost layer, as it is placed first, had the highest water content, and as the soil of the following layers had waited longer, evaporating, they had gradually decreasing preparation water contents. To remove this error and to prepare uniform specimens, material is mixed in five different dishes, each with the desired water content (Figure 4a). A syringe is used for precise control of the amount of water added to the soil. Before placing each layer's material into the mold, its water content is measured prior to placement. Then each layer is placed and compacted to the desired density by controlling the thickness of the layer for a given mass of soil (Figure 5).

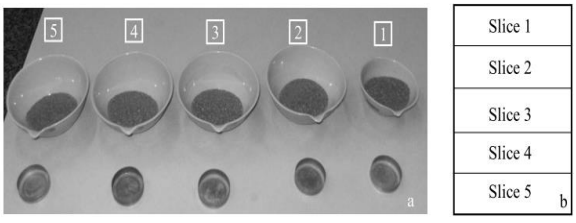


Figure 4. Specimen layers, a) soil prepared for each layer b) order of layers

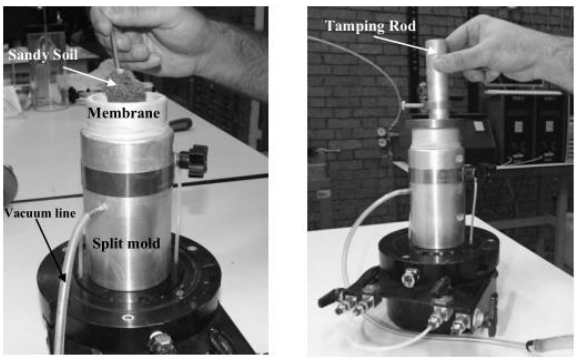


Figure 5. Preparing unsaturated specimen with desired water content

Then the triaxial cell is filled with distilled de-aired water. Next, the test conditions are specified and triaxial test is controlled by the computer from start to finish. In the CW tests, the air-phase line is kept open to atmospheric pressure throughout the test procedure. However, this air-drainage line is placed into a water container with atmospheric pressure, to avoid evaporation of water from the specimen. In order to prevent water flow between the phreatic water container and specimen (which was observed in earlier tests), a water trap was added on this line.

The specimen is then consolidated to desired isotropic stress, and then sheared at a constant rate of strain of 0.217 %/min. The same strain rate was used during shear in all triaxial tests presented here. The entire test takes 2-3 hours. At the end of test, air drainage valve is closed immediately and the specimen is removed as quickly as possible from the triaxial cell. Then, the specimen is divided into five slices and final moisture content of each slice is measured. The order of slices is shown in Figure 4b.

Nineteen triaxial tests were carried out at different confining pressures, initial water contents and drainage conditions. In these tests, specimens were prepared in both loose and dense condition, with dry density of 1.35 and 1.70 g/cm3, and initial water contents of 5, 10 and 15%. These are summarized in Table 2.

Two UU tests were done by initial water content of 5% under 40 and 80 kPa, sealing the specimen by placing wax paper at its boundaries. There was a little variation in water content of the layers (Figure 6), but the results are within the acceptable absolute error margin of water content measurements (0.5% according to ASTM D2216). However, due to inability of this system to keep air pressure constant, these types of tests were abandoned in favor of CW tests.

Table 2. Summary of triaxial test conditions

Drainage Conditions	Initial Water content (%)	Dry density (gr/cm^3)	Confining pressure (kPa)	Operator initials	Porous stone moisture
UU	5	1.7	40	RAN	-
UU	5	1.7	80	RAN	-
CW	10	1.7	30	RAN	10% wc
CW	10	1.7	70	RAN	10% wc
CW	10	1.7	140	RAN	10% wc
CW	15	1.7	30	RAN	15% wc
CW	15	1.7	140	RAN	15% wc
CW	5	1.7	30	RAN	5% wc
CW	5	1.7	70	RAN	5% wc
CW	5	1.7	140	RAN	5% wc
CW	5	1.35	30	MAA	5% wc
CW	5	1.35	70	RAN	5% wc
CW	5	1.35	140	MAA	5% wc
CW	5	1.7	140	MAA	5% wc
CW	5	1.7	140	RAN	Saturated
CW	5	1.7	140	MAA	dry
CW	5.5	1.7	70	RAN	dry
CW	5.5	1.7	30	RAN	dry
CW	5.5	1.35	140	RAN	dry

Afterwards, five CW tests were performed on specimens prepared at 10% and 15% water contents and sheared under 30, 70 and 140 kPa cell pressures. The water content values are found to increase from top of the specimen to bottom (Figure 7), indicating a trend of water migration within the specimen. The variation of water content is slightly more pronounced in the specimens with 15% percent preparation water content compared to the specimens with 10% water content. In specimens with high water content, the water phase permeability is also greater, while gravity becomes significant compared to suction. Both of these mechanisms contribute to water migration from top to down.

The tests were performed by two different operators. Comparing the results of the tests with preparation water content of 5%, there is an operator error in preparing specimens where the final water content of the specimens prepared by one of the operators are slightly lower and these of the other are slightly higher than 5%. However, this doesn't affect the uniformity of water content, as the difference between the water contents of layers of each test are within the acceptable absolute error margin of 0.5% (Figure 8).

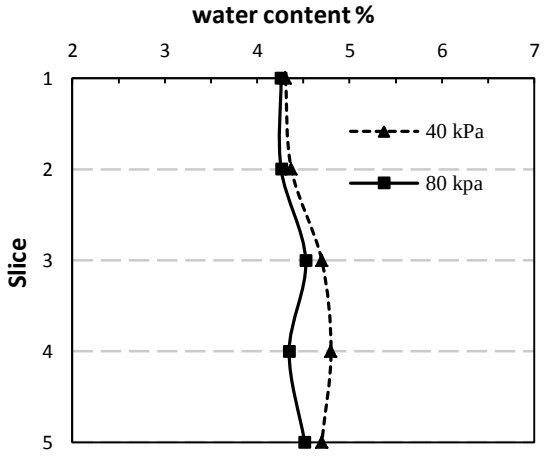


Figure 6. Water content variation at the end of the UU tests with preparation water content of 5%, at two different cell pressures

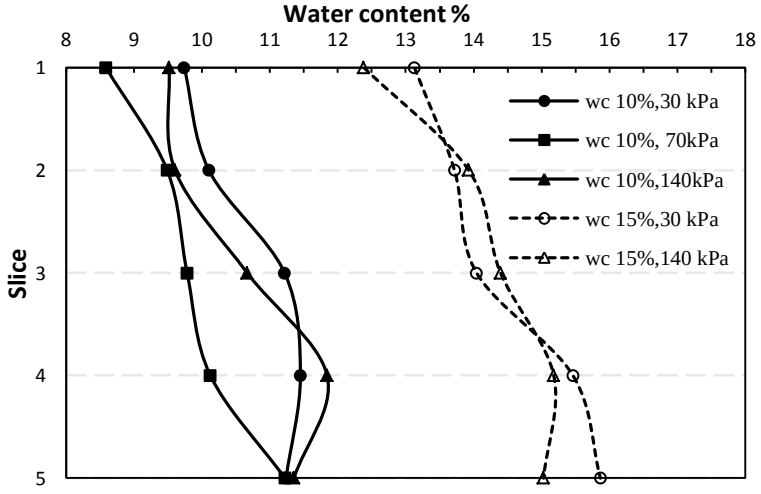


Figure 7. Variation of final water content, for specimens prepared at dry density of 1.7 g/cc, with 10 and 15% preparation water contents. The porous stones have 10 and 15% water content, respectively, at the start of test

To consider the effect of porous stone moisture on specimen water content, three CW triaxial tests were done. These tests were performed on specimens with 5% preparation water content, using dry, 5% moist and saturated porous stones. Results are shown in Figure 9.

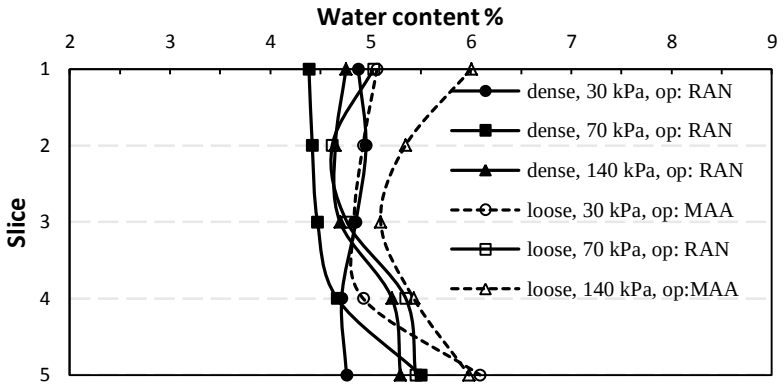


Figure 8. Effect of operator on variation of final water content in CW triaxial tests with 5% initial water content

The final water contents of the specimen with saturated porous stones are significantly higher than its preparation water content, and are maximum at the ends where the specimen is in contact with the porous stones. This indicates that some of the water was sucked into the soil from the fully saturated porous stone. When the porous stone is 5% moist, again a small amount of water is sucked by the top and bottom of the specimen. This is related to difference between the SMC curve of porous stone and soil sample. The test with dry porous stone gives most uniform water content values throughout the specimen. In addition, in all mentioned tests, specimens were observed to lose 0.5% water content at average, due to evaporation, during the specimen preparation. Therefore, performing the tests with dry porous stone and preparing the specimens with a preparation water content that is 0.5% higher than the desired value is the next step.

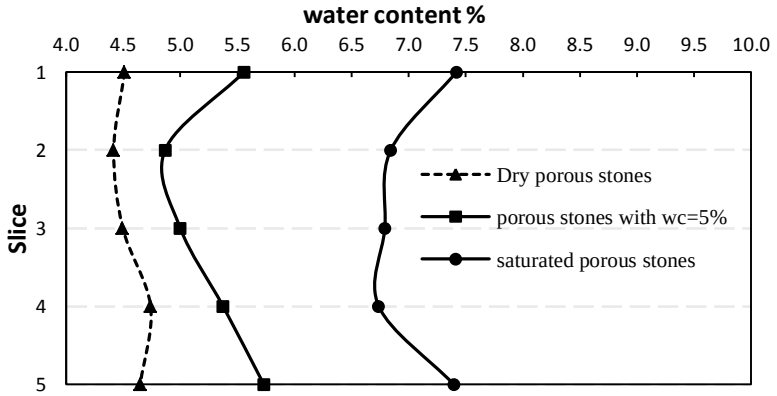


Figure 9. The effect of stone moisture on specimen water content. All of the three specimens have 5% preparation water content and 1.7 g/cc dry density

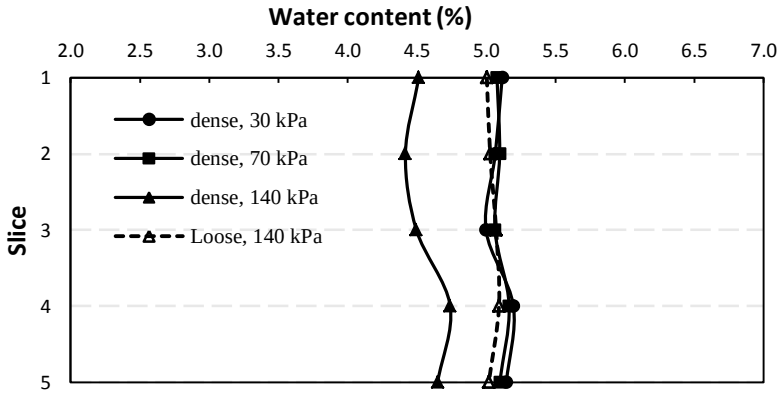


Figure 10. Variation of final water content with height, for tests with dry porous stones

Considering all of the previous tests, the least spatial variability of water content was observed in the test with dry porous stones. Therefore, three more triaxial tests with dry porous stones were performed to verify the repeatability of the procedure. To remedy the difference between the desired and measured water content (the "dry porous stone" curve in Figure 9 is about 4.5% whereas the desired water content was 5%), the preparation water content of the specimen was increased by the difference (0.5%). These test were done on both loose and dense specimens, and are presented alongside the initial dry porous stone test (labeled as 5%) in Figure 10. The results in Figure 10 show that there is no significant variation in water content from layer to layer. Moreover, confining pressure and density does not seem to noticeably affect water content homogeneity through the layers. All of the specimens with dry porous stones have lost 0.4 – 0.5% water content during preparation. For the three tests with 5.5% preparation water content, the mean difference between the measured and desired water content is 0.08% with a standard deviation of 0.06%, accounting for each layer's water content separately.

4. Direct Shear Tests

In order to do the direct shear tests, two significant modifications were made on the standard setup: replacing the porous stones with impermeable discs, and keeping the shear box humid.

In preliminary DST tests water content was found to have spatial variation at the end of the test. This variation in water content was observed in the form of dryness near the upper and lower porous stones. Soil water content changes due to the difference of suction between the specimen and the porous stones because of difference in SMC curve of the stone and soil sample. As a solution, in water controlled DSTs, the top and bottom boundaries are made impermeable, by replacing the porous stones with Plexiglas plates. The authors used two Plexiglas platens with the same thickness instead of the porous discs (Figure 11a). Note that the ribbed metal plates that provide the rough boundary surfaces above and below the specimen in conventional DST are not removed from the setup.

An additional observation during preliminary tests was reduced water content at the end of the test. This hints at evaporation during shearing, probably through the gap between two halves of the shear box, and possibly through the drainage ducts on the shearing box. In order to prevent evaporation, humidity around the shearing box was preserved by enveloping it within a wet cloth or towel (Figure 11b).

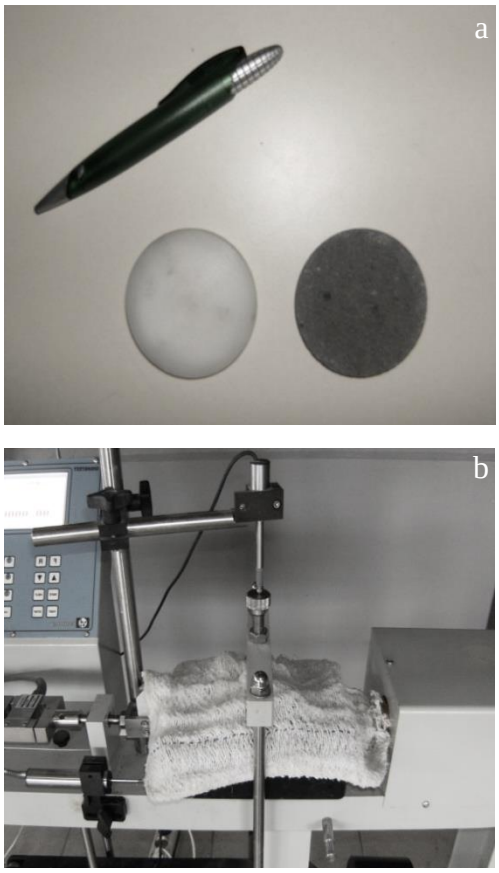


Figure 11. Modifications to DST. a) Plexiglas replacing porous stone, b) Shear box clothing

This moisture blanket covers the shear box loosely, in order not to introduce errors to the shear force measurements. However, it must be noted that this procedure was applicable only for the tests in cohesionless material since shearing rate was high enough to let the test finish before there is any significant transfer of humidity between the cloth and the specimen, or the cloth dries.

As the specimen of DST is relatively thin, the entire specimen is prepared at the desired initial water content and placed at intended dry density at once, as a single layer. Shearing rate is selected as in conventional DSTs (ASTM D3080), which in this case is horizontal displacement rate of 0.1 mm/min.

In order to study the water content changes in specimen through the tests multiple samples obtained for water content measurement from each of upper and lower (in some tests, only middle) parts of the specimen at the end of tests and compared with water content before the tests. Combinations of three preparation water contents (approximately 5, 10 and 15% – the preparation water contents were not precisely controlled as single-layer specimens are initially assumed as homogeneous), two initial dry

densities (1.35 & 1.8 gr/cm3) and four normal stresses (20, 40, 80 & 150 kPa) were tested (24 tests in total).

Mostly two samples from each of upper and lower parts are taken for water content determination (W_{up} and W_{bot}). Investigating each test or set of tests shows that; as a general rule, water contents in the samples with lower initial moisture (5 for both densities and 10% for dense specimen) tend to stay uniform. The variations of water content at the end of the test (W_f) from initial water content (W_i) are shown in Figure 12. Water content of specimens with 5% preparation moisture deviated at most 0.3% from the initial value.

Water contents of specimens with about 10% preparation moisture deviated by up to 0.5% from the initial value during the test (Figure 12), and the water content was detected to increase with depth. In the dense specimens, this increase is no greater than 0.2% from top to bottom of the specimen, while it can be as large as 1.7% in the loose specimens (Figure 13). This may be because the SWCC of a dense specimen is lower on the water content axis compared to a loose specimen of the same soil (for a given suction, dense specimen can hold more water). The boundary between bulk drainage regime and pendular regime being around 9.5% would also cause this difference between the two sets of tests, whose preparation water contents are around 9% and 10%.

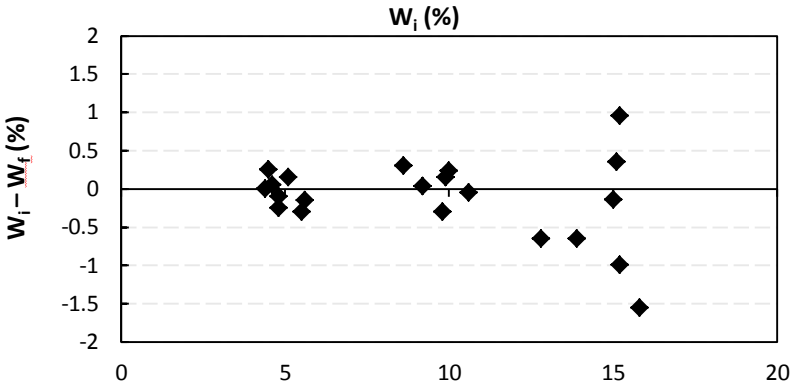


Figure 12. Variation of water content at the end of the test from initial value

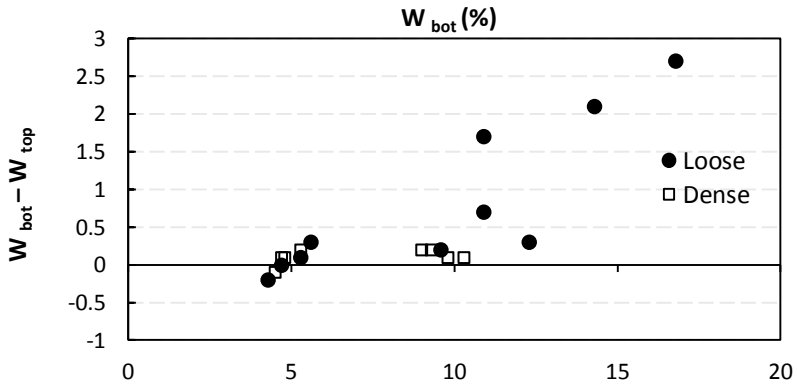


Figure 13. Variation of water content from top to bottom

In the specimens with high (15%) preparation water content, the range of differences between preparation and final water contents increase to ±1.6% (Figure 12), while downward transport of water (Figure 13) becomes more pronounced (up to 2.7% difference between top and bottom water contents at the end of test). The latter result (increased downward flow of water) is expected as the water phase permeability and relative effect of gravitational forces both increase with increasing water content. This result was also encountered in the triaxial tests where the preparation water content lies in the bulk drainage range.

4. Conclusion

For each laboratory tests type (triaxial and direct shear), a series of experiments were conducted on granular specimens, under various water-content and stress conditions. Based on the water content measurements before and after each test, the best

procedure to keep the specimen moisture uniform and constant throughout each test type is devised, for test durations within the range of a few hours.

In order to carry out such a constant water content test in either test type, water content of the specimen must be within the pendular regime (high suction, low water content portion of SWCC). For larger water contents, the extra (bulk) water is significantly affected by gravity, causing in a downward moisture gradient within the specimen.

Differences of the final procedure to run a CW triaxial test, compared to a standard CD test (ASTM D 7181) are: (i) the pore pressure-volume measurement/control system is removed from the setup. (ii) The specimen is prepared at a water content that is 0.5% higher than the desired value, which must be within the pendular regime. (iii) The porous stones must be dry. (iv) The pore drainage line of the triaxial setup must be connected to the air phase of a sealed container of air and water, which in turn must be connected to a phreatic water reservoir. (v) If desired, specimen volume changes may be measured by calibrated measurements of cell fluid volume (Ahmadi-Naghadeh and Toker, 2012).

In order to perform CW tests, the following modifications were made to the direct shear setup and test procedure: (i) The specimen is prepared at the desired water content. (ii) Drainage through the top and bottom must be prevented by replacing the porous discs with two plexiglas platens of the same dimensions. (iii) In order to prevent evaporation, air surrounding the shear box has to be kept humid by loosely covering the shear box with a wet cloth or towel.

For both test types, the modifications outlined above result in the water content to be controlled throughout the specimen dimensions and testing duration, within the acceptable absolute error margin of water content measurements (0.5% according to ASTM D 2216).

Acknowledgements:

We would like to thank the Scientific and Technical Research Council of Turkey (TÜBİTAK) for supporting this project financially under PhD Fellowship Program for Foreign Citizens grant 2215.

Corresponding Author:
Reza Ahmadi-Naghadeh
Department of Civil Engineering, METU, Ankara, Turkey
E-mail: e176494@metu.edu.tr

2/10/2013

References

1. Ahmadi-Naghadeh, R. and Toker, N. K. (2012). Volume change measurement in triaxial testing of unsaturated soils, 3rd International Conference on New Developments in Soil Mechanics and Geotechnical Engineering, Nicosia, North Cyprus.
2. ASTM D2216 (2010). Standard Test Methods for Laboratory Determination of Water (Moisture) Content of Soil and Rock by Mass, Annual Book of ASTM Standards, American Society for Testing and Materials, Easton, MD.
3. ASTM D3080 (2011). Standard Test Method for Direct Shear Test of Soils Under Consolidated Drained Conditions, Annual Book of ASTM Standards, American Society for Testing and Materials, Easton, MD.
4. ASTM D7181 (2011). Method for Consolidated Drained Triaxial Compression Test for Soils, Annual Book of ASTM Standards, American Society for Testing and Materials, Easton, MD.
5. Bishop, A. W., Alpan, I., Blight, G. E., and Donald, I. B. (1960). Factors controlling the shear strength of partly saturated cohesive soil. ASCE Research Conference on Shear Strength of Cohesive Soils, pp. 503-532, Boulder, Colorado.
6. Bishop, A. W., and Donald, I. B. (1961). The experimental study of partly saturated soil in the triaxial apparatus. 5th International Conference on Soil Mechanics and Foundation Engineering, Vol. 1, pp. 13-21, Paris.
7. Cunningham, M. R., Ridley, A. M., Dineen, K., and Burland, J. J., (2003). The mechanical behavior of a reconstituted unsaturated silty clay. Geotechnique, Vol. 53, No. 2, pp. 183-194.
8. Geocomp Corp. (2010). TRIAXIAL Software Manual.
9. Jotisankasa, A., Coop, M., Ridley, A. (2007). The development of a suction control system for a triaxial apparatus, Geotechnical Testing Journal, Vol. 30, No. 1, pp. 69-75
10. Ladd, R.S., (1978). Preparing test specimens using under compaction. Geotechnical Testing Journal, Vol.1, No.1, pp.16-23.
11. Rahardjo, H., Heng, O. B., and Leong, E. C. (2004). Shear strength of a compacted residual soil from consolidated drained and constant water content triaxial tests. Canadian Geotechnical Journal, 41, pp.1-16.
12. Teng, F. and Ou, C., (2011). Application of a suction control system in the method of specimen saturation in triaxial tests. Geotechnical Testing Journal, Vol. 34, No. 6, pp.1-10.
13. Vanapalli, S. K., Fredlund, D. G., Pufahl, D. E. (1996). The relationship between the soil-water characteristic curve and the unsaturated shear strength of a compacted glacial till. Geotechnical Testing Journal, Vol. 19, No. 3, pp. 259-268.

Antihypertensive Prescribing Pattern and Blood Pressure Control among hypertensive patients over a Ten Year period in a Primary Care Setting in Malaysia

Chia Yook Chin [1, 2 *] Victoria L Keevil[3] Ching Siew Mooi [4]
[1] Department of Primary Care Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, 50603, Malaysia.

[2] Curtin Health Innovation Research Institute, Faculty of Health Sciences, University of Curtin, GPO Box U1987, Perth, Western Australia 6845, Australia. chiayc@um.edu.my
[3]University of Cambridge: Strangeways Research Laboratory; Wort's Causeway, Cambridge. CB1 8RN, UK vlk20@cam.ac.uk
[4]Department of Family Medicine, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Serdang Selangor, 43400, Malaysia siewmooi@medic.upm.edu.my

Abstract: Suboptimal control blood pressure (BP) leads to multiple complications. This study aims to examine BP control and the change in prescribing pattern of antihypertensive agents over a 10-year period. Data was obtained from the 10-year retrospective cohort of randomly selected adult patients registered with the Department of Primary Care Medicine Clinic at the University of Malaya Medical Centre. Demographic data, BP and anti-hypertensive drug use in 1998, 2002 and 2007 were captured from patient records. Target BP control was defined as BP <140/90mmHg for those with hypertension alone and <130/80mmHg for those hypertensives with concomitant diabetes mellitus or chronic kidney disease. A total of 886 hypertensives patients were recruited. The mean age was 57.2 years (SD±9.6); 63.1% were female. The mean BP at baseline and at the end of 10-year were 146 / 87 (18/10) mmHg and 136/80 (16/9) mmHg respectively. In 1998, 74.3%, 22.5% and 1.6% were on monotherapy, 2 agents and ≥3 agents respectively. In 2007 after 10 years, 24.9%, 46.5% and 26.9% were on monotherapy, 2 agents and ≥3 agents respectively. At the end of 10 years there was improvement in overall blood pressure control, increasing from 15.6% in 1998 to 43.7% in 2007. However, the control rate of BP is still far from optimal in spite of an increase in the number of agents per patients used over a10 year follow-up. Based on our study the majority of patients with hypertension will need 2 or more agents to achieve target BP.
[Chia Yook Chin,Victoria L Keevil, Ching Siew Mooi. **Antihypertensive Prescribing Pattern and Blood Pressure. Control among hypertensive patients over a Ten Year period in a Primary Care Setting in Malaysia.** *Life Sci J* 2013;10(1):2031-2035] (ISSN:1097-8135). http://www.lifesciencesite.com. 289

Keywords: Hypertension; Blood pressure control; Antihypertensive ; Prescribing; Cohort; Primary care; Malaysia.

1. Introduction

Hypertension has been recognised as a modifiable risk factor for cardiovascular diseases such as stroke, heart disease and renal disease since the 1950s [1]. It is highly prevalent in populations worldwide and effective evaluation, treatment and control of high blood pressure to target levels (<140/90mmHg) has unquestionable health benefits [2]. Studies have shown that effective treatment significantly reduces cardiovascular disease mortality and morbidity and the deterioration of kidney function among patients with hypertension [2-6].

However, despite extensive public health campaigns, primary and secondary intervention strategies, hypertension and its negative health consequences continue to pose a worldwide problem. In total, hypertension is estimated to account for 4.5% of the global disease burden [7].

The concept of cardiovascular disease as a problem restricted to the industrialised world is outdated. It is the leading cause of death across Asia and much needed research into cardiovascular disease in the Asia-Pacific region is beginning. A number of longitudinal cohort studies, investigating the associations between cardiovascular risk factors and stroke, coronary heart disease and mortality are currently active [8]. These aim to characterise the differences and similarities between Western and Asian populations. Such knowledge is vital to allow the design and implementation of effective local intervention strategies. Presently, evidence emerging from these cohort studies suggests the hazards of high blood pressure are similar between East and West [9].

Malaysia, a middle income country in South East Asia, has a high and rapidly rising prevalence of hypertension. Information from cross sectional population surveys has shown the prevalence of hypertension in adults (≥ 30 years old) has increased from 32.9% in 1996 to 42.6% in 2006[10, 11]. This relative increase of 28.6% combined with the high absolute prevalence is alarming, but similar trends have been observed in other low or middle income

Asian countries, such as China [12]. In comparison high income countries have seen less dramatic rises or have even seen falls. For example, in the USA the age-adjusted prevalence of hypertension was 24.4% in the 1988-1994 National Health and Nutrition Examination Survey, compared to 28.9% in the 1999-2004 survey; a relative increase of 18%[13]. More locally, in New Zealand and Australia, a downward trend in population systolic blood pressure has been observed, and in New Zealand this trend has been estimated to account for up to 42% of the observed decline in coronary heart disease mortality from 1980 to 2004[14].

Despite the high absolute prevalence of hypertension in Malaysia, improvements in awareness, treatment and control of hypertension have been limited. In 2006, only 26.3% achieved target blood pressure control amongst the respondents who were aware of their hypertensive state and currently receiving treatment [11]. These figures have only marginally improved from the 1996 national survey [10] and are comparable with the control rates reported in the Chinese national survey (2001-2002)[12]. However, these figures fall short of those reported for high income countries. In the USA 35.1% of the patients with hypertension achieved target blood pressure control (1999-2004), a relative increase of 34.5% in those achieving control since 1988-1994[13].

Therefore, more work is needed to address the imbalance between the high prevalence of hypertension and its control especially in view that control rate is generally less than 50%[2, 15]. To date information gathered on hypertension and its management in Malaysia is derived only from cross sectional population snapshots. To our knowledge, no information is available on the presence and management of hypertension from longitudinal cohort studies. Here we report findings from a retrospective cohort study of primary care patients attending an urban clinic in Malaysia over ten years. This retrospective cohort study aimed to examine blood pressure control and the use of antihypertensive agents amongst a hypertensive population treated at a university hospital primary care clinic in Malaysia. It is important to look at how some improvements have been made and where future efforts and resources should be directed.

2. Material and Methods

2.1 Setting

This data is part of a 10-year retrospective cohort study of patients registered with the Department of Primary Care Medicine Clinic at the University of Malaya Medical Centre (UMMC). This clinic besides offering the usual primary care services also provides substantial care for patients with chronic diseases like hypertension, diabetes and dyslipidaemia. It serves around 450,000 inhabitants. This clinic is run by family medicine specialists, vocational trainees in family medicine and other medical officers.

2.2 Inclusion criteria

The cohort was randomly selected from the clinic patient records based on numbers generated by a computer program. Baseline data was collected in 1998, and follow-up data collected in 2002 and 2007 at five-year intervals. Out of this original cohort, all adult patients aged 18 with diagnosis of hypertension i.e. BP $\geq$ 140/90 mmHg or on antihypertensive agents were identified and included in this analysis.

2.3 Data collection

Socio-demographic data and co-morbidities from patient records were captured. Documented blood pressure measured by mercury sphygmomanometer as well as the patient's height and weight were captured. Target control BP was defined as BP < 140/90mmHg for non-diabetics and < 130/80mmHg for patients with diabetes mellitus (DM). Body mass index (BMI) was calculated as weight in kilograms per square meter (kg/m^2).

Diabetes mellitus was those clinically diagnosed as diabetes or those taking the diabetics medications. Chronic kidney disease is defined as the eGFR < 60ml/min based on Cockcroft-Gault formula[16].. Anti-hypertensive drug use was also captured and classified into the following classes; RAS (renin-angiotensin system: i.e. angiotensin converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARB); beta-blockers (ß-blockers); calcium-channel blocker (CCB); diuretics and alpha-blockers (α-blockers).

2.4 Classification of Hypertension

We categorised the patient into three stages: Stage 1 HPT defined as BP of 140-159/90-99 mmHg; Stage 2 hypertension as BP 160-179/100-109 mmHg and Stage 3 hypertension when BP is $\geq$ 180/110mmHg [17].

2.5 Statistical analysis

Analysis was performed using SPSS version 19 (SPSS IBM New York, United States). Continuous data is described as mean and standard deviation (SD) when the distribution is normal. However when the data is a skewed distribution, median and interquartile range (25-75th percentiles) are used. Categorical data is reported as proportions (percentage). Chi-square test or Fisher exact tests are used for the categorical or dichotomous predictors. All analyses are done at 95% confidence intervals (CI), and a p- value of less than 0.05 is indicates statistical significance.

3. Results

A total 1547 patients were in the original cohort and 886 patients were hypertensive. After excluding missing data on BP in 1998 (n=1) and missing data on BP in 2007(n=63), finally 822 patients who had complete data and who fulfilled the inclusion criteria were entered into our analysis. Table 1 shows the demographics of patients with hypertension. In 1998, the mean age was 57.2 ± 9.6 years, 63.1% were female and 22.4% were aged ≥65 years. The mean BMI was 27.1 ± 4.6 kg/m^2. Chinese were the largest ethnic groups among the hypertensive patients. 6.7% were smokers. The obesity among patients less than 65 years old was 31.7% (n= 202) compared to 22.8% (n=42) in those 65 years and older. The mean BP (Table 2) at baseline and at the end of 10-year were 146 / 87 (18/10) mmHg and 136/80 (16/9) mmHg respectively.

Table 2 shows the prescribing pattern of antihypertensive agents over the 10-years.

In 1998 three quarters of the patients were on monotherapy and this was reduced by two thirds to 24.9% being on monotherapy at the end of 10 years. The commonest prescribed monotherapy at baseline was ß-blockers and this changed to CCB 10-year later. The number of patients on 2 agents doubled at the end of 10 years. There was an increase in the use of three or more agents at the end of 10 years. The commonest prescribed agent in 1998 was ß-blockers. However, at the end of 10-years, it changed to CCB. The use of RAS agents which was low at baseline increased fourfold at the end of 10-year. The commonest drugs combination prescribed throughout the 10 years was ß-blocker and CCB

Table 3 shows the BP control rates in patients with hypertensive with or without diabetes over the last 10 years. Overall, BP control improved from 15.6% to 20.0 % after five years and further increased to 43.7% at the end of 10-years. However, the blood pressure control was still very poor among hypertensive patients with underlying diabetes achieving only 11.9% at the end of 10-year. Over the time, we observed more antihypertensive agents were required to achieve target. Nearly one drug is needed to be added in to achieve the same target.

Table 1. Characteristics of hypertensive patients in UMMC (N=822) at baseline

Variables	Total	Value
Mean Age in 1998, years	822	57.2 ± 9.6
Mean BMI in 1998, kg/m2	623	27.1 ± 4.6
Male gender, n (%)	822	303(36.9)
Race , Chinese, n (%)	822	405(49.3)
Malays, n (%)	822	216(26.3)
Indian, n (%)	822	188(22.9)
Co- morbidities , n (%)		
Diabetes	822	262(31.9)
Dyslipidaemia	822	63(7.7)
Obesity	623	244(39.8)
Coronary heart disease	822	78(9.5)
Cerebrovascular accident	822	35
Chronic kidney disease	765	187
Peripheral arterial disease	822	2
Heart failure	822	3

Table 2. Antihypertensive prescribing patterns over the last 10-years in UMMC

	1998	2002	2007
Mean systolic BP (mmHg)	146 ± 18	143 ± 17	136 ± 16
Mean diastolic BP (mmHg)	87 ± 10	84 ± 9	80 ± 9
Monotherapy (n, %)	611 (74.3)	380 (46.2)	205 (24.9)
Two drugs (n, %)	185 (22.5)	351 (42.7)	382 (46.5)
≥Three drugs (n, %)	13 (1.6)	77 (9.4)	221 (26.9)
Frequency of drugs prescribed overall			
ß-blockers (n, %)	433 (52.7)	450 (54.7)	432 (52.6)
CCB (n, %)	349 (42.5)	413 (50.2)	515 (62.6)
Diuretics (n, %)	102 (12.4)	231 (28.1)	299 (36.4)
RAS (n, %)	90 (10.9)	184 (22.4)	379 (46.1)
α-blockers (n, %)	46 (5.6)	39 (4.7)	39 (4.7)

BP: blood pressure; CCB: calcium-channel Blocker; RAS: renin-angiotensin system

Table 3 Control rate among patients with hypertension over the last 10-year

	1998	2002	2007
BP controlled in patients with hypertensive with or without diabetes, n (%)	128/822 (15.6)	164/822 (20.0)	359/822 (43.7)
BP controlled in patients with hypertensive alone, n (%)	123/560 (22.0)	149/458 (32.5)	300/382 (78.5)
BP controlled in patients with hypertensive with diabetes, n (%)	5/262 (1.6)	7/342 (2.0)	50/419 (11.9)
BP controlled in patients with hypertensive with CKD, n (%)	34/187 (18.2)	48/287 (16.7)	138/295 (46.8)
Average number of drugs used in those patients who achieved target BP	1.2	1.2	2.2
on monotherapy, n (%)	103 (80.5)	81(49.4)	85 (23.7)
on two drugs, n (%)	22 (23.2)	68 (41.5)	186 (51.8)
on three drugs, n (%)	2 (1.6)	10 (6.1)	83 (23.1)

CKD: chronic kidney disease

4. Discussions

In this study, although the overall control of BP improved from 15.6. % to 43.7% over a10-year period, it is far from best practice. However, this result is in keeping with findings even in developed countries where BP control rates varied from 10% to 37% [13, 18-20]. A few possible reasons explain the improvement in BP control seen in this clinic. Firstly, there was a substantial increase by twofold and 17 times in the use of two and three antihypertensive agents respectively over the 10 year period. In parallel, the mean number of drugs needed to achieve target BP also increased. These findings are consistent with previous studies that showed that most patients require two or more drugs to achieve BP control [21-23]. Secondly, this clinic is based in a teaching hospital where there is greater availability of the newer agents which are subsidised. Furthermore, trainees are more likely to adhere to guidelines in achieving BP targets.

In this study, CCB and ß- blockers still remain the most frequently prescribed antihypertensive agents. This finding is consistent with studies done in other countries[24, 25]. Nevertheless, there is a decreasing trend in the usage of ß- blockers and this most likely is due to the more recent guidelines which no longer recommend it as the first line monotherapy[17, 26] in view of its unfavourable outcomes[27-31].

Although the prescription of ACEI/ ARB increased over time, its use is still low compared to some countries where ACEIs constitute the most frequently prescribed antihypertensive drug class[32, 33] . The use of ACEI was low in this study because while available, its use was heavily controlled in our institution. However, with the availability of generic preparations, we predict that the use of not only ACEI, but ARB will increase dramatically in the next decade. Finally, this is a retrospective study. Some data were missing in particular blood pressure reading, weight and biochemical parameters needed to define chronic kidney disease. However these will not affect our findings in any substantial way.

In conclusion, this retrospective study shows that there is improvement in blood pressure control over time. However there is a further need for improvement. Hopefully this can be achieved in a shorter period of time. Our results indicate that physicians should use at least two or more agents to achieve target BP.

Acknowledgements

The author would like to acknowledge University of Malaya and the British Geriatrics Society for providing the research grant (UMRG 116/09HTM) as well as **D**epartment of **P**rimary **C**are at the University of Malaya for providing the support during the data collection.

Corresponding Author:
Dr. Chia Yook Chin,
Department of Primary Care Medicine,
Faculty of Medicine,
University of Malaya, 50603 Kuala Lumpur,
Malaysia
Email: chiayc@um.edu.my

References:

1. Kannel WB, Schwartz MJ, McNamara PM. Blood pressure and risk of coronary heart disease: the Framingham study. Dis Chest 1969;56(1):43-52
2. Chobanian AV, Bakris GL, Black HR, et al. The seventh report of the joint National Committee on Prevention, Detection, Evaluation, and treatment of high Blood pressure: the JNC7report. JAMA 2003;289:2560-72
3. Neal B, MacMahon S,Chapman N. Blood Pressure Lowering Treatment Trialists' Collaboration. Effects of ACE inhibitors, calcium antagonists, and other blood-pressure-lowering drugs: results of prospectively designed overviews of randomised trials. Lancet 2000;356(9246):1955-64
4. Prospectives Studies Collaboration. Age-specific relevance of usual blood pressure to vascular mortality: a meta-analysis of individual data for one million adults in 61 prospective studies. Lancet 2002;360(9349):1903-13
5. Hansson L, Zanchetti A, Carruthers SG, Dahlöf B, Elmfeldt D, Julius S, Ménard J, Rahn KH, Wedel H, Westerling S. Effects of intensive blood-pressure lowering and low-dose aspirin in patients with hypertension: principal results of the Hypertension Optimal Treatment (HOT) randomised trial. Lancet 1998;351(9118):1755-62
6. Chia Y, Ching S: Hypertension and the development of New onset chronic kidney disease over a 10year period: a retrospective cohort study in a primary care setting in Malaysia. *BMC Nephrology*, 13(1):173.
7. Whitworth JA; World Health Organization, International Society of Hypertension Writing Group:2003 World Health Organization (WHO)/International Society of Hypertension (ISH) statement on management of hypertension. J Hypertens 2003;21(11):1983-92
8. Woodward M, Barzi F, Martiniuk A, et al. Cohort profile: the Asia Pacific Cohort Studies Collaboration. Int J Epidemiol 2006;35(6):1412-16
9. Asia Pacific Cohort Studies C. Joint Effects of Systolic Blood Pressure and Serum Cholesterol on Cardiovascular Disease in the Asia Pacific Region. Circulation 2005;112(22):3384-90

10. T O Lim, Z Morad. Hypertension Study Group. Prevalence, Awareness, Treatment and Control of Hypertension in the Malaysian Adult Population: Results from the National Health and Morbidity Survey 1996. Singapore Med J 2004;45(1):20-27
11. Public Health Institute. Malaysia National Health And Morbidity Survey III Ministry of Health, 2006.
12. Gu D, Reynolds K, Wu X, et al. Prevalence, awareness, treatment, and control of hypertension in China. Hypertension 2002;40(6):920-27
13. Cutler JA, Sorlie PD, Wolz M, et al. Trends in Hypertension Prevalence, Awareness, Treatment, and Control Rates in United States Adults Between 1988-1994 and 1999-2004. Hypertension 2008;52(5):818-27
14. Tobias M, Taylor R, Yeh LC, et al. Did it fall or was it pushed? The contribution of trends in established risk factors to the decline in premature coronary heart disease mortality in New Zealand. Aust N Z J PublicHealth 2008;32(2):117-25
15. Guidelines Committee: 2003 European Society of Hypertension European Society of Cardiology guidelines for the management of arterial hypertension. Hypertension 2003;21:1011–53.
16. Chia Yook Chin, Ching Siew Mooi: Concordance of Serum Creatinine to Estimated Glomerular Filtration Rate in Determining Early Chronic Kidney Disease in Malaysia *Life Science Journal* 2012, 9(3):453-457
17. Ministry of Health Malaysia. Clinical Practice Guideline on Management of Hypertension (3rd edition). Kuala Lumpur, Malaysia, 2008.
18. Morgado MP, Rolo SA, Pereira L, et al. Blood pressure control and antihypertensive pharmacotherapy patterns in a hypertensive population of Eastern Central Region of Portugal. BMC Health Serv Res 2010;10:349
19. Wolf-Maier K, Cooper RS, Kramer H, et al. Hypertension Treatment and Control in Five European Countries, Canada, and the United States. Hypertension 2004;43(1):10-17
20. Rodriguez Roca GC, Artigao Rodenas LM, Llisterri Caro JL, et al. Control of Hypertension in Elderly Patients Receiving Primary Care in Spain. Rev Esp Cardiol (English Edition) 2005;58(4):359-66
21. Black HR, Elliott WJ, Neaton JD, et al. Baseline Characteristics and Early Blood Pressure Control in the CONVINCE Trial. Hypertension 2001;37(1):12-18
22. William C Cushma, Charles E Ford, Paula T Einhorn, et al. Blood pressure control by drug group in the antihypertensive and lipid-lowering treatment to prevent heart attack trial (ALLHAT). J Clin Hypertens 2008;10(10):751-60
23. Björn Dahlöf, Peter S Sever, Neil R Poulter, et al. Prevention of cardiovascular events with an antihypertensive regimen of amlodipine adding perindopril as required versus atenolol adding bendroflumethiazide as required, in the Anglo-Scandinavian Cardiac Outcomes Trial-Blood Pressure Lowering Arm (ASCOT-BPLA): a multicentre randomised controlled trial. Lancet 2005;366(9489):895-906
24. Crucitti A, Cecchi E, Gensini GF et al. Use of antihypertensive drugs in the Italian hospitals. Pharmacol Res 2000;41:249-53
25. Walley T, Duggan AK, Haycox AR, et al. Treatment for newly diagnosed hypertension: patterns of prescribing and antihypertensive effectiveness in the UK. J R Soc Med 2003;96:525-31
26. National Collaborating Centre for Chronic Conditions, National Institute for Clinical Excellence (NICE). Hypertension: management in adults in primary care: pharmacological update. London: Royal College of Physicians 2006.
27. Lindholm LH, Carlberg B, Samuelsson O. Should beta-blockers remain first choice in the treatment of primary hypertension? A meta-analysis. Lancet 2005;366:1545-53
28. Khan N, McAlister FA. Re-examining the efficacy of beta-blockers for the treatment of hypertension: a meta-analysis. CMAJ 2006;174(12):1737-42
29. Elliot WJ, Meyer PM. Incident diabetes in clinical trials of antihypertensive drugs: a network meta-analysis. . Lancet 2007;369:201-7
30. Ram CVS. Beta-Blockers in Hypertension. Am J Cardiol 2010;106(12):1819-25
31. Pischon T, Sharma AM. Use of beta blockers in obesity hypertension: potential role of weight gain. Obes Rev 2001;2(4):275-80
32. Siegel D, Lopez J, Meier J, et al. Changes in the pharmacologic treatment of hypertension in the Department of Veterans Affairs 1997–1999: decreased use of calcium antagonists and increased use of -blockers and thiazide diüretics. Am J Hypertens 2001;14:957–62
33. Siddiqui S, Ogbeide DO, Karim A, et al. Hypertension control in a community health centre at Riyadh,Saudi Arabia. Saudi Med J 2001; 22:49-52.

2/2/2013

Designing and Validating Standards of Nursing Practice in Radiology Department of El-Manial University Hospital

Elham Youssef Elhanafy[1] and Touto Abdel-Hamid Ismail[2]

[1]Nursing Administration, Faculty of Nursing, Damanhour University. Egypt
[2] Nursing Manager of Specialized Medical Center, Ministry of Health. Egypt
elham_youssef2006@yahoo.com

Abstract: The standards of practice describe a competent level of radiology nursing care as determined by the critical thinking module known as the nursing process. The aim of this study was to design standards of nursing practice in radiology department at El Manial University Hospital, Cairo University. This cross-sectional descriptive operational study was conducted at the radiology department of El-Manial University Hospital. It involved 40 staff nurses, and a jury group of 24 nursing and medical faculty members and nursing administrators in the field of radiology. The data collection tools included an opinionnaire sheet for staff nurses, an opinionnaire form for validation, an observation checklist for staff nurses' performance, and structure inventory checklist for the settings. The study findings revealed majority agreement of nurses upon the importance of structure and process criteria of the standard, and 100.0% agreement upon applicability. Almost jury group members agreed upon the face and content validity of the proposed standard. Staff nurses' performance was generally low before dissemination of the standard, but showed statistically significant improvements after standards dissemination ($p<0.001$). The study settings were deficient regarding availability of mission or vision, or performance appraisal. In conclusion, a standard for nursing care in radiology department was developed and face and content validated. Its applicability was shown through observation of nurses' performance, and settings structure. It is recommended to apply the developed protocols in the study settings, with training staff nurses in its implementation. Quality improvement programs should be available in the radiology department to improve the quality of care provided.

[Elham Youssef Elhanafy and Touto Abdel-Hamid Ismail. **Designing and Validating Standards of Nursing Practice in Radiology Department of El-Manial University Hospital.** *Life Sci J* 2013;10(1):2036-2047] (ISSN:1097-8135). http://www.lifesciencesite.com. 290

Keywords: Radiology nurse, standards, Validity.

Introduction

Radiology, formerly called roentgenology, is the branch or specialty of medicine that deals with the study and application of imaging technology like X-ray and radiation to diagnosing and treating disease (1). The Radiology Department has inpatient and outpatient services. The inpatient is a 24-hour imaging service as prescribed by physicians, to assist in the diagnosis of disease or injury. Meanwhile, radiology provides complete outpatient diagnostic and therapeutic imaging procedures (2).

Radiology is increasingly playing a major role in the early management of emergency patients to identify patients who require immediate intervention, hospital monitoring or early discharge. Evolving and more sophisticated technologies may aid in the diagnosis and management of diseases and trauma in ways that were previously not possible. Along with such developments, it is critical that radiology staffing needs and services are optimized to meet ever-increasing demands (3).

Imaging nurses influence patient care in a variety of settings and nursing roles. Imaging nurses are involved in the assessment, care planning, and direct care of patients before, during, and after diagnostic and therapeutic imaging procedures. Imaging nurses promote high quality patient care in those environments. Each imaging nurse is charged with providing safe patient care according to the standards of nursing practice (4).

A quality imaging service will consistently perform the right procedure at the right time for the right patient, refusing requests for inappropriate examinations; the imaging report will be timely and accurate; and the patient will receive optimal personal care (5).

Standards of care are one way for an individual, or a group (department) or the entire healthcare facility to objectively measure how well they are doing in terms of quality and performance. Measurement capability is generally built into professional standards of care (6). Quality of care is the degree to which health services for individual and populations increase the likelihood of desired health outcomes and are consistent with current professional knowledge (7).

Professions, including the healthcare professions, have standards of practice. Standards of practice establish minimum practice guidelines and expectations. They reflect the standard in terms of what

should be done and how it should be done. They establish and document what is considered acceptable practice within the profession (8).

Knowledge of standards of care and standards of professional practice assist the nurse in identifying and setting goals for professional growth within radiology and imaging nursing practice (9,10 4, 2). Moreover, the ***Joint Commission*** (11) as set forth standards of care which include performance evaluation, establishment of policies and procedures for nurses, oversight authority in providing patient care, and improvement of patient outcomes.

The Association for Radiologic and Imaging Nursing believes that quality care for all patients is a primary responsibility of nurses. The imaging nurse can only be evaluated by another registered nurse who holds a leadership role. This nurse leader has the ability to determine if the imaging nurse's performance meets the standards of nursing care. The imaging nurse can not be under the supervision or be evaluated by a technical staff member in any imaging environment. Such situations may create a conflict in the delivery of quality patient care (11).

Additionally, standards set out the legal and professional basis for nursing practice. They help to identify for nurses, the public, government, and other stakeholders the desired and achievable level of performance expected of nurses in their practice, against which actual performance can be measured 12).

Therefore absence of standard was associated with many work problems such as role ambiguity, overlapping of responsibilities, and lack of qualified staff. These would lead to role conflict that jeopardizes the provision of quality nursing services in the department of radiology. Therefore, this study is an attempt to develop and validate this standard, which is deemed necessary for the study settings.

Aim of this study

The aim of this study was to design and validate standard of nursing practice in radiology department through:

- Investigating the performance of the nurses working in the radiology department
- Designing standard of nursing practice in radiology department based on national and international sources
- Assessing the validity of the proposed standard based on experts' viewpoints
- Assessing the opinions of the nurses working in radiology department toward the developed standard.
- Assessing applicability of the developed standard.

Study Methods:

Design

A cross-sectional descriptive study design was used for achieving the study aim.

Study setting

The study was conducted at the radiology department of El-Manial University Hospital, affiliated to Cairo University. The department building consists of three floors; the first one contains two units of magnetic resonance imaging (MRI); the second has two computerized tomography (CT) units; in the third there are five units: one there is one unit of angiography, one unit of mammography, one unit of Dual Energy X-ray Absorpometry (DEXA), in addition to an ultrasound unit equipped with six ultrasound machines. The department provides diagnostic radiological investigations that include computerized tomography (CT), magnetic resonance imaging (MRI), Angiography, Ultrasound, and Mammography in addition to other conventional diagnostics.

Sample

Two groups of subjects were included in the study, namely staff nurses and jury group.

- Staff nurses group: the total number of staff nurses included in the study sample was 40. These nurses were either nursing diploma (27) or nursing baccalaureate graduates (13). They were representing all available nurses in the study setting at the time of the study with the only inclusion criterion of having at least one year in the field of work.

- Jury group: This group served as jury to assess the content and face validity of the proposed standard. The group included three categories of jury:

- Nursing faculty members from administration and medical-surgical nursing departments at the Faculties of Nursing at Ain Shams and Cairo Universities. Their number was eight.
- Medical faculty members from the radiology department at El-Manial University Hospital and Faculty of medicine in Cairo University. Their number was eight.

Head nurses representing nursing managers in the field of radiology nursing. They were recruited from the radiology departments in Nasser Institute, Cairo Scan Radiology Center, and Cairo-University Hospital. Their number was eight head nurses.

Tools of data collection

Four types of data collection tools were used in this study. These included an opinionnaire sheet for validating the proposed standards, an opinionnaire sheet for staff nurses, an observation checklist to assess staff nurses' performance, and a structure inventory checklist for assessment of the settings.

Jury opinionnaire sheet: This tool was designed by the researcher for testing the validity of the

proposed standard through jury group opinions. It consisted of the following three parts:

- Part 1: for personal data such as category, job position, and years of experience.
- Part 2: This was designed to assess the face validity of the proposed standard. It included questions about the format appropriateness, clarity, understandability, measurability, and achievability. It also tested the relevance of the format to the title and content.
- Part 3: This part was intended to assess the content validity of the proposed standard. It included the same criteria as those in the staff nurses' opinionnaire form regarding structure and process of nursing practice in radiology department. The response to each item was either agree or disagree, with a space for any comments.

Scoring: For each item the responses "agree" and "disagree" respectively scored 1 and 0. The scores of the items were summed-up and the total divided by the number of the items, giving a mean score. These scores were converted into a percent score, and means and standard deviations were computed. The jury opinion was considered as agreeing with importance if the percent score was 75% or more and disagreeing if less.

Staff nurses' opinionnaire sheet: This tool was designed by the researcher to solicit staff nurses' opinions regarding the importance and applicability of the proposed standard. It was proposed after reviewing pertinent literature related to standards of nursing practice for patients undergoing radiology examinations ***ARNA-American Radiological Nurses Association and American Nurses Association*** (9) ; Caprich(13); ***USAID-United State Agency International Development*** (14); ***Kirschner*** (15); ***RCN-Royal College of Nursing*** (16). The form consisted of three parts:

- Part1: This entailed personal data such as age, qualification, job position, years of experience, workplace, and attendance of training courses in radiology and administration.
- Part 2: This part was designed for assessing the items of the initial list of the proposed structure standard of radiology department. It includes 35 criteria as following:

Vision and mission: e.g. presence of written vision and mission in accordance with hospital vision and mission.

Policies and procedures: e.g. the department has clear written consistent with vision and mission.

Human resources: e.g. appropriate human resources including nurses with qualification meeting job needs.

Organizational structure: e.g. the organizational chart is clear, determines responsibilities and authorities in the department.

Job descriptions: e.g. the department has job descriptions which are clear, compatible with nursing specialties.

Procedural protocols: e.g. the department has protocols for angiogram, CT, MRI.

Other resources: e.g. the department has adequate physical and financial resources for quality care.

Work environment and safety: e.g. there are radiation safety protocols, warning signs are posted on X-ray rooms.

Infection control: e.g. the department has written guidelines for infection control, known to all nursing personnel.

Performance appraisal system: e.g. the department has a clear performance appraisal system based on job descriptions.

- Part 3: This was proposed for the purpose of assessing staff nurses' opinions regarding the importance and applicability of criteria of nursing practice in radiology department. It includes 71 criteria under the main heading that identify body system for nursing practice in radiology department as following:

  Nursing assessment
  Nursing diagnosis
  Nursing planning
  Nursing interventions:
  Intervention nursing care pre, during, and after procedure
  Intervention in case of use of dye
  Integrating ethical provisions in all areas of practice
  Evaluation.

Scoring: For the importance, each item had 3 levels of answers: "agree", "uncertain", and "disagree." These were respectively scored 2, 1, and 0. The scores of the items were summed-up and the total divided by the number of the items, giving a mean score. These scores were converted into a percent score, and means and standard deviations were computed. The nurse opinion was considered as agreeing with importance if the percent score was 75% or more and disagreeing if less. This was based on mathematical calculation taking the midpoint between agree (2) and uncertain (1) as a cutoff point, i.e. 1.5. This represents 75% of the maximum score of 2. For the applicability, the scores were one for applicable, and zero for not applicable. The same procedure was followed as with importance.

- **Observation checklist**: This tool was designed by the researcher for assessment of staff nurses' performance of the criteria of the proposed

standard. The tool was used twice: once before dissemination of the standard, and the second time after its dissemination. It consisted of the following two parts:

- Part 1: for personal data such as department, age, sex, qualification, years of experience, and job position.
- Part 2: This part was intended to assess the actual staff nurses' performance of the process items of the proposed standard. It included the same criteria as those in the staff nurses' opinionnaire form regarding process of nursing practice in radiology department. Each criterion was checked during observation as either done or not done, or not applicable.

Scoring: The items "not done" and "done" were scored "0" and "1", respectively. For each part, the scores of the items were summed-up and the total divided by the number of the items, giving a mean score for the part. These scores were converted into a percent score, and means and standard deviations were computed. The performance was considered adequate if the percent score was 60% or more and inadequate if less than 60**%** ***Butsashvili et al,*** (17).

- **Structure inventory checklist**: This checklist was designed by the researcher for assessment of the structure criteria of the proposed standard in the study settings. The tool consisted of the following two parts:
  - Part 1: for personal data of the interviewee who helped in the observation of the setting. This included the department, age, sex, nursing qualification, job position, and years of experience.
  - Part 2: This part was intended to assess the structure criteria of proposed standard in the study settings. It included the same criteria as those in the staff nurses' opinionnaire form regarding structure elements of the radiology department. Each criterion was checked during observation as either present or absent or not applicable.

Pilot study

It was done on a sample of 10% from the sample. The results were used for finalization of the tools. Modifications included re-phrasing of certain items. The time needed for filling the forms was also estimated based on the pilot results.

Fieldwork

The actual fieldwork of the study started on the beginning of December 2010 and ended in June 2012. Fieldwork included two methods to collect the data needed for the development of the proposed nursing practice standards for the radiology department. These two methods were self-administration of the opinionnaire forms, and observation of the staff nurses' performance and of the structure of the settings.

Data collection started with eliciting staff nurses' opinions regarding the proposed standard importance and applicability. After securing official permissions, the researcher met with the staff nurses, explained the aim of the study to obtain their verbal informed consent for participation in the study. Upon acceptance, they were handled the forms and asked to fill them. The researcher was present all the time to answer any questions raised.

Then, the researcher started the observation process. This was done using the designed observation checklist, and before disseminating the proposed standard. Each staff nurse was observed during her routine work for all the criteria of the process standard. The aim of the observation was to assess the applicability of the standard. Each item in the checklist observed to be performed by the nurse was recorded as done. This was done in two shifts. The morning shift started from 8:30 till 13:30, and the afternoon shift started from 13:30 till 18:30. The average duration of the observation was four hours in each shift. This was done six days per week. Each nurse was observed until the checklist items were totally fulfilled. The duration of observation was about five months.

Upon ending with the staff nurses' opinionnaires and observation, testing of the validity of the proposed standard was done. The researcher met with the jury group members individually, explained the objectives of the study for each one, and asked them to express their opinions and suggestions regarding the proposed standard. They gave some comments on the clarity of some criteria of the constructed standard and its coverage of all aspects related to process and structure. According to the comments and recommendations of jury members, the standard was modified. Then the proposed standard was considered valid.

Then, assessment of staff nurses' performance was done again after disseminating the standard using the same observation checklist.

Standard development

Based on the validity and applicability data obtained from various study tools, the nursing practice standard was proposed. The researcher divided the proposed standard of nursing practice in radiology according to Donabedian's models into two parts, namely structure standards and process standards

Administrative design

To carry out the study at the selected settings, official letters were issued from the Faculty of Nursing, Damanhour University to get permission from the hospital administration, and the nursing director. The purpose of the study and its procedures were explained to them to get their consent and cooperation.

Ethical considerations

Oral informed consent was obtained from the participants included in the study sample. They were reassured about the confidentiality of the obtained information. They were informed about their rights to refuse participation or withdraw at any time. The study maneuvers could not entail any harm on participants.

Statistical design

Data entry and statistical analysis were done using SPSS 14.0 statistical software package. Data were presented using descriptive statistics in the form of frequencies and percentages for qualitative variables, and means and standard deviations for quantitative variables. Qualitative categorical variables were compared using chi-square test. Whenever the expected values in one or more of the cells in a 2x2 tables was less than 5, Fisher exact test was used instead. In larger than 2x2 cross-tables, no test could be applied whenever the expected value in 10% or more of the cells was less than 5. Statistical significance was considered at p-value <0.05.

Results

Part I. Validation of standards by jury group.

According to the general profile of the of jury group members, data in Table (1) showed that: two-thirds of them (66.7%) were nursing. The sample was equally distributed among professors, assistant professors, and nursing directors, 33.3% each. Their years of experience were mostly more the ten years (87.5%).

Table 1. Socio-demographic characteristics of jury group members (n=24)

	Frequency	Percent
Category:		
Nursing	16	66.7
Medical	8	33.3
Job position:		
Professor	8	33.3
Assistant professor	8	33.3
Nursing director	8	33.3
Experience (years):		
<10	3	12.5
10+	21	87.5
Range	5.0-39.0	
Mean±SD	18.8±8.3	

Regarding to the total agreement of jury groups upon the areas of the proposed standards was presented in table (2) It points to a unanimous agreement of both nursing and medical groups upon all areas, with only one exception in the nursing group, and three in the medical group. These were the areas of non-human resources in both groups, 93.8% and 87.5%, respectively, as well as work environment and safety (87.5%), and infection control (87.5%) in the medical group.

Part II. Nurses' opinion regarding importance and applicability of standards.

The personal characteristics of nurses in the opinionnaire sample showed at table (3), More than half of them (55.0%) were diploma nurses. The majority were more than 30 years old (82.5%), with more than ten years experience in nursing (85.0%) and in radiology (62.5%). About one third attended training courses in radiology (32.5%), and only two of them (5.0%) had training in management.

Table (4) summarizes nurses' opinions regarding the importance and applicability of total standards criteria. Their agreement upon importance ranged between 70.0% and 97.5%. The corresponding figures for applicability were 85.0% and 100.0%. A statistically significant difference was revealed between them importance and applicability of evaluation (p=0.01), where agreement upon applicability exceeded that upon importance. Overall, only two nurses had a total disagreement for importance, and one for applicability of the standards.

Part III. Applicability of standards through observation of nurses' performance.

Table (5) describes the socio-demographic characteristics of nurses in the observation sample before and after dissemination of the proposed standards. Their mean age was around 37 years. Their mean years of experience in radiology and in total nursing were about 13 and 16 years, respectively.

On other hand, Figure 1 illustrated total nurses' performance before and after dissemination of the standards. It points to very low percentages of adequate performance in all areas, especially regarding planning, which was adequately performed by only one nurse (2.5%). After dissemination of the standards, statistically significant improvements were demonstrated in all areas (p<0.001). The total adequate performance increased from 5.0% to 80.0%.

Part IV. Inspection of structure criteria of study settings

According to the socio demographic characteristics of nurses who were interviewed in site inspection, Table (6) showed that, they were mostly head nurses, 40 years age or older, females, with 20 or more experience years.

Table (7) describes the total presence of structure standards in the five study settings. It indicates that most of them had adequate structures related to human resources, procedures and standards, and work environment and safety. On the other hand, none of them had adequate mission or vision, or performance appraisal. Overall two of the five settings (40.0%) had total adequate structure.

Table 2 . Agreement of jury group upon total standards criteria

	Category				X^2 Test	p-value
	Nursing (n=16)		Medical (n=8)			
	No.	%	No.	%		
Vision	16	100.0	8	100.0	0.00	1.00
Policies and procedures	16	100.0	8	100.0	0.00	1.00
Human resources	16	100.0	8	100.0	0.00	1.00
Organizational structure	16	100.0	8	100.0	0.00	1.00
Job descriptions	16	100.0	8	100.0	0.00	1.00
Procedures protocols	16	100.0	8	100.0	0.00	1.00
Non-human resources	15	93.8	7	87.5	Fisher	1.00
Work environment and safety	16	100.0	7	87.5	Fisher	0.33
Infection control	16	100.0	7	87.5	Fisher	0.33
Performance appraisal	16	100.0	8	100.0	0.00	1.00
Total structure	16	100.0	8	100.0	0.00	1.00
Assessment	16	100.0	8	100.0	0.00	1.00
Diagnosis	16	100.0	8	100.0	0.00	1.00
Planning	16	100.0	8	100.0	0.00	1.00
Implementation	16	100.0	8	100.0	0.00	1.00
Evaluation	16	100.0	8	100.0	0.00	1.00
Total procedures	16	100.0	8	100.0	0.00	1.00
Total	16	100.0	8	100.0	0.00	1.00

Table 3. Socio-demographic characteristics of nurses in the opinionnaire sample (n=40)

	Frequency	Percent
Age (years):		
<30	7	17.5
30+	33	82.5
Range	17.0-54.0	
Mean±SD	37.2±9.1	
Nursing qualification:		
Nursing school diploma	22	55.0
Specialty diploma	4	10.0
Technical institute diploma	1	2.5
Bachelor of nursing	13	32.5
Job position:		
Staff nurse	16	40.0
Radiology technician	22	55.0
Head nurse	2	5.0
Total experience (years):		
<10	6	15.0
10+	34	85.0
Range	1.0-30.0	
Mean±SD	16.5±7.6	
Experience in radiology (years):		
<10	15	37.5
10+	25	62.5
Range	0.0-30.0	
Mean±SD	12.8±7.8	
Attended training courses in:		
Radiology	13	32.5
Management	2	5.0

Table 4. Nurses' opinions regarding importance and applicability of total standards criteria

	Agree upon				X^2 Test	p-value
	Importance		Applicability			
	No.	%	No.	%		
Vision/mission	35	87.5	40	100.0	Fisher	0.055
Policies and procedures	36	90.0	40	100.0	Fisher	0.12
Human resources	36	90.0	34	85.0	0.46	0.50
Organizational structure	32	80.0	36	90.0	1.57	0.21
Job descriptions	39	97.5	38	95.0	Fisher	1.00
Procedures protocols	39	97.5	39	97.5	Fisher	1.00
Non-human resources	36	90.0	35	87.5	Fisher	1.00
Work environment and safety	36	90.0	36	90.0	Fisher	1.00
Infection control	38	95.0	40	100.0	Fisher	0.49
Performance appraisal	38	95.0	39	97.5	Fisher	1.00
Total structure	37	92.5	39	97.5	Fisher	0.62
Assessment	39	97.5	39	97.5	Fisher	1.00
Diagnosis	36	90.0	36	90.0	Fisher	1.00
Planning	36	90.0	37	92.5	Fisher	1.00
Implementation	39	97.5	39	97.5	Fisher	1.00
Evaluation	28	70.0	37	92.5	6.65	0.01*
Total procedures	38	95.0	38	95.0	Fisher	1.00
Total	38	95.0	39	97.5	Fisher	1.00

(*) Statistically significant at p<0.05

Table 5. Socio-demographic characteristics of nurses in the observation and applicability sample (n=40)

	Observation			
	Before (n=40)		After(n=40)	
	No.	%	No.	%
Department:				
MRI	8	20.0	8	20.0
CT	10	25.0	10	25.0
Radiology	19	47.5	17	42.5
Angiography	3	7.5	5	12.5
Age (years):				
<30	7	17.5	7	17.5
30+	33	82.5	33	82.5
Range	19.0-54.0		19.0-54.0	
Mean±SD	37.4±8.7		37.3±8.7	
Sex:				
Male	5	12.5	6	15.0
Female	35	87.5	34	85.0
Nursing qualification:				
Nursing school diploma	24	60.0	26	65.0
Specialty diploma	3	7.5	2	5.0
Bachelor of nursing	13	32.5	12	30.0
Experience in radiology (years):				
<10	14	35.0	15	37.5
10+	26	65.0	25	62.5
Range	1.0-30.0		1.0-30.0	
Mean±SD	13.1±7.7		12.9±7.8	
Total experience (years):				
<10	7	17.5	6	15.0
10+	33	82.5	34	85.0
Range	1.0-30.0		1.0-30.0	
Mean±SD	16.6±8.0		16.7±7.7	

Table 6. Socio-demographic characteristics of nurses in the site inspection interview sample (n=5)

	Frequency	Percent
Department:		
MRI	1	20.0
CT	1	20.0
Radiology	2	40.0
Angiography	1	20.0
Age (years):		
<40	1	20.0
40+	4	80.0
Sex:		
Female	5	100.0
Nursing qualification:		
Nursing school diploma	3	60.0
Bachelor of nursing	2	40.0
Experience in radiology (years):		
<20	2	40.0
20+	3	60.0
Experience in nursing (years):		
<20	1	20.0
20+	4	80.0
Job position:		
Head nurse	3	60.0
Radiology technician	2	40.0

Table7. Availability of total standards criteria in the study settings

	Departments					
	MRI	CT	Radiology (n=2)	Angiography	Total	
	No.	No.	No.	No.	No.	%
Vision/mission	0	0	0	0	0	0.0
Policies and procedures	1	1	0	0	2	40.0
Human resources	1	1	1	1	4	80.0
Organizational structure	0	1	0	0	1	20.0
Job descriptions	1	0	0	1	2	40.0
Procedures protocols	1	1	1	1	4	80.0
Non-human resources	1	0	1	1	3	60.0
Work environment and safety	1	1	1	1	4	80.0
Infection control	1	0	0	1	2	40.0
Performance appraisal	0	0	0	1	0	0.0
Total structure	1	1	0	0	2	40.0

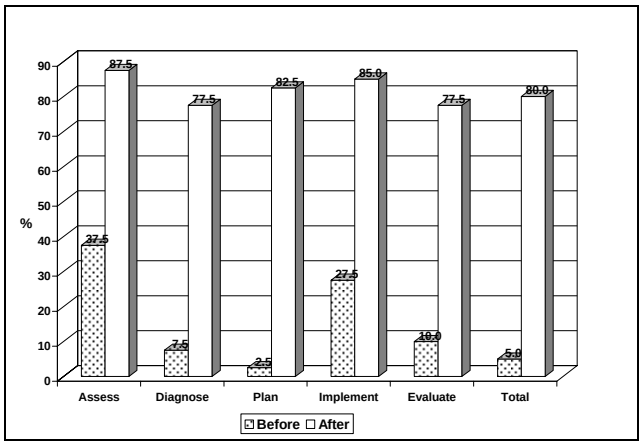


Figure1. Nurses' total performance before and after dissemination of standards

Discussion

In this study, the evidence supporting the content validity of proposed tool was based on literature review, clinical observation, and the judgment of the jury group. In the process of construction of standard, the viewpoints of jury were solicited to ensure validity, while jobholders' views were sought to assess the importance and applicability of these standards. According to the study findings, there was a majority agreement among jury group regarding proposed standards' face and content validity. This step is considered an important criterion for adoption of standards. Content validity is the degree to which the items adequately represent the universe of content of tasks and responsibilities. This ensures a match between target and developed standard (18, 19).

Concerning the validation of the checklist entailing structure items for radiology department, all jury members in the present study have agreed upon the importance of the presence of the vision, mission, and policy and procedures, and that they should be available to all members of the radiology department. Also the majority of nurses agreed upon the importance as well as the applicability of these same items. These findings imply that nurses in the study sample have good awareness of these components of the structure standard, and realize their importance. Also, they do not perceive any problems with their application as the results demonstrated higher percentages of agreement upon applicability compared to importance.

In agreement with these present study findings, ***El-Guindy*** (20) reported a unanimous consensus of jury group members upon the importance of having written policies for nursing personnel in the operation department, and upon their availability to all members. Similarly, in the study of ***Ahmed et al.*** (21) all jury group members agreed upon policies and procedures, and suggested that it should be collected in a policy manual accessible to nursing personnel; these policies and procedures must be regularly updated. The findings are also in congruence with ***El Hanafy*** (22) and ***Tantawy*** (23) who reported majority agreement of jury upon the importance of the availability of policies and procedures at emergency and intensive care units.

The present study finding showed that all the jury group members agreed upon the importance of organizational structure and job description items. They were also agreed upon by the majority of nurses regarding their importance and applicability. These components of the structure standard are essential as they define clearly the lines of commands and the roles of each member of the team, which would preclude any role ambiguities with consequent conflict. Due to the importance of these components, very high percentages of agreement were reported upon them in similar previous studies ***Sidani et al,*** (24); ***Ahmed et al.,*** (21); ***El Hanafy*** (22***)***. All these studies insisted that job description must be clear, available, and reviewed periodically.

Concerning structure standards related to safety and infection control, the present study results indicated that all jury group members and the majority of nurses agreed upon the importance and applicability of all items. These two components have a special importance in radiology department where serious hazardous exposures to ionizing radiation can occur. Also, the risk of nosocomial infection could be high, especially in invasive procedures. The findings reflect a high level of awareness among nurses regarding such hazards.

In agreement with these present study results, ***Ismail*** (25), ***Ahmed et al.*** (21) and ***El-Guindy*** (20) reported majority agreements among jury group members upon the necessity of written guidelines for infection control available in operating rooms and intensive care units, and that they should be known to all nursing personnel. Similarly, ***El-Hanafy*** (22) reported a unanimous agreement upon the presence of written guidelines for infection control that must be available in the immediate postoperative hepatic patient units.

Despite the high awareness about safety and infection control, ***Grant*** (26) reported that although the incidence of infection in intensive care unit is one of the highest in the hospital, facilities to prevent infection are often inadequate in these important areas. Therefore, ***Potter and Perry*** (27) emphasized that the nurse must follow general guidelines for controlling infection and use programs according to hospital policy. In addition, nurses should use barrier precautions to prevent the occurrence of infection for patients and for themselves.

The second part of the standards developed in the present study was concerned with process standards. These were based on the steps of the nursing process, which is a systematic, deliberate problem-solving approach to meet the healthcare and nursing needs of patients' assessment, diagnosis, planning, implementation, and evaluation of outcomes. In the specialty of nursing radiology, the ***ARNA-American Radiological Nurses Association and American Nurses Association*** (9) outlined the use of the nursing process for patients undergoing diagnostic and therapeutic imaging procedures as follows: collection of ongoing data, synthesis and analysis of data to determine outcomes, development of an age appropriate plan of care, implementation of the nursing plan, evaluation of the patient's responses

to plan, and reassessment and revision of the plan and goals as indicated.

The first step included in the process standard was that of nursing assessment. All jury group members in the present study have agreed upon all proposed items for radiology patients. Similarly, the majority of nurses agreed upon the importance and applicability of all items. The high agreement of both jury and nurses reflect the major importance of this step at the start of patient encounter. This has been emphasized by ***Phipps et al.*** (28) who mentioned that the nurse should perform a complete physical assessment of the patient, which would include collecting data about the onset of disease and its progress. As stressed by ***Ellis and Hartley*** (29), each nurse must demonstrate competencies that meet minimum criteria in the performance of taking efficient and complete physical assessment. This is vital to construct a database to formulate the nursing diagnosis (patient problem) and in order to delineate competent nursing actions.

Due to the importance of this step of the nursing process, the ***RCN-Royal College of Nursing*** (16) emphasized that the comprehensive pre-procedural care of patients requiring vascular and/or non-vascular interventional radiology is essential in the work of the radiology nurse. It involves assessing the physical and psychological wellbeing of the patient through pre-procedural interviewing. At this time, drug, medical, and social histories can be obtained. Most patients, despite their best efforts, have little understanding of the procedures they are about to undergo. Thus, experienced nurses are often in a position to assess the degree of knowledge and anxiety prior to the procedure, thereby allaying patient fears through explanation and reassurance.

The last item in assessment is getting the patient to sign a consent form. All jury group members in the present study agreed upon this step. Meanwhile, still about one-fourth of the nurses could not perceive the importance of this step. This could be explained by their lack of knowledge, as well as their traditional thinking that the patient should be passive in his/her treatment plan, and should cede all decisions to the healthcare provider. The finding is in congruence with ***El Hanafy*** (22) who showed that the lowest percentage of agreement was related to the consent form.

Another explanation of the relatively low agreement of nurses upon getting the consent signed by the patient could be that they might consider this step as one of the physician's responsibilities. In fact, the ***Court of Appeal of Louisiana*** (30) had a ruling that the physician has the legal responsibility to communicate with the patient about the benefits, risks, and alternatives of the proposed procedure, and to obtain the patient's informed consent before any intervention procedure.

The second step of the nursing process is nursing diagnosis. The present study has demonstrated that all the jury group members agreed upon all the proposed items of nursing diagnosis for the radiology patients. Also the majority of nurses agreed upon their importance and applicability, with higher agreement upon applicability. The findings are in congruence with ***El Hanafy*** (22) and ***Nasr El din*** (31) who has also demonstrated a majority agreement of jury group members regarding nursing diagnosis items.

Nevertheless, the actual observation of nurse in the present study was very low before dissemination of standards. These results are consistent with ***El Hanafy*** (22) who found that less than one third of the studied nurses made nursing diagnosis. On the same line, ***Mohammed*** (32) and ***Nasr El din*** (31) reported that most of the nurses did not formulate nursing diagnosis.

However, statistically significant improvements were demonstrated in the formulation of nursing diagnosis by nurses in the present study after dissemination of the standards. This shows that the lack of performance was due to lack of knowledge among study nurses. It also implies that that the standards are applicable, and that their implementation will lead to better performance since nurses realized the importance of setting a nursing diagnosis for better further nursing care for the patient. In this regard, ***Tatiana et al.*** (33) highlighted that nursing diagnosis allows defining the profile of needs of patients, thus making the global focus of nursing interventions easy. Similarly, ***Gordon (in: Mohammed*** (32) emphasized that in order to make continuous nursing assessment easier over the several shifts, language standardization in the form of clear nursing diagnosis is required.

The third step of the nursing process is planning. According to ***Flippo*** (34) patient's care plan is a very effective method of monitoring standards. The findings of the present study showed that all jury group members and the majority of the nurses agreed upon all criteria of the planning standards. This included both their importance and their applicability. These results are congruent with ***El-Guindy*** (20) study in which all the jury group members gave their agreement upon all items related to planning. They are also in consistence with ***El-Hanafy*** (22).

The fourth step of the nursing process is that of implementation of the nursing care plan. Its items included items similar to those listed by ***Catherine and Linton*** (35) which involve, but are not limited to, health teaching, direct client care, medical treatments,

medications, and dressing changes. These all aim to achieve established goals of care.

All jury group members in the present study agreed upon all the items of the proposed standards. Similarly, the majority of the nurses expressed their agreement upon the importance and applicability of these items. This is expected since this is the part of the nursing process that is mostly realized and known to nurses because they consider it as their main job. In this respect, the ***ARNA-American Radiological Nurses Association and American Nurses Association*** (9) indicated that the radiology nurse should focus on patient and patient's responses to radiological interventions or physiologic changes while in radiology department or under the care of the radiologist. The high consensus among jury group members is in congruence with ***El-Guindy*** (20) and ***El-Hanafy*** (22) findings. Also, the agreement of the great majority of the nurses upon most implementation items is in consistence with ***Mosa*** (36).

Despite their high agreement upon the importance and applicability of all implementation items, their performance of nursing interventions before of the standards was low. However, it was better when compared to the performance in the first three steps of the nursing process. This might be explained by that these direct patient care items constitute the daily work of nurses. The deficiency in their performance, however, could be related to lack of standards to be followed, as well as lack of facilities and supplies. This would lead to poor quality of care rendered to the patients as clarified by ***Thompson*** (37). Nurses' performance demonstrated statistically significant improvements after dissemination of the standards, which shows the importance and potential impact of its application.

Concerning nurses' performance in case of use of contrast media before dissemination of the standards, the present study findings showed it was high in some of the items, and low in others. This could be attributed to rules and regulations concerning nurse's role in administration of such materials, which should be clarified by standards. In this regard, ***Eliker et al*** (38) pointed out that a radiology nurse may administer intravenous contrast media under supervision of physician, and she/he must first assess the patients for risk factors predisposing them to adverse reactions. Furthermore, ***Fajardo and Roe*** (39) added that during and following the injection, the administering individual will remain with the patient to observe for possible reactions. Therefore, when the actual roles of the radiology nurse were made known through dissemination of the developed standards, their performance significantly improved.

One of the most important tasks and responsibilities of the radiology nurse is the monitoring of patients in interventional radiology departments after the use of contrasts or similar interventions. For all patients under angiography during the initial post procedural period, a skilled nurse should periodically monitor blood pressure, record vital signs, and maintain intravenous access for administration of fluids and medications as needed ***Spiese et al***, (40). This makes the radiology nurse a vital member of the interventional radiology team that should be available during all procedures. Due to the major importance of this role, the present study findings indicated high levels of adequate performance before dissemination of the developed standard, and this further improved after dissemination, but without statistically significant difference.

Integrating ethical provisions in areas of practice was one of the least adequately performed by nurses in the present study before dissemination of the developed standards. This indicates low awareness about patient rights. It could also be attributed to low empowerment among these nurses, which would hinder their ability to play the role of patient advocate as determined by the ***ARNA-American Radiological Nurses Association and American Nurses Association*** (9), which stressed the role of the radiology nurse in helping the patient to make decisions regarding own health. On the same line, the ***American Nurses Association [ANA]*** (41) indicated that the radiology nurses are the primary patient advocates in the radiology department; they should protect patient's privacy and dignity, and ensure focus on the patient. This has been observed among the present study nurses after dissemination of the standards.

The last step of the nursing process, nursing evaluation, was also agreed upon by all jury group members, and the majority of the nurses confirmed its importance and applicability. However, their agreement upon applicability was significantly higher than their agreement upon the importance of its items. This reflects that these nurses were not highly convinced with this step of the nursing process, which is in contradiction with ***Catherine and Linton*** (35) who mentioned that evaluation enables the nurse to determine what progress the patient has made in meeting the goals for care, and is a measure for determining outcomes of care. Thus, the performance of the present study nurses of the nursing evaluation items before dissemination of the developed standards was very low, but it significantly improved after dissemination of the standards.

Lastly, inspection of the study settings revealed that some of the structure standards were

present, while others were lacking in most of the settings. These were mainly related to mission, vision, and performance appraisal. The developed structure standards would amend this deficiency. However, other structure items related to direct patient as human and non-human resources must be made available to ensure that implementation of the developed standard would have a positive impact on radiology nurses' performance.

Conclusion

In the light of the study findings, it is concluded that the majority of staff nurses working in radiology department in El-Manial University Hospital agree upon the importance and applicability of the criteria of structure and process protocol, with applicability being higher than importance. However, their performance of these criteria is mostly inadequate. The proposed protocol has been face and content validated through majority agreement of nursing and medical jury group members. Dissemination of the protocols among staff nurses improved their performance, which points to its applicability. Additionally, the study settings have adequate structures that would guarantee the possibility of implementation of the protocol in these settings.

Recommendations

Based on the finding of the study, the following is recommended:

- The developed standards for nursing practice for patients in radiology department are recommended to be applied in the radiology department.
- The developed standards should be made known and available to all staff nurses working in the radiology department.
- The developed nursing practice standards should be explained in details for all nurses working in the radiology department, emphasizing its importance.
- The mission and vision of radiology department should be written and posted to be available for all staff members.

References

1. ***Herman G.T. (2009)***: Fundamentals of computerized tomography: Image reconstruction from projection, 2nd edition, Springer.
2. ***Coastal Bend College (2005)***: "Radiologic technology program 'handbook program and clinical polices and procedures, Beeville, texas78102.
3. ***William C., and Savvas N. (2002)***: " Emergency radiology in Canada: a national survey". Canadin Association Radiol J; 53(3):160-7.
4. ***Joint Commission. (2009)***: The Joint Commission's standards: DF.1-DF.4; PM.1-PM.4. 2009 comprehensive accreditation manual for hospitals: The official handbook (CAMH). Oakbrook Terrace, IL: Author.
5. ***Thrall J.H. (2004)***: Quality and safety revolution in health care. Radiology; 233: 3–6.
6. ***McGlynn E.A., Asch S.M., and Adams J. (2003)***: The quality of health care delivered to adults in the United States. N Engl J Med.; 348: 2635-2645.
7. ***Donald B., and Maulik S. (2006)***: 'Health care quality book" health care quality and the patient, Chicago, Washington D.C. Illinois-AUPH: Health Administration Press, chapter(1) .
8. ***Scott-Tilley D. (2008)***: Competency in nursing: a concept analysis, J Contin Educ Nurs 39 (2) 58–64.
9. ***ARNA-American Radiological Nurses Association and American Nurses Association. (2007)***: Radiology nursing: Scope and standards of practice. Silver Spring, MD: Authors.
10. ***Joint Commission. (2009)***: The Joint Commission's standards: DF.1-DF.4; PM.1-PM.4. 2009 comprehensive accreditation manual for hospitals: The official handbook (CAMH). Oakbrook Terrace, IL: Author.
11. ***Madewell J.E., Hattery R.R., and Thomas S.R. (2005)***: American Board of Radiology: maintenance of certification. Radiology; 234:17–25.
12. ***CNO-College of Nurses of Ontario, (2002)***: Professional Standards for Registered Nurses and Registered Practical Nurses in Ontario. (Rev. Ed.). Toronto: Author
13. ***Caprich S. (2003)***: Nursing news quarterly. Radiology Nursing Team Building; 4.
14. ***USAID-United State Agency International Development (2005)***: &partner for health reformplus, Ministry of health and population. "Egyptian hospital accreditation program: standards, May 2005 6th ed.
15. ***Kirschner R. (2006)***: 'Creating circle of trust" Journal of Radiology Nursing; (25): 52.
16. ***RCN-Royal College of Nursing (2006)***: Guidelines for nursing care in interventional radiology "the roles of the registered nurse and nursing support. BFCR (06)7 approved by the Board of the faculty of clinical radiology.
17. ***Butsashvili M., Kamkamidze G., Umikashvili L., Gvinjilia L., Kankadze K., Berdzuli N. (2010)***: Knowledge of health care-associated

infections among Georgian obstetricians and gynecologists. J Infect Dev Ctries; 4(5):329-333

18. ***Portney L., and Watkins M. (2000)***: Foundations of clinical Research, Application to Practice .2nd ed., Upper saddle River, New Jersy, .U.S.A.: Prentice-Hall, Inc., pp. 70-90.
19. ***Polite D.F., and Back C. (2006)***: Essentials of Nursing Research, Methods, Appraisal, and Utilisation 6th ed. Lippincott, Williams and Wilkins. Philadelphia, London: A wolter Kluwer Co., pp.328-331.
20. ***El Guindy A.M.H. (2008)***: Developing standards of nursing care for nurses working in the intensive care unit. Un puplished PHD thesis submitted to faculty of nursing Ain Shamc University.
21. ***Ahmed S., Abdel-aziz M., Mohammed Dsoky G.M. (2007)***: Study of nurses's performance regarding infection control for patients with central venous catheter, Unpublished master degree.
22. ***EL-Hanafy Y.E. (2008)***: Developing Nursing care standards for postoperative Hepatic Patients. Unpublished PHD Ain shams university.
23. ***Tantawy N. (2004)***: Developing of Nursing care standards for emergency surgical patients. Thesis submitted to the Faculty of Nursing, Ain shams University for fulfillment of the master degree.
24. ***Sidani S., Doran D., Mitchell P.H. (2004)***: A Theory-Driven Approach to Evaluating Quality of Nursing Care. Journal of Nursing Scholarship; 36(1): 60–65.
25. ***Ismail T. (2000)***: Assessment of nurse's awareness and attitudes toward infection control concept at el Manial University Hospital. Unpublished Master, Faculty of Nursing, Cairo University.
26. ***Grant (2007): Iin Abdelmoneim S., Abdelaziz M., Mohammed G., Dsoky M.*** Study of nurses performance regarding infection control for patients with central venous catheter Unpublished master, faculty of nursing, Zagazeg University,pp.148, 194-150
27. ***Potter P.A., and Perry A.G. (2007)***: Basic Nursing, Essentials for practice. 6th ed., St Louis: Mosby.
28. ***Phipps W., Monahan F., and Sand J. (2003)***: Medical surgical nursing, 7th ed., Toronto: Mosby, pp. 1363-1383.
29. ***Ellis J, and Hartley C. (2000)***: Management & coordinating nursing care. 3rd ed., New York: J.B. Lippincott co.
30. ***Court of appeal of Louisiana (2006)***: Surgical consent forms, informed consent: Appropriately. Http://www.nursinglaw.com/nov06pageone.pdf.
31. ***Nasr El din W. (2007)***: Establishing standard of nursing care for patients with upper gastrointestinal bleeding. Unpublished master thesis, Faculty of Nursing, University of Alexandria.
32. ***Mohammed N. (2004)***: Development of nursing care standards for emergency surgical units. Unpublished PhD thesis submitted to Faculty of Nursing, Ain Shams University
33. ***Tatiana R., Rachel C., Luzia E. (2004)***: Nursing diagnosis for the immediate postoperative period of patients submitted to liver transplantation Einstein; 2(2):100-104.
34. ***Flippo E. (2000)***: Personal management, 6th ed., Singapore: McGraw-hill Book Co., pp. 77-88.
35. ***Catherine K., and Linton A.D. (2007)***: Introduction to Medical-Surgical Nursing, 3rd ed. - ISBN 0-7216-9527-2.
36. ***Mosa H.H. (2002)***: Assessing nurses performance at intensive care. Unpublished Master Thesis, Faculty of Nursing, Ain Shams University
37. ***Thomson S. (2005)***: Food for Thought: Giving feedback to staff is a great test of a manager's skill. ***Nursing Standard***; Accessed in September 2010
38. ***Eliker B.M., Cypel Y.S., and Weinreb J.C. (2006)***: "IVcontrast administration for CT: a survey of practices for screening and prevention of contrast nephropathy. Am J Roentgenol; 186: 1651-1658.
39. ***Fajardo L., and Roe J. (2007)***: Department of radiology policy on intravenous contrast in adults ,university of lowa department of radiology, city lowa, I.A. http://www medicine, lowa ed/radiology/healthcare providers /documents/contrast –policy pdf.
40. ***Spiese J.B., Bakal C.W., Burke R.D., and Cardella J.F. (2003)***: Angioplasty standard of practice "Society of interventional radiology standards of practice committee. Journal of Interventional Radiology; 14:s219-s221.
41. ***American Nurses Association, [ANA] (2001)***: Code of ethics for nurses with interpretive statements. Washington, DC: Nuresbooks.org.

Biochemical and histopathological effects of melamine on liver, spleen, heart and testes in male rats

Abdulbasit I. Al- Sieni [1], Haddad A. El Rabey*[1] and Abdullah A. Majami[2]

[1] Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.
Permanent address: Bioinformatics Department, Genetic Engineering and Biotechnology Research Institute, Minufiya University, Sadat City PO Box 79, Egypt.
[2] FDA, Jeddah, Saudi Arabia
elrabey@hotmail.com

Abstract: This study aimed to evaluate the toxic effect of different melamine doses (5000, 10000, 15000 and 20000 ppm), supplemented orally in the diet for 28 days to male rats, on biochemical parameters and the histopathology of liver, testes, spleen. The complete blood count (CBC), serum proteins, serum bilirubin, serum liver enzymes, sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of serum proteins, histopathological examinations of four organs (liver, spleen, heart and testes) were investigated. The CBC showed non significant changes in the melamine supplemented groups. Liver function enzymes were slightly affected. Serum protein was decreased and serum bilirubin was increased. The SDS-PAGE showed induction of two new high molecular weight bands and another low molecular weight band as a result of melamine supplementation. The histopathological examination of the four organs under study showed adverse pathological signs according to the melamine dose.
[Abdulbasit I. Al- Sieni, Haddad A. El Rabey and Abdullah A. Majami **Biochemical and histopathological effects of melamine on liver, spleen, heart and testes in male rats.** *Life Sci J* 2013;10(1):2048-2059] (ISSN:1097-8135). http://www.lifesciencesite.com. 291

Keywords: electrolytes, bilirubin, melamine, liver, histopathology.

1.Introduction

Melamine [$C_3N_3(NH_2)_3$] is a white color, crystalline, nitrogen rich organic base, slightly soluble in water, used in organic synthesis and in the manufacture of resins. Melamine was considered non-toxic, until significant morbidity and mortality related to crystalluria, nephrolithiasis and nephrotoxicity resulted from pet food and milk-based products contamination in China in 2008, children in Taiwan, Hong Kong and Macau (Hau *et al*., 2009 and Skinner *et al.,* 2010). Clinical signs of melamine poisoning in cats and dogs were inappetence, vomiting, polyuria, polydipsia and lethargy. Urine and kidneys from affected animals contained large golden-brown birefringent crystals consisting of melamine and co-contaminant cyanuric acid (Gossner *et al.,* 2009).

The European Union set a standard for acceptable human consumption (Tolerable Daily Intake) of melamine at 0.5 milligrams per kg of body mass. It was reduced to 0.2 mg per kg (Harrington, 2010). Canada declared a limit of 0.35 mg and the US FDA's limit was put at 0.63 mg, but was later reduced to 0.063 mg daily. The World Health Organization's food safety director estimated that the amount of melamine a person could stand per day without incurring a bigger health risk, the "tolerable daily intake" (TDI), was 0.2 mg per kg of body mass (Endreszl, 2008).

The Melamine Incident in China surfaced in September 2008 was one of the largest food adulterations for profit that affected about 300,000 Chinese infants and young children, with six reported deaths. The impacts were not limited in China but raised global food safety issues of milk and milk-containing products exported from China (Gossner *et al.,* 2009). More than 150 pet food products were identified as containing contaminated ingredients and were recalled. Analysis revealed that these products contained up to approximately 3200 ppm melamine and 600 ppm cyanuric acid (Cianciolo *et al*., 2008 and Skinner *et al*., 2010).

Melamine cyanurate is more toxic than either melamine or cyanuric acid alone (Babayan and Aleksandryan, 1985 and Puschner and Reimschuessel, 2011). Diagnosis of melamine poisoning is based on the presence of melamine and cyanuric acid in samples of urine or kidney from affected animals, or contaminated food or feed. Gas chromatograph/mass spectroscopy (GC/MS) and liquid chromatography/tandem MS are used to analyze for melamine and cyanuric acid (Cianciolo *et al*., 2008; Hon *et al*., 2011 and Puschner and Reimschuessel, 2011). Schnackenberg *et al*. (2012) found that the male rats were slightly more affected than female rats following dosing with the 120 and 180 ppm melamine. On the other hand, Ren *et al*. (2012) concluded that the rapid development and rapid regression, and melamine withdrawn plays a key role in the stone dissolution-discharge necessary for BEH regression.

On the other hand, Bai *et al.* (2010) stated that the highest tissue melamine concentrations in chickens fed melamine-containing diets were found in the kidneys, with lower concentrations in the liver and muscle. Whereas Jingbin *et al.* (2010) reported that, tissue residues were depleted 10 to 20 days after exposure ceased. Osborne *et al*. (2008) and Bhalla *et al*., (2009) stated that melamine and cyanuric acid crystallize; forming a lattice structure at the molecular level, at a pH of 5.8. On the other hand, melamine contained crystals recovered from kidneys and urine of cats that ingested melamine-contaminated food contained 70% cyanuric acid and 30% melamine based on infrared spectra results (Osborne *et al*., 2008 and Thompson *et al*., 2008).

Several analytical methods were developed to determine melamine in the serum and organs; after intravenous administration (Wu *et al.*, 2009), whereas Huang *et al.* (2012) developed a method for determination of melamine and related triazine by-products based on capillary electrochromatography-mass spectrometry using poly(divinyl benzene-alkene-vinylbenzyl trimethylammonium chloride) monolithic stationary phases. Liu *et al.* (2012) developed a new method for detection of melamine in a human renal uric acid stone by matrix-assisted laser desorption / ionization time-of-flight mass spectrometry (MALDI-TOF MS).

The toxic effect of different melamine doses (5000, 10000, 15000, and 20000 ppm), supplemented to male rats orally in the diet for 28 days was studied as revealed by biochemical parameters and histopathology of liver, testes, spleen and heart in male rats.

2.Materials and methods

The toxic effect of melamine was tested in male Albino Wister rats (*Rattus rattus*) of an average of 200-225 g were obtained from King Fahd Centre for Medical Research, King Abdulaziz University, KSA. Rats were housed five per cage and held for approximately two weeks before the study began for acclimatization. Cages, bedding, and glass water bottles (equipped with stainless steel sipper tubes) were replaced twice per week. Test diets, control diets, and tap water are available *ad libitum.* Conventional animal basal diet was obtained from Grain Silos & Flour Mills Organization–Makkah branch in Jeddah- Saudi Arabia and Melamine (99%) was purchased from Sigma-Aldrich Cat. No. M2659-5G. The rats were distributed into five groups; rats of the first group were fed normal basic diet as a (-)ve control and four melamine supplemented groups; G1 were supplemented with 5,000 ppm, G2 were supplemented with 10,000 ppm, G3 were supplemented with 15,000 ppm and G4 supplemented with 20,000 ppm. Test diets were prepared by mixing the suitable melamine weight according to the dose to the basal diet. The rats were observed twice daily for mortality and morbidity and were weighed every week and at the end of the study. Necropsies were performed on all animals at the end of the study (28 days). All rats were dissected, photographed and the target organs for histopathological investigations were kept in 10% formalin.

Complete blood counts (CBC)

The following CBC parameters were measured using Flex reagent cartridge, URCA method of Dimension Vista System, Siemens Health Care, Diagnostics Inc., Newark, DE 19714, USA, according to the instruction of the supplier: GRP (granulocytes), Hb (hemoglobin), HCT (hematocrit), LY (lymphocytes), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), MCV (mean corpuscular volume), MO (monocytes), MPV (mean platelet volume), PCT (platelet-Crit), PDW (platelet distribution width), PLT (platelet count), RBC (red blood cell), RDW (red cell distribution width), WBC (white blood cell).

Serum bilirubin (direct and total) and serum liver enzymes (ALT, AST, ALP and GGT) were measured also using Flex reagent cartridge, URCA method of Dimension Vista System, Siemens Health Care, Diagnostics Inc., Newark, DE 19714, USA, according to the instruction of the supplier. The Sodium dodecyl sulphate polyacrlamide gel electrophoresis (SDS-PAGE) was performed using a modified method from Laemmli (1970).

Histopathological investigations

The liver, spleen, heart and testes were dissected out and fixed in 10% formalin, dehydrated in gradual ethanol (50-99%), cleared in xylene, and embedded in paraffin. Sections were prepared and then stained with hematoxylin and eosin dye for microscopic investigation (Drury *et al.,* 1976). The stained sections were examined and photographed under a light microscope to detect histopathological changes.

Data analysis

All data were analyzed using the SPSS (Statistical Program for Sociology Scientists) Statistics Version 17.0 for computing the mean values, the standard errors (SE), and the test of significance (t-test).

3. Results

Complete blood count (CBC)

Data in Table (1), shows the effect of melamine supplementation on white blood cell count. The mean values of WBC in all melamine supplemented groups (G1, G2, G3 and G4) were non significantly lower than that of the negative control (17.36±0.64, 19.86±1.89, 16.62±0.77, 17.84±1.91

and 20.91±3.98 X10^ 3 / uL, respectively). The mean values of LY% in all treatments (G1, G2, G3 and G4) were lower than that of the negative control (78.58±2.37, 70.02±2.72, 79.28±0.33, 78.88±3.37 and 86.63±3.16 %, respectively). Whereas differences were non significant in G1 and G4; significant at 5% (P <0.05) in G3 and highly significant at 1% (P <0.01) in G2, when compared to negative control. The mean values of MO% in all treatments (G1, G2, G3 and G4) were higher than that of the negative control (14.32±1.36, 19.38±1.71, 14.52±0.90, 11.32±1.50 and 7.71±0.49%, respectively). The differences were significant at 5% (P <0.05) in G4, highly significant at 1% (P<0.001) in G1 and very high significant at 0.1% (P <0.0001) in G2 and G3, when compared to the negative control.

Table (1): Effect of melamine supplementation for 28 days on white blood cell (WBC).

Parameter	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
WBC X10^ 3 / uL	Mean±SE	20.91±3.98	17.36±0.64	19.86±1.89	16.62±0.77	17.84±1.91
	T-test		-0.98 NS	-0.21 NS	-1.28 NS	-0.86 NS
LY %	Mean±SE	86.63±3.16	78.58±2.37	70.02±2.72	79.28±0.33	78.88±3.37
	T-test		1.63 NS	2.95**	2.42*	1.56 NS
MO %	Mean±SE	7.71±0.49	14.32±1.36	19.38±1.71	14.52±0.90	11.32±1.50
	T-test		-4.28**	-5.40***	-11.54***	-2.19*
GR %	Mean±SE	6.75±2.41	7.10±1.11	10.56±1.15	7.28±0.39	9.80±3.15
	T-test		-0.11 NS	-1.11 NS	-0.25 NS	-0.76 NS
LY X10^ 3 / Ul	Mean±SE	23.96±5.14	13.62±0.50	13.74±1.05	12.98±0.48	13.94±1.42
	T-test		2.02	2.06	2.20*	2.02
MO X10^ 3 / uL	Mean±SE	1.73±0.42	2.48±0.30	3.92±0.57	2.44±0.25	4.04±2.19
	T-test		-1.42	-2.41*	-3.29**	-0.95
GR X10^ 3 / uL	Mean±SE	1.92±0.95	1.94±0.21	2.16±0.38	3.22±0.10	3.84±2.25
	T-test		0.64	-0.19	0.80	-0.72

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P<0.05), ** means high significant at 1% (P<0.01), *** means very high significant at 0.1% (P<0.001) and NS means non significant.

The mean values of GR% in all the groups (G1, G2, G3 and G4) were non significantly higher than that of the negative control (7.10±1.11, 10.56±1.15, 7.28±0.39, 9.80±3.15 and 6.75±2.41%, respectively). The mean values of LY in all treatments (G1, G2, G3 and G4) were non significantly lower than that of the negative control (13.62±0.50, 13.74±1.05, 13.94±1.42 and 23.96±5.14X10^3/Ul, respectively). The mean values of MO in all treatments (G1, G2, G3 and G4) were higher than that of the negative control (2.48±0.30, 3.92±0.57, 2.44±0.25, 4.04±2.19 and 1.73±0.42X10^3/Ul, respectively). Differences were non significant in G1 and G4; significant at 5% (P <0.05), in G2 and highly significant at 1% (P <0.01) in G3. The mean values of GR in G1 and G3 were non significantly lower than that of the negative control (1.94±0.21, 2.16±0.38, 3.22±0.10, 3.84±2.25 and 1.92±0.95X10^3/Ul, respectively).

Table (2), shows the effect of melamine supplementation on red blood cell indices in rats. The mean value of RBC in G1, G2, G3 and G4 were higher than that of the negative control (7.90±0.004, 7.86±0.09, 7.75±0.01, 7.81±0.03 and 7.46±0.09 X10^ 6/uL, respectively). However, differences were highly significant at 1% (P <0.01) in G1, G2, G3 and G4, when compared to the negative control. The mean values of hemoglobin (Hgb) in G1, G2, G3 and G4 were higher than that of the negative control (15.40±0.61, 16.08±0.44, 15.26±0.37, 16.18±0.24 and 14.71±0.28 g/dl, respectively). The differences were non significant in G1 and G3, and significant at 5% (P <0.05) in G2, when compared to negative control.

The mean values of HCT% in G1, G2, G3 and G4 were lower than that of the negative control (41.80±0.26, 41.56±0.06, 41.90±0.35, 40.77±0.12 and 43.07±0.56%, respectively). The differences were non significant in G1 and G3; highly significant at 1% (P <0.01) in G4 and very high significant at 0.1% (P <0.001) in G2, when compared to the negative control.

Table (2): Effect of melamine supplementation on red blood cell indices (RBC).

Parameter	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
RBC X 10^ 6/uL	Mean±SE	7.47±0.10	7.90±0.004	7.86±0.09	7.75±0.01	7.81±0.03
	T-test		-4.32**	-3.19**	-3.26**	-2.95**
Hgb g/dl	Mean±SE	14.71±0.28	15.40±0.61	16.08±0.44	15.26±0.37	16.18±0.24
	T-test		-1.48 NS	-2.30*	-1.13 NS	3.47**
HCT %	Mean±SE	43.07±0.56	41.80±0.26	41.56±0.06	41.90±0.35	40.77±0.12
	T-test		2.01 NS	-4.85***	1.733 NS	4.04**
MCV fl	Mean±SE	56.26±0.69	54.18±0.66	54.32±0.99	54.24±0.56	52.28±0.30
	T-test		1.55*	1.18*	2.35*	5.82***
MCH pg	Mean±SE	19.02±0.05	17.65±0.06	19.00±0.17	18.70±0.13	17.85±0.14
	T-test		16.74***	-3.50**	3.35**	6.69***
MCHC g/dl	Mean±SE	3.32±0.09	3.33±0.15	3.46±0.06	3.45±0.10	3.42±0.26
	T-test		-5.2***	-9.8***	-10.99***	-4.75***
RDW %	Mean±SE	1.54±0.37	1.55±0.69	1.84±3.09	1.57±0.44	2.11±0.65
	T-test		-0.02 NS	-0.90 NS	-0.87 NS	-8.45***

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P<0.05), ** means high significant at 1% (P<0.01), *** means very high significant at 0.1% (P<0.001) and NS means non significant.

The mean values of MCV in all treatments (G1, G2, G3 and G4) were lower than that of the negative control (54.18±0.66, 54.32±0.99, 54.24±0.56, 52.28±0.30 and 56.26±0.69 fl, respectively), however differences in G1, G2 and G3 were significant at 5% (P <0.05) and in G4 was very high significant at 0.1 (P <0.001), when compared to the negative control. The mean MCH values of G1, G2, G3 and G4 were lower than that of the negative control (17.65±0.06, 19.00±0.17, 18.70±0.13, 17.85±0.14 and 19.02±0.05 pg, respectively), whereas the MCH value in G2 was higher than that of the negative control (19.65±0.17 and 19.00±0.04 pg, respectively). Differences of G2 and G3 were highly significant at 1% (P <0.01) and very high significant at 0.1% (P <0.001) in G1 and G4, when compared to negative control. The mean MCHC values in all treatments (G1, G2, G3 and G4) were higher than that of the negative control (3.33±0.15, 3.46±0.06, 3.45±0.10, 3.42±0.26 and 3.32±0.09 g/dl, respectively), whereas differences were very high significant at 0.1% (P <0.001) in all treatments, when compared to the negative control. The mean values of RDW % in G1, G2, G3 and G4 were higher than that of the negative control (1.84±3.09, 1.55±0.69, 1.57±0.44, 2.11±0.65 and 1.54±0.37%, respectively). The differences were non significant in all treatment except for G4 which were very high significant at 0.1% (P <0.001), when compared to the negative control.

Table (3), illustrates the effect of melamine supplementation on platelet indices in rats under study. The mean values of platelet indices of all treatments (G1, G2, G3 and G4) were non significantly higher than that of the negative control (1.03±25.11, 1.04±52.77, 1.05±86.55, 1.17±66.70 and 1.01±17.31 X10^ 3 / uL, respectively). The mean values of MPV in all melamine supplemented groups were higher than that of the negative control (5.10±0.07, 5.22±0.08, 5.45±0.07, 6.42±1.01 and 5.01±0.06 fL, respectively). Differences were non significant in all treatments except for G3, which was very high significant at 0.1% (P <0.001), when compared to the negative control.

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P<0.05), ** means high significant at 1% (P<0.01), *** means very high significant at 0.1% (P<0.001) and NS means non significant.

The mean values of PCT% in G1, G2, G3 and G4 were higher than that of the negative control (0.51±0.02, 0.54±0.01, 0.55±0.04, 0.59±0.02 and 0.48±0.03%, respectively). Differences were highly significant at 1% (P <0.01) in G4 and non significant in all other groups, when compared to the negative control. The mean values of PDW% in all treatments (G1, G2, G3 and G4) were lower than that of the negative control (1.61±0.42, 1.58±0.30, 1.60±0.28, 1.63±0.38 and 1.72±0.48%, respectively), however, differences were non significant in G4; highly significant at 1% (P <0.01) in G1 and G2, and very

high significant at 0.1% (P <0.001) in G3, when compared to the negative control.

Serum proteins

Table (4), shows the effect of melamine supplementation for 28 days on serum protein (albumin and total protein) in rats under study. As shown, the mean values of albumin in all melamine supplemented groups were non significantly lower than that of the negative control (10.80±0.20, 10.60±0.24, 11.20±0.20, 10.60±0.40, and 11.18±0.4 g/L, respectively).

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P<0.05), ** means high significant at 1% (P<0.01), *** means very high significant at 0.1% (P<0.001) and NS means non significant.

The mean values of total protein (TP) in all melamine supplemented groups (G1, G2 and G4) were lower than that of negative control (62.60±1.12, 62.00±1.00, 61.60±2.01, 61.60±2.01 and 63.20±1.04 g/L, respectively). However, differences were non significant in all groups, except in G3 that was significant at 5% (P <0.05).

Serum bilirubin

Table (5), illustrate the effect of melamine supplementation for 28 days on serum bilirubin (total and direct bilirubin) in rats. As shown, the mean values of the total bilirubin in G1, G2, G3 and G4 were higher than that of the negative control. (2.60±0.24, 3.20±0.37, 2.98±0.45, 3.00±0.31 and 2.15±0.22 mmol/L, respectively). However, differences were non significant in G1 and G2, significant at 5% (P <0.01) in G3 and G4.

Table (3): Effect of melamine supplementation for 28 days on platelets indices (PLT).

Parameter	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
PLT index X 10^ 3 / uL	Mean±SE	1.01±17.31	1.03±25.11	1.04±52.77	1.05±86.55	1.17±66.70
	T-test		0.72 NS	-0.15 NS	-0.24 NS	-1.51 NS
MPV fL	Mean±SE	5.01±0.06	5.10±0.07	5.22±0.08	5.45±0.07	6.42±1.01
	T-test		0.00 NS	-1.90 NS	-5.03***	-1.02 NS
PCT %	Mean±SE	0.48±0.03	0.51±0.02	0.54±0.01	0.55±0.04	0.59±0.02
	T-test		-1.21 NS	0.84 NS	-1.75 NS	-4.03**
PDW %	Mean±SE	1.72±0.48	1.61±0.42	1.58±0.30	1.60±0.28	1.63±0.38
	T-test		3.83**	2.81**	5.24***	1.145 NS

Table (4): Effect of melamine supplementation for 28 days on serum protein (albumin and total protein), in rats under study.

Parameters	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine G3	G4 20,000 ppm melamine
ALB g/L	Mean±SE	11.18±0.20	10.80±0.20	10.60±0.24	11.00±0.20	10.60±0.40
	T-test		1.63 NS	1.50 NS	1.01 NS	1.50 NS
TP g/L	Mean±SE	63.20±1.04	62.60±1.12	62.00±1.00	61.60±2.01	62.80±1.24
	T-test		0.24 NS	1.53 NS	2.76*	0.09 NS

Table (5): Effect of melamine supplementation for 28 days on serum bilirubin (total and direct bilirubin), in rats under study.

Parameters	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
Total bilirubin mmol/L	Mean±SE	2.15±0.22	2.60±0.24	3.20±0.37	2.98±0.45	3.00±0.31
	T-test		-1.75 NS	-1.86 NS	-2.17*	-2.13*
Direct bilirubin mmol/L	Mean±SE	7.17±0.37	38.44±4.19	81.22±10.07	56.00±4.78	66.30±8.36
	T-test		-7.57***	-7.19***	-9.91***	-6.93***

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P<0.05), ** means high significant at 1% (P<0.01), *** means very high significant at 0.1% (P<0.001) and NS means non significant.

The mean values of direct bilirubin in G1, G2, G3 and G4 were higher than that of the negative control (38.44±4.19, 81.22±10.07, 56.00±4.78, 66.30±8.36 and 7.17±0.37 mmol/L, respectively). However, the differences were very high significant at 0.1% (P <0.001), when compared to the negative control.

Serum liver enzymes

Table (6), shows the effect of melamine supplementation for 28 days on serum liver enzymes (ALT, AST, ALP, GGT) in rats under study. The mean values of ALP in all groups (G1, G2, G3 and G4) were lower than that of the negative control (147.80±20.75, 100.40±15.06, 114.80±17.93, 116.00±12.13 and 152.21±13.08 U/L, respectively. However, the paired T-test did not show any significant differences in G1, G3 and G4, whereas the value of G2 was significant at 5% (P <0.05), when compared to that of the negative control. The mean values of AST in all groups (G1, G2, G3 and G4) were lower than that of the negative control (80.60±16.75, 106.00±23.83, 84.00±8.27, 76.60±9.37, and 107.22±3.38 U/L, respectively. However, the paired sample T-test did not show any significant in G1 and G2, whereas differences in G3 and G4 were they were significant at 5% (P <0.05), when compared to the negative control. Similarly for ALT, the mean values of all groups (G1, G2, G3 and G4) were lower than that of the negative control (56.00±10.45, 31.40±5.93, 29.40±3.04 and 69.61±3.95 U/L, respectively). However, differences were non significant in G1, and very high significant at 0.1% (P <0.001) in G2, G3 and G4, when compared to the negative control. The mean values of Gamma-glutamyl Transpeptidase (GGT) in all supplemented groups (G1, G2, G3 and G4) were non significantly higher than that of the negative control (3.08±0.03, 3.10±0.03, 3.12±0.03, 3.16±0.02 and 3.07±0.03 U/L, respectively).

Table (6): Effect of melamine supplementation for 28 days on serum liver enzymes (ALT, AST, ALP, GGT), in rats under study.

Parameters	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine G3	G4 20,000 ppm melamine
ALB g/L	Mean±SE	11.18±0.20	10.80±0.20	10.60±0.24	11.00±0.20	10.60±0.40
	T-test		1.63 NS	1.50 NS	1.01 NS	1.50 NS
TP g/L	Mean±SE	63.20±1.04	62.60±1.12	62.00±1.00	61.60±2.01	62.80±1.24
	T-test		0.24 NS	1.53 NS	2.76*	0.09 NS

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P<0.05), ** means high significant at 1% (P<0.01), *** means very high significant at 0.1% (P<0.001) and NS means non significant.

SDS-PAGE of serum proteins

Figure (1), shows the serum protein electrophoretic profile of the four groups supplemented with melamine compared to protein marker and the negative control. Five major protein bands of a molecular weight of about 200, 180, 60, 29, 26 kDa and one minor band of a molecular weight of about 24 kDa, were appeared common in all treatments. Two new high molecular weight (at about 160 and 140 kDa, respectively) bands appeared in the high doses of melamine fed groups (15,000 and 20,000 ppm). Another band of a molecular weight about 22 kDa appeared in all melamine fed rats.

Histopathology

Liver

The rat liver of G1 and G2 groups which were regularly supplied with 5,000 and 10,000 ppm melamine, respectively showed massive fatty changes, necrosis, and broad infiltration of the lymphocytes (Figure 2, B & C), comparable to those of the control group (Figure 2, A).

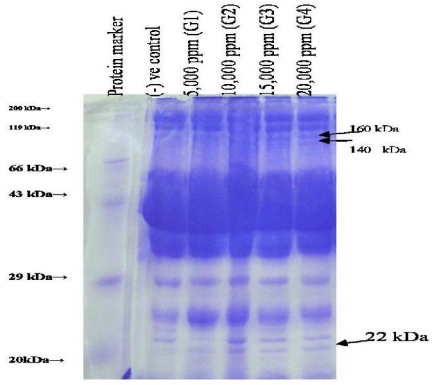


Figure (1): SDS-PAGE of serum proteins. Lanes were arranged as follows: M: protein markers, (-)ve control, G1: fed on 5000 ppm melamine, G2: fed on 10,000 ppm melamine, G3: fed on 15,000 ppm melamine, G4: fed on 20,000 melamine.

The histological architecture of liver sections of the rats supplemented with moderate doses of melamine (15,000 and 20,000 ppm) showed more or less abnormal patterns, with a mild degree of necrosis and slightly lymphocyte infiltration, almost

comparable to those of the control group (Figure 2, D & E).

Spleen

The spleen of the control group contains hematopoietic and lymphoid elements, is a primary site of extramedullary hematopoiesis, and removes degenerate and aged red blood cells as well as particulate materials and circulating bacteria from the blood supply. Lesions of this important component of the immune system may center on the red pulp, the white pulp or involve both compartments (Figure 3, A). The appearance of pigment in the red pulp (Figure 3, B & C) is a common background lesion in 5,000 and 10,000 ppm melamine treated rats. Pigment, can be ceroid/lipofuscin or hemosiderin, is usually harbored in macrophages and may be present in the marginal zone in addition to being in the red pulp. In both rat groups, hemosiderin is more prominent in females. Iron stains, e.g., Perl's Prussian blue, can demonstrate the iron in hemosiderin-containing macrophages. After rats were supplemented with high doses (15,000 and 2,0000 ppm), they showed large amounts of pigment present as in this example. It can usually be detected at low magnification in the red pulp. Higher magnification of Figure (3, D & E) shows brown granular hemosiderin pigment predominantly within macrophages in the red pulp.

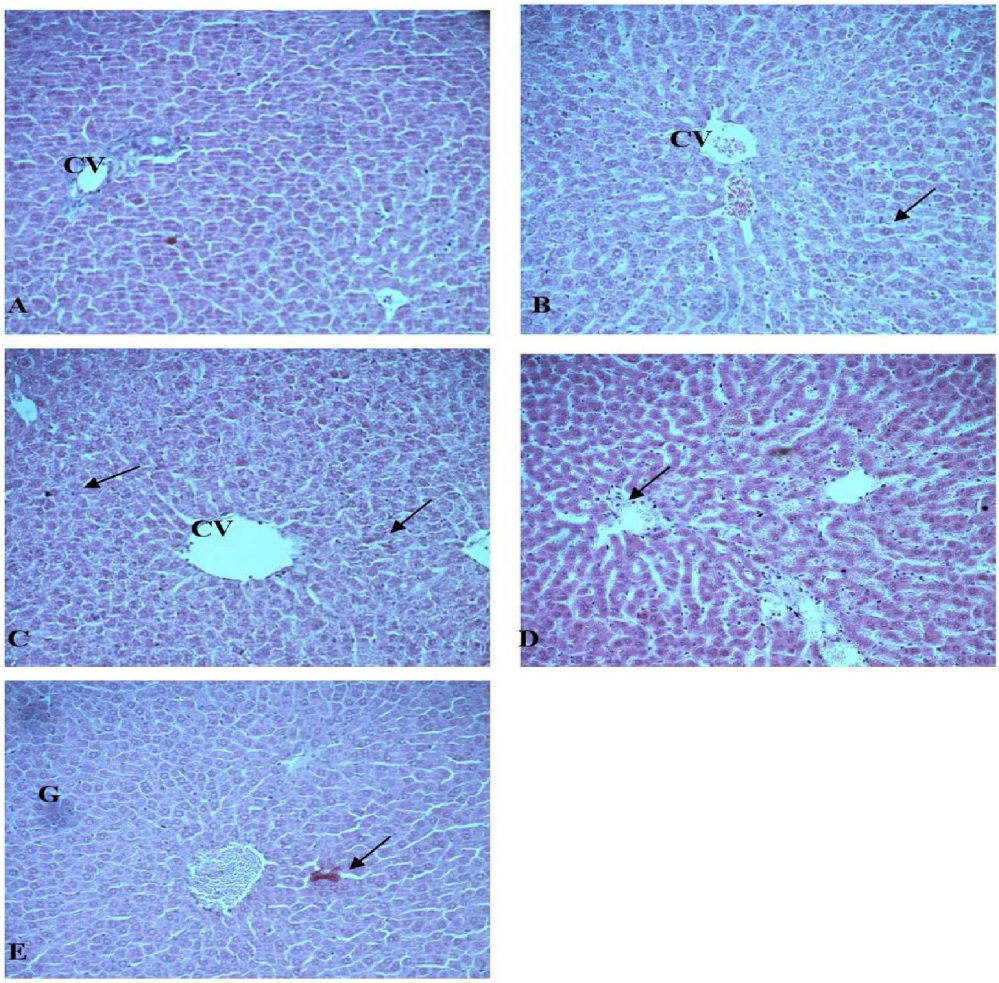


Figure (2): A; Normal hepatic tissues showing hepatic strands of cells around the central vein (CV) leaving blood sinusoids (S), **B;** hepatic tissues of 5,000 ppm melamine fed group showing cellular necrosis around the central veins (arrow), **C;** hepatic tissues of 10,000 ppm melamine fed group showing mild cellular necrosis, **D;** hepatic tissues of 15,000 ppm melamine fed group showing highly degree of necrosis and accumulation of melamine salt particles (arrow), **E;** hepatic tissues of 20,000 ppm melamine showing highly degenerated hepatic tissues and accumulation of granules in hepatic tissues (G). X 200 (H&E stains).

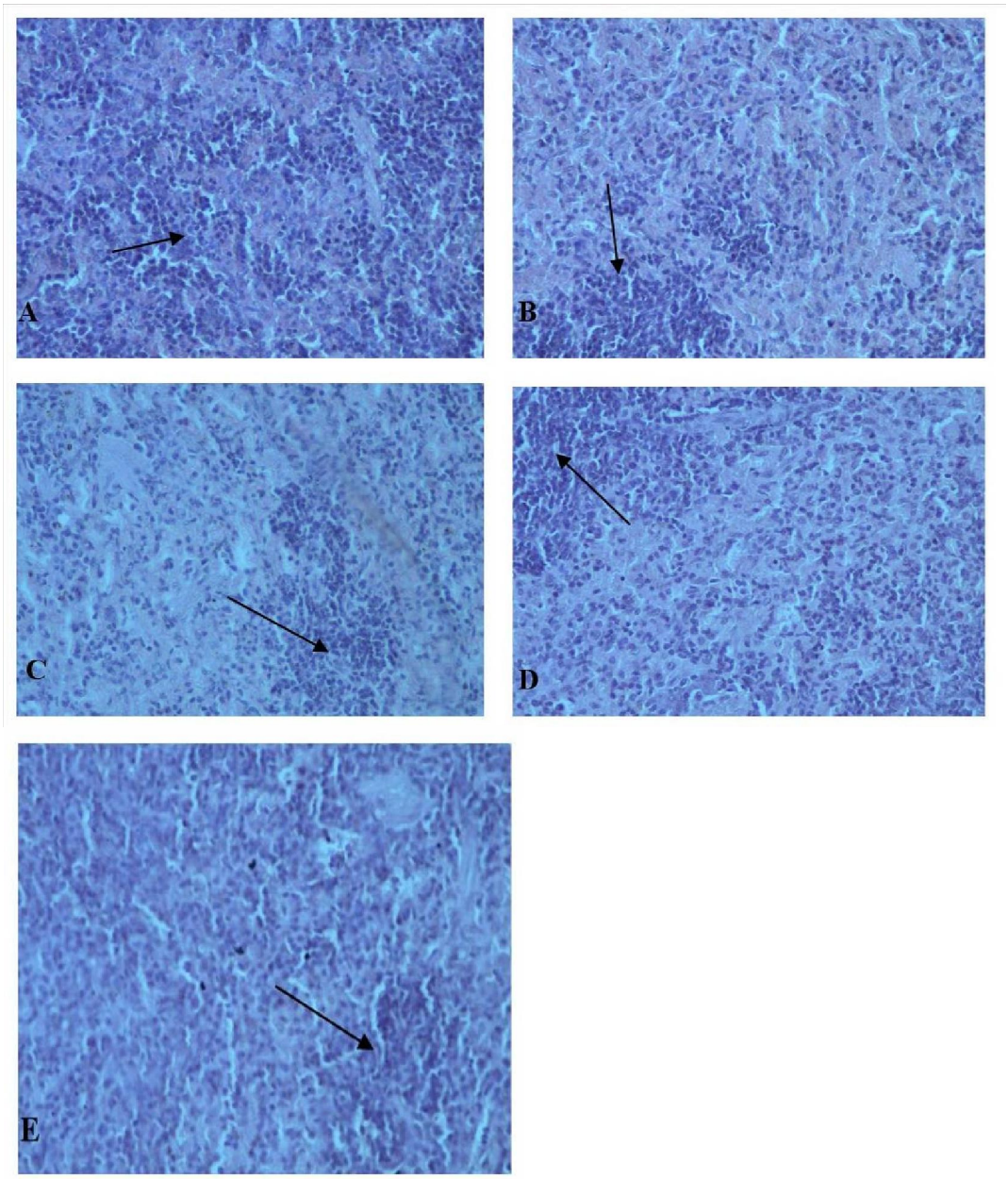


Figure (3): A; Normal spleen tissues showing hematopoietic and lymphoid elements (arrow), **B;** spleen tissues of 5,000 ppm melamine fed group showing the appearance of pigment in the red pulp (arrow), **C;** spleen tissues of 10,000 ppm melamine fed group showing hemosiderin-containing macrophages (arrow), **D;** spleen tissues of 15,000 ppm melamine showing increases in the incidences of hyperplasia (arrow), **E;** spleen tissues of 20,000 ppm melamine showing highly granular hemosiderin pigment in spleen tissues (arrow). X 200 (H&E stains).

Heart

The normal structure of cardiac muscles is shown in control group (Figure 9, A).

Hyalinization in the cardiac muscles appeared in the low melamine doses which were supplemented 5,000 and 10,000 ppm (Figure 4, B & C, respectively) groups, but minimal cardiac muscles damage in the melamine-supplemented rats that received 10,000 ppm melamine (Figure 4, C). In addition, heart tissues of 15,000 ppm melamine supplemented rats, showed pathological changes such as congestion and slight infiltration, focal parenchymatous degeneration of cardiomyocytes and the presence of single basophils was observed (Figure 4, D), but the heart tissue of the rats given 20,000 ppm (Figure 4, E) indicated maximal hyalinization of

muscle fibers, with focal cellular infiltration or necrosis of muscle fibers.

Testes

The light microscopy examination of the testis of the control rats had normal structure, completely enveloped by a thick capsule, tunica albuginea, which is composed mainly of dense collagenous fibrous connective tissue. The structural components of the testis are the seminiferous tubules (ST) and interstitial tissues (IT). The seminiferous tubules are two types of cells, the Sertoli cells, resting on the thin basal lamina (basement membrane) and the spermatogenic cells (SC). These cells are many layers, namely, the spermatogonia (S), primary spermatocytes (PS); secondary spermatocytes (SS); spermatoids (SP) and finally mature spermatozoa (Z), (Figure 5 A).

In the low doses of melamine supplemented groups (5,000 and 10,000 ppm melamine, respectively), showed cellular changes. The seminiferous tubules had thickened in basement membrane together with focal areas of vacuolar degenerative changes appeared in the cytoplasm of the spermatogenic epithelium (Figure 5, B & C).

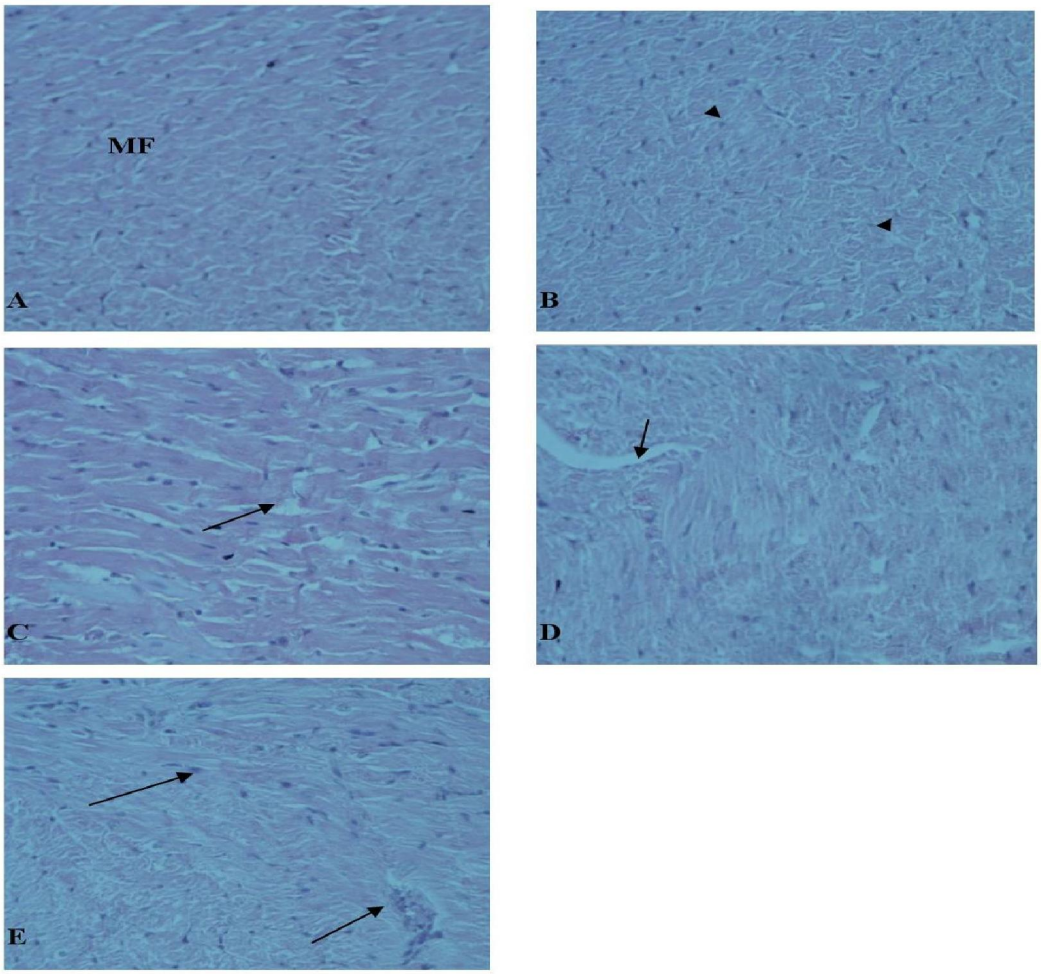


Figure (4): A; Normal cardiac tissues showing normal structure of cardiac muscles consists of muscle fibers (MF), **B;** cardiac tissues of 5,000 ppm melamine fed group showing increased hyalinization (arrow heads), **C;** cardiac tissues of 10,000 ppm melamine fed group showing minimal cardiac muscles damage (arrow), **D;** cardiac tissues of 15,000 ppm melamine fed group showing degeneration of cardiomyocytes (arrow), **E;** cardiac tissues of 20,000 ppm melamine showing necrosis of muscle fibers (arrow). X 200 (H&E stains).

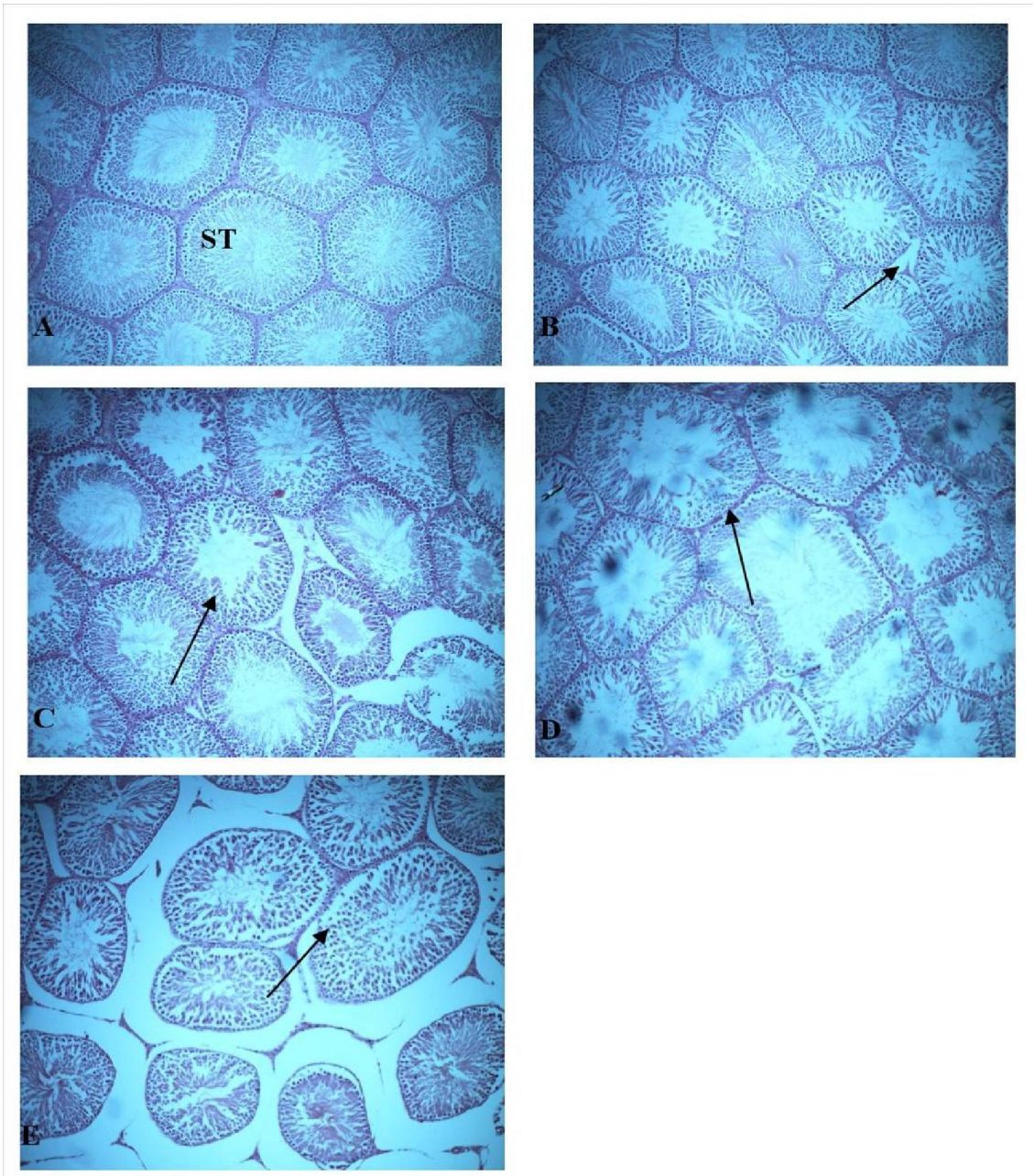


Figure (5): A; Testicular tissues of control group showing structural components of the testis which are the seminiferous tubules (ST), **B;** testicular tissues of 5,000 ppm melamine fed group showing the seminiferous tubules had thickened in basement membrane together (arrow), **C;** testicular tissues of 10,000 ppm melamine fed group showing vacuolar degenerative changes appeared in the cytoplasm of the spermatogenic epithelium (arrow), **D;** testicular tissues of 15,000 ppm melamine fed group showing the form of degenerative changes (arrow) and **E;** testicular tissues of 20,000 ppm melamine fed group showing highly degenerated testicular tissues and few fragmented sperms in the lumen (arrow). X 200 (H&E stains).

After rats had been supplemented with higher doses (15,000 and 20,000 ppm) of melamine, they showed more exaggerated features of focal areas of spermatogenesis, arrest at the spermatid level, in the form of degenerative changes in the germinal cells together with few fragmented sperms in the lumen and acquired a thick, irregular basement membrane (Figure 5, D & E).

4. Discussion

The present investigation shows the effect of an oral supplementation of four melamine doses (5,000,

10,000, 15,000 and 20000 ppm) in the diet on the biochemical indices and histopathology of liver, spleen, heart and testes in male rats.

The CBC analysis showed that mean values of WBC were non significantly lower than that of the negative control. On the other hand, the mean values of RBC were slightly higher than that of the negative control. In addition, the mean values of platelet indices were non significantly higher than that of the negative control. This result agrees with that of Chen *et al.* (2009), who noticed that rats fed with higher melamine doses diets experienced only small changes in hematological parameters.

The serum protein (albumin and total protein) were non significantly increased compared to the negative control. This result is in concordant with similar results of Puschner *et al.* (2007) and Chen *et al.* (2009), who noted that a significant decline in albumin levels were detected.

The serum bilirubin (total and direct bilirubin) showed significant increase in the mean values than that of the negative control. This was expected due to the toxicity of melamine and the yellowish color appeared in the melamine treated rats. In contrast, Puschner *et al.* (2007) found that bilirubin in cats supplemented with melamine was not affected.

The non significant decrease in liver enzymes activity (ALT, AST, ALP, GGT), revealed that melamine supplementation for 28 days have not greatly affected the studied liver enzymes in studied male rats. The current liver enzymes results are consistent with those of Jeong *et al.* (2006) and Chen *et al*., 2009), who found that no significant change occurred in the levels of aspartate aminotransferase, alanine aminotransferase, or other examined enzymatic parameters. Furthermore, Bai *et al.* (2010) stated that the highest tissue melamine concentrations in chickens fed melamine-containing diets were found in the kidneys, with lower concentrations in the liver and muscle. It could be said that in spite of the high toxic effect of melamine on kidney as revealed by the very high significant differences of the kidney functions, it did not greatly affect the liver.

On the other hand, (Lv *et al.*, 2010) stated that melamine concentrations in the kidney were higher than concentrations in the skeletal muscle or liver of lambs. This result is supported by other investigations in rats (Wu *et al.*, 2009 and Ding *et al.*, 2012).

The SDS-PAGE showed induction of two new high molecular weight bands (at about 160 and 140 kDa, respectively) and a low molecular weight protein band (ca 22 kDa), as a result of melamine supplementation for 28 days. These bands may be induced due to the expression of antitoxicity genes. The current results are supporting to Camacho *et al.* (2011) who noted that histopathology and clinical chemistry examination indicated marked toxicity only in the animals exposed to two high combined doses of melamine and cyanuric acid and consistent with these observations, quantitative real-time polymerase chain reaction analysis of kidney tissue indicated increased expression of all genes analyzed relative to the control in both male and female rats fed daily with 229 or 694 ppm melamine and cyanuric acid.

In spite of the dramatic pathological changes of liver tissues and accumulation of hepatic granules and melamine crystals in most tissue, this is not reflected on the liver enzymes. This may be due to the chelating of enzymes by cyanuric acid that resulted from the contamination of melamine. The non significant changes in liver enzymes result is consistent with that of Lv *et al.* (2010), who reported that melamine concentrations in the kidney were higher than concentrations in the skeletal muscle or liver of lambs; and Bai *et al.* (2010) who stated that the highest tissue melamine concentrations in chickens fed melamine-containing diets were found in the kidneys, with lower concentrations in the liver and muscle. On the other hand, Chen *et al*. (2009) found that liver weight in male rat was not affected as a result of melamine toxicity.

Liver, heart, testes and spleen tissues showed dramatic pathological changes. Testes showed decrease in the number of spermatogonia. The cardiac tissues showed pathological changes and damage. Spleen showed vascular obstruction, hemorrhage and accumulation of melamine crystals. This result is consistent with Gao *et al.* (2012) who reported pathological changes in the heart, testes, spleen and urinary bladder of rats as a result of long term melamine supplementation. On the other hand, Chen *et al.* (2009) found that testes weight in male rats was increased as a result of melamine toxicity.

Based on the current results, it could be said that the toxic effect of melamine increases with increasing the melamine dose, as revealed from the histopathological investigations and SDS-PAGE of serum proteins. In addition, melamine supplementation affected other organs beside the kidney as revealed from the biochemical and histopathological investigations.

Acknowledgement

This project was financed by King Abdulaziz City for Science and Technology for supporting and funding this project (Grant No. 0736 -11 - ط - ت), therefore the authors are greatly indebted for that.

References

1. Babayan AA and Aleksandryan AV (1985). "Toxicological characteristics of melamine cyanurate, melamine and cyanuric acid", Zhurnal Eksperimental'noi i Klinicheskoi Meditsiny, 25: 345-9. Original article in Russian, English abstract retrieved from SciFinder on 2007-05-07.
2. Bai X, Bai F, Zhang K, Lv X, Qin Y, Li Y, Bai S and Lin S (2010). Tissue deposition and residue depletion in laying hens exposed to melamine-contaminated diets. J Agric Food Chem. 58: 5414–5420.
3. Bhalla V, Grimm P, Chertow GM and Pao AC (2009). Melamine nephrotoxicity: an emerging epidemic in an era of globalization. Kidney Internat 7: 774–779.
4. Camacho L, Kelly KP, Beland FA and da Costa GG (2011). Gene expression of biomarkers of nephrotoxicity in F344 rats co-exposed to melamine and cyanuric acid for seven days. Toxicology Letters 206: 166-171.
5. Chen K, Liao C, Cheng F *et al.* (2009). Evaluation of Subchronic Toxicity of Pet Food Contaminated with Melamine and Cyanuric Acid in Rats. Toxicologic Pathology, 37: 959-968.
6. Cianciolo RE, Bischoff K, Ebel J, Van Winkle TJ, Goldstein RE and Serfilippi LM (2008). Clinicopathologic, histologic, and toxicologic findings in 70 cats inadvertently exposed to pet food contaminated with melamine and cyanuric acid. J Am. Vet. Med. Assoc. 333: 729–737.
7. Ding X, Bai S, Zhang K, Wang L, Wu C, Chen D, Jia G and Bai J (2012). Tissue Deposition and Residue Depletion in Broiler Exposed to Melamine-Contaminated Diets. Journal of Integrative Agriculture 11(1): 109-115.
8. Drury RA, Wallington EA and Cancerson R (1976). Carlton's histopathological techniques, 4th edn. Oxford University Press, Oxford
9. Endreszl L (2008). "Safe Melamine Levels Named by World Health Organization". Health News. http://www.healthnews.com/alerts-outbreaks/safe-melamine-levels-named-world-health-organization-2252.html.
10. Gao H, Xu Y, He J, He X, Ma Y, Qin C, Gao J and Qin C (2012). Toxicity and prognosis of melamine and melamine analogues in rats after oral administration for eight weeks. International Journal of Automation and Computing, 26 (4): 555-562.
11. Gossner CM, Schlundt J, Ben Embarek P, Hird S, Lo-Fo-Wong D, Beltran JJ, Teoh KN and Tritscher A (2009). The melamine incident: implications for international food and feed safety. Environ. Health Perspect. 117, 1803–1808.
12. Harrington R (2010). "EFSA cuts melamine TDI by 60 per cent". FoodQualityNews.com. http://www.foodqualitynews.com/Legislation/EFSA-cuts-melamine-TDI-by-60-per-cent?utm_source=RSS_text_news. Retrieved 2010-04-16.
13. Hau AK, Kwan TH and Kam-tao P (2009). Melamine toxicity in the kidney. J Am Soc Nephrol 20: 245–250.
14. Hon PY, Chu PW, Cheng CH, Lee TC *et al.* (2011). Development of melamine certified reference material in milk using two different isotope dilution mass spectrometry techniques. J Chromatogr A 1218: 6907–6913.
15. Huang H, Lin C, Jiang S, Singco B and Cheng Y (2012). Capillary electrochromatography-mass spectrometry determination of melamine and related triazine by-products using poly(divinyl benzene-alkene-vinylbenzyl trimethylammonium chloride) monolithic stationary phases. Analytica Chimica Ada, 719: 96-103.
16. Jeong WI, Do SH, Jeong H, Chung JY, Yang HJ, Yuan DW, Hong IH, Park JK, Goo MJ and Jeong KS (2006). Canine renal failure syndrome in three dogs. J Vet Sci. 7: 299–301.
17. Jingbin W, Ndong M, Kai H, Matsuno K and Kayama F (2010). Placental transfer of melamine and its effects on rat dams and fetuses. Food Chem Toxicol, 48, 1791–1795.
18. Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 15; 227 (259): 680-685.
19. Liu C, Wu C, Shiea J *et al.* (2012). Detection of Melamine in a Human Renal Uric Acid Stone by Matrix-Assisted Laser Desorption / Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). Clin Chim Acta. 9- 413 (19-20): 1689-95.
20. Lv, X, JWang J, Wu L, Qiu J, Li J, Wu Z and Qin Y (2010). Tissue deposition and residue depletion in lambs exposed to melamine and cyanuric acid-contaminated diets. J Agric Food Chem 58: 943–948.
21. Osborne, CA, Lulich JP and Ulrich LK (2008). Melamine and cyanuric acid-induced crystaluria, uroliths, and nephrotoxicity in dogs and cats. VetClin N Am Small Anim, 39: 1–14.
22. Puschner B and Reimschuessel R (2011). Toxicosis caused by melamine and cyanuric acid in dogs and cats:uncovering the mystery and subsequent global implications. Clin Lab Med 31: 181–199.
23. Puschner B, Poppenga R, Lowenstine L, Filigenzi MS and Pesavento PA (2007). Assessment of melamine and cyanuric acid toxicity in cats. J Vet Diagn Invest 19:616–624.
24. Ren S, Xu C, Du Y, Gao X, Sun Y and Jiang Y (2012). The natural outcome of melamine-induced bladder stones with bladder epithelial hyperplasia after the withdrawal of melamine in mice. Food and Chemical Toxicology 50: 2318–2324.
25. Schnackenberg L, Sun J, Pence L, Bhattacharyya S, da Costa G and Beger R (2012). Metabolomics evaluation of hydroxyproline as a potential marker of melamine and cyanuric acid nephrotoxicity in male and female Fischer F344 rats. Food and Chemical Toxicology 50:3978–3983.
26. Skinner CG, Thomas JD and Osterloh JD (2010). Melamine toxicity. J Med Toxicol 6: 50–55.
27. Thompson ME, Lewin-Smith MR, Kalasinsky K, Pizzolato KM, Fleetwood ML, McElhaney MR and Johnson TO (2008). Characterization of melamine-containing and calcium oxalate crystals in three dogs with suspected pet food-induced nephrotoxicosis. Vet Pathol 55: 417–426.
28. Wu Y, Huang C, Lin C, Ho W, Lin L, Chiu T, Tarng D, Lin C and Tsai T (2009). Determination of melamine in rat plasma, liver, kidney, spleen, bladder and brain by liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 1216: 7595–7601.

2/2/2013

Unusual Abdominal Metastasis from Marjolin's Ulcer (Case Report and Review of Literature)

Munaser S. Alamoodi

Assistant Professor and Consultant General Surgeon, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.
munas88@yahoo.com

Abstract: Marjolin's ulcer is a malignant transformation of a long standing scar tissue into squamous cell carcinoma that can often be aggressive if not dealt with early. We are dealing with a 34 year old patient who presented with Marjolin's ulcer with metastasis to the peritoneal cavity.
[Munaser S. Alamoodi. **Unusual Abdominal Metastasis from Marjolin's Ulcer (Case Report and Review of Literature**. *Life Sci. J* 20132;10(1):2060-2062]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 292

Key words: Marjolin's ulcer, metastasis, Peritoneal cavity

Introduction

Marjolin's ulcer is a malignant transformation of a chronic ulcer usually of a burn scar first described by Jean-Nicholas Marjolin in 1828.Over 90% of malignancies are of aggressive squamous cell carcinomas. To my knowledge this is the first report in the literature of Marjolin's ulcer metastasizing to the peritoneal cavity.

Case Report

34 year old male presented to the ER department with abdominal pain, distension and vomiting secondary to small bowel obstruction.

He presented 5 months prior to the above episode with a non healing ulcer over the left popliteal fossa where he had a burn scar at the age of 4 years old. Biopsy of the ulcer confirmed squamous cell carcinoma for which he underwent excision and full thickness skin grafting. The histopathology of the specimen confirmed a well differentiated squamous cell carcinoma with free margins.

CT scan of the abdomen was carried out which showed free abdominal fluid with dilated proximal small bowel and collapsed distal ileal loops with thickened wall (figure1).

The decision to perform a laparatomy was taken. At laparatomy there were multiple deposits involving the peritoneum, small bowel and omentum(Figure 2), with multiple small perforations in the small bowel. The loop where these perforations were, was resected and primary anastomosis was carried out. Biopsies from the omentum, peritoneum, small bowel mesentery and small bowel serosa were taken. The histopathology showed metastatic squamous cell carcinoma involving the omentum and predominantly the serosal surface of the small bowel.

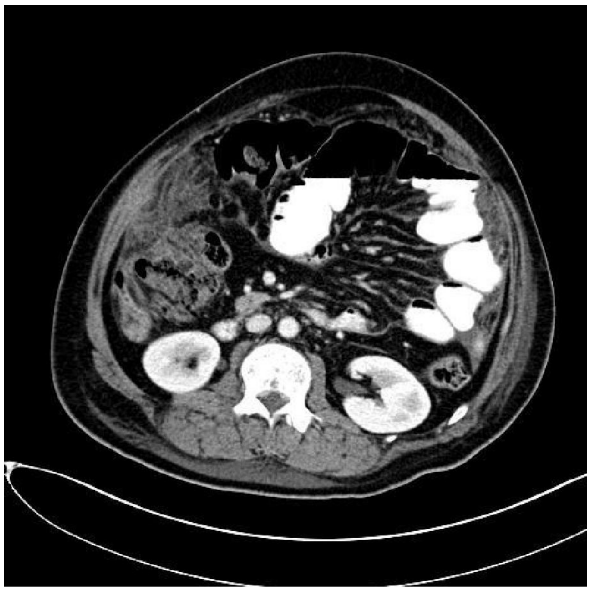


Figure 1: CT Abdomen showing small bowel thickening suggesting tumor invasion.

Figure 2: Picture showing thickened Greater Omentum due to metastasis.

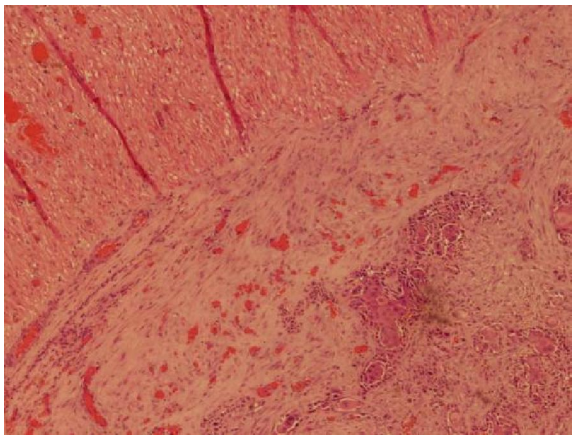


Figure 3:Image showing invasion of serosal layer of the small bowel by tumor cells

Discussion

Marjolin's ulcer is a rare but aggressive disease occurring years post a burn scar, but has been known in very rare cases to have occurred over chronic skin diseases such as hydradenitis and pilonidal sinus amongst other conditions. Commonest mode of metastasis is locally and through lymphatics.

It was first described in 1928 by Jean Nicholas Marjolin. Marjolin's ulcer make up 1.2% of all skin cancers (1,2). It has 1-2% incidence in all burn scars but can also develop from traumatized and scared tissue of other etiologies as stated above.

The mechanism of malignant transformation of Marjolin's ulcer remains unclear and controversial (3,4). The latency period from time of injury to onset of malignant transformation averages 36 years(5). In my patient it was almost 30 years since his initial burn. Having said that it is also known to arise earlier (6). Although the average age of diagnosis is usually the fifth decade, my patient was in his 3rd. It is 3 times commoner in males than in females (6). Most lesions occur on the extremities(60%) , with ulcers on the head and face occurring less frequently(30%) and lowest frequency in the trunk(10%)(7).

The diagnosis in this case was made early and treatment plan initiated but due to the disease's aggressiveness the survival of the patient was not possible. In usual circumstances early diagnosis and treatment is the goal standard for increased survival ratio(8). Most Marjolin's ulcers 75%-96%(9) present as squamous cell carcinoma. Other neoplasms have also been reported and these include basal cell carcinoma, melanoma, osteogenic sarcoma, fibrosarcoma and liposarcoma(10).

The recognition of malignant transformation can be confused with infection, but changes such as appearance of flat non-healing ulcers enlarging in circumference with elevated and indurated borders, foul smelling, painful with exudates and bloody drainage suggest malignant transformation(11,12).

Macroscopically, Marjolin's ulcer has been reported to exist in two forms which are prognostically important,(a) exophytic and (b)infilterative. The former is less likely to metastasize and leads a benign course while the later is more aggressive (13).

Marjolin's ulcers have been reported to have an aggressive course and a much greater tendency to metastasize than other types of skin cancer, which makes early diagnosis a must. Metastasis to the brain, liver, lung, kidney and distant lymph nodes has been commonly reported (14,15,16,17). In my case it was found to metastasize to the peritoneal cavity, which to my knowledge has not been reported before.

The treatment of Marjolin's ulcers requires a multidisciplinary approach. Treatment modalities include wide local excision, block dissection of regional lymph nodes, amputation in advanced lesions of limbs, radiotherapy and chemotherapy given as neo or adjuvant therapy(17). In my patient excision with a surgical margin plus primary skin graft was carried out but no lymph node dissection was necessary since non were felt.

Recurrence and fatality rates are higher due to the aggressive nature of this tumour (18&19). This was evident in my patient who within a very short period after his presentation with Marjolin's ulcer presented with peritoneal cavity metastasis that resulted in small bowel perforation.

Conclusion

Although metastasis from Marjolin's ulcer to the peritoneal cavity is rare, it should be considered in patients with previous history of the disease presenting with abdominal symptoms. Unfortunately the survival is poor due to the aggressive nature of the disease.

Corresponding author
Munaser S. Alamoodi,
Assistant Professor, Faculty of Medicine,King Abdulaziz University,P.O.BOX 80215,Jeddah 21589, Saudi Arabia.
munas88@yahoo.com

Reference

1. Er-Fan X, Li AO, Shi-ling W, Shao-Yu K, Guang-Xiu C. Burn scar carcinoma: Case reports and review of the literature. Annals of the MBC. 1992;5:2
2. Daya M, Balakrishan T. Advanced Marjolin's ulcer of the scalp in a 13-year-old boy treated by excision and free tissue transfer: Case report and

review of literature. Indian J Plast Surg. 2009;42:106-. doi: 10.4103/0970-0358.53020.
3. Tan O, Atik B, Bekerecioglu M, Tercan M, Bayram I. Squamous carcinoma in a pressure sore with a very short latency period. Eur J Plast Surg. 2003;26:360–2. doi: 10.1007/s00238-003-0565-y.
4. Thio D, Clarkson JH, Misra A, Srivastava S. Malignant change after 18 months in a lower limb ulcer: Acute Marjolin's ulcer revisited. British Journal of Plastic Surgery. 2003;56:825–8. doi: 10.1016/j.bjps.2003.08.016.
5. Ochenduszkiewicz U, Matkowski1 R, Szynglarewicz1 B, Kornafel J. Marjolin's ulcer: malignant neoplasm arising in scars. Rep PractOncolRadiother. 2006;11(3):135–138. doi: 10.1016/S1507-1367(06)71058-6.
6. Dávalos BA, Cortés-Flores AO, Bandera-Delgado A. Malignant neoplasm in burn scar: Marjolin's ulcer: Report of two cases and review of the literature. Cir Cir. 2008;76:329–31.
7. Treves N, Pack GT. The development of cancer in burn scars. SurgGynecol Obstet. 1930;51:749–782.
8. Calikapan GT, Akan M, Karaca M, Aköz T. Marjolin ulcer of the scalp: intruder of a burn scar. J Craniofac Surg. 2008;19:1020–1025. doi: 10.1097/SCS.0b013e31814b2a1f.
9. Ozek C, Celik N, Bilkay U, Akalin T, Erdem O, Cagdas A. Marjolin's ulcer of the scalp: Report of 5 cases and review of theliterature. J Burn Care Rehabil. 2001;22:65–9. doi: 10.1097/00004630-200101000-00013.
10. Suhag V, Singh S, Nimbrian VK, Dimri K, Sharma N. Marjolin's Ulcer developing in electrical burns: A rare case report. Pakistan Journal of Medical Science. 2005;21:375–6.
11. Celik E, ErogSlu S, KaracaogSlan N, Uzunismail A. Case report: Early arising Marjolin's ulcer in the scalp. Ann Burns Fire Disasters. 2003;16:217–21.
12. Malheiro E, Pinto A, Choupina M. Marjolin's ulcer of the scalp: case report and literature review. Ann Burns Fire Disasters. 2001;14:39–42.
13. Hahn SB, Kim DJ, Jeon CH. Clinical study of Marjolin's ulcer. Yonsei Med J. 1990;31(3):234–41.
14. Aydogdu E, Yildirim S, Akoz T. Is surgery an effective and adequate treatment in advanced matjolin'sulcer. Burns. 2005;31:421–31. doi: 10.1016/j.burns.2005.02.008.
15. Tutela RR Jr, Granick M, Benevenia J. Marjolin's ulcer arising in pressure ulcer. Adv Skin Wound Care. 2004;17:462–7. doi: 10.1097/00129334-200411000-00010.]
16. Kowal-Vern A, Criswell BK. Burn scar neoplasms: a literature review and statistical analysis. Burns. 2005;31:403–13. doi: 10.1016/j.burns.2005.02.015.
17. Gamatsi E, McCulloch TA, Bailie FB, Srinivasan JR. Malignant melanoma in a skin graft: burn scar neoplasm or a transferred melanoma. Br J Plast Surg. 2000;53:342–52. doi: 10.1054/bjps.2000.3322.
18. Copcu E, Aktas A, Sismant N, Oztan Y. Thirty-one cases of Marjolin's ulcer. Clinical and Experimental Dermatology. 2003;28:138–41. doi: 10.1046/j.1365-2230.2003.01210.x.
19. Gupta SK, Sandhir RK, Jaiswal AK, Kumar S. Marjolin's ulcer of the scalp invading calvarial bone, dura and brain. J ClinNeurosci. 2005; 12:693–696. doi: 10.1016/j.jocn.2004.08.030.

2/5/2013

Effect of Breast-feeding and Formula- feeding on Antibody Response of Hepatitis B Vaccination

Mohsenzadeh A[1], Ahmadipour SH[1] , Firouzi M[1], Babaei Homa[2] , Anbari KH[3]

1-Department of Pediatrics, Shahid Madani Hospital, Lorestan University of Medical Sciences, Khorram Abad, Iran.
2-Department of Pediatrics, Imam Reza Hospital, Kermanshah University of Medical Sciences, Kermanshah, Iran.
3-Community medicine specialist, Lorestan University of Medical Sciences, Khorram Abad, Iran.
Corresponding Author: Ahmadipour Shokoufeh, Email: shokof57@yahoo.com

Abstract: Background and aim: About one-third of the world's population has some serologic evidence of suffering infection by hepatitis B virus (HBV). One of the effective measures for reducing the mortality because of hepatitis B virus is vaccination at birth time, 2 months and 4 months old. The criterion for effectiveness of hepatitis B vaccine is enough production of antibody. The aim of this study is to investigate the immunity response to hepatitis B vaccination in breast-fed and formula-fed infants vaccinated at the age of 12-24 months. **Material and methods:** This descriptive study is conducted using accessible sampling based on entrance criteria on healthy 12-24 months old infants who received 3 dose of hepatitis B vaccine. one group was 51 formula-fed infants and the other 56 breastfed infants. Blood sample was taken from both groups to measure the antibody titer of hepatitis B using ELISA experimental method, and finally both groups were compared based on their antibody level and age, sex and the type of feeding. **Results:** Both groups were the same with regard to age average and sex. Overall 9 infants (8.4%) had antibody titer of lower than 10 mIU/ml and didn't response well to vaccine, among whom 1 was breast-fed and 8 ones were formula-fed infants. HBS-Ab average was 124.4±55.9 mIU/ml and 91.7±51.1mIU/ml in the first and second group respectively (p=0.002).The response in the first group (98.2%) had a meaningful difference with the second group (84.3%) (p=0.009). In each group, there was no significant statistical difference between girls and boys in responding the vaccine, but in both groups overall, the boys had more immune response to hepatitis B vaccine compared to girls (p=0.004). **Conclusion:** The results of this study showed that breastfed infants respond well to hepatitis B vaccine and the average antibody titer is higher in boys.
[Mohsenzadeh A, Ahmadipour SH, Firouzi M, Babaei Homa, Anbari KH**. Effect of Breast-feeding and Formula-feeding on Antibody Response of Hepatitis B Vaccination.** *Life Sci J* 2013;10(1):2063-2068] (ISSN:1097-8135). http://www.lifesciencesite.com. 293

Keywords: Hepatitis B; Antibody; Breastfeeding; Formula

Introduction

One of the common viral infections in the world is hepatitis B virus and about 1/3 of the world's population have the serologic evidence of being infected by hepatitis B virus (HBV) (1). In 1-5% of adults and 90% of infants infected with hepatitis B virus, there is chronic infection (2). Although right now there is no definitive treatment for hepatitis B complications like hepatic cirrhosis and carcinoma, but one of the basic steps to reduce the mortality and complications related to HBV, is timely vaccination, and according the recommendation of the World Health Organization (WHO) hepatitis B vaccination is performed routinely in 3 dose, at the birth time, 2 months and 6 months old (0.5cc intra muscular) but for infants lower than 2 kg birth weight ,it is performed in 4 doses (3,4). About 5-15% of infants do not respond well to this vaccine (5) and the effectiveness criteria for hepatitis B vaccination is producing enough antibodies (HBS-Abs). If the antibody titer in 10 mule/ml or higher, the body has responded to the vaccine appropriately and the higher antibody titer, means the more immune response (6,7).

HBV is not transferred through breastfeeding, so in mothers with HBS-Ag^+ there is no limitation for breastfeeding, but the hepatitis B immunoglobulin (HBIG) should be injected to the infants in addition to Hepatitis B vaccine in the first hours after birth to ensure enough immunity against hepatitis B (8). In addition to the role that breastfeeding plays in nutrition and growth of infants, it is full of different antibodies, cytokines and immune cells and results in the evolution of immune and nervous systems and protection against infections and allergies in infants (9,10,11).

By increasing the period of breastfeeding, 1.5 million mortality is prevented in the countries with high mortality rate (12,13).

Oligosaccharides play an important role in promoting the immune system quality and human milk has higher neutral and acidic oligosaccharide compared to the cow milk (14,15).

During pregnancy, IgG antibodies from mother enter the fetus body through placenta and give immunity to the infant. 6-12 months after birth the antibodies with maternal origin decrease in the infants' body, although by appropriate and enough

breastfeeding the infant can receive enough antibody from mother, (16). In 1903, Schlossman and Moro detected antibodies in mothers' milk, which play an important role in the immune system of the infants (17).

Breastfeeding has important effects on the first six months' vaccination of infants and helps the mother and infant to bear the stressful situations when prescribing injective vaccines and also promotes the effects of vaccines, elevates the immunologic and Entero-hepatic system in infants and can alter the ethyl-mercury metabolism derived from some vaccines (18). There is much evidence, which shows that breastfeeding can empower the immunologic response after vaccination (19).

With regard to the mothers' milk and the importance of hepatitis B infection and the possibility of inadequate response after hepatitis B vaccination in some infants, this study has been conducted to investigate and compare the immune response after full hepatitis B vaccination in breastfed and formula-fed infants.

Materials and methods

This study is a partial descriptive research conducted using accessible sampling based on the entrance criteria on the healthy infants of 12-24 months old referred to Pediatrics clinic of Shahid Madani educational hospital, Khorram Abad, Lorestan,Iran.

In this study, 56 and 51 infants who were breastfed and formula-fed respectively from birth were investigated. All of them were term infants with the gestational age of ≥37 weeks and ≥2500 gr birth weight.

In both of the groups no hospitalization, Immunodeficiency disorder and immunoglobulin or blood products receiving was found and their mothers were HSBS-Ag negative. All of the infants had received hepatitis B vaccine (Hepavax-Gene, made in Korea) according to the national routine for about 0.5 cc intra- muscular at the birth time, 2 months and 6 months.

The information related to the negativity of HSBS-Ag in mothers and full hepatitis B vaccination was received from infants' health care records.

In the case of the parents' written consent to conduct the test, 1cc of blood clot was taken in Sina laboratory in aseptic conditions and HSB-Ab level was calculated through ELISA method (Biomerieux, USA). If the HSB-Ab titer was 10mlU/ml or more, it was taken as the appropriate response to the vaccine.

The test was conducted free and no cost was incurred for parents. The information was analyzed using SPSS software and statistical tests of Chi-squared test and t-test and Pearson's correlation coefficient were analyzed statistically. In this study, the significance level of lower than 0.05 is considered.

Results

In the population under study, 80 ones (74.8%) were under 18 months and 27 subjects (25.2%) were between 18-24 months. The average age in breastfed and formula-fed groups was 15.3±4.1 and 16.2±4.2 months, respectively.

In breastfed group, there was 31 girls (55.4%) and 25 boys (44.6%), while in formula-fed group there was 25 girls (49%) and 26 boys (51%) and according to the statistical t-test and Chi-squared test, there is no statistical difference between two groups with regard to age (p=0.35) and sex (p=0.41).

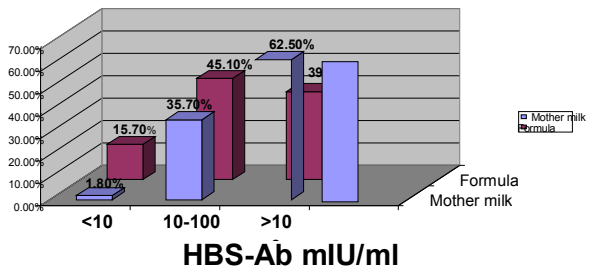
Hepatitis B antibody titer in both groups is divided into 3 levels of 10, 10-100 and above 100 mlU/ml.

The study of frequency distribution in different levels of antibody according to the feeding type and sex in all of the infants under study showed antibody response was lower than10 mlU/ml in 10% of girls and 7% of boys.

In 107 infants studied, 9 ones responded inadequate to hepatitis B vaccine, in a way that in 1 of the breastfed infants (1.8%) and 8 ones in formula-fed group (15.7%) the HBS-Ab titer was lower than 10 mlU/ml.

In 20 breast-fed infants (35.7%) and 23 formula-fed ones (45.1%) the hepatitis B antibody titer was between 10-100 mlU/ml.

In 35 breastfed infants (62.5%) and 20 formula-fed ones (39.2%) the HBS-Ab titer was above 100 mlU/ml. (graph 1)



Graph 1: Relative frequency distribution of Anti HBS-Ab level in the infants under study based on feeding type

Fisher exact tests the difference in antibody level of infants under study is significant based on the feeding type (p=0.009), but it is not significant based on their sex (p=0.35) (table 1).

Table 1- Frequency distribution of different levels of Anti HBS-Ab in the infants under study based on sex and feeding type

Pvalue	total	>100	10-100	<10	HBS-Ab levels mIU/ml	
0.35	50(100%)	22(44%)	23(46%)	5(10%)	Female	Sex
	57(100%)	23(57.9%)	20(35.1%)	4(7%)	Male	
0.009	56(100%)	35(62.5%)	20(35.7%)	1(1.8%)	Breastfed	Feeding type
	51(100%)	20(39.2%)	23(45.1%)	8(15.7%)	formula	

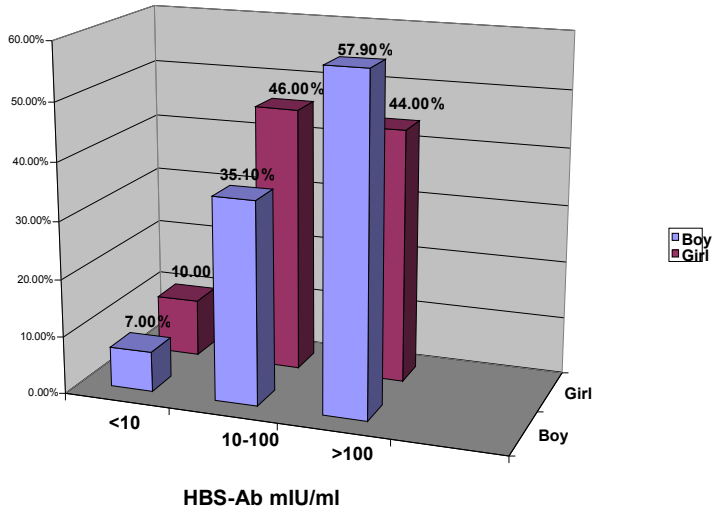
In table 2, the comparison between average and standard deviation of HBS-Ab values is presented for all of the infants under study based on age, sex and feeding type.

In the infants under 18 months the average antibody was higher than 18-24 months infants, but based on the statistical independent t-test this difference was not significant statistically (p=0.11). In addition, by comparing the average antibody value based on nutrition according to independent t-test the average antibody value in breastfed infants is higher than formula-fed ones (p=0.002).

Table 2- Comparison between the average and standard deviation values of anti HBS-Ab in infants based on age, sex and feeding type

P value	The type of statistical test		variable	
0.11	T test	113.1±59.8	Under 18	Age (month)
		96.4±40.8	18-24	
0.044	T test	97.3±53.1	female	Sex
		119±56.9	Male	
0.002	T test	124.4±55.9	breastfed	Feeding Type
		91.7±51.1	formula	

The difference between antibody values average is significant based on sex, in such as a way that in girls the antibody average is lower than boys are (p=0.044) (graph 2).



Graph 2: The relative frequency distribution of anti HBS-Ab levels in infants under study based on sex

In table 3 and 4 the average antibody values in breastfed and formula-fed infants is studied based on age and sex; although in breastfed and formula-fed group the antibody value is higher in boys than girls, but based on independent t-test this difference is not significant (p=0.15) and (p=0.18).

The average antibody is significantly higher in breastfed infants younger than 18 months infants are (p=0.45), but in formula-fed infants the difference was not statistically significant (p=0.86).

Table 3- Comparison between average and standard deviation of anti HBS-Ab values in breastfed infants based on age and sex

P value	The type of statistical test		variable	
0.045	T test	128.9±59.9	Under 18	(month) Age
		104.1±25.1	18-24	
0.15	T test	112.6±51.6	female	sex
		134±58.2	male	

Table 4- Comparison between average and standard deviation of anti HBS-Ab values in formula-fed infants based on age and sex

Pvalue	The type of statistical test		variable	
0.86	T test	94.6±53.4	Under 18	Age (month)
		89.5±47.7	18-24	
0.18	T test	82.1±51.7	female	sex
		101±50.7	male	

In all of the infants in both groups, there is a reversed linear relationship between age and antibody titer based on Pearson correlation coefficient and the antibody value decreases by age (P=0.024, r= -0.31).

Discussion

The results of this study showed that the response to hepatitis B vaccine is higher in infants who are breastfed from birth compared to formula-fed infants.

In the study conducted by Hahn and colleagues the antibody value in infants was measured after OPV vaccine injection and is spite of the fact that the antibody value was higher in low-protein formula-fed infants than the group who received conventional formula, but it was lower compared to the solely breastfed infants (20). In addition, the immunity value was higher in breastfed infants after diphtheria, tetanus and pertussis vaccination (DTap).

Azzari and colleagues in Italy measured the subclasses of HBSAb IgG in healthy infants born from HBS-Ag positive mothers and received full hepatitis B vaccine, they found that the HBS-Ab value in lgG2 subclass was 3 times higher in breastfed group compared to formula-fed infants but HBS-Ab IgG1 in formula-fed group was higher. There was no difference in lgG3 subclass between two groups (21). In this study, the total HBS-Ab titer in healthy 12-24 months infants with HBS-Ag negative mothers was measured and antibody titer was significantly higher in the group who were solely breastfed for 6 months compared to formula-fed group.

In a study, Pichichoro found that breast-feeding is very effective in increasing the response of oral rhesus rotavirus vaccine sero-conversion (22).

In a study by John et al, no difference was found in the response to the first and third doses of oral live poliovirus vaccine type 1,2 and 3 (OPV) between breastfed and formula-fed infants and breastfeeding has no frenum effect on the amount of antibody production in infant after receiving OPV vaccine (23). However, Chandra reported in 1978 that poliovirus antibodies in mother's milk might interfere with live attenuated poliovirus vaccine (24).

The results of the study by Silfverdal et al. shows that infant solely breastfed for 90 days or more had better serologic response to conjugate Hib and pneumococcal serotype 6B and 14 vaccines compared to the infants breastfed less than 90 days (25). In this study, HBS-Ab production in infants solely breastfed for at least 6 months is higher than bottle-fed ones.

In a research by West et al., the immunologic response to Hib vaccine in breastfed infants for less than 6 months is significantly higher than the infants who were breastfed for 6 months or more (26).

In a study by Wold et al. antibody production against live viral vaccines (MMR) is lower in breastfed compared to bottle-fed infants and it is probably because of the present of lgA in mother's milk which has antiviral feature and the virus in the vaccine cannot replicate in the digestive system and stimulate the infant immune system (27).

Furthermore in a study in Kermanshah carried out by Hemmati et al, on premature infants born from HBS-Ag$^-$ mothers, 1 month after injecting the last turn of hepatitis B vaccine(in month 7) 86.8% of premature infants responded 100% to hepatitis B vaccine (28).

In a study by Zahedpasha et al. in Babol, on the amount of response to hepatitis B vaccine in premature and mature infants of 12-24 months, it was evaluated that both groups responded 100% to the vaccine and in premature infants there was no

statistical difference according to antibody titer in boys and girls, but in term infants boys had higher titer than girls. In addition, in all of the infants the average antibody titer was significantly higher in boys than girls (29).

In this study in term infants, 91.6% responded well to the hepatitis B vaccine, but in 8.4% there was inadequate response (titer less than 10 mlU/ml) which was found more in the group who did not receive their mother's milk, in addition the average antibody value was higher in all of the boys compared to girls, but no significant difference was found between infants in breastfed and formula-fed groups according to their sex.

In the study conducted by Yang et al. 37% and 94% of premature and mature infants responded well to hepatitis B vaccine at the age of 4-7 years old (30).

In a study on term infants by Pickering at el, the antibody titer of tetanus and OPV was higher in infants who were fed by human milk compared to those who were fed by formula containing nucleotide, but antibody titer of Hib and Diphtheria was higher significantly in nucleotide-formula-fed (31).

In this study, although antibody decreases by age and antibody titer in less than 18 months infants is higher than 12-24 months infants, but this difference is not significant statistically. In comparing sex with average hepatitis B antibody, in all of the infants studied in this research, boys had more antibody production compared to girls and there is a meaningful relationship between the amount of hepatitis B antibody (HBS-Ab) and the feeding type. In the solely breastfed group, the higher antibody titer was seen compared to the formula-fed infants who did not receive their mother's milk. The reason for higher average antibody in boys compared to girls is not clear and it needs to be investigated in future researches.

Acknowledgments

We thank the research assistant of Lorestan Medical School for confirming and funding this project. In addition, we thank and welcome the Sina Laboratory and Shahid Madani hospital personnel.

References

1. Goldstein ST, Fiore AE.Toward the global elimination of hepatitis B virus transmission. J Pediatr 2001; 139 (3):343-5.
2. Mackie CO, Buxton JA, Tadwalker S, Patrick DM. Hepatitis B immunization strategies: timing is everything. CMAJ 2009; 180 (2): 196-202.
3. Committee on infectious disease. American Academy of pediatrics Universal Hepatitis B Immunization. Pediatrics 1992; 89:795-800.
4. Kligman RM, Behrman RE, Jenson HB. Nelson textbook of pediatrics, 19th Edition. 2011;1399-1400.
5. Chang MH. Poor response to HBV vaccination and strategies to enhance immunogenicity. Journal of Gastroentrology and Hepatology 2004; 19: 131-132.
6. Hollinger FB, Liang TJ. Hepatitis B Virus. In: Knipe DM et al., eds. *Fields Virology,* 4th ed. Philadelphia, Lippincott Williams & Wilkins 2001:2971-3036.
7. Greenberg DP, Vadheim CM, Wong VK, et al. Comparative safety and immunogenisity of two recombination hepatitis B vaccines given to infant at 2, 4, 6 months of age. Pediatr Infect Dis J 1996; 15 (7): 590-596.
8. Shepard CW, Simard EP, Finelli L, Fiore AE, Bell BP. Hepatitis B virus infection: epidemiology and vaccination. Epidemiol Rev 2006; 28(1): 112-125.
9. Anatolilou F. Human milk benefits and breastfeeding. J Pediatric and Neonatal Individualized Medicine 2012; 1(1): 11-18.
10. Stahl B. The Relevance of Human Milk Research today. In: 44th Annual Meeting of ESPGHAN, Italy 2011.
11. Paramasivam K, Michie C, Opera E. Human breast milk immunology(a review).Int J Fertil Womens Med 2006;51(5): 208-217.
12. Jones G, Steketee RW, Black RE, Bhutta ZA, Morris SS; Bellagio Child Survival Study Group. How many child deaths can we prevent this year? Lancet 2003; 362(9377):65-71.
13. Lauer JA, Betran AP, Barros AJ, de Onis M. Deaths and years of life lost due to suboptimal breast-feeding among children in the developing world: a global ecological risk assessment. Public Health Nutr 2006; 9(6):673-685.
14. Stahl B, Thurl S, Zeng J, Karas M, Hillenkamp F, Steup M Sawatzki G. Oligosaccharides from human milk as revealed by matrix-assisted laser desorption/ionization mass spectrometry .Anal Biochem 1994; 223(2):218-226.
15. Boehm G, Stahl B. Oligosaccharides In: Mattila-Sandholm T .Functional dairy products. Cambridge: Woodhead Publishers 2003; 203-243.
16. Kelly M, Andrea M. Breastfeeding, The Immune Response, and Long Term Health.The Journal of American Osteopathic Association 2006; 106(4): 203-207.
17. Laura M, Arjen P, Gunther B, John G .Breastfeeding and its Role in Early Development of the Immune system in infants.

American Society for Nutrition 2008; 138(9): 1782s-1790s.
18. Dorea JG. Breastfeeding is an essential complement to vaccination. Acta Pediatrica 2009; 98(8): 1244-1250.
19. Duijts L, Jaddoe VWV, Hofman A, & Moll HA. Prolonged and exclusive breastfeeding reduces the risk of infectious diseases in infancy. *Pediatrics* 2010; 126: e18-e25.
20. Hahn-Zoric M, Fulconis F, Minoli I, Moro G, Carlsson B, Bottiger M, Raiha N, & Hanson LA. Antibody responses to parenteral and oral vaccines are impaired by conventional and low protein formulas as compared to breastfeeding. *Acta Paediatrica Scandinavia* 1990; 79:1137-1142.
21. Azzari C, Resti M, Rossi ME, Lami CA, Vierucci A.Modulation by human milk of IgG subclass response to hepatitis B vaccine in infants. Journal of Pediatric Gastroenterol Nutr 1990; 10(3):310-315.
22. Pichichoro ME. Effect of Breast-Feeding on Oral Rhesus Rotavirus Vaccine Sero-conversion: A Metaanalysis.Journal of Infect Dis 1990; 162(3): 753-755.
23. John TJ, Devarajan LV, Luther L. Effect of breast-feeding on Seroresponse of infants to oral poliovirus vaccination. *Pediatrics*1976; 57: 47-53.
24. Chandra RK. Immunological Aspects of Human milk .Nutr Rev.1978; 36(9): 265-272.
25. Silfverdal SA, Ekholm L, & Bodin L. Breastfeeding enhances the antibody response to Hib and Pneumococcal serotype 6B and 14 after vaccination with conjugate vaccines. *Vaccine* 2007; 25(8): 1497-1502.
26. West CE, Gothefors L, Granstrom M, Kayhty H, Hammarstrom ML. Effects of feeding probiotics during weaning on infections and antibody responses to diphtheria, tetanus and Hib vaccines. *J Pediatric Allergy and Immunology* 2008; 19(1): 53-60.
27. Wold AE, Adlerberth I. Does breastfeeding affect an infant's immune responsiveness? *Acta Paediatrica* 1998; 87(1): 19-22.
28. Hemmati M, Ahmadipour SH, Babaei H. The Effect of Oral Zinc sulfate On Hepatitis B vaccine Immunogenicity in Premature Infants. Life Science Journal 2012; 9(4):3366-3368.
29. Zahedpasha Y, Ahmadpour K, Pournasrollah M, Bijani A. Immune Response in Preterm infants to Hepatitis B Vaccine. Journal of Babol Univ Med 2011; 13(4):34-39.
30. Yang YJ, Lin CH, Tai YT, Hung SC. The clinical efficacy and immunologic responses of hepatitis B vaccination in very low birth weight infants. Pediatr Infect Dis J 2008; 27(8):768.
31. Pickering LK, Granoff DM, Erickson JR, Masor ML. Modulation of the immune system by human milk and infant formula containing nucleotides. Pediatrics 1998; 101:242–249.

2/2/2013

Attenuation of specific CTL responses by highly efficient transduction of the recombinant adenovirus expressing His-tag-ICP47 fusion gene

Wang Peng [1], Zhang Zhenxiang [1*], Kan Quancheng [2], Yu Zujiang [2], Li Ling [3], Pan Xue [1], He Hongjun [1], Feng Ting [1], Li Xiangnan [1], Jiang Li-li [1], Zhai Guangyu [1] and Cui Guanglin [4]

1. Nursing College, Zhengzhou University, Zhengzhou, Henan 450052, China
2. Clinical Pharmacology Base, First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan 450052, China
3. Department of Palliative Care and Hospice Care, the Ninth People's Hospital of Zhengzhou, Zhengzhou, Henan 450053, China 4. Department of Gastroenterology, Second Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan 450052, China. zhangzx6666@126.com

Abstract: Hepatocyte transplantation (HT) has been proposed as an alternative therapy to orthotopic liver transplantation (OLT) for patients with acute liver failure and metabolic disorders and hepatocytes are attractive targets for gene therapy. Adenovirus vector is considered a safe and efficient way to introduce foreign genes into several kinds of cells and is widely used in the various fields of gene therapy. But the response of host immune systems against gene products expressed by genetically modified cells is a major obstacle to successful gene therapy. Major histocompatibility complex (MHC) class I antigen presenting pathway is very important in acute allograft rejection and blocking MHC I antigen expression is becoming a research hotspot of inducting immune tolerance. Infected cell protein 47 (ICP47) expressed by herpes simplex virus type 1 (HSV-1), inhibits MHC I antigen presentation pathway by binding to host transporter associated with antigen presentation (TAP), and thereby attenuates of specific cytotoxic T lymphocytes（CTL）responses by virus-infected cells and evades the host immune clearance. This subject was designed to construct a recombinant adenovirus expressing His-tag-ICP47 fusion protein to investigate further the role of ICP47 in the elimination of transgene expression.
[Wang Peng, Zhang Zhenxiang, Kan Quancheng, Yu Zujiang, Li Ling, Pan Xue, He Hongjun, Feng Ting, Li Xiangnan, Jiang Li-li, Zhai Guangyu and Cui Guanglin. **Attenuation of specific CTL responses by highly efficient transduction of the recombinant adenovirus expressing His-tag-ICP47 fusion gene.** *Life Sci J* 2013;10(1):2069-2074] (ISSN:1097-8135). http://www.lifesciencesite.com. 294

Keywords: recombinant adenovirus; His-tag-ICP47 fusion gene; cytotoxic T lymphocytes

1. Introduction

Organ transplantation is one of the most important treatments of end-stage organ failure, yet graft rejection and shortage of organ donors are the two major problems persisting in the treatments. Hepatocyte transplantation (HT) may serve as an alternative to orthotopic liver transplantation (OLT) for patients with end-stage liver disease because the most important advantage of its decreasing mortality in the waiting list and allowing more patients to be treated[1-2], but immune rejection of HT is still an important problem to be solved[3]. With recent advances in transgenic technology, the availability of transgenic HT evading the clearance of host immune systems could be a critical subject of HT.

Adenovirus vector is considered a safe and an efficient way to introduce foreign genes into several kinds of cells and is widely used in the various fields of gene therapy[4]. However, the response of host immune systems against foreign gene products expressed by genetically modified cells and/or vector-encoded proteins is a major obstacle to successful gene therapy [5]. Therefore, prevention of an immune response against the product of introduced genes and transplanted cells could be as critical as circumventing allograft rejection for the long-term success of gene therapy[6].

On the cellular level, major histocompatibility complex (MHC) class I antigen presenting pathway is very important in acute allograft rejection and has been an attractive target for immune rejection, and blocking MHC I antigen expression is becoming a research hotspot of inducting immune tolerance[7].

Many viruses have evolved mechanisms to evade clearance of host immune systems by blocking MHC I antigen presentation pathway. ICP47, an 88–amino acid cytosolic polypeptide, which is an immediate-early protein expressed by herpes simplex virus type 1(HSV-1), inhibits MHC I antigen presentation pathway by binding to host transporter associated with antigen presentation (TAP), and thereby attenuates of specific CTL responses by virus-infected cells and evades the host immune clearance[8].

Based on these studies, this subject was designed to construct a recombinant adenovirus vector expressing His-tag-ICP47 fusion protein to investigate further the role of ICP47 in the elimination of transgene expression. We expect this finding should have important implications for analyzing the

mechanisms of immune tolerance as well as human gene therapy.

2. Material and Methods

Construction and purification of recombinant adenovirus

The adenovirus vector AdEasy-1 system was used to prepare the recombinant adenovirus expressing His-tag-ICP47 fusion gene or the control empty recombinant adenovirus r-Track as previously described[9]. Firstly, His-tag-ICP47 fusion gene was cloned into the pAdTrack-CMV vector, then the gene fragments digested by restriction endonuclease Pme I were co-transformed in E.coli BJ5183 cells with adenoviral backbone vector pAdEasy-1 to produce recombinant adenovirus vector pAdEasy-H-ICP47. The His-tag-ICP47 fusion gene nucleotide sequence of final constructs was determined to confirm that no mutation was introduced. Linearized with Pac I, the recombinant adenovirus vector was subsequently transfected into human embryonic kidney 293 cells to product r-H-ICP47. Meanwhile, the control empty recombinant adenovirus r-Track was generated in the same way. Finally, the viruses were amplified, purified by ultracentrifugation on a cesium chloride (CsCl) step gradient, dialyzed, and stored in -80 ℃

Efficiency of transfection of HL-7702 Cells In Vitro.

Adenovirus efficiency of transfection was quantified by monitoring the expression of GFP in r-H-ICP47 or r-Track infected cells. Briefly, HL-7702 cells were plated on 12-well plates at a density of 1×10^5 cells/ml, allowed to adhere overnight and removed nonadherent cells by gentle washing with phosphate-buffered saline (PBS). Subsequently, wells were then randomly assigned to one of four experimental groups: multiplicity of infection (MOI) of 0, 50, 100, 200 in triplicate, respectively. HL-7702 cells exposed to viruses at various MOI of r-H-ICP47 or r-Track in 1mL RPMI1640 and incubated for 48 h at 37℃ under air plus 5% CO_2 conditions.

The total cell number was calculated using a standard haemacytometer following cell detachment with 0.1% trypsin plus 1 mM ethylenediamine tetraacetic acid (EDTA) in PBS and the GFP-positive cells were counted with a fluorescent light microscope. Efficiency of transfection (ET) was calculated according to the following formula: ET= number of GFP-positive cells/ number of total cell ×100%.

Western blot analysis

The expression of the proteins produced by recombinant adenovirus was analyzed by Western blot analysis. Briefly, the total proteins were extracted from r-H-ICP47-infected, r-Track-infected, and mock-infected HL-7702 cells, respectively. The proteins were separated by SDS-PAGE, and at the end of the run, polypeptide bands in the gel were electrophoretically transferred to a PVDF membrane (Bio-Rad). The membrane was incubated for 1 h at room temperature with rabbit anti-6×His antibody, rabbit anti-GFP antibody or rabbit anti-β-tubulin antibody (Bioss Inc.), respectively. On the membrane, the binding of antibody to the specific protein band was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bioss Inc.) and an ECL Western blotting detection system.

Growth curves of HL-7702 cells with or without adenovirus infection

HL-7702 cells were plated on 96-well plates at a starting number of 1000 cells/well, allowed to adhere overnight and removed nonadherent cells by gentle washing with phosphate-buffered saline (PBS). Subsequently, HL-7702 cells were infected with 100 µl of r-H-ICP47 or r-Track at a MOI of 100 in triplicate and incubated at 37℃ under air plus 5% CO_2 conditions. The cell proliferation rate was determined every other day for 7 days after infection by an 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously. Briefly, 20 µl MTT solution (5 mg/ml) were added to the culture medium. After 4 h at 37℃ the medium was removed and 150 µl DMSO was added to each well. The colour was allowed to develop for 5 min and optical density at 570 nm was determined.

CTL assay

Briefly, HL-7702 cells were plated on 12-well plates at a density of 1×10^6 cells/ml, allowed to adhere overnight and removed nonadherent cells. On the following day, HL-7702 cells were infected with r-H-ICP47 or r-Track at a MOI of 100 in triplicate and incubated for 48 h at 37℃ under air plus 5% CO2 conditions. Subsequently, r-H-ICP47-infected, r-Track-infected, and mock-infected HL-7702 cells were treated with mitomycin C (50 µg/ml) at 37°C for 45 min to inhibit proliferation, respectively and then washed three times to remove residual mitomycin C.

Lymphocytes were generated from PBMCs. Briefly, human PBMCs were isolated from healthy donors obtained from first affiliated hospital of Zhengzhou University, China, upon ethical approval for the use of such materials. Subsequently, PBMCs were allowed to differentially adhere to 6-well plates by culturing 5×10^6cells /mL in 1 ml of complete RPMI-1640medium/well for 4h at 37°C. Nonadherent cells were then removed by gentle rinsing and lymphocytes were harvested.

The lymphocytes were stimulated in vitro by Mitomycin C-treated cells at an effector: stimulator ratio of 10:1 in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 20U/mL IL-2. Seven days later, The effector cells

were harvested, counted, and mixed with target cells at an effector: target ratio of 20:1 and incubated overnight at 37℃.

Finally, specific cytotoxic activity was determined by an MTT assay and optical density at 490 nm was determined. Specific cytotoxic activity was calculated as follows: specific lysis (%)=(OD_{target}-OD_{mock}-$OD_{control}$)/ OD_{target}×100%.

Statistical analysis

Quantitative results were expressed as mean ± standard error of the mean ($\bar{x} \pm s$). Means between two groups were compared using a two-tailed, unpaired Student's *t* test, Statistical analysis was performed by SPSS 10.0 software. *P* value of 0.05 or less was considered statistically significant.

3. Results

Sequencing identification of recombinant adenovirus and fluorescence photomicrograph observation

The results of sequencing identification of recombinant adenovirus and fluorescence photomicrograph observation was reported in our previous study[9]. The results verified that His-tag-ICP47 fusion gene fragment had been correctly cloned into the recombinant adenovirus vector. And the recombinant adenoviruses of r-H-ICP47 and r-Track were successfully constructed and successfully transducted into 293 cells.

Western blot analysis

Proteins producted by mock-infected, r-Track-infected or r-H-ICP47-infected HL-7702 cells were confirmed by Western blot analysis (Fig.3). In all cells extracts, the blots probed with anti-β-tubulin antibody were detected at approximately 55 kDa molecular mass. Bands of extracts of r-Track-infected and r-H-ICP47-infected HL-7702 cells were recognized at approximately 27 kDa molecular mass when the blots were probed with anti-GFP antibody, but no bands were recognized in the extracts of mock-infected HL-7702 cells. When blots were probed with anti-6×His antibody, the identical band of His-tag-ICP47 fusion protein (11kDa) was recognized in extracts of r-H-ICP47-infected HL-7702 cells, but no bands were recognized in the extracts of mock-infected and r-Track-infected HL-7702 cells.

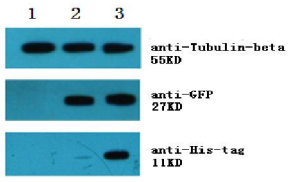
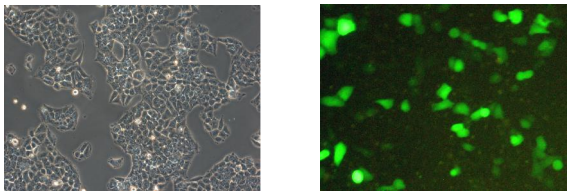


Fig.1 Western-blot assay

Lane1: mock-infected HL-7702 cells; Lane 2: r-Track-infected HL-7702 cells; Lane 3: r-H-ICP47-infected HL-7702 cells

Efficiency of transfection of HL-7702 Cells In Vitro

HL-7702 cells were exposed to viruses at various MOI of r-H-ICP47 or r-Track in 1mL RPMI1640 and incubated for 48h and the green fluorescence could be seen under a fluorescence microscope (Fig. 4). The results of fluorescence photomicrograph observation verified that the recombinant adenoviruses of r-H-ICP47 and r-Track were successfully transducted into HL-7702 cells. Adenovirus efficiency of transfection was quantified by monitoring the expression of GFP in r-H-ICP47 or r-Track infected cells. As MOI increased, efficiencies of transfection were increasing (Fig.5). There was no difference in efficiencies of transfection with r-H-ICP47 between MOI 100 group and MOI 200 group, which were 86.87±3.14% and 88.53±3.69%, respectively (*P*>0.05). Whereas they were significantly higher than the transfection efficiency of MOI 50 group (29.52±5.22%) (*P*<0.05). Similarly, 32.12±2.27% r-Track infected HL-7702 cells expressing *GFP* with a MOI of 50, 90.32±2.25% at a MOI of 100 and 90.64±3.65% at a MOI of 200. On the basis of these studies, all subsequent studies were carried out with a MOI of 100.



A B

Fig. 2 Morphological identification of HL-7702 cells

A. Normal HL-7702 cells before transfected with r-H-ICP47/r-Track（200×）

B. HL-7702 cells transfected with r-H-ICP47/r-Track at the 48th h（200×）

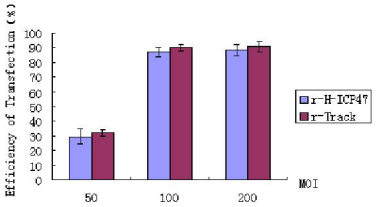


Fig. 3 Efficiency of transfection with r-H-ICP47/r-Track in HL-7702 cells（%, $\bar{x} \pm s$, n=3）

Growth curves of HL-7702 cells with or without adenovirus infection

The cell proliferation rate was determined every other day for 7 days after infection by an MTT assay and the results showed that the kinetics of cell proliferation in HL-7702 cells after exposure to r-H-ICP47 or r-H-ICP47 were similar to that in mock-infected HL-7702 cells as Fig.6 showed（*P*>0.05）.

Moreover, the accelerated phase of the growth curve was initiated from the 3rd day after transduction in three groups of HL-7702 cells. The results verified that the growth of HL-7702 cells was not inhibited by either of the two recombinant adenoviruses.

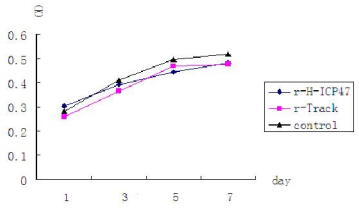


Fig. 4 Growth curves of HL-7702 cells with or without recombinant adenovirus infection.

CTL assay

After HL-7702 cells were transfected with either r-H-ICP47 or r-Track at a MOI of 100, the cytotoxic activity of CTL was determined by using MTT assay. The results showed that the percent cytolysis of HL-7702 cells transfected with r-H-ICP47 by CTL （20.67±3.54%）was reduced comparing with those of the r-track group （36.91±5.36%）and the control group （34.84±4.59%）as Tab.1 showed (P<0.05) .

Tab. 1 Cytotoxicity of CTL activated by HL-7702 cells transfected with r-H-ICP47/r-Track (%, $\bar{x} \pm s$, n=3）

Group	Cytotoxicity of CTL
r-H-ICP47	20.67±3.54*▲
r-Track	36.91±5.36
Control	34.84±4.59

Compared with the control group,* P <0.05; compared with the r-Track group, ▲P <0.05

4. Discussions

Liver pathologies affect hundreds of millions of patients worldwide and can lead to progressive liver injury, liver fibrosis, cirrhosis, and ultimately liver failure, and in some instances, cancer[10]. One of the most common causes of hepatopathy is chronic hepatitis virus infection and one of the most curative therapy is orthotopic liver transplantation (OLT)[11]. But the shortage of organ donors and the high costs are worldwide problems of OLT, and nearly 15% of adult patients with life threatening liver diseases are going to die while on the waiting list[1-2]. Thus, the development of human hepatocyte transplantation (HT) for the treatment of end-stage hepatic diseases is currently under investigation all over the world. Compared with OLT, HT is less expensive, less invasive and relieving shortages of donor organs and it has been proposed as an alternative or a “bridge” therapy for patients with acute liver failure and metabolic disorders[12]. However the procedure of HT is still limited by the immune rejection and the lifelong immunosuppressive treatments, which are not always effective and associated with the risk of infectious complications, and other regimen-related toxicities[13-14].

Adenovirus vectors offer many advantages, including efficient gene transfer, high titer, limited pathological potential, broad range of infectivity, and feasibility for delivery in vivo compared to plasmid vector [4,15]. They have become versatile tools for gene delivery and expression and been used extensively in the treatments of genetic disease[16] and cancer[17]. However, the response of host immune systems against foreign gene products expressed by genetically modified cells and/or vector-encoded proteins is a major obstacle to successful gene therapy. Therefore, prevention of an immune response against the product of introduced genes and transplanted cells could be as critical as circumventing allograft rejection for the long-term success of gene therapy,.

On the cellular level, MHC class I–restricted antigen processing pathway is critical for elimination of most tumor surveillance, transplantation rejection, graft-versus-host reactions, virus infections, and has been an attractive target for immune rejection and blocking MHC Ⅰ antigen expression is becoming a research hotspot of inducting immune tolerance[7]. Efficient antigen presentation restricted by MHC class I is associated with TAP[18], which is a member of the ATP binding cassette protein family. TAP plays a critical role in transporting cytosolic peptides across the membrane of endoplasmic reticulum (ER) for combined with MHC class I heavy chain (HC) and β_2-microglobulin (β_2m). In the absence of antigenic peptides and functional TAP, most MHC class I molecules should be eventually redirected to the cytosol and degraded by proteasomes[19-22].

Many viruses have evolved mechanisms to evade clearance of host immune systems by blocking MHC Ⅰ antigen presentation pathway. ICP47, an 88–amino acid cytosolic polypeptide, which is an immediate-early protein expressesd by HSV-1 and binds to the TAP1–TAP2 heterodimer in human cells and inhibits transport of antigenic peptides from mostly cytosolic proteins into ER, where they would be loaded onto freshly synthesized MHC class I molecules. Consequently, the empty MHC class I molecules are retained in the ER, and the presentation of epitopes to $CD8^+$ T cells is abolished in HSV-infected human cells [8-9]. $CD8^+$CTLs are important for viral clearance in many virus systems. And it recognizes MHC class I molecules bound to small peptides 8 to 10 residues in length derived from viral proteins on the surfaces of virus-infected cells. Because priming of CTL responses requires MHC class I-restricted presentation of the relevant antigen,

the HSV-infected human cells are not lysed by CTL and effectively evade the immune response in humans just as during HSV infection in vivo[23-24].

Recently, the immune response to adenovirus has become increasingly important because adenovirus vectors have become versatile tools for gene gene therapy. It is reported that the CTL response to adenovirus vectors contributes to the elimination of transgene expression in vivo[5].

Thereby, the vector-encoded proteins as well as therapeutic proteins in models of gene replacement therapy are a potential problem. Meanwhile, hepatocytes are attractive targets for gene therapy because a variety of genetic disorders play an important role in hepatic metabolic processes. With recent advances in transgenic technology, the availability of transgenic HT evading the clearance of host immune systems could be a critical subject of HT for the long-term success of gene therapy as well as circumventing allograft rejection.

Based on these studies, we constructed an adenovirus vector expressing the His-tag-ICP47 fusion protein to investigate further the role of ICP47 in the elimination of transgene expression. In our study, the shuttle plasmid pAdTrack-CMV of AdEasy-1 system contains a GFP gene allowing direct observation of the efficiency of transduction, which is very convenient for operation[25], and 6×His tag also facilitates detection using biotinylated anti-6×His antibody and enables purification and detection of recombinant adenovirus without affecting tropism or production[26].

Consequently, a recombinant adenovirus expressing the His-tag-ICP47 fusion protein was successfully constructed and the proteins producted by r-H-ICP47-infected HL-7702 cells were confirm by Western blot analysis[9]. Moreover, efficiencies of transduction in HL-7702 with the recombinant adenoviruses at various multiplicity of infection (MOI) were analyzed and the cytotoxic activity of CTL was determined by using a MTT assay. The results verified that recombinant adenovirus expressing His-tag-ICP47 fusion gene could efficiently and safely transfer genes into the HL-7702 cells and the expression of introduced genes was at a desired level without effecting the growth of HL-7702 cells. Most important of all, recombinant adenovirus r-H-ICP47 had the abilities of attenuating of specific CTL responses by HL-7702 cells transfected with r-H-ICP47 comparing with those of the r-track group and the control group.

To some extents, these results could predict an absence of deleterious host immune responses against the transplanted cells or gene products and indicate that recombinant adenovirus expressing His-tag-ICP47 fusion gene and transplanted cells could confer immune tolerance and lead to long-term cell survival in recipients. The experiments lay a good foundation to carry out *in vitro* and animal studies of experimental gene therapy trial for immunological activities of the His-tag-ICP47 fusion protein and we expect those findings should have important implications for analyzing the mechanisms of immune tolerance as well as human gene therapy. Moreover, we expect these studies should open up new horizons for expanding the fields of viral immunology, exploring the interactions between host immune systems and viruses, and enable us to explore more effective preventions and treatments for clinical diseases.

Acknowledgements:

Foundation item: National Natural Science Foundation of China (No.30472031) , Foundation of He'nan Educational Committee (12A310010), Foundation for University Key Teacher of He'nan Educational Committee(2011GGJS-013) , Science and Technology Planning Project of Henan 122300410338, and Science and Technology Planning Project of Zheng zhou(10PTGS484-7 and 121PPTGG507-22). Authors are grateful for the financial supports to carry out this work.

Corresponding Author:
Professor Zhang Zhenxiang
Nursing School
Zhengzhou University, Zhengzhou University
Zhengzhou, Henan 450052, China
Email: zhangzx6666@126.com

References

1. Pietrosi G, Vizzini GB, Gruttadauria S, et al. Clinical applications of hepatocyte transplantation. World J Gastroenterol, 2009, 15(17): 2074-2077.
2. Song X, Guo Y, Duo S, et al. A Mouse Model of Inducible Liver Injury Caused by Tet-On Regulated Urokinase for Studies of Hepatocyte Transplantation. Am. J. Pathol., Nov 2009; 175(5): 1975-83.
3. Brückner S, Dollinger MM, Stock P, et al. Allogeneic hepatocyte transplantation in the rat.FASEB J, 2009; 23: 1004.6.
4. Silva AC, Peixoto C, Lucas T, et al. Adenovirus vector production and purification. Curr Gene Ther, 2010, 10(6): 437-455.
5. Holst PJ, Ørskov C, Thomsen AR, et al. Quality of the Transgene-Specific CD8+ T Cell Response Induced by Adenoviral Vector Immunization Is Critically Influenced by Virus Dose and Route of Vaccination . J Immunol., 2010, 184 (8): 4431- 4439.

6. Aisenbrey C, Sizun C, Koch J, Meike Herget, Rupert Abel, Burkhard Bechinger,and Robert Tampe. Structure and Dynamics of Membrane-associated ICP47,a Viral Inhibitor of the MHC I Antigen-processing Machinery . J. Biol. Chem., 2006; 281(41): 30365 - 30372.
7. Felix NJ, Brickey WJ. Griffiths R, et al. H2-DMα-/- Mice Show the Importance of Major Histocompatibility Complex–bound Peptide in Cardiac Allograft Rejection . J. Exp., 2000.192(1):192: 31-40
8. Lampen MH, Verweij MC, Querido B, et al. CD8+ T Cell Responses against TAP-Inhibited Cells Are Readily Detected in the Human Population . J. Immunol., 2010, 185(11): 6508 - 6517.
9. Wang P, Kan QC, Yu ZJ, et al. Construction and identification of a recombinant adenovirus vector expressing His-tag-ICP47 fusion gene. Life Science Journal. 2012, 9(1): 756-763.
10. Piscaglia AC, Campanale M, Gasbarrini A, et al. Stem cell-based therapies for liver diseases: state of the art and new perspectives. Stem Cells Int, 2010, 2010: 259461.
11. Kawahara T, Toso C, Douglas DN, et al. Factors affecting hepatocyte isolation, engraftment, and replication in an in vivo model. Liver Transpl, 2010, 16(8): 974-82.
12. Skvorak KJ, Paul HS, Dorko K, et al. Hepatocyte transplantation improves phenotype and extends survival in a murine model of intermediate maple syrup urine disease. Mol Ther, Jul 2009; 17(7): 1266-73.
13. Christians U, Schmitz V, Schoning W, et al. oxicodynamic therapeutic drug monitoring of immunosuppressants: promises, reality, and challenges. Ther Drug Monit, 2008, 30(2): 151-158.
14. Albert MH, Anasetti C, Yu XZ. T regulatory cells as an immunotherapy for transplantation. Expert Opin Biol Ther. 2006, 6(4):315-324.
15. Raviprakash K, Wang D, Ewing D, et al. A tetravalent dengue vaccine based on a complex adenovirus vector provides significant protection in rhesus monkeys against all four serotypes of dengue virus. J Virol. 2008, 82 (14):6927-6934.
16. Eto Y, Yoshioka Y, Asavatanabodee R, et al. Development of pegylated adenovirus vector for cancer gene therapy.. Yakugaku Zasshi. 2008, 128(12):1733-1742.
17. Furukawa K, Ohashi T, Haruki K, et al. Combination treatment using adenovirus vector-mediated tumor necrosis factor-alpha gene transfer and a NF-κB inhibitor for pancreatic cancer in mice . Cancer Lett. 2011, 306(1):92-98.
18. Lybarger L, Wang X, Harris M, et al. Viral immune evasion molecules attack the ER peptide- loading complex and exploit ER-associated degradation pathways . Curr Opin Immunol, 2005, 17 (1): 71-78.
19. Ressing ME, Keating SE, Leeuwen D, et al. Impaired Transporter Associated with Antigen Processing-Dependent Peptide Transport during Productive EBV Infection. J. Immunol., 2005, 174 (11): 6829-6838.
20. Koppers-Lalic D, Reits EAJ, Ressing ME, et al. Varicelloviruses avoid T cell recognition by UL49.5-mediated inactivation of the transporter associated with antigen processing . PNAS, 2005, 102 (14): 5144- 5149.
21. Nozawa N, Inoue N. Mechanisms of congenital CMV infection . Nippon Rinsho, 2006, 64 (Suppl 3): 446-450
22. Pande NT, Powers C, Ahn K, et al. Rhesus Cytomegalovirus Contains Functional Homologues of US2, US3, US6, and US11. J. Virol., 2005, 79 (9): 5786 - 5798.
23. Hong M, Li WZ, Li QH. The immune escape mechanism of herpes simplex virus type 1 suppression of transporter associated with antigen processing (TAP) by ICP47 . Bing Du Xue Bao, 2007, 23 (1): 72-75.
24. Oosten LEM, Koppers-Lalic D, Blokland E, et al. TAP-inhibiting proteins US6, ICP47 and UL49.5 differentially affect minor and major histocompatibility antigen-specific recognition by cytotoxic T lymphocytes. Int. Immunol., 2007, 19 (9): 1115 - 1122.
25. Zhang XW, Meng ZH, Zhao JS, et al. Gene therapy targeting for carcinoma regulated by E2F-1 promoter. Zhonghua Wai Ke Za Zhi. 2006, 44(23):1636- 1639.
26. Wu Q, Fu Q, Chen Q, et al. Prokaryotic expression, identification and bioinformatics analysis of fbpB-esxA fusing gene from Mycobacterium tuberculosis. Asian Pac J Trop Med. 2011, 4(7):530-534.

2/2/2013

Growth of the green alga *Chlorella vulgaris* as affected by different carbon sources

[1]Battah M. G. [2]El-Sayed, A.B. and [1]El-Sayed, E.W

[1] Botany Department, Faculty of Science, Benha University, Egypt.
[2] Algal Biotechnology Unit, National Research Centre, Dokki- Cairo, Egypt.
bokhair@msn.com

Abstract: The green alga *Chlorella vulgaris* was grown under BG-II growth medium conditions comparing with such media enrichment with different carbon sources. The tested carbon sources were carbon dioxide, sodium acetate, acetic, oxalic and citric-acids. The effect of urea carbon was also eliminated using urea free carbon dioxide cultures. Dry weight, total chlorophyll and carotenes were daily determined. Urea nitrogen cultures; as a carbon source; exhibited more dry weight comparing with those of nitrate cultures. Concerning other carbon source in spit urea, acetate salt cultures surpassed all other examined carbon sources. An opposite manner was observed on chlorophyll accumulation, where, nitrate cultures represented the maximum yield. Maximum carotene content was observed with sodium acetate cultures. On the average, biomass growth rate was higher with urea nitrogen and acetate followed by carbon dioxide. The lowest dry weight, total chlorophyll and carotene accumulation was observed by cultures that grown with oxalic carbon which might ascribe to the harmful acidic action.
[Battah M. G.; El-Sayed, A.B. and El-Sayed, E.W. **Growth of the green alga *Chlorella vulgaris* as affected by different carbon sources**. *Life Sci J* 2013;10(1):2075-2081] (ISSN:1097-8135). http://www.lifesciencesite.com.295

Keywords: *Chlorella vulgaris*, nitrate, urea, organic carbon, growth parameters

1. Introduction

Photosynthesis is the key tune making solar energy available in useable forms for all organic life, where photosynthetic microorganisms including algae are able to use such energy to combine water and carbon dioxide to create life biomass. It has long been recognized that a significant portion of the inorganic carbon fixed in actively growing photosynthetic algae is released as dissolved organic carbon (Bertilsson and Jones, 2002). Maximum releasing of total fixed carbon as extracellular dissolved organic carbon (DOC) is closely depending on the nutrients availability that increased by nutrient limitation (Andersson and Zeutschel, 1970; Mague *et al*., 1980; Fogg, 1983; Lancelot, 1983; Obernosterer & Herndl, 1995; Malinsky-Rushansky and Legrand, 1996; Moran and Estrada, 2002). Most of microalgae are photo-autotrophs, while some microalgae can use organic carbon substances as a source of energy and carbon for cell growth. For some microalgae, photosynthesis and the oxidative phosphorylation of organic carbon substances seem to function independently. The growth rate in mixotrophic conditions is approximately the same as a sum of the growth rate in the photo-autotrophic and heterotrophic cultures, such as *Chlorella regularis* (Endo *et al*., 1977); *Chlorella vulgaris* (Ogawa and Aiba 1981); *Spirulina platensis* (Marquez *et al*., 1993).

Organic carbon metabolism may exert an opposing influence on photosynthesis. Glucose can reduce the apparent affinity for CO_2 in CO_2 fixation in some algae species such as *Chlorella sp*. (Lalucat *et al*., 1984), and *Chlorella vulgaris* (Martinez and Orus, 1991). Growth of cells under increasing concentrations of acetate culture reduced the photosynthetic CO_2 fixation and net O_2 evolution, without effects on respiration and photosystem II (PS II) efficiency (Heifetz *et al*., 2000). Acetate can also reduce carbonic anhydrase (CA) activity (Fett and Coleman, 1994). On the other hand, Kindle (1987); Goldschmidt-Clermont (1986) showed that acetate can inhibit the light-harvesting chlorophyll a/b binding gene. In the unicellular green alga *Chlorogonium elongatum*, acetate inhibited the synthesis of Calvin cycle enzyme ribulose-bisphosphate carboxylase/oxygenase (RuBPCase) and its mRNAs (Steinbiß and Zetsche, 1986). For microalgae that are able to survive hetero-trophically, exogenous carbon sources offer prefabricated chemical energy, which the cells often store as lipid droplets (Ratledge, 2004).

Hetero-trophically cultivated *Chlorella protothecoides* has been shown to accumulate as much as 55% of its dry weight as oil, compared to only 14% in cells that grown photo-autotrophically (Wu and Miao, 2006). Another natural mechanism through which microalgae can alter lipid metabolism is the stress response owing to a lack of bio-available nitrogen (Tornabene *et al*., 1983). Moreover, other factors triggered the oil accumulation were also early recognized. Although nitrogen deficiency appears to inhibit the cell cycle and the production of almost all cellular components, the rate of lipid synthesis remains higher, which leads to the accumulation of oil in starved cells (Sheehan *et al*., 1998). Interestingly,

nitrogen deprivation also promotes the accumulation of the antioxidant pigment astaxanthin in the green alga *Haematococcus pluvialis* (Boussiba, 2000). Both of these adaptive responses help to ensure the cells' survival during times of stress, while lipids serve as energy stores, astaxanthin seems to play a role in the protection against reactive oxygen species.

Microalgae are expensive to produce, and different systems have been designed for the growth and handling of microalgae on a large scale (Gudin and Chaumont 1980; Richmond 2004; Tredici, 2004; Weissman *et al*., 1988). From an industrial standpoint, these 'lipid triggers' can be useful for the production of polyunsaturated fatty acids (*e.g.* omega-3) and biodiesel from algal triacylglycerides. Addition of at least 10% of nutrients mainly nitrogen are required to overcome the dry weight accumulation failure (El-Shafey *et al*., 1999). The present work was achieved to determine the effect of different carbon sources; versus industrial carbon dioxide; on growth metabolites mainly dry weight and pigmentation of the green alga *Chlorella vulgaris*.

2. Materials and Methods

Alga and growth conditions

The green alga *Chlorella vulgaris* (NRC); was heterotrophically grown under optimum conditions of BG-II nutrient solution (Stainer *et al*., 1971) to obtain the proper inoculums. Continuous light illumination was provided from day light lamps (5x40w) from one side. Aeration was performed by free oil compressed air from the upper hold throughout 3mm polyethylene tube. Room temperature was recorded as 27±2^{o}C during the whole incubation period. Incubation was employed within fully transparent polyethylene bags (75cm length x75mm diameter and 100µ thickness) containing 2.5 L of the algal broth (El-Sayed and El-Fouly, 2005).

Carbon Sources

The original concentration of nitrogen (1.5 g.l^{-1} $NaNO_3$) was substituted by urea at 0.53 g.l^{-1} (El-Sayed *et al*., 2001). The initial carbon content of such urea concentration was the base of calculation in concerning other carbon sources including acetic acid, sodium acetate, citric acid and oxalic acid. The initial carbon content of 0.53 g.l^{-1} urea was found to be 0.2473 g carbon monoxide (CO) equal to 0.5622 g of acetic acid (the-mono-carboxylic); 0.417 g of oxalic acid (the di-carboxylic) and 0.5943 g citric acid (the tri-carboxylic). Concerning sodium acetate that used as a part of growth media for vegetative growth enhancement it was also used based on its carbon content at 0.804 g.l^{-1}.

Growth measurements

The investigated parameters were dry weight, total chlorophyll and total carotene. For dry weight estimation, 5ml from each replicate were filtered over a pre-weighted Whatman sterile membrane filters (pore size 0.45µm , 0.47 mm in diameter and white grade). After filtration, filters were left to dry for 30 minutes at 105 °C by circulated oven and then kept over anhydrous calcium chloride till room temperature and then re-weighted. The difference between weights monitored the net dry weight of the grown alga within defined sampling time .the dry weight was calculated as g.l^{-1}.

Total chlorophyll was extracted by dimethyl sulfoxide (DMSO) according to Burnison (1980). Five ml of algal suspension was centrifuged at 3500 rpm for 5 minutes. The supernatant was discarded and the residual pellet was re-suspended in 5 ml of 95% DMSO, homogenized and kept for 5 minutes at 70 °C water bath. Such extract contains total chlorophyll and cell carotenoids. The extracted cells were re-centrifuged again at 3500 rpm for 5 minutes. The extracted solution was measured by reading the absorbance (A) of the pigment extract by spectrophotometer at 666m. Total chlorophyll content was calculated (mg l^{-1}) according to Seely *et al*. (1972). To recover carotenes, saponification of the algal pellet was performed by 5% KOH/30% MeOH and the residual was re-extracted by DMSO after the addition of 5 drops of concentrated acetic acid (Boussiba *et al*., 1992). Carotenes absorbance was measured at 468nm and concentration was calculated (mg.l^{-1}) according to Davies (1976). Growth rate (µ) was calculated using the methods adopted by Pirt (1973)**.**

3. Results and Discussion

Dry weight

Growth dry weight of the green alga *Chlorella vulgaris* (Fig.1a) was dramatically varied as they grown under different carbon sources even with non-universal carbon source like urea. Thus, it could be mentioned that urea surpasses nitrate growth own to the initial carbon generated from urea utilization. This finding is in the harmony with those early obtained by El-Shafey *et al*. (1999) who reported that urea at 0.53g.l^{-1} could replace the nitrate content at 1.5g.l^{-1}.

When such cultures were supported by gaseous carbon source (1.5% CO_2); a considerable increase in dry weight accumulation was observed. The variation between the two results might be return to the effect of carbon on alga growth. Data also showed a slight inhibitory effect on dry weight accumulation by such alga as they fed by carbon in the presence of nitrate

nitrogen. The inhibitory effect could be ascribed to the liberated sodium as cells stimulate nitrate nitrogen.

Regarding the effect of some organic carbon sources, variable results were obtained. Nitrate nitrogen cultures under CO_2 exhibited the greatest positive effect as compared with acetic carbon result. On the contrary, carbon as sodium acetate represented slightly lower result which might goes back to liberated sodium ion (El-Shafey *et al*., 1999). In this connection, supplying growth media by super optimal concentrations of urea as N source enhanced vegetative growth of *Scenedesmus* sp. This might be led to increase some nutrient solubility or decrease pH values to near acidic reaction, Here; urea also acts as a complementary source of carbon (El-Sayed. *et al*., 2010).

The high initial carbon content of urea (46.5% CO) with high solubility rate to form carbonic acid might be enhanced the vegetative growth of algae. Furthermore, the cleavage of urea molecule led to the fast utilization of ammonia nitrogen part by the used alga. Urea degradation as a nitrogen source involves two specific enzymatic systems (urease and urea amydolayase); which are absent in algal metabolic matrix. The degradation might be achieved by bacteria; or due to the media reaction, mainly acid reaction, light, aeration and/or hydrolysis by extracellular algal excretions. Other organic carbon including citric and oxalic-acids showed the lowest increase on dry weight accumulation. Here, the slight inhibitory effect of oxalic and citric acids on growth could be return to their effect on the acid reaction of growth media.

Also, growth as dry weight was enhanced at first by the addition of citrate wastes even at the higher concentrations. This would be mainly due to the presence of organic carbon that allows the fast carotenoids accumulation on the expense of carbohydrate metabolism and green pigments. Addition of extra citrate amount would increase the salt potential of the growth medium; even though they are serve as utilizable nutrients as a source of different macronutrients including carbon, nitrogen, phosphorous, potassium and some micronutrients (El-Sayed, 2010 and El-Sayed *et al*., 2012). The dry weight in case of oxalic acid was lower than in case of $NaNO_3$ and this might due to acidity of the solution which negatively affects the biomass accumulation.

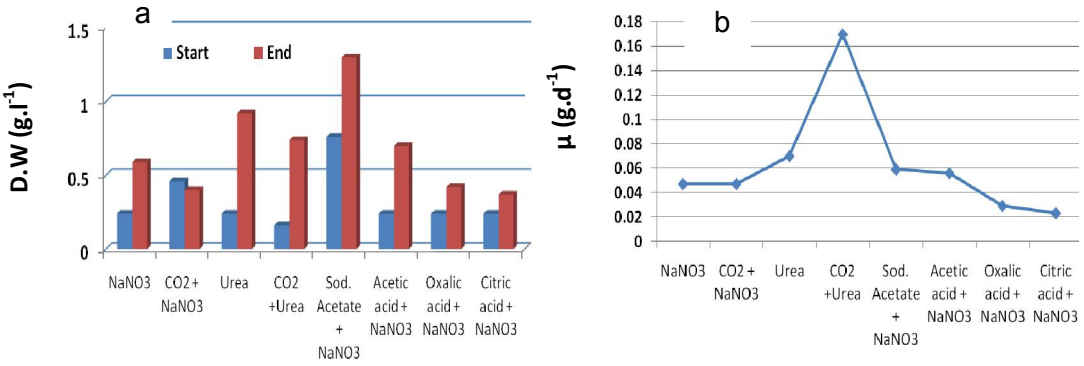


Fig. 1. a) Dry weight (g.l^{-1}) and b) growth rate of *Chlorella vulgaris* under different carbon sources.

On the average, growth rate was higher with urea nitrogen cultures as compared with nitrate cultures due to the extra nitrogen quantities from urea metabolism (Fig.1a). This finding could be confirmed as both cultures were enriched by carbon dioxide. Among the various organic carbon sources used, acetate carbon represented the maximum. The enhancing effect of acetate might be goes back to their lower molecular weight that making it easy to stimulate via carotene fixation as compared with oxalic or citric acids.

Total Chlorophyll

On the contrary with dry weight accumulation data, nitrate nitrogen surpasses ammonical nitrogen regarding chlorophyll accumulation (Fig.2). The effect of CO_2 supplementation was similar in both nitrogen sources (nitrate and urea). On the other hand, acetate carbon represented wide variations, where acetic

carbon enhanced the biosynthesis of chlorophyll as compared with urea cultures, but less in nitrate case. Furthermore, sodium acetate enhanced chlorophyll accumulation of nitrate cultures and inhibited those of urea cultures. This finding was in agreement with Belcher (1968); who reported that 10 mM of acetate increased oxygen consumption by *Botrycoccus braunii*; however Weetall (1985) showed that sodium acetate had no effect on the growth of *Botrycoccus braunii*. In addition, Tenaud *et al*. (1989) reported that 5 mM acetate did not affect growth, but significantly increased respiration and inhibited photosynthesis. Sodium acetate effect seems to be concentration depending, where its variable concentration used 5-45mM depending on growth stage. Low concentration enhanced vegetative growth, while higher concentration resulted in chlorophyll de-composition especially in the presence of oxygen species agent like ferrous sulfate. As early observed by Droop (1954) and Borowitzka *et al*. (1991), acetate at small quantities; appeared to be an important carbon source; enhancing both growth and carotenogensis, however, the effect of acetate was concentration dependent. Higher concentrations inhibiting growth, but markedly increasing astaxanthin content per cell. Acetate addition in excess may generate a relative shortage of nitrogen inducing cyst formation and astaxanthin accumulation triggered by a high carbon/nitrogen (C/N) ratio (Kakizono *et al*., 1992). The algal cells seem to reduce their nitrogen uptake and begin to use the cellular nitrogen as in typical N-deficiency conditions, despite presence of nitrogen in the culture medium.

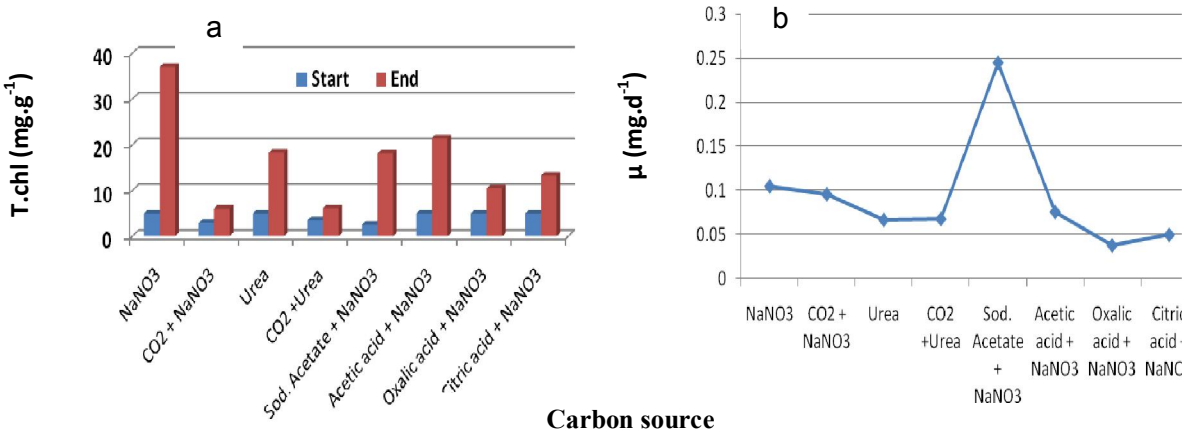


Fig. 2. a) Total chlorophyll (mg.g^{-1}) and b) growth rate of *Chlorella vulgaris* under different carbon sources.

Addition of more than 10 mM sodium acetate to the cultures increased chlorophyll fluorescence intensity, while it decreased with 20 mM acetate. However, in the presence of glucose under both light and dark culture conditions, the ratios of chlorophyll to dry weight were lower than those under autotrophic culture. The decline in chlorophyll content might be caused by inhibition of chloroplast development by the presence of glucose or by a relative increase in the level of other substances other than chlorophyll (Tanoi *et al*., 2011). Other organic acids such as oxalic and citric enhanced the chlorophyll accumulation. The last effect could be attributed to the possible assimilation of such organic acid in the absence of chlorophyll function.

On the contrary with dry weight results, growth rate; on the average; was found to be the maximum with nitrate cultures free or enriched by carbon dioxide suggesting the trigger effect of nitrate on chlorophyll accumulation rather than urea. Acetate urea culture also represented the lowest. In general, Nitrate acetate cultures surpass all of the examined cultures.

Carotenes

Carotene's pigment in the green alga *Chlorella vulgaris* was affected by different carbon sources (Fig. 3). A slight difference was observed on carotene content between cultures of nitrate or urea nitrogen due to the same nitrogen content regardless the liberated sodium ions. When nitrate cultures were

enriched by carbon dioxide, a slight reduction was observed as compared with both nitrate or urea nitrogen free carbon. This might be goes back to the formation of carbonate compounds mainly sodium carbonate. Also, Carotene bio-accumulation is not conditioned by the presence of carbon dioxide, but di-carbon fragment is very necessary for fatty acid accumulation via carotene metabolism. This hypothesis could be confirmed by the obtained result of acetic acid supplementation of nitrate cultures that reduces pH values and also inhibit carotene formation comparing with carbon cultures.

Oxalic and citric acid cultures exhibited extra inhibitory response on carotene formation. The most effective enhancing action was observed with cultures that fed with sodium acetate plus nitrate nitrogen. Influence of adding an organic carbon source such as acetate increases the growth rate and carotene level this agree with Borowitzka *et al*. (1991); Calo *et al*. (1995); Kobayashi *et al*. (1993); Meyer and Du Preez (1993) and Orosa *et al*. (2005) who, stated that acetate and malonate appear to be an interesting carbon sources, enhancing both growth and carotenogenesis. Moderate carotene production was observed when acetic acid and citric acid were used, but depression was occurred when oxalic acid was used due to the high acidic effect which lacking carotenogensis.

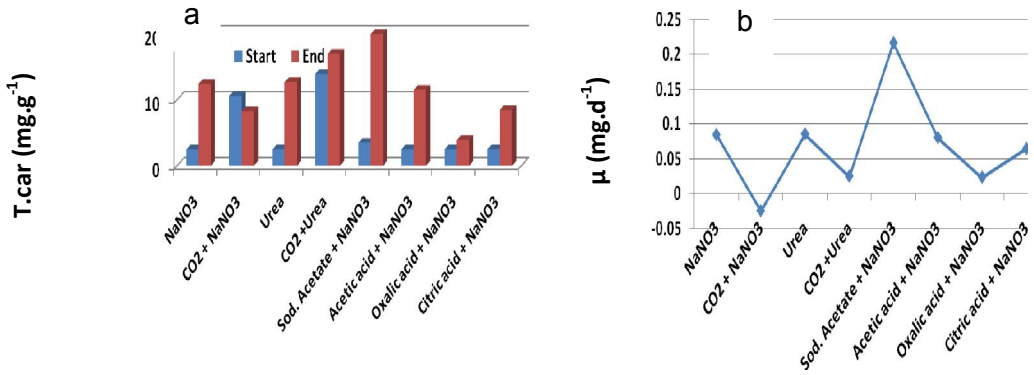


Fig. 3. a) Carotenes (mg.g^{-1}) and b) growth rate of *Chlorella vulgaris* under different carbon sources.

4. Conclusion

Nitrate was early recognized as the prefer nitrogen source for many algal species. Here, urea seems to be the best as providing algal growth media by extra carbon amount. *Chlorella vulgaris* able to grow and survive under the entire examined carbon source, but acetate carbon (acetic or sodium acetate) was the more efficient. This finding opens the way to use organic carbon wastes (starch effluent) for algae biomass production which in turn reduces the production costs.

Acknowledgments

The authors express all thanks to the stuff member of Algal Biotechnology Unit, National Research Center for scientific and technical supporting.

Corresponding author:
El-Sayed, A. B.
bokhair@msn.com

References

Anderson, G.C. and Zeutschel, R.P. (1970). Release of dissolved organic matter by marine phytoplankton in coastal and off shore areas of the northern Pacific Ocean. Limnol. Oceanogr.; 15: 402- 407.

Belcher, J.H. (1968). Note on the physiology of *Botryococcus braunii* Kützing. Arch. Mikrobio.; 61 :335-346.l

Bertilsson, S. and Jones, J.B. (2002). Supply of Dissolved Organic Matter to Aquatic Ecosystems: Autochthonous Sources, *In* Findlay SEG, Sinsabaugh, R.L (eds); Aquatic Ecosystems: Interactivity of Dissolved Organic Matter, Academic Press, New York: 3-24.

Borowitzka, M.A.; Huisman, J.M. and Osborn, A. (1991). Culture of the astaxanthin producing green alga *Haematococcus pluvialis*. 1. Effects of nutrients on growth and cell type, J. Appl. Phycol.; 3: 295–304.

Boussiba, S. (2000). Carotenogenesis in the green alga *Haematococcus pluvialis*: cellular physiology and stress response. Physiol. Plant.; 108:111-117.

Boussiba, S.; Fan, L. and Vonshak, A. (1992). Enhancement and determination of astaxanthin accumulation in green alga *Haematococcus pluvialis*. Methods in Enzymology, 213, Carotenoids Part A, Lester Packer (ed.), Academic Press: 386-371.

Burnison, K. (1980). Modified dimethyl sulfoxide (DMSO) extraction for chlorophyll analysis of phytoplankton. Can. J.Fish. Aquat. Sci.; 37:729-733.

Calo, P.N.; Miguel, T. and Vel´azquez J.B. (1995). Astaxanthin accumulation in the green alga Villa T. G.: Mevalonic acid increases trans-astaxanthin and carotenoid biosynthesis in *Phaffia rhodozyma*, Biotechnol. Lett.; 17: 575–578.

Davis, H. (1976). Carotenoids. In Chemistry and Biochemistry of Plant Pigments.2nd Eddition, vol.2.ed. Goodwin, T. W. pp.38-165. Academic Press.

Droop, M.R. (1954). Conditions governing haematochrome formation in *Haematococcus pluvialis*. Arch. Mikrobiol.; 20, 391.

El-Sayed, A. B. (2010). Carotenoids accumulation in the green alga *Scenedesmus* sp. incubated with industrial citrate waste and different inductions stress. Nature and Science 2010; 8(10):34 40.

El-Sayed, A. B. and El-Fouly, M. M. (2005). Recovery of outdoor mass culture bleached *Scenedesmus* sp. Pakistan Journal of Biological Sciences (8,3) 470-474.

El-Sayed, A.B.; Hoballah, E.M. and Khalafallah, M.A. (2012). Utilization of citrate wastes by *Scenedesmus* sp. I- Enhancement of vegetative growth. Journal of Applied Sciences Research, 8(2): 739-745.

El-Shafey, Y.H.; El-Fouly, M.M.; Khalil, M.M.; Abdalla, F.E. and El-Sayed, A.B. (1999). Secondary carotenoid accumulation in some green algae species. The First Congress on the Recent Technologies in Agriculture. 27-29 Nov., 1999, Fac. of Agric., Cairo Univ. Cairo, Egypt.

Endo, H.; Sansawa, H. and Nakajima, K. (1977). Studies on *Chlorella regularis*, heterotrophic fast-growing strain. II. Mixotrophic growth in relation to light intensity and acetate concentration. Plant Cell Physiol.; 18:199–205.

Fett, J.P. and Coleman, J.R. (1994). Regulation of periplasmic carbonic anhydrase expression in *Chlamydomonas reinhardtii* by acetate and pH. Plant Physiol.; 106:103–108.

Fogg, G.E. (1983). The ecological significance of extracellular products of phytoplankton photosynthesis. Botanica Marina, 26: 3-14.

Goldschmidt-Clermont M (1986). The two genes for the small subunit of RuBp carboxylase/oxygenase are closely linked in *Chlamydomonas reinhardtii*. Plant Mol. Biol.; 6:13–21.

Gudin, C. and Chaumont, D. (1980). A biotechnology of photosynthetic cells based on the use of solar energy. Biochem Soc. Trans.; 8:481–482.

Heifetz, P.B.; Foster, B.; Osmond, C.B.; Giles, L.G. and Boynton, J.E. (2000). Effects of acetate on facultative autotrophy in *Chlamydomonas reinhardtii* assessed by photosynthetic measurements and stable isotope analyses. Plant Physiol.; 122:1439–1445.

Kakizono, T.; Kobayashi, M. and Nagai, S. (1992). Effect of carbon/nitrogen ratio on encystment accompanied with astaxanthin formation in a green alga, *Haematococcus pluvialis*. J. Ferment. Bioeng.; 74, 403–405.

Kindle, K.L. (1987). Expression of a gene for a light-harvesting chlorophyll a/b-binding protein in *Chlamydomonas reinhardtii*: effect of light and acetate. Plant Mol. Biol.; 9:547–563.

Kobayashi, M.; Kakizono, T. and Nagai, S. (1993). Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis*, Appl. Environ. Microbiol.; 59:867–873.

Lalucat, J.; Imperial, J. And Pares, R. (1984). Utilization of light for the assimilation of organic matter in *Chlorella* sp. VJ79. Biotechnol. Bioeng.; 26:677–681.

Lancelot, C. (1983). Factors affecting phytoplankton extracellular release in the Southern Bight of the North Sea. Mar. Ecol. Progr. Ser.*;* 12: 115-121.

Mague, T.H.; Friberg, E.; Hughes, D.J. and Morris, I. (1980). Extracellular release of carbon by marine phytoplankton: a physiological approach. Limnol. Oceanogr.; 25: 262-279.

Malinsky-Rushansky, N.Z. and Legrand, C. (1996). Excretion of dissolved organic carbon by phytoplankton of different sizes and subsequent bacterial uptake. Mar. Ecol. Progr.; Ser. 132: 249-255.

Marquez, F.J.; Saski, K.; Kakizono, T.; Nishio, N. and Nagai, S. (1993). Growth characteristics of *Spirulina platensis* in mixotrophic and

heterotrophic conditions. J. Ferment. Bioeng.; 76:408–410.

Martinez, F. and Orus, M.I. (1991). Interactions between glucose and inorganic carbon metabolism in *Chlorella vulgaris* strain UAM101. Plant Physiol.; 95:1150–1155.

Meyer, P.S. and Du Preez, J.C. (1993). Effect of acetic acid on astaxanthin production by *Phaffia rhodozyma*, Biotechnol. Lett.; 15, 919–924.

Morán, X.A.G. and Estrada, M. (2002). Phytoplankton DOC and POC production in the Bransfield and Gerlache straits as derived from kinetic experiments of 14C incorporation. Deep Sea Research II, 49: 769-786

Obernosterer, I. and Herndl, G.J. (1995). Phytoplankton extracellular release and bacterial growth: dependence on the inorganic N: P ratio. Mar. Ecol. Progr.; Ser. 116: 247-257.

Ogawa, T. and Aiba, S. (1981). Bioenergetic analysis of mixotrophic growth in *Chlorella vulgaris* and *Scenedesmus acutus*. Biotechnol. Bioeng.; 23:1121–1132.

Orosa, M.; Franqueira, D.; Cid, A. and Abalde, J. (2005). Analysis and enhancement of astaxanthin accumulation in *Haematococcus pluvialis*, Bioresour. Technol.; 96: 373–378.

Pirt, S.J. (Ed.) (1973). Principle of Microbe and Cell Cultivation. Blackwell Sientific Publication, pp: 4-7.

Ratledge, C. (2004). Fatty acid biosynthesis in microorganisms being used for single cell oil production. Biochimie.; 86:807-815.

Richmond A. (2004). Handbook of Microalgal Culture-Biotechnology and Applied Phycology. Blackwell Publishing, Malden, MA, 566 pp.

Seely, G. R.; duncan, M. j. and Widaver, W. E. (1972). Preparative and analytical extraction of pigments from brown algae with dimethyl sulfoxide .Mar. Biol.; 12:184-188.

Sheehan, J.; Dunahay, T.; Benemann, J. and Roessler, P. (1998). A Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae Golden, Colorado: TP-580-24190, National Renewable Energy Laboratory.

Silva, H.J.; Italiano, M.C. and Ferrari, S.G. (1994). Improved biomass production of cyanobacteria by reutilization of culture media Biotechnology Techniques. 8 (12):889-894.

Stainer. R.Y.; Kunisawa, R.; Mandel, M. and Cohin-Bazire, G. (1971). Purification and properties of unicellular blue-green algae (order *Chrococcales*). Bacteriol Rev.;35:171-205.

Steinbiß, H.J. and Zetsche, K. (1986). Light and metabolite regulation of the synthesis of ribulose-1,5-bisphosphate carboxylase/ oxygenase and the corresponding mRNAs in the unicellular alga *Chlorogonium.* Planta 167:575–581.

Tanoi, T.; Kawachi, M. and Watanabe, M, M. (2011). Effects of carbon source on growth and morphology of *Botryococcus braunii*. J. Appl. Phycol.; 23:25–33.

Tenaud, M.; Ohmori, M. and Miyachi, S. (1989). Inorganic carbon and acetate assimilation in *Botryococcus braunii* (Chlorophyta). J. Phycol.; 25:662–667.

Tornabene, T.G.; Holzer, G.; Lien, S. and Burris, N. (1983). Lipid composition of the nitrogen starved green alga *Neochloris oleoabundans*. Enzyme Microb. Technol.; 5:435-440.

Tredici, M. R. (2004). Mass production of microalgae: Photobioreactors. In Handbook of microalgal culture. A. Richmond(ed); Chapter 9, 178–214. Oxford, UK: Blackwell Science Ltd.

Weetall, H.H. (1985). Studies on the nutritional requirements of the oil producing alga *Botryococcus braunii*. Appl. Biochem. Biotech.; 11:377–391.

Weissman, J.C.; Goebel, R.P. and Benemann, J.R. (1988). Photobioreactor design: mixing, carbon utilization and oxygen accumulation. Biotech. Bioengng.; 31: 336-344.

Wu, Q. and Miao, X. (2006). Biodiesel production from heterotrophic microalgal oil. Bioresour. Technol.; 97:841-846.

2/2/2013

Estimation of soil Fertility and Yield Productivity of Three Alfalfa (*Medicago sativa* L.) Cultivars Under Sahl El-Tina Saline Soils Conditions

Abd El-Naby, Zeinab, M[1], Nabila, A. Mohamed[1] and Kh. A. Shaban[2]

[1]Forage Res. Dept., Field Crop Res. Inst., Agric. Res. Center, Giza, Egypt
[2]Soil, Water and Environment Res. Inst. Agric. Res. Center, Giza, Egypt
zeina_bree@yahoo.com

Abstract: Salt stress is a serious environmental problem throughout the world which may be partially relieved by breeding cultivars that can tolerate salt stress. Three alfalfa cultivars, i.e., Ismaelia, New Valley and Siwa, were sown under three levels of salinity (8.73, 10.63 and 15.31dSm^{-1})**.** A field experiment was carried out in sandy clay loam soil at North Sinai Governorate during two successive seasons from mid-spring 2011 to late-Autumn 2012. The results of this study indicated that long growing duration of alfalfa cultivars under different soil salinity levels led a slight decrease in soil pH and soil EC. The available macronutrients (N, P and K) and micronutrient (Fe, Mn and Zn) contents accumulated in soil recorded positive increasing effects, depending on the length of alfalfa cultivation period. Evaluation at the germination stage is absolutely critical in developing cultivars that can establish a good stand. Natural selection through frequent cutting and salinity stress is an excellent means to screen large sets of salt tolerant genotypes and get an idea for regrowth potential under high salinity levels. Siwa cultivar was the highest yielding across all seasons and salinity levels; it also recorded the highest mean of total fresh and dry forage yield (53.06 and 12.60 t fed^{-1}, respectively), while New Valley cultivar recorded the lowest means (40.66 and 10.12 t fed^{-1}, respectively) across all salinity levels. Siwa cultivar was superior over Ismaelia and New Valley in most of the agronomical characters under study. The high level of salinity (EC= 15.31dSm^{-1}) depressed the number of tillers plant^{-1}, fresh and dry forage weight (g) plant^{-1}. New developed alfalfa population (Sinai-1) was the most tolerant and adaptable to saline soils in Sahl El-Tina conditions.

[Zeinab M. Abd El-Naby, Nabila, A. Mohamed and Kh. A. Shaban. **Estimation of soil Fertility and Yield Productivity of Three Alfalfa (*Medicago sativa* L.) Cultivars Under Sahl El-Tina Saline Soils Conditions.** *Life Sci J* 2013;10(1):2082-2095] (ISSN:1097-8135). http://www.lifesciencesite.com. 296

Key words: *Alfalfa, Midicago sativa, soil Salinity, Macronutrients, Micronutrients, Forage, Fresh, Dry yield.*

Introduction

In Egypt the total cultivated area is approximately 3.15 million hectare (hectare =2.385 Feddan); comprised of 2.35 million hectare of old fertile and and 0.8 million hectare of new reclaimed land. The country has a rapid population growth rate; the population is expected to reach about 110 million inhabitants by the year 2025. Sinai represents 6% of the total area of Egypt (Hafez 2005). Sahl El-Tina plain is situated in the North Western part of Sinai Peninsula. Jing *et al*. (2012) reported that the EC in the soil profile is gradually decreased with the duration of alfalfa cultivation. The effect of successive alfalfa planting for an improvement of soil fertility across the years is considerably significant in salt-affected soils. In comparison with the control, EC in the 0–20 cm soil layer decreased significantly, after 1 and 2 years, respectively (*Zhang et al.* 2007). Fan *et al*. (2002) showed that a greater effect on the soil physico-chemical properties was found with increasing the duration of alfalfa cultivation. Tang *et al*. (1999) found that the pH in the soil layer of 0–20 cm was decreased by 0.15 units as compared to that before alfalfa planting. Alfalfa species relying on symbiotic N_2 fixation acidified their rhizosphere, causing a considerable H^+ extrusion in the rhizosphere to decrease soil pH. Qadir *et al*. (2008) indicated that the effect of successive alfalfa planting for soil fertility improvement across the years is considerably significant in salt-affected soil.

Alfalfa (*Medicago sativa* L.), is a perennial warm-season legume, that is grown on 30 million hectares worldwide. It has been characterized as moderately sensitive to salts (Maas and Hoffman, 1977). In contrast, alfalfa has also been characterized as tolerant to salts with a range of EC values from 6.0 to 8.0 (3840 to 5120 ppm) at which some reduction in growth and yield can be expected (Longenecker and Lyerly, 1974). Alfalfa yields can be expected 7% decrease with each dSm^{-1} unit increase in saturation extract (Rawlins, 1979). Alfalfa was as tolerant as barley (*Hordeum vulgare*) and cotton (*Gossypium spp*.), however the growth of alfalfa was still retarded in saline conditions (Munns, 2005). Alfalfa can be successfully grown on most soils, and can tolerate some level of salinity during the germination stage, but once established it will tolerate higher levels of salinity up to 8 dSm^{-1}, (Sheard, 2007) and 10.3 dSm^{-1} (Shaban and El-Sherief, 2007). In addition, alfalfa fixes its own nitrogen with the help of *Rhizobium meliloti* bacteria on its roots (Bauder, 2007).

Local alfalfa cultivars (Siwa and Ismaelia), are characterized with a higher level of tolerance to salinity,

as been recognized in Argentina (El-Nahrawy *et al*., 1995), which has resulted in increasing the demand for seed export. Argentina asked Egyptian authorities for importing about 2500 tons of alfalfa seed annually of the two mentioned cultivars, if available. There is a high tendency to expand in alfalfa cultivation in the reclaimed lands of the Egyptian desert. Now its cultivated area is about 63.000 ha (El-Nahrawy *et al*., 1995).

Sahl El-Tina is situated in the North Western part of Sinai Peninsula, covers about 50.000 fed lying between longitudes 30° 51 South and 31° 15 North. Experiments of this study were located at the southern part of Sahl El-Tina, which composed of silty clay soil intercalated with salts and evaporates in the western part. Our breeding efforts have been directed at creating salt-tolerant populations capable of surviving high salinity levels and producing high yields in the same time.

This research was conducted to: a) evaluate and select new alfalfa composite populations of more adaptability to salinity stress, b) evaluate alfalfa productivity under different salinity levels, and c) effect of alfalfa long term stand on soil fertility (the main physical and chemical properties of the cultivated soils and also their content of some macro and micro-nutrients).

Materials and Methods

A field experiment was carried out in sandy clay loam soil of a private farm at Gelbana, east Suez Canal at North Sinai Governorate during two successive seasons from mid-spring 2011 to late-autumn 2012. Three salinity levels were measured 8.73 dSm^{-1} (S1), 10.63 dSm^{-1} (S2) and 15.31 dSm^{-1} (S3). The three Egyptian alfalfa studied cultivars, i.e., Ismaelia, New Valley and Siwa were obtained from Field Crops Research Institute, Agricultural Research Center, Giza, Egypt. Cultivars had sown in a split block design in randomized complete blocks arrangement with three replications. Salinity levels were considered the main plots, while the cultivars were distributed in the sub-plots. The plot size was 50 m^2 (5X10 m). Seeds were sown with the rate of 20 Kg fed^{-1}. Super phosphate (15.5 % P_2O_5) was applied at a rate of 200 kg fed^{-1} during tillage then 100 kg super-phosphate was added every four months. Urea (46% N) was applied as N fertilizer at a rate of 30 kg N fed^{-1} on three equal doses after 21, 42 and 62 days from planting. Potassium sulphate (48% K_2O) was applied at a rate of 100 kg fed^{-1} with two doses, after 21and 42 days, and then 50 kg fed^{-1} were added every four months. The first irrigation was applied after eight days from sowing. The following irrigations were applied each eight days during summer and fifteen days during winter seasons. Irrigation water was applied from Salam Canal.

Soil studies

Soil samples from the surface (0-30 cm) were collected from each treatment before and after alfalfa harvesting, ground and subjected to determination of available nutrients N, P and K as outlined by Page *et al*. (1982). Soil pH was determined using a 1 :2.5 soil water suspension

Electrical conductivity and soluble cations and anions of soil saturation paste extract were determined (Page *et al*., 1982**)**. The soil samples were air dried, crushed and finely ground, then sieved through a 2mm sieve and kept for analysis. Available micronutrients were extracted from soil samples by Diethyline tetra amin penta Acitic acid (DTPA) according to Lindsay and Norvell (1978) and determined by Atomic Absorption Spectrophotometer model GBC 932.

The obtained data were statistically analysed using COSTAT program and L.S.D. value at the probability levels of 5% was calculated according to Gomez and Gomez (1984).

Table 1. Physical and chemical properties in soil before planting.

	Coarse sand (%)	Fine sand (%)	Silt (%)	Clay (%)	Texture		O.M* (%)		$CaCO_3$ (%)
Mean	2.16	65.83	10.60	21.41	Sandy clay loam		0.45		5.92
Salinity levels EC (dS/m)	pH (1:2.5)		Cations (meq^{-1})				Anions (meq^{-1})		
			Ca^{++}	Mg^{++}	Na^{+}	K^{+}	HCO^{-}_3	Cl^{-}	SO^{--}_4
15.31	8.12		10.49	22.69	119	0.92	10.20	98	44.90
10.63	8.07		12.68	14.67	78	0.95	5.63	52	48.67
8.73	8.05		10.36	8.97	62	0.97	4.82	44	40.09
	Available nutrients ($mgkg^{-1}$ soil)								
	N	P	K		Fe		Mn		Zn
15.31	34	3.36	180		1.84		6.26		0.85
10.63	37	3.69	188		1.92		6.31		0.88
8.73	40	3.77	193		1.95		6.36		0.91

*.= organic matter %.

Table 2. Mean Chemical composition of El-Salam Canal water.

pH	EC (dSm^{-1})	Soluble anions (meq/l)				Soluble cations (meq/l)				SAR*
		Ca^{2+}	Mg^{2+}	Na^{+}	K^{+}	CO^{-2}_3	HCO^{-}_3	CL^{-}	SO^{2-}_4	
8.20	1.93	3.57	5.30	10.10	0.16	0.00	2.83	8.40	8.10	5.22

Macronutrients (mg/l)		Micronutrients (mg/l)					
NO_3-N	NH_4 N	P	Fe	Mn	Zn	Ca	B
4.03	12.57	5.10	0.51	0.31	0.40	0.55	0.05

*= Sodium adsorption ratio.

Agronomical traits

Fifteen cuts were taken according to dates presented in Figure (1). In Figure 1 shows cutting dates after sowing in March 2011 till July 2012, then plants were prevented from irrigation and left for flowering for four months to seed set.

Percentage of plant stand establishment recordings were made after the first cut (75 days from sowing). Cutting was done at 8-centimeter height at 28-30 day intervals. Fresh forage weight was recorded in the field immediately after cutting. Fresh forage $plot^{-1}$ weighed and air dried to calculate dry matter %. Samples of ten plants from each plot were collected for agronomical observations $plant^{-1}$ on height (cm), stem diameter (cm), number of tillers fresh (g) and dry weight (g), root length (cm), crown diameter (cm), and root weight (g) for fifteen cuts $plot^{-1}$ for each cultivar and each salinity level. Also, seed characters were recorded for 1000 seed weight (g), number of seeds g^{-1}, number of default seeds g^{-1}, seed weight (g) m^{-2} and seed weight (kg) fed^{-1} after harvesting.

Laboratory analysis

Samples of ten plants were collected randomly from each plot before cutting and then oven dried at 70 ^{o}C. The plant samples were ground, 0.5 g of each sample was digested using H_2SO_4 and $HClO_4$ mixture according to the methods described by Black, (1965). The plant content of nitrogen was determined by Kjedahl method (Chapman and Partt,1961**).** P, K, Fe, Mn and Zn were determined in plant digestion was determined by Inductively Coupled Plasma Spectrometer (ICP) plasma 400 according using the methods described by Cottenie *et al*. (1982) and Page *et al.* (1982).

Statistical methods.

Data were recorded and submitted to ANOVA using SAS 9.2 TS Level 2M3 (Der and Everitt, 2008). Fresh and dry forage weights $plot^{-1}$ across the fifteen cuts were adjusted to t fed^{-1} for each cultivar and productivity was calculated. Statistical analysis used the general linear model (GLM) for repeated measures to account for the split block design ($\alpha = 0.05$) under each salinity level and least significant differences were calculated according to according to Waller and Duncan (1969).

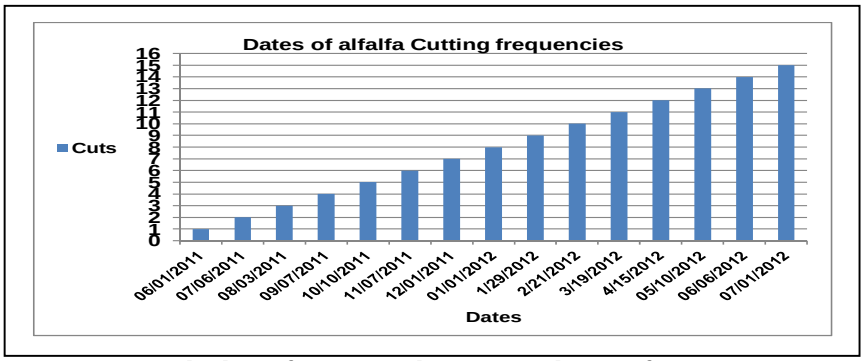


Figure 1. The dates of agronomical practices and cutting frequencies.

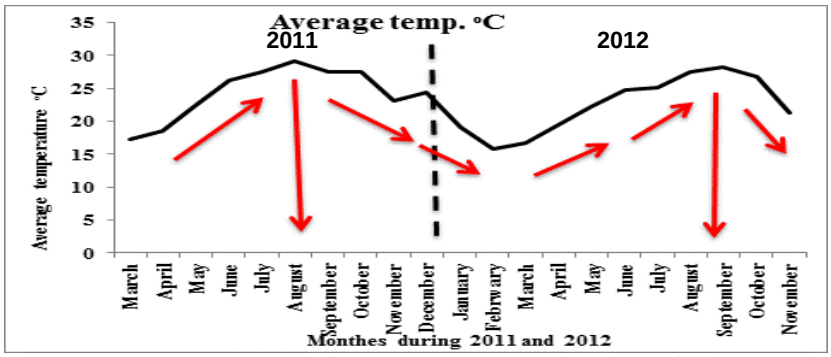


Figure 2. Average temperature °C during sowing, growing, cutting and blooming months.

Results and Discussion

Soil evaluation

Effect of growing alfalfa on soil EC (dSm^{-1}) and pH

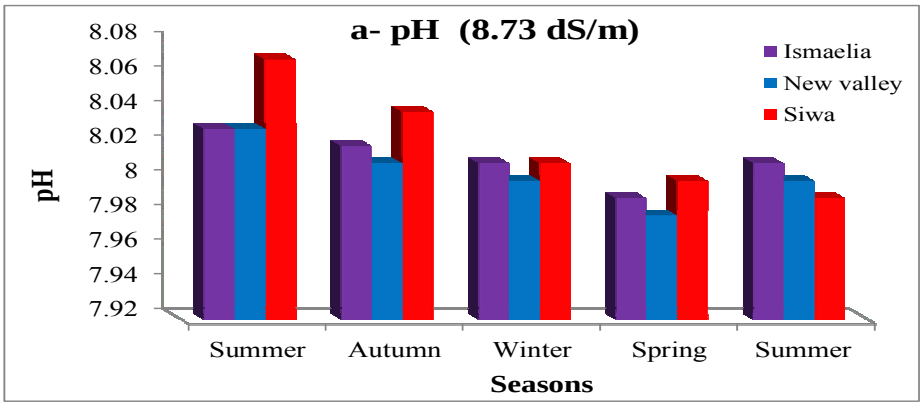
The effects of alfalfa cultivation for long duration period (summer 2011, autumn 2011, winter 2012, spring 2012 and summer 2012) on soil salinity are illustrated in Figure (3) Table (3). The soil EC was decreased with increasing the growing periods. All cultivars were efficient in decreasing EC values after alfalfa cutting compared with the initial soil EC (Table-3). The highest mean value of EC was 9.84 dSm^{-1} for Ismaelia cultivar, while the lowest mean values of EC was 7.62 dSm^{-1} for Siwa cultivar after cutting. However, the greatest reduction in EC values was recorded in winter 2012, hence the mean values of soil EC were 6.97, 6.83 and 6.92 dSm^{-1} for Ismaelia, New Valley and Siwa cultivars, respectively. The relative decreases of mean values of soil EC as affected by Ismaelia, New Valley and Siwa cultivars were (35.73, 49.31 and 40.04 %) for S1, (25.50, 27.00 and 30.95 %) for S2 and (25.66, 26.28 and 27.49 %) for S3, compared with the initial soil EC. (Table-3) These results are in agreement with Jing *et al.* (2012) who indicated that planting alfalfa led to a significant decrease in salt content in upper soil layer. Zhang and Ming (2004) found that the effect of alfalfa on saline soil improvement was significant.

Table 3. Soil salinity levels EC (dSm^{-1}) after alfalfa cutting.

Cultivars	Initial salinity levels Ec (dSm^{-1})	Soil EC (dSm^{-1}) throught growing seasons					
		Summer	Autumn	Winter	Spring	Summer	Mean
Ismaelia	8.73	7.94	6.88	5.73	5.88	6.01	6.49
	10.63	9.41	8.33	6.97	7.05	7.82	7.92
	15.31	12.59	10.98	8.20	8.45	8.96	9.84
Mean		9.98	8.75	6.97	7.13	7.60	8.08
New Valley	8.73	7.56	6.71	5.84	6.00	6.14	6.45
	10.63	9.14	8.17	6.55	6.96	7.96	7.76
	15.31	12.20	10.22	8.10	8.20	8.75	9.49
Mean		9.63	8.37	6.83	7.05	7.62	7.90
Siwa	8.73	7.31	6.49	5.80	5.98	6.09	6.33
	10.63	8.55	7.99	6.52	6.68	6.97	7.34
	15.31	11.85	9.79	7.95	8.10	8.22	9.18
Mean		9.24	8.09B	6.76	6.92	7.09	7.62
LSD (0.05)		0.57	0.85	2.92	2.06	0.10	n.s.

.n.s. : Insignificant differences at level of probability (0.05).

The changes in soil pH play a key role in the bioavailability of insoluble elements in soil. Data presented in Figure (3-a, b and c) show that the three alfalfa cultivars grown under different soil salinity levels decreased slightly the soil pH values during the growing seasons. The highest value of soil pH was 8.10 in summer 2011 for Siwa cultivar. At the highest level of soil salinity, the lowest value of pH was 7.98 in Spring 2012 for New Valley cultivar. In other words the pH values ranged between 8.10 and 7.98. The relative reduction of soil pH after cutting of the three alfalfa cultivars were 1.11 %, 4.95 % and 1.24 % for Ismaelia, New Valley and Siwa cultivars compared with initial pH, respectively. These results agreed with those reported by Shaban and Omar (2006), who indicated that the high activity of dehydrogenase enzyme and the released carbon dioxide in the rhizosphere cause the formation of carbonic acids and thus the decrease of pH of the root zone.



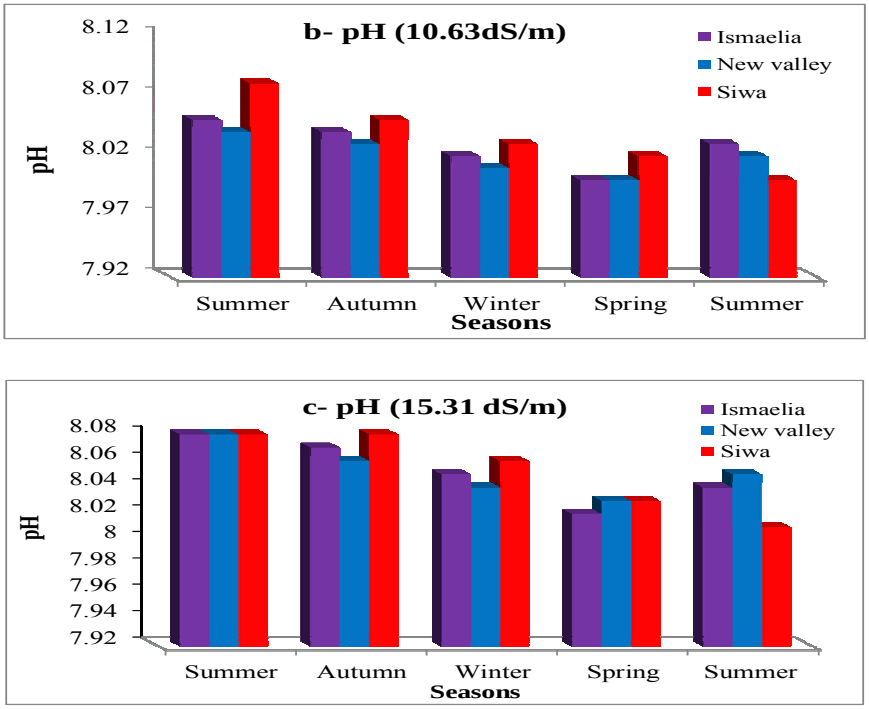


Figure 3. Soil pH after alfalfa cutting under different levels of salinity: a) Ec =8.73 dSm^{-1}, b) Ec =10.63 dSm^{-1} and c) Ec =15.31 dSm^{-1}.

Available macronutrients

a) In soil

Different alfalfa cultivars caused a positive increase in macronutrients (N, P and K available in soil under conditions of saline soil levels. Data presented in Table (4) indicated an increase in available N, P and K in soil by increasing growing duration of alfalfa. The highest mean value of N, P and K content in soil was 52.77, 3.97 and 205 mg kg^{-1}, respectively, after cutting of Siwa cultivar compared with the other cultivars. On the other hand the best mean value of N content in soil was 56.78 mg kg^{-1} recorded for Spring 2012 season, while the mean values of P and K contents in soil recorded (4.01 and 208 mg kg^{-1} soil) for winter 2012 season, respectively (Table 4).

Results indicated that highest relative increased of N content was in soil planted with New Valley compared other cultivars, while the soil planting with Ismaelia cultivar showed the highest increase of P and K content in soil compared to other cultivars. Therefore, the effect of long period of alfalfa planting for improving soil fertility is considerably significant in salt-affected soil. N content in the soil increased due to N_2 fixation by alfalfa roots. These results agreed to those of Xue *et al*. (2010) who noticed that the available N content in the soil decreased in the first year of cultivation. Such a result could be attributed to the uptake of nutrients from the soil in the early growth stages of alfalfa, as well as decrease of N fixation under the high level soil salinity. With the growth and development of alfalfa, its capacity for N fixation enhances; furthermore, due to decomposition and turnover of root residues, total N content in the soil increases with the extension of cultivation time. The effects of different periods of planting all alfalfa cultivars on available P content in saline soil indicated an increase in P content with decreasing soil pH and soil salinity. These results are in agreement with Jing *et al*. (2012) who reported that the effect of successive alfalfa planting for an improvement of soil fertility over the years is considerably significant in salt-affected soils. The general tendency was that EC and salt ions gradually declined yearly, and soil organic matter, N storage and P availability significantly increased with the extension of cultivating ages as compared to the no-planted soils.

b) Concentration of macronutrients in alfalfa plant tissues

Data presented in Table (4) show that the macronutrients (N, P and K) concentration in plants per alfalfa cultivars, after cutting under three soil salinity conditions, were significantly ($p< 0.05$) increased by increasing time periods of planting alfalfa. The N, P and K concentration (%) on plants of the three studied cultivars of alfalfa were clear by increased with the decrease of soil salinity level. Such increase was found in long planting periods. The Mean values content of N concentration (3.81 %) in the winter 2012 for New

Valley Cultivar was higher than other cultivars. The highest mean values of P concentration (0.48 %) of alfalfa for New Valley cultivar during spring 2012 period planting compared with other cultivars. On the other hand, the mean values of K concentration (2.77 %) of dry matter of alfalfa for New Valley cultivar during spring 2012 planting period compared with other cultivars. Also the mean values of N, P and K concentrations in alfalfa New Valley cultivar were higher than that of all cultivars. These results may be attributed to improving soil properties and increasing available nutrients contents by growing alfalfa.

The relative increases of mean values of N, P and K contents in soil after alfalfa cutting for N in soil planting of Ismaelia, New Valley and Siwa cultivars were 43.44, 49.17, and 32.00 %, respectively, compared with N initial mean in soil (37 mg/kg). Also, the corresponding relative increases of mean values of available P content in soil were 15.77, 6.23 and 5.30 % Ismaelia, New Valley and Siwa cultivars, respectively compared with the initial (4.61mg kg^{-1}) . The relative increases of mean values of K content in soil were 11.67, 8.00 and 6.22% for Ismaelia, New Valley and Siwa cultivars, respectively (Table 4).

Across salinity levels, N content in plant tissue recorded 19.35 % in New Valley and 9.35 % in Siwa cultivars compared with Ismaelia cultivar (P content was 41.37 % in Ismaelia and 48.27 % in New Valley cultivars compared with Siwa cultivar. K content recorded 1.2 % in Ismaelia and 4.8 % in New Valley cultivars compared with Siwa cultivar (Table 4).

These results are in agreement with those of Jing *et al*. (2012) who reported that alfalfa caused a variable degree of soil nutrients, which was influenced by the development stage and physiology change of the root system as well as the degree of P utilization by the root system at different soil layers. Khorshidi *et al*. (2009) found that increasing salinity decreased Ca^{2+}, K^{+} and N concentration but increased significantly Mg^{2+}, P, Na^{+} and Cl^{-}. Tolerant alfalfa cultivars can absorb more K^{+} and Ca^{2+} ions under saline conditions and prevent Na^{+} absorption and then increase K^{+}/Na^{+} and Ca^{2+}/Na^{+} ratios.

Table 4. Macronutrient contents in soil just before alfalfa cutting ($mgkg^{-1}$) and its concentration (%) in alfalfa tissues after cutting

Cultivars	Salinity levels EC (dSm^{-1})	In soil						In plant tissues					
		Summer	Autumn	Winter	Spring	Summer	Mean	Summer	Autumn	Winter	Spring	Summer	Mean
		N ($mgkg^{-1}$)						N %					
Ismaelia	8.73	46.16	50.13	53.03	55.33	52.12	51.35	2.88	3.25	2.95	3.31	2.70	3.02
	10.63	45.29	49.07	51.20	53.41	51.20	50.03	2.81	3.10	2.89	3.25	2.65	2.94
	15.31	38.14	42.95	46.77	48.86	47.86	44.92	3.13	3.57	3.21	4.02	2.85	3.36
	Mean	43.20	47.38	50.33	52.53	50.39	48.77	2.94	3.31	3.02	3.53	2.73	3.10
New Valley	8.73	40.09	45.28	48.18	51.91	48.77	46.85	3.68	3.62	3.75	3.60	3.56	3.64
	10.63	46.28	51.53	53.02	55.34	52.27	51.69	3.72	3.69	3.81	3.68	3.66	3.71
	15.31	49.10	53.17	55.31	57.22	53.32	53.62	3.77	3.72	3.86	3.73	3.70	3.76
	Mean	45.16	49.99	52.17	54.82	51.45	50.72	3.72	3.68	3.81	3.67	3.64	3.70
Siwa	8.73	52.47	54.74	57.30	59.30	53.41	55.44	3.42	3.55	3.64	3.51	3.39	3.50
	10.63	48.29	52.34	54.46	56.35	50.34	52.36	3.35	3.41	3.49	3.42	3.26	3.39
	15.31	44.46	48.70	52.81	54.70	51.85	50.50	3.28	3.32	3.36	3.30	3.22	3.30
	Mean	48.41	51.93	54.86	56.78	51.87	52.77	3.35	3.43	3.50	3.41	3.29	3.39
LSD. (0.05)		n.s.	1.153	1.151	n.s.	0.33	-	0.91	0.85	0.83	0.48	1.03	-
		P ($mgkg^{-1}$)						P %					
Ismaelia	8.73	3.94	3.95	3.98	3.91	3.87	3.93	0.41	0.45	0.48	0.45	0.40	0.44
	10.63	3.89	3.91	3.97	3.87	3.85	3.90	0.38	0.43	0.45	0.42	0.39	0.41
	15.31	3.79	3.85	3.94	3.82	3.81	3.84	0.35	0.41	0.42	0.40	0.36	0.39
	Mean	3.87	3.90	3.96	3.87	3.84	3.89	0.38	0.43	0.45	0.42	0.38	0.41
New Valley	8.73	3.96	3.98	4.00	3.94	3.91	3.96	0.38	0.49	0.43	0.51	0.43	0.45
	10.63	3.93	3.96	3.97	3.92	3.89	3.93	0.36	0.39	0.47	0.48	0.35	0.41
	15.31	3.82	3.87	3.93	3.88	3.86	3.87	0.39	0.44	0.42	0.46	0.38	0.42
	Mean	3.90	3.94	3.97	3.91	3.89	3.92	0.38	0.44	0.44	0.48	0.39	0.43
Siwa	8.73	3.99	4.02	4.05	4.01	3.97	4.01	0.28	0.26	0.28	0.32	0.27	0.28
	10.63	3.97	3.99	4.02	3.99	3.95	3.98	0.26	0.30	0.30	0.33	0.23	0.28
	15.31	3.89	3.92	3.96	3.95	3.90	3.92	0.24	0.28	0.32	0.36	0.25	0.29
	Mean	3.95	3.98	4.01	3.98	3.94	3.97	0.26	0.28	0.30	0.34	0.25	0.29
LSD. (0.05)		0.570	1.153	1.150	n.s.	n.s.	-	0.079	0.082	0.120	0.082	0.068	-
		K ($mgkg^{-1}$)						K %					
Ismaelia	8.73	198	205	209	205	202	204	2.50	2.63	2.39	2.60	2.36	2.50
	10.63	195	201	206	203	201	201	2.44	2.65	2.40	2.69	2.41	2.52
	15.31	189	197	203	201	199	198	2.56	2.74	2.52	2.72	2.31	2.57
	Mean	194	201	206	203	201	201	2.50	2.67	2.44	2.67	2.36	2.53
New Valley	8.73	205	207	209	207	206	207	2.45	2.65	2.43	2.73	2.51	2.55
	10.63	202	203	206	204	202	203	2.63	2.72	2.51	2.74	2.49	2.62
	15.31	195	199	204	202	201	200	2.59	2.81	2.63	2.83	2.43	2.66
	Mean	201	203	206	204	203	203	2.56	2.73	2.52	2.77	2.48	2.61
Siwa	8.73	208	209	210	206	205	208	2.50	2.52	2.56	2.58	2.51	2.53
	10.63	206	207	209	205	203	206	2.48	2.46	2.53	2.56	2.48	2.50
	15.31	200	203	205	202	200	202	2.43	2.45	2.52	2.54	2.43	2.47
	Mean	205	206	208	204	203	205	2.47	2.48	2.54	2.58	2.47	2.50
LSD. (0.05)		n.s.	6.50	5.00	1.22	2.59	-	0.83	0.87	0.72	0.85	n.s.	-

Micronutrients content

a) In soil

Available micronutrient contents (Fe, Mn and Zn mg kg^{-1}) in salinity affected soil under alfalfa growing are presented in Table (5). Results from the three levels of soil salinity grown with three alfalfa cultivars after two growing seasons, indicated low changes in micronutrient contents were obtained. Micronutrients (Fe, Mn and Zn) contents in soil under three levels of salinity were significantly increased with increase in duration period. The highest mean values of Fe Mn and Zn contents in soil were 2.07, 6.50 and 0.98 mg kg^{-1}, respectively. The increase of micronutrients content in soil depended on the decrease of soil pH and EC (dSm^{-1}) and increase of growing
duration of alfalfa. These results are agreement by Johan *et al*. (2012). Availability of soil nutrients is determined by the form and chemical properties of the element, the soil pH, and interactions with soil colloids, microbial activity and soil physical conditions such as aeration, compaction, temperature, and moisture. On the other hand, the increase of microelements in soils depends on long duration of growing period. The distribution pattern of available Fe, Mn and Zn in soil may be due to the increase of soil organic matter in the surface layer. These results are in agreement with those obtained by Shaban (2005), who found that increasing in upper soil layer microelements were depending on long time of leached periods after rice and wheat. The distribution pattern of available Fe, Mn, Zn, B and Pb in soil may be due to the increase of soil organic matter in the surface layers.

Also, the relative increases of Fe content in soil as affected by different alfalfa cultivars under three levels of soil salinity were 8.69, 7.81 and 3.59% for Ismaelia, New Valley and Siwa cultivars, compared with initial soil salinity, respectively. Moreover, the relative increases of Mn content in soil compared with initial soil salinity were 3.35, 3.01 and 2.20 % for planting by Ismaelia, New Valley and Siwa cultivars, respectively. On the other hand, the relative increases of Zn content in soil were 9.41 %, 7.95 % and 8.90 % for Ismaelia, New Valley and Siwa cultivars compared with initial soil salinity, respectively (Table 5). It is worthy to mention that in this study contents of available microelements, in general, lay within the sufficient limits of Fe, Mn and Zn (FAO, 1992).

b) Concentration of micronutrients in alfalfa tissues.

Micronutrients concentrations in plant tissues of different alfalfa cultivars under levels of soil salinity are presented in Table (5). Data showed that the amounts of Fe, Mn and Zn in tissues were increased over all alfalfa cultivars by increasing alfalfa growing duration. The highest mean values of Fe concentration was in Ismaelia cultivar tissues (82.69 mg kg^{-1}) for spring season compared with other treatments. However, Mn content was maximum (61.51mg kg^{-1}) in New Valley cultivar during winter season. On the other hand, the best mean values of Zn concentration was 38.24 mg kg^{-1} for Siwa cultivar in winter season, compared with other cultivars. Concentrations of Fe, and Mn in alfalfa tissues across cultivars were maximum in winter and spring seasons, while the Zn concentration in plant tissues was insignificant in summer 2012. Also, data recorded increasing of Fe, Mn and Zn concentrations in alfalfa tissues across all cultivars by decreasing soil salinity. These results are in agreement with those of Wang and Han (2007), who reported that, salinity reduced Cu uptake and concentrations in alfalfa tissues but significantly increased the Zn content in the roots, shoots and leaves. Moreover, soil salinity significantly increased uptake and concentration of Mn in the shoots and leaves of alfalfa plants. Murat *et al*. (2011) found that the salinity treatment caused significant increases in the Fe content of the leaves, shoots and roots across all cultivars.

From the previous results, it could be concluded that planting alfalfa under saline soil conditions in the newly reclaimed areas is very important to improve soil fertility. Also, the results showed that New Valley and Siwa cultivars had a significant positive effect on soil fertility.

Plant evaluation

1-Plant establishment

Seed germination is highly affected by salinity levels, seedling growth and plant recovery after cutting. Figure (5) show that the percentages of plants establishing gradually decreased by increasing in salinity level. Although tolerance at the germination and seedling development stages is highly desirable at high salinity levels, tolerance of the resultant adult plants is of equivalent importance. Siwa cultivar showed the best stand plants across all salinity levels followed by Ismaelia cultivar. The New Valley cultivar was the most sensitive with high salinity levels. Natural selection plays an important role in our study.

The stand of residual plants was amazing and incredible vigorous after the fourth cut; they were getting more tillers, branches and sub branches. Soil salinity may affect the germination of seeds either by creating low osmotic potential to the seeds preventing water uptake or through the toxic effects of Na+ and Cl- ions on germinating seed (Khaje-Hosseini *et al.*, 2003; Atak *et al*., 2006; Kaya *et al*., 2006 and Golbashy *et al.*, 2010).

Table 5. Micronutrient contents in soil just before alfalfa cutting (mgkg^{-1})and its concentration (%) in alfalfa tissues after cutting.

Cultivars	Salinity levels EC (dSm^{-1})	In soil						In plant tissues					
		Summer	Autumn	Winter	Spring	Summer	Mean	Summer	Autumn	Winter	Spring	Summer	Mean
		Fe (mgkg^{-1})						Fe %					
Ismaelia	8.73	57.19	62.11	74.10	81.00	70.14	68.91	2.03	2.05	2.08	2.12	1.99	2.05
	10.63	64.24	69.10	75.37	83.02	72.29	72.80	1.97	1.96	2.02	2.04	1.97	1.99
	15.31	62.33	63.25	77.21	84.05	66.34	70.64	1.91	1.94	1.98	1.99	1.95	1.95
	Mean	61.25	64.82	75.56	82.69	69.59	70.78	1.97	1.98	2.03	2.05	1.97	2.00
New Valley	8.73	74.29	75.00	75.07	77.22	62.08	72.73	2.01	2.07	2.09	2.08	1.98	2.23
	10.63	65.17	63.06	70.36	74.26	58.14	66.20	1.99	2.06	2.08	1.96	1.95	2.01
	15.31	60.08	68.22	66.20	72.11	63.32	65.99	1.94	2.03	2.06	1.95	1.93	1.98
	Mean	66.51	68.76	70.54	74.53	61.18	68.31	1.98	2.05	2.08	1.99	1.95	2.07
Siwa	8.73	64.19	68.04	77.22	79.10	63.33	70.38	2.03	2.04	2.07	2.07	2.01	2.04
	10.63	59.42	63.41	72.18	76.43	66.20	67.53	1.98	1.99	2.05	2.06	1.99	2.01
	15.31	62.31	67.63	66.15	71.11	62.38	65.92	1.96	1.98	2.02	2.03	1.98	1.99
	Mean	61.97	66.36	71.85	75.55	63.97	67.94	1.99	2.00	2.05	2.05	1.99	2.02
LSD. (0.05)		8.34	n.s.	6.01	n.s.	5.38	-	n.s.	0.011	1.15	n.s.	0.015	-
		Mn (mgkg^{-1})						Mn %					
Ismaelia	8.73	44.36	48.41	52.25	47.38	46.22	47.72	6.50	6.51	6.54	6.57	6.47	6.52
	10.63	40.20	46.18	49.00	45.43	43.01	44.76	6.46	6.48	6.52	6.56	6.43	6.49
	15.31	43.19	44.22	47.06	44.50	38.23	43.44	6.35	6.39	6.44	6.47	6.40	6.47
Mean		42.58	46.27	49.44	45.77	42.49	45.31	6.44	6.46	6.50	6.53	6.43	6.47
New Valley	8.73	52.00	54.23	64.18	59.06	42.17	54.33	6.47	6.51	6.56	6.56	6.57	6.53
	10.63	49.19	52.28	62.13	57.41	38.26	51.85	6.44	6.48	6.53	6.53	6.54	6.50
	15.31	46.15	49.30	58.22	56.23	36.11	49.20	6.39	6.43	6.48	6.51	6.49	6.46
	Mean	49.11	51.94	61.51	57.57	38.85	51.79	6.43	6.47	6.52	6.53	6.53	6.50
Siwa	8.73	49.33	53.22	58.36	55.09	45.27	52.25	6.48	6.53	6.57	6.58	6.49	6.53
	10.63	46.52	47.35	56.25	52.00	43.36	49.10	6.46	6.51	6.55	6.55	6.47	6.51
	15.31	43.05	42.39	50.10	47.26	41.24	44.81	6.41	6.45	6.52	6.50	6.45	6.47
	Mean	46.30	47.65	54.90	51.45	43.29	48.72	6.45	6.50	6.55	6.54	6.47	6.50
LSD.(0.05)		-	8.15	n.s.	5.23	7.95	8.11	0.57	0.86	0.91	0.75	n.s.	1.88
		Zn (mgkg^{-1})						Zn %					
Ismaelia	8.73	21.71	23.69	26.59	28.81	23.38	24.84	0.93	0.95	0.99	0.96	0.93	0.95
	10.63	22.96	21.45	23.70	24.46	19.17	22.35	0.90	0.93	0.98	0.94	0.92	0.93
	15.31	18.68	19.20	20.24	22.20	17.09	19.48	0.89	0.92	0.94	0.92	0.90	0.91
	Mean	21.12	21.45	23.51	25.16	19.88	22.22	0.91	0.93	0.97	0.94	0.92	0.93
New Valley	8.73	33.29	35.18	37.66	39.59	35.28	36.20	0.93	0.97	0.99	0.98	0.95	0.96
	10.63	28.60	34.41	35.54	37.14	29.34	33.01	0.92	0.96	0.98	0.96	0.94	0.95
	15.31	24.52	31.33	33.18	32.56	27.55	29.83	0.88	0.94	0.96	0.95	0.91	0.93
	Mean	28.80	33.64	35.46	36.43	30.72	33.01	0.91	0.96	0.98	0.96	0.93	0.95
Siwa	8.73	35.38	37.65	40.25	38.10	37.83	37.84	0.97	0.99	1.04	1.03	0.98	1.00
	10.63	30.52	33.28	38.77	35.09	31.75	33.88	0.95	0.97	1.02	1.00	0.97	0.98
	15.31	27.33	32.67	35.69	34.02	28.71	31.68	0.93	0.96	0.98	0.99	0.94	0.96
	Mean	31.08	34.53	38.24	35.74	32.76	34.47	0.95	0.97	1.01	1.01	0.96	0.98
LSD. (0.05)		2.41	7.15	8.43	3.48	n.s.	-	n.s.	0.021	0.026	0.022	0.031	-

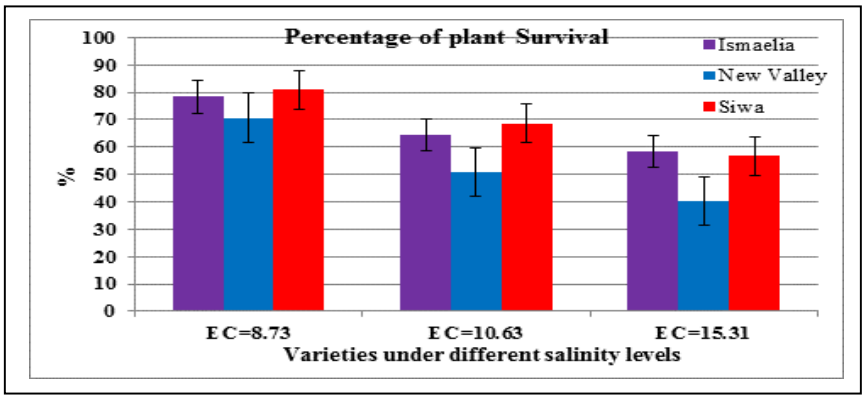


Figure 5. Percentages of plant survival (percentages±SE) of three alfalfa cultivars sown under three levels of salinity after 65-day from sowing.

2-Forage and dry yield

The analysis of variances of forage fresh and dry yield with three local cultivars, under three salinity levels and their interaction. The focus of forage fresh and dry yield were significantly (P<0.01) affected by salinity levels, cultivars and their interaction except for spring 2011 season, where differences between cultivars

were significant. Salinity levels recorded highly significant differences in fresh and dry yield across all seasons, whereas insignificant affected of fresh forage yield in spring and summer 2011. Fresh forage and dry yield analysis indicated highly significant differences ($P<0.01$) for all seasons except for spring 2011 season across cultivars. Which reversed any difference in dry yield interaction effects in spring 2011 season and for fresh forage yield in summer 2011season.

Table (6) shows forage fresh and dry yield means (t.fed^{-1}) at different growing seasons. Summer (2012) season recorded the best means of forage fresh and dry yield for all studied seasons. Siwa cultivar was superior yielding cultivar over all seasons and salinity levels. Also, it recorded the best average of total fresh forage yield (55.91, 53.17 and 50.09 t fed^{-1}) and total dry forage yield (13.57, 12.45 and 11.77 t fed^{-1}) for the three levels of salinity (EC's = 8.73, 10,63 and 15.31 dSm^{-1}), respectively (Table 6 and Figure 6). Ismaelia cultivar followed Siwa cultivar in total forage fresh and dry yield with an average fresh yield of 52.07, 47.03 and 42.38t fed^{-1} and dry yield of 12.93, 11.23 and 9.38 t fed^{-1}, respectively. New Valley cultivar was the lowest yielding among cultivars, and thus it was the most sensitive cultivar in this study to higher salinity levels. Summer (2012) season recorded the highest mean of forage fresh and dry yield (t fed^{-1}), this may be due to temperature effects.

High phenotypic variation between genotypes was found in the second and third levels of salinity (10.73 and 15.31 dSm^{-1}). Monirifar *et al*. (2004) reported the presence of phenotypic variation between some alfalfa cultivars at different salinity levels expressed in forage yield. Epstein *et al*. (1980) and Shannon (1984) reported that the development of salt tolerance in crops depends on the availability of genetic variation by screening and selection of those plants with superior performance. Our results were in agreement with the phenotypic variation of alfalfa cultivars for salt tolerance reported by Al-Khatib *et al*. (1993) and Noble *et al*. (1984).

Dry yield was increased according to the decrease of salinity levels across the three studied cultivars of alfalfa. Such increase was found in long duration period of plant standing. Ismaelia cultivar recorded high dry forage yield (t fed^{-1}) in summer 2012 season under 8.73 and 10.63 dSm^{-1} salinity levels. New Valley cultivar showed the best forage dry yield (t fed^{-1}) in winter (2011-2012) under 8.73 dSm^{-1} level of salinity and in summer 2012 season under 10.63 dSm^{-1} level of salinity. Siwa cultivar was the best forage yield performances for all growing seasons and salinity levels (Figure 6 and Table 6).

Table 6. Means of forage fresh and dry yield (t fed^{-1}) for natural selected alfalfa population through different seasons.

Populations	Salinity levels Ec (dSm^{-1})	Spring (2011) - 1 cut		Summer (2011) -3 Cuts		Autumn (2011) - 3 Cuts		Winter (2011/12) - 3 Cuts		Spring (2012) - 3 Cuts		Summer (2012) - 2 Cuts		Total	
		Fresh Yield	Dry Yield	Fresh Yield	Dry Yield	Fresh Yield	Dry Yield	Fresh Yield	Dry Yield	Fresh Yield	Dry Yield	Fresh Yield	Dry Yield	Fresh Yield	Dry Yield
Ismaelia	8.73	4.467	0.853	7.689	2.304	8.044	2.131	9.333	2.524	9.644	2.286	10.889	2.737	52.07	12.93
	10.63	3.233	0.580	6.089	1.973	7.178	1.840	8.100	2.150	8.933	2.062	10.000	2.291	47.03	11.23
	15.31	3.133	0.478	5.711	1.371	5.533	1.167	6.533	2.045	7.867	1.936	9.200	2.085	42.38	9.38
Mean		3.611	0.637	6.496	1.883	6.918	1.713	7.989	2.240	8.815	2.095	10.030	2.371	47.160	11.180
New Valley	8.73	3.933	0.680	7.689	2.203	5.956	1.748	7.917	2.636	9.467	2.340	9.822	2.377	46.92	12.20
	10.63	2.433	0.387	6.867	2.005	5.244	1.507	6.750	1.850	7.022	1.362	8.978	2.576	42.19	10.09
	15.31	2.000	0.312	4.400	1.712	5.178	1.289	6.000	1.708	5.711	1.148	6.844	1.938	32.87	8.070
Mean		2.789	0.460	6.319	1.973	5.459	1.515	6.889	2.065	7.400	1.617	8.548	2.297	40.660	10.120
Siwa	8.73	5.233	0.921	8.978	2.866	8.733	1.716	9.550	2.836	11.156	2.468	10.889	2.671	55.91	13.57
	10.63	3.560	0.606	8.578	2.546	8.667	1.927	9.033	2.557	9.378	2.382	10.044	2.119	53.17	12.45
	15.31	3.200	0.433	7.711	2.412	7.511	1.852	8.533	2.622	9.089	2.018	10.044	2.104	50.09	11.77
Mean		4.031	0.653	8.422	2.608	8.304	1.832	9.039	2.672	9.874	2.289	10.326	2.298	53.057	12.597
Grand mean		3.478	0.573	7.035	2.155	6.894	1.686	7.972	2.325	9.052	2.111	9.635	2.322	45.19	11.39
LSD (0.05)	Salinity	1.789	0.290	2.780	1.138	1.890	0.433	2.059	0.631	1.98	0.650	2.97	1.140	1.801	0.290
	Cultivars	0.449	0.049	1.350	0.679	0.499	0.111	0.981	0.220	1.00	0.260	0.822	0.240	0.450	0.049
	Interaction	0.770	0.185	2.340	0.389	0.861	0. 201	1.360	0.390	1.731	0. 442	1.420	0.411	0.771	0.098

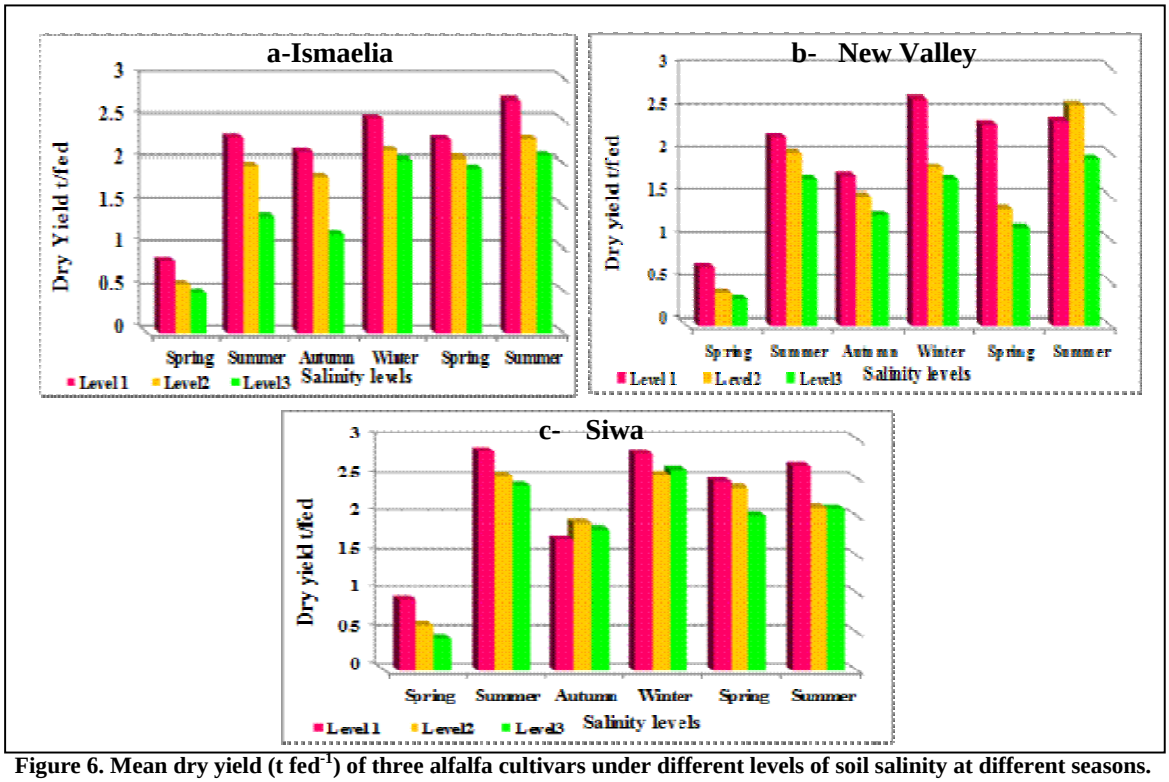


Figure 6. Mean dry yield (t fed^{-1}) of three alfalfa cultivars under different levels of soil salinity at different seasons.

3-Seed Characters:

Data showed the studied seed characters showed significant differences (P<0.05) among salinity stress levels. Cultivars had significant differences for all studied characters except for thousand seed weight g^{-1}. Variance due the interaction between salinity and cultivar was significant for No. of seed g^{-1}, No. of default seeds g^{-1}and seed weight kg fed^{-1}.

The highest weight of of thousand seed weight was recorded with EC= 8.73 dSm^{-1} (2.31, 2.21` and 2.28) for Ismaelia, Siwa and New Valley cultivars, respectively (Table 7). Siwa cultivar had the greatest weight of thousand seed (1.86 g) at (EC= 15.31) level of salinity, therefore it was the highest seed number g^{-1} (491.3, 358.3 and 370.0 in salinity levels (EC= 8.73, 10.63 and 15.31 dSm^{-1}, respectively). New Valley cultivar recorded the highest number of default seeds g^{-1} at the three levels of salinity (97.3, 112. 7 and 124.0, respectively). Siwa cultivar was superior in both of weight seeds (g) m^{-2} and (kg) fed^{-1}over all levels of salinity (74.13 g and 281.7 kg) in EC= 8.73 dSm^{-1}, (58.57g and 222.5 kg) in EC= 10.63 dSm^{-1} and (55.37g and 210.4 kg at EC=15.31 dSm^{-1}), respectively. Ismaelia cultivar behaved similar to Siwa cultivar for the three salinity levels. However, New Valley cultivar recorded the lowest means of seed characters and was superior in weight of seeds (g) m^{-2} and (kg) fed^{-1} at the high level of salinity EC= 15.31 dSm^{-1} (Table 7).

Table 7. Means of seed characters of three alfalfa cultivars across three salinity levels.

Cultivars	Salinity Levels (dSm^{-1})	1000 seed weight (g^{-1})	No. seed (g^{-1})	No. default seeds (g^{-1})	Seed weight (g m^{-2})	Seed weight (kg fed^{-1})
Ismaelia	8.73	2.31	473.3	77.33	70.50	267.9
	10.63	2.11	351.3	94.00	49.80	189.2
	15.31	1.637	351.7	94.00	37.87	143. 9
Mean		2.02	392.1	88.44	52.72	200. 4
New Valley	8.73	2.21	373.0	97.33	60.00	228.8
	10.63	1.67	348.3	112.67	45.50	172.9
	15.31	1.06	292. 7	124.00	40.60	154.3
Mean		1.64	338.0	111.33	48.70	185.3
Siwa	8.73	2.28	491.3	50.67	74.13	281.7
	10.63	2.28	358.3	63.33	58.57	222.5
	15.31	1.86	370.0	98.67	55.37	210.4
Mean		2.142	406.5	70.89	62.69	238.2
Grand mean		1.93	378. 9	90.22	54.72	208.0
L.S.D (0.05)	Salinity	0.280	95.00	62.38	20.72	78.74
	Cultivars	n.s.	32.99	19.79	4.140	15.73
	Interactions	n.s.	57.14	34.29	n.s.	27.25

n.s.= insignificant

Table 8. Means of individual plant shoot and root characters as affected by cultivars and salinity across growing seasons.

Cultivar	Salinity levels dSm^{-1}	Shoot characters $plant^{-1}$				Root characters $plant^{-1}$		
		Plant height (cm)	Stem diameter (cm)	No. tillers	Fresh weight (g)	Length (cm)	Diameter (cm)	Weight (g)
Ismaelia	8.73	52.31	0.43	16.29	75.40	27.97	2.91	28.10
	10.63	55.95	0.33	14.49	75.01	29.81	2.45	30.49
	15.31	53.85	0.32	11.75	58.07	27.48	1.97	23.49
Mean		54.04	0.356	14.18	69.49	28.42	2.81	27.36
New Valley	8.73	55.00	0.37	16.79	71.45	30.28	2.77	27.53
	10.63	57.11	0.34	14.11	70.97	28.92	2.48	26.00
	15.31	52.80	0.32	11.33	54.99	28.98	1.35	19.03
Mean		54.96	0.342	14.08	65.80	29.39	1.83	24.19
Siwa	8.73	55.25	0.36	18.19	83.35	30.71	2.72	34.64
	10.63	57.76	0.35	13.24	66.98	29.36	2.49	25.37
	15.31	56.61	0.34	12.53	63.61	29.35	2.33	24.14
Mean		56.54	0.439	14.35	71.31 A	29.81	2.53	28.05
Grand mean		**55.18**	**0.38**	**13.97**	**68.87**	**29.21**	**2.72**	**26.53**
L.S.D (0.05)	Cultivars	5.11	0.13	1.67	12.28	n.s.	n.s.	n.s.
	Salinity	n.s.	n.s.	0.53	19.30	n.s.	n.s.	n.s.
	Interactions	n.s.	n.s.	1.64	21.14	n.s.	n.s.	6.09

n.s.= insignificant differences

Selection of highly salinity tolerant genotypes between and within open pollinated cultivars could be expected to provide useful material for further breeding, and for experimental comparisons (Al-Khatib *et al.*, 1993). Selected seeds were blend after harvesting to contain new developed alfalfa population (Sina-1) was extra tolerant and adaptable to saline soils in Sahl El-Tina conditions. Now developed population (Sina-1) under evaluation with their parents by the same levels of salinity in Sahl El-Tina conditions

Plant shoot and root characters

Although high salinity levels reduced shoot growth of all cultivats, the magnitude of the reduction varied among them. Data recorded significant differences ($p<0.01$) for plant height, No. tillers, plant fresh weight of all cultivars and ($p< 0.05$) for stem diameter. Root characters recorded insignificant differences over cultivars, salinity levels and their interactions among growing seasons except for root weight indicated significant differences ($p<0.01$) for the interaction between cultivar and salinity levels dSm^{-1}. Levels of salinity dSm^{-1} recorded significant differences ($p= 0.01$) for No. tillers and fresh weight $plant^{-1}$ across growing seasons.

Results presented in Table (8) indicated that Siwa cultivar was superior to Ismaelia and New Valley in most of studied shoot and root characters when estimated across the three salinity levels. However, root length and diameter insignificantly affected number of tillers. Plant fresh weight (g) was significantly affected by salinity levels. The high salinity (EC= 15.31dSm^{-1}) reduced tillers number $plant^{-1}$ and fresh weight (g) (11.75, 11.33, 12.53 tillers $plant^{-1}$ and 58.07, 54.99, 63.61g $plant^{-1}$ for Ismaelia, New Valley and Siwa cultivars, respectively (Table 8). Means of studied characters were significantly reduced as salinity levels were increased. The same trend was observed for root fresh weight (g). These results were in agreement with those reported by Monirifar and Barghi (2009).

Figure 7. Shows alfalfa plants growing under salt soils in: a) spring 2011 season after 45 from sowing, b-3^{ed} cut in summer 2011 season , c) 8th cut in winter 2012 season , d) 12th cut in spring 2012 season and e) 15 th cut in summer 2012 season.

Conclusions:

Three alfalfa cultivars were evaluated through three levels of salinity. Comparison of screening is difficult due to the fact that field conditions are extremely difficult because of the high salinity concentration and the limited layer of growing soil (30-50 cm) in Sahl El-Tina soil conditions.

At different duration of planting, alfalfa caused a variable degree of soil salinity amelioration. The general tendency was that EC and salt ions gradually declined yearly, and soil organic matter, N storage and P availability significantly increased with the extension of growing periods as compared to non-planted alfalfa. The schedule of 15 cuts of reclamation via alfalfa growing achieved and maintained a good soil quality with adequate structure and nutrient availability status, but the change of solute ion, pH, organic matter, total N and available P in the soil profile presented a spatial and temporal variability due to its being affected by the interaction between soil and plant.

Evaluation at the germination stage is absolutely critical in developing cultivars that can establish a good stand. This is the key starting point to the identification of salt tolerant genotypes. Natural selection through successive cutting with selection for high yield and vigour plants under salinity stress is an excellent method to screen large sets of salt tolerant genotypes and get an idea for regrowth potential under high salinity levels. This study has reaffirmed the difficulty in correlating different screening procedures for salt tolerance in alfalfa. The importance of researching, developing and marketing salt tolerant alfalfa cultivars

is substantial, especially when we consider the economic advantages to growers in salt affected regions. Siwa cultivar had the top yielding ability, comparing to Ismaelia and New Valley under Sahl El-Tina conditions.

1- It was possible in this study to develop a high tolerant composite population may be considered as a new cultivar available to grown in salt affected regions in Egypt.
2- This study raised the idea of role of sowing alfalfa in saline soils for increasing the chance to improvement of soil physical and chemical properties and thus soil fertility.
3- New developed alfalfa population (Sina-1) was resulted from this study; it was the most tolerant and adaptable to saline soils in Sahl El-Tina conditions.

Corrersponding author
Zeinab M. Abd El-Naby
Forage Res. Dept., Field Crop Res. Inst., Agric. Res. Center, Giza, Egypt
zeina_bree@yahoo.com

References

Al-Khatib, M., T. McNeilly and J.C. Collins (1993). The potential of selection and breeding for improved salt tolerance in Lucerne (*Medicago sativa* L.). Euphytica, 65:43-51.

Atak M, M.D. Kaya, G. Kaya, Y. Çıkılı and C.Y. Çiftçi (2006). Effects of NaCl on the germination, seedling growth and water uptake of triticale. Turk. J. Agric. For. 30: 39-47.

Black, C. A. (Editor**) (1965).** Methods of Soil analysis . Soil Science Society of American Inc ublisher, Madison, Wisconsin, U.S.A

Chapman, H.D. and F.P. Pratt (1961). Ammonium vandate-molybdate method for determination of phosphorus. In: Methods of analysis for soils, plants and water. 1st Ed. California: California University, Agriculture Division, pp: 184-203.

Cottenie, A.; verloo, M.; Velghe, G. and Cameriynck, R. (1982) "Chemical Analysis of plant and soil." Laboratory of analytical and Agrochemistry, State Univ., Ghent , Belgium.

Der, G., and B. S. Everitt, (2008). *A handbook of statistical analyses using SAS, third edition.* SAS Publishing. Cary, NC, USA.

El-Nahrawy, M.A., L. A. Hanna, E.Elbrougy, H.Yousri, and O. Niemelainen (1995). Quality parameters of Egyptian clover seed lots used on newly reclaimed area in Egypt. Proceeding of 3rd International Herbage Seed Conference. Halle (Saale.), Germany, June 18 – 23. : p 33- 34.

Epstein, E. and J. J. Norlyn, G. W. Rush, R. W. Kingsbury, D. W. Kelly, G. A. Cunningham and A. F. Wrona (1980). Saline culture of crops: a genetic approach. Science. 210:399-404.

Fan, Z. L., J.Y. Ma, Y.J. Ma, (2002) Assessment and prediction of developing trend of soil salinization of the cultivated land in west china, Arid Land Geography, 2 (25): 97–101.

FAO, (1992). Waste water treatment and use in agriculture. FAO Soils Bull. No.47, Rome.

Grattan , S. R. and C.M. Grieve, (1999). Salinity – mineral nutrient relations in horticultural crops. J. Scientia Horticulturae (78): 127 – 157.

Golbashy, M., S. K. Khorasani, M. Ebrahimi and R. Choukan (2010). Study of response of corn hybrids to limited irrigation. 11th Iranian Crop Science Congress Tehran, 24-26 July. University of Shahid Beheshti, Iran, p. 218.

Gomez, K.A. and A.A. Gomez (1984). Statistical Procedures of Agricultural Research. Second Ed. Wielly inter Science Publ. John Wiley and Sons, New York, 357-423.

Hafez, A. (2005). Investigation of El-Salam Canal project in northern Sinai, Egypt**.** Phase I- : Environmental Baseline, soil and water quality studies**.** Ninth International Water Technology Conference, IWTC9, Sharm El-Sheikh, Egypt

Bauder, J.W. (2007). Fertilizing alfalfa forage **.** Extension Soil and Water Quality Montana state Univ Communication Service**,** Bazeman Mt page 8366.

Jing, C., L.Xianting, K.Xiaole, L.Rengel and D.Liping (2012). Using Alfalfa (*Medicago sativa*) to ameliorate salt- affected soils in Yingda irrigation district Northwest China. Ecological Society of China. (32): 68 – 73.

Jackson, M.L. (1973). Soil Chemical Analysis. Prentic Hall Inc., N.J., USA.

Johan , D.S., T. Calvin, and A. F. Mike (2012). Evaluating alfalfa (*Medicago sativa*, L) cultivars for salt tolerance using laboratory, greenhouse and field methods. J. Agric. Sci., 4 (6): 90 – 103.

Kaya, M.D., G. Okçu, M. Atak, Y. Çıkılı and O. Kolsarıcı (2006). Seed treatments to overcome salt and drought stress during germination in sunflower (*Helianthus annuus* L.). Eur. J. Agron., 24: 291-295.

Khaje-hosseini M, A.A. Powell and I.J. Bingham (2003). The interaction between salinity stress and seed vigour during germination of soybean seeds. Seed Sci. Technol., 31: 715-725.

Khorshidi, M. B., M.Yarnia and D.Hassanpanah (2009). Salinity effect on nutrients accumulation in alfalfa shoots in hydroponic condition. J. Food. Agric. and Envi., 7 (3and 4) : 787 – 790.

Lindsay, W.L. and Norvell, W.A. (1978). Development of DTPA test for zinc, iron, manganese and copper. Soil Sci. Soc. Am. J., 42: 421-429.

Longenecker, D.E. and P.J. Lyerly (1974). B-876 Control of Soluble Salts in Farming and Texas Agricultural Experiment Station, Texas A and M University System, College Gardening. Station. June. 36 pp.

Maas, E. V. and G. J. Hoffman (1977). Crop salt tolerance, current assessment. Journal of Irrigationand Drainage, 115.

Monirifar H. and M. Barghi (2009). Identification and selection for salt tolerance in alfalfa (*Medicago sativa* L.) ecotypes *via* physiological traits. Not. Sci. Biol., 1 (1) :63-66.

Monirifar, H., M. Valizadeh, R. Mohammadian, M. S. Abedi and A. O. Milani (2004). Variation for tolerance in five alfalfa cultivars. Azerbaijan International Meeting on Soil Fertility Land Management and Agroclimatology. Turkey, 2008. p: 709-713

Munns, R. (2005). Response of crops to salinity. In Abstracts, International Salinity Forum Proceedings, pp. 339, Riverside, CA. 25-27 Apr. 2005. Riverside, CA. USA.

Murat , T., T Ruveyde, Y.Sunyamin and C.Vahdettin (2011). Changes of micronutrients, dry weight and plant development in canola (*Brassica napus* L.) cultivars under salt stress. African Journal of Biotechnology. 10 (19) : 3726 – 3730.

Noble, C. L., G. M. Halloran and D. W. West (1984). Identification and selection for salt tolerance in Lucerne (*Medicago sative* L.). Aust. J. Agric. Res., 35: 239-252.

Page, A.I.; R.H. Miller and D.R. Keeney (1982). Methods of Soil Analysis, Part- 2.Chemical and Microbiological Properties.2nd (Ed.), Amer. Soc. of Agron., Madison, Wisconsin, U S A

Qadir, M., A.Tubeileh, J.Akhtar, A.Larbi, S.Minhas and M.A. Khan (2008). Productivity enhancement of salt-affected environments through crop diversification. Land Degradation and Development, 19: 429–453.

Rawlins, S. L. (1979). Irrigation to miminize salt problems. In Abstracts, 9th California Alfalfa Symposium Proceedings, Fresno, CA. pp. 68-71. [Online] http://alfalfa.ucdavis.edu/+symposium/proceedings/2005-237.pdf

Shaban, KH. A. (2005) " Effect of different irrigation water resources on properties and productivity of salt affected soils. Ph.D. Thesis , Fac .of Agric ., Monufiya University .Egypt.

Shaban, Kh, A. and A. A. El-Sherife (2007). Evaluation of alfalfa productivity and its response to bio-fertilizer, mineral nitrogen and sulphure under saline condition in newly reclaimed area**.** Egypt. J. Soil Sci., 47, (4): 347 -366.

Shaban, Kh . A. and M. N. Omar (2006). Imrovement of maize yield and some soil properties by using nitrogen mineral and PGPR group fertilization in newly cultivated saline soils. Egypt. J. Soil, Sci., 46 (3): 329 – 342.

Shanon, M. C. (1984). Breeding, selection and genetics of salt tolerance, pp.231- 254. In: Salt salinity tolerance in plants. (John Wiley and Sons Eds.), R.C. staples and G.H toenniessen.

Sheard , R.W. (2007). Fertilizer practices for alfalfa production**.** Published in Plant and fertilizer. Department Land Source Science, Univ. of Guelph, (2) : 1-5.

Tang, C., M.J. Unkovich and J.W. Bowden (1999). Factors affecting soil acidification under legumes III. Effects of nitrate supply. J. New Phytologist, 143: 513–521.

Waller, R.A. and D.B. Duncan (1969). A bays rule for the symmetric multiple comparison problem. Amer. Stat. Assoc. J., 64: 1485.

Wang X .S. and J .G. Han (2007). Effects of NaCl and silicon on ion distribution in the roots, shoots and leaves of two alfalfa cultivars with different salt tolerance. Soil Sci. Plant Nutr., 53(3): 278-285.

Xue, Z.Y., Z.Y.Zhou, Y.Y. Zhan and W.. Ren, (2010) Characteristics of phosphorus content in the rhizosphere under xeromorphic shrubs in arid deserts, Acta Ecologica Sinica, 30 (2): 341–349.

Zhang, X. Q. and U. G. Ming (2004). The effect of alfalfa on physical and chemical properties of saline soil. J. Pratacultural Science. (11):7 – 11.

Zhang, G., Z.Y. Zhou, C.P. Zhang (2007). The effect of land use on the levels of salt andorganic matter in saline soil, Acta Prataculturae Sinica, 16 (4): 15–20.

2/2/2013

Distinctive Features of the Professional Nursing Practice Environment As Perceived By Bachelor Nursing Students and Nurses at University of Dammam – Saudi Arabia

Dr. Sana A. Al-Mahmoud

University of Dammam – College of Nursing, Kingdom of Saudi Arabia
saalmahmoud@ud.edu.sa

Abstract: Although the Saudi Arabian society is considered to be protective, and a traditional society with many obstacles when woman employment in question; the nursing profession is considered a female-profession. In the kingdom of Saudi Arabia nursing is one of the fast growing fields. As more numbers of students enrolling for studying nursing, more emphasis is given to nursing as a career and during the score of the academic study. One aspect for studying the phenomenon of increasing Saudi female students in the field of nursing is a quantitative study of their perception of the practice environment of nursing. Expectations in general determine the amount of involvement and the amount of effort applied from the side of the employee (the nurse). This study, points upon the expectations of the nurses regarding the environment where they will practice their career. The study is an analysis of the responses provided by senior nursing students and the nurses doing their field training on an eight-parts questioner, The questionnaire collected the nurses' responses and feelings regarding; the philosophy of clinical care, nurses knowledge, promotion of lead nurses, nurses participation in decision making, clinical development, professional development, teams collaboration and technology utilization. On every part of the aforementioned aspects, a group of questions in the form of assuring sentence and five options starting from strongly agree to strongly disagree format was used. And the responses on the 39 questions were analyzed using SPSS. Generally positive attitude towards the practice environment of nursing and career was shown, however, in some specific points related to nursing practice the results showed some disagreement. The study assured that nursing is a favorable career path, because its environment offers positive opportunities, services and human interaction, technologies and chances for development and the quality of human relationships.

[Sana A. Al-Mahmoud. **Distinctive Features of the Professional Nursing Practice Environment As Perceived By Bachelor Nursing Students and Nurses at University of Dammam – Saudi Arabia.** *Life Sci J* 2013;10(1):2096-2106] (ISSN:1097-8135). http://www.lifesciencesite.com. 297

Keywords: Nursing Practice, Saudi Arabia, Quality Assurance in Nursing, Nursing Human Resource, Nursing Students, hallmark.

1. Introduction

The Kingdom of Saudi Arabia has recently witnessed a rapid comprehensive and continuous development in life sciences and modern technology. This has its reflection on nursing profession and nursing education as well. The problem of national nursing shortages in Saudi Arabia is made up of many factors. The dependency on expatriates originated with the rapid expansion of the Saudi Health System and the introduction of new technologies in the 1980s. Too few nationals were available to cope with the expansion or with the skills to operate the newly introduced technologies. In order to keep up with this rapid development, it is necessary to prepare professionals for providing high quality nursing care in different settings to all citizens of the Kingdom.

Saudi nurses have a key role to play in the provision of high quality nursing care to the growing population of Saudi Arabia. These Saudi nurses are assumed to be able to communicate more efficiently with patients and their families, which will be reflected in the quality of care and counseling provided compared to the vast majority non-Arabic-speaking nurses working in the Kingdom. Non-Arabic-speaking nurses are at a disadvantage as care providers because of language barriers, cultural differences and their relatively short-term commitment. Thus, taking account also of the increased demand for health care personnel, there is a need, not only to provide additional places, but also more attention to attracting Saudi nationals to, and retaining them in the nursing profession (Al-Mahmoud *et al.*, 2012). The work environment for the practice of nursing has long been cited as one of the most demanding across all types of work settings. Nurses provide the vast majority of patient care in hospitals and other health care settings (AONE, 2000). The "hallmarks" may inform students and new graduates, nurse educators, executives, and practicing nurses about key characteristics of health care settings that promote professional nursing practice (AACN, 2001).

An example of the development is the program of nursing education at the University of Dammam

which had started in 1989 as one of the departments of Medical College with enrolment and graduation of six female nursing students only. In 2003 it was recognized as separate Nursing College which provides education to female and Saudi students. In 2011, 30 male students and 150 female students were enrolled at the college. The qualities of teaching staff, materials and facilities have improved dramatically with establishment of very high quality skills laboratories for undergraduate practice prior to their exposure to clinical settings. This is considered being the best skills labs in the Middle-East. With all these efforts and improvements, there is a real problem of retention in the workforce.

Purpose of this Study

In order to further Quality Assurance in the nursing programs, this study aims to study the attributes (features) of professional environments to practice the nursing profession from the perspective of undergraduate Bachelor Nursing Students and Interns Students at the University of Dammam– Nursing College – to give incentive and motivation for Saudi students to continue working in this institution, and to make the nursing profession for a long time is the career for them. It was well observed the high-drop-out rate and low-retention-rate during the course of study and more critical, after graduation. It was noticed that only about 10 % of graduates resume to work as staff nurses in hospitals and as bed-side nurses in clinical setting. Miller-Rosser *et al.* (2006) state that traditionally, and largely because of cultural values, Saudi women have not undertaken employment. It is only recently and with limited relaxation of cultural beliefs that Saudi women have actively sought employment.

Objectives of the Study

The objectives of the study were:

1. To identify Nurses' opinions towards "Philosophy and professional accountability"
2. To determine Nurses' opinions towards "Recognition to contributions of nurses' knowledge and expertise to clinical care quality and patient outcomes."
3. To identify Nurses' opinions toward "Promoting executive level nursing leadership."
4. To identify Nurses' opinions towards "Empower nurses' participation in clinical decision-making and organization of clinical care systems."
5. To determine Nurses' opinions towards "Maintaining clinical advancement programs based on education, certification, and advanced preparation."
6. To determine Nurses' opinions towards "Demonstrate professional development support for nurses."
7. To identify Nurses' opinions towards "Creating collaborative relationships among members of the health care provider team."
8. To determine Nurses' opinions towards "Utilizing technological advances in clinical care and information systems."

2. Material and Methods

A survey was used in this research because it was believed that this is the best way to explore - as much as possible - the opinions of the nurses and students freely in a very limited period of time through administering questionnaires. Therefore, this section elaborates on the main survey parts used for this research, data collection method, target population, mode of administering the questionnaires, and the instrument used for data analysis.

The survey was based on two parts:

1. Demographic factors (B.Sc. student before graduation, Intern student), the University of Dammam.
2. Themes "Hallmarks" of the study (8 themes) composed of several sub-expressions are complementary to each other to reflect the total expressed in the theme.

To study this research questionnaire was designed containing the eight themes, the questions for each theme, there were thirty-nine questions and the quantitative variables of the five weights are (Strongly agree, Agree, I do not know, Disagree and Strongly Disagree), also the questionnaire contained the degree of education (undergraduate or intern student).

Data Collection

After the distribution of the questionnaire on the target sample to answer them, data was collected and there were 109 questionnaires, of which 49 undergraduate students and 60 Intern students.

Target Populations

The target population is: The fourth year nursing students (academic year 2008-2009) and the nursing interns (academic year 2008-2009).

Administration

The questionnaires were sent by e-mail to the students and interns.

Themes "Hallmarks" of the Survey

1. Manifest a philosophy of clinical care emphasizing (quality, safety, interdisciplinary collaboration, continuity of care, professional accountability): the target population was asked to give their opinions if the organization has a philosophy and mission statement that reflects these criteria, nursing staff have meaningful input into policy development and operational management of issues related to clinical quality, safety, and clinical outcomes evaluation, Nurse

staffing patterns have an adequate number of qualified nurses to meet patients' needs, including consideration of the complexity of patient conditions, nursing is represented on the organization's staff committees that govern policy and operations, the organization has a formal program of performance improvement that includes a focus on nursing practice, safety, continuity of care, and outcome and nursing staff assume responsibility and accountability for their own nursing practice.

2. Recognize contributions of nurses' knowledge and expertise to clinical care quality and patient outcomes: the target population was asked to give their opinions on if the organization differentiates the practice roles of nurses based on educational preparation, certification, and advanced preparation, the organization's performance improvement program has criteria to evaluate whether nursing care practices are based on the most current research evidence, professional and educational credentials of all disciplines, including nurses, are recognized by title on nametags and reports, nurses and other disciplines participate in media events, public relations announcements, marketing of clinical services, and strategic planning, nurses are encouraged to be mentors to less experienced colleagues and to share their enthusiasm about professional nursing within the organization and the community and advanced nursing roles, including clinical nurse specialists, nurse practitioners, scientists, educators, and other advanced practice roles, are utilized in the organization to support and enhance nursing care.
3. Promote executive level nursing leadership: the target population was asked to give their opinions on the nurse executive participates on the governing body, nurse executive reports to highest level operations or corporate officer, nurse executive has the authority and accountability for all nursing or patient care delivery, financial resources, and personnel and nurse executive is supported by adequate managerial and support staff.
4. Empower nurses' participation in clinical decision-making and organization of clinical care systems: the target population was asked to give their opinions on the decentralized, unit-based program or team organizational structure for decision making, organization or system-wide committee and communication structures include nurses, demonstrated leadership role for nurses in performance improvement of clinical care and the organization of clinical care systems, Utilization review system for nursing analysis, and correction of clinical care errors and patient safety concerns and staff nurses have the authority to develop and execute nursing care orders and actions and to control their practice.
5. Maintain clinical advancement programs based on (education, certification, advanced preparation): the target population was asked to give their opinions on the financial rewards available for clinical advancement and education, opportunities for promotion and longevity related to education, clinical expertise and professional contributions, peer review, patient, collegial, and managerial input available for performance evaluation on annual or routine basis and individuals in nursing leadership/management positions have appropriate education and credentials aligned with their role and responsibilities.
6. Demonstrate professional development support for nurses: the target population was asked to give their opinions on the professional continuing education opportunities available and supported, resource support for advanced education in nursing, including RN-to-BSN completion programs and graduate degree programs, preceptor ships, organized orientation programs, re-tooling or refresher programs, residency programs, internships, or other educational programs available and encouraged, Incentive programs for registered nursing education for interested licensed practical nurses and non-nurse health care personnel, long-term career support program targeted to specific populations of nurses, such as older individuals, home care or operating room nurses, or nurses from diverse ethnic backgrounds, specialty certification and advanced credentials are encouraged, promoted, and recognized, APNs, nurse researchers, and nurse educators are employed, and utilized in leadership roles to support clinical nursing practice and linkages are developed between health care institutions and baccalaureate/graduate schools of nursing to provide support for continuing education, collaborative research, and clinical educational affiliations.
7. Create collaborative relationships among members of the health care provider team: the target population was asked to give their opinions on the professional nurses, physicians, and other health care professionals practice collaboratively and participate in standing organizational committees, bioethics committees, the governing structure, and the institutional review processes, professional nurses have appropriate oversight and supervisory authority of unlicensed members of the nursing care team, and interdisciplinary

team peer review process is used, especially in the review of patient care errors.

8. Utilize technological advances in clinical care and information systems: the target population was asked to give their opinions on the documentation is supported through appropriate application of technology to the patient care process, appropriate equipment, supplies, and technology are available to optimize the efficient delivery of quality nursing care, and resource requirements are quantified and monitored to ensure appropriate resource allocation.

3. Results

The collected responses were entered and visualized using SPSS; the questioner of 39 statements (questions) was distributed among the population, averages for each statement were calculated, tabulated and visualized.

Likert Scale

The averages of each of the themes then were calculated and using Likert scale the generalized averages are found table 1.

Table 1: General Based on Likert Scale

Level	Average
Strongly Disagree	From 1 to 1.79
Disagree	From 1.8 to 2.59
I don't Know	From 2.6 to 3.39
Agree	From 3.4 to 4.19
Strongly Agree	From 4.2 to 5

The stability of the questionnaire statements was assured using the Cronbach's Alpha factor, which was calculated to be 0.923 for 39 statements, this is a highly positive number near to one, this indicate high stability of the data.

The Cronbach's Alpha factor

The Cronbach's Alpha factor was calculated for each theme on its own, and the calculated results for the factor were as in table 2.

Table 2: Stability for Each Theme

Theme	Stability
1	0.735
2	0.759
3	0.690
4	0.748
5	0.622
6	0.828
7	0.539
8	0.252
Total	0.932

Chi – Square Test

The Chi – Square Tests showed that there is significance and statistical relationship between the various themes; this is shown in table 3

Table 3 Chi -Square Results

Theme	Chi - Square	*p* if less than 0.05
1	58.037	.000
2	67.697	.000
3	40.862	.000
4	46.725	.000
5	45.266	.000
6	72.101	.000
7	26.936	.003
8	78.000	.000

t-Tests and Correlations

For $\alpha = 0.05$ the t-tests were implemented, with the null hypotheses; there are no differences between responses and the level of the students. The value of Levene test was 14.859 with significance 0.000, t-test gave 4.898 with $\alpha = 0.000$, the results of tests deduced the rejection of the null hypothesis, and showed there is a difference between the answers of the students according to their level (senior or graduated students).

The t-tests were made to see the relationship between the themes, and they showed that all themes are related, and except for theme 5 and theme 8, there is statistical significance between themes relations. This is shown in table 4.

Response Analysis

As the study is performed on a targeted sample some biased responses would be expected due to their lack of field experience and short term field training. Some responses emphasized this fact through the results, as an example; 17% of the sample responded that; they don't know if educational programs are available and encouraged within a health care institution. The rest of section contains the general results which were obtained from the responses of the study sample:

On the philosophy of clinical care emphasizing: quality, safety, interdisciplinary collaboration, continuity of care and professional accountability, most of the sample agreed on that; the organization has a philosophy and mission statement that reflects the above listed criteria. Added to that, they agreed on nursing staff have meaningful input into policy development and operational management of issues related to clinical quality, safety, and clinical outcomes evaluation. The organization has a formal program of performance improvement. And Nursing staff assume responsibility and accountability for their own nursing practice. On the other hand, the population responded that Nurses staffing who acquires an adequate number of qualified nurses to meet patients' needs is not enough

and does not cover the needs. The population had mixed responses and no clear attitude whether Nursing is represented on the organization's staff committees that govern policy and operations. Details are in table 5.

Table 4:Correlations between Themes

Themes		Themes 1	Themes 2	Themes 3	Themes 4	Themes 5	Themes 6	Themes 7	Themes 8
1	**Pearson Correlation**	1	.674(**)	.528(**)	.619(**)	.187	.544(**)	.368(**)	.278(**)
	Sig. (2-tailed)		.000	.000	.000	.052	.000	.000	.003
	N	109	109	109	109	109	109	109	109
2	**Pearson Correlation**	.674(**)	1	.610(**)	.715(**)	.292(**)	.599(**)	.504(**)	.209(*)
	Sig. (2-tailed)	.000		.000	.000	.002	.000	.000	.029
	N	109	109	109	109	109	109	109	109
3	**Pearson Correlation**	.528(**)	.610(**)	1	.609(**)	.344(**)	.491(**)	.345(**)	.225(*)
	Sig. (2-tailed)	.000	.000		.000	.000	.000	.000	.019
	N	109	109	109	109	109	109	109	109
4	**Pearson Correlation**	.619(**)	.715(**)	.609(**)	1	.370(**)	.553(**)	.470(**)	.241(*)
	Sig. (2-tailed)	.000	.000	.000		.000	.000	.000	.012
	N	109	109	109	109	109	109	109	109
5	**Pearson Correlation**	.187	.292(**)	.344(**)	.370(**)	1	.566(**)	.330(**)	.442(**)
	Sig. (2-tailed)	.052	.002	.000	.000		.000	.000	.000
	N	109	109	109	109	109	109	109	109
6	**Pearson Correlation**	.544(**)	.599(**)	.491(**)	.553(**)	.566(**)	1	.370(**)	.542(**)
	Sig. (2-tailed)	.000	.000	.000	.000	.000		.000	.000
	N	109	109	109	109	109	109	109	109
7	**Pearson Correlation**	.368(**)	.504(**)	.345(**)	.470(**)	.330(**)	.370(**)	1	.310(**)
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000		.001
	N	109	109	109	109	109	109	109	109
8	**Pearson Correlation**	.278(**)	.209(*)	.225(*)	.241(*)	.442(**)	.542(**)	.310(**)	1
	Sig. (2-tailed)	.003	.029	.019	.012	.000	.000	.001	
	N	109	109	109	109	109	109	109	109

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Table 5:(Theme 1) Nurses' opinions towards "Philosophy and professional accountability"

characteristics of the Professional Nursing Practice Environment		Strongly agree	Agree	Neutral	Disagree	Strongly Disagree	Mean	St. Deviation
The organization has a philosophy and mission statement that reflects these criteria.	%	26.6	56.9	2.8	.0	2.8	3.94	0.99
	No.	29	62	3		3		
Nursing staff have meaningful input into policy development and operational management of issues related to clinical quality, safety, and clinical outcomes evaluation.	%	38.5	44.0	1.8	.7	0.9	4.05	1.04
	No.	42	48	2		1		
Nurse staffing patterns have an adequate number of qualified nurses to meet patients' needs, including consideration of the complexity of patient condition.	%	8.3	12.8	2.8	.0	20.2	2.33	1.18
	No.	9	14	3		22		
Nursing is represented on the organization's staff committees that govern policy and operations.	%	24.8	20.2	24.8	.7	4.6	3.35	1.24
	No.	27	22	27		5		
The organization has a formal program of performance improvement that includes a focus on nursing practice, safety, continuity of care, and outcome.	%	38.5	36.7	2.8	.8	9.2	3.83	1.32
	No.	42	40	3		10		
Nursing staff assume responsibility and accountability for their own nursing practice.	%	47.7	33.0	0.0	.2	10.1	3.99	1.33
	No.	52	36	0		11		
Total	**%**	**30.7**	**33.9**	**5.8**	**.6**	**8**	**3.58**	**0.78**
	No.	**201**	**222**	**38**	**1**	**52**		

Table 6, shows responses whether there is recognition for the contributions of nurses' knowledge and expertise to clinical care quality and patient outcomes, a general agreement among the population on that fact was collected, however, mixed responses were observed for the point. The organization's performance improvement program has criteria to evaluate whether nursing care practices are based on the most current research evidence, and some negative response were collected regarding the participation of nurses in various events related to knowledge transfer.

Table 6: (Theme 2) Nurses' opinions towards "Recognition to contributions of nurses' knowledge and expertise to clinical care quality and patient outcomes"

characteristics of the Professional Nursing Practice Environment		Strongly agree	Agree	Neutral	Disagree	Strongly Disagree	Mean	St. Deviation
The organization differentiates the practice roles of nurses based on educational preparation, certification, and advanced preparation.	%	25.7	40.4	0.0	22.0	11.9	3.46	1.39
	No.	28	44	0	24	13		
The organization's performance improvement program has criteria to evaluate whether nursing care practices are based on the most current research evidence.	%	18.3	25.7	17.4	23.9	14.7	3.09	1.35
	No.	20	28	19	26	16		
Professional and educational credentials of all disciplines, including nurses, are recognized by title on nametags and reports.	%	25.7	36.7	13.8	16.5	7.3	3.57	1.24
	No.	28	40	15	18	8		
Nurses and other disciplines participate in media events, public relations announcements, marketing of clinical services, and strategic planning.	%	14.7	29.4	13.8	29.4	12.8	3.04	1.31
	No.	16	32	15	32	14		
Nurses are encouraged to be mentors to less experienced colleagues and to share their enthusiasm about professional nursing within the organization and the community.	%	22.0	48.6	0.0	15.6	13.8	3.5	1.36
	No.	24	53	0	17	15		
Advanced nursing roles, including clinical nurse specialists, nurse practitioners, scientists, educators, and other advanced practice roles, are utilized in the organization to support and enhance nursing care.	%	34.9	41.3	1.8	13.8	8.3	3.81	1.28
	No.	38	45	2	15	9		
Total	**%**	**23.5**	**37**	**7.8**	**20.2**	**11.5**	**3.41**	**0.89**
	No.	**154**	**242**	**51**	**132**	**75**		

The responses showed that the population believed that nurses executives has an important contact with highest level management, and that they have authority over financial resources, personnel, and patient care delivery, however, they have less participation with governing bodies.

Table 7: (Theme 3) Nurses' opinions toward "Promoting executive level nursing leadership"

characteristics of the Professional Nursing Practice Environment		Strongly agree	Agree	Neutral	Disagree	Strongly Disagree	Mean	St. Deviation
Nurse executive participates on the governing body.	%	27.5	29.4	19.3	21.1	2.8	3.58	1.18
	No.	30	32	21	23	3		
Nurse executive reports to highest level operations or corporate officer.	%	30.3	32.1	7.3	22.9	7.3	3.55	1.33
	No.	33	35	8	25	8		
Nurse executive has the authority and accountability for all nursing or patient care delivery, financial resources, and personnel.	%	29.4	36.7	12.8	17.4	3.7	3.71	1.17
	No.	32	40	14	19	4		
Nurse executive is supported by adequate managerial and support staff.	%	12.8	46.8	8.3	23.9	8.3	3.32	1.21
	No.	14	51	9	26	9		
Total	**%**	**25**	**36.2**	**11.9**	**21.3**	**5.5**	**3.54**	**0.88**
	No.	**109**	**158**	**52**	**93**	**5.5**		

The existence of high awareness and initiative can be deduced from the responses related to empower nurses' participation in clinical decision-making and organization of clinical care systems, all responses lead to an agreement of the importance and need for nurses involvement in the decision making process.

Table 8: (Theme 4) Nurses' opinions towards "Empower nurses' participation in clinical decision-making"

Characteristics of the Professional Nursing Practice Environment		Strongly agree	Agree	Neutral	Disagree	Strongly Disagree	Mean	St. Deviation
Decentralized, unit-based program or team organizational structure for decision making.	%	10.1	62.4	1.8	12.8	12.8	3.44	1.22
	No.	11	68	2	14	14		
Organization or system-wide committee and communication structures include nurses.	%	26.6	42.2	6.4	19.3	5.5	3.65	1.22
	No.	29	46	7	21	6		
Demonstrated leadership role for nurses in performance improvement of clinical care and the organization of clinical care systems.	%	24.8	46.8	1.8	19.3	7.3	3.62	1.25
	No.	27	51	2	21	8		
Utilization review system for nursing analysis and correction of clinical care errors and patient safety concerns.	%	39.4	27.5	7.3	15.6	10.1	3.71	1.39
	No.	43	30	8	17	11		
Staff nurses have the authority to develop and execute nursing care orders and actions and to control their practice.	%	25.7	44.0	2.8	14.7	12.8	3.55	1.36
	No.	28	48	3	16	14		
Total	**%**	**25.3**	**44.6**	**4**	**16.3**	**9.7**	**3.59**	**0.91**
	No.	**138**	**243**	**22**	**89**	**53**		

An amount of dissatisfaction was concluded from the answers related to the fifth theme, Maintain clinical advancement programs based on: education, certification, advanced preparation. Nurses showed disagreement and satisfaction financial rewards, the availability of opportunities related to educational and clinical expertise in their organizations. Less amount of criticism was shown toward the evaluation of performance, yet the responses showed no satisfaction, same was the case for leader nurses and their education. See table 9.

Table 9: (Theme 5) Nurses' opinions towards" Maintaining clinical advancement programs based on education, certification, and advanced preparation"

Characteristics of the Professional Nursing Practice Environment		Strongly agree	Agree	Neutral	Disagree	Strongly Disagree	Mean	St. Deviation
Financial rewards available for clinical advancement and education.	%	21.1	11.9	5.5	23.9	37.6	2.55	1.59
	No.	23	13	6	26	41		
Opportunities for promotion and longevity related to education, clinical expertise and professional contributions.	%	15.6	29.4	0.9	33.9	20.2	2.86	1.44
	No.	17	32	1	37	22		
Peer review, patient, collegial, and managerial input available for performance evaluation on annual or routine basis	%	27.5	34.9	3.7	22.9	11.0	3.45	1.39
	No.	30	38	4	25	12		
Individuals in nursing leadership/management positions have appropriate education and credentials aligned with their role and responsibilities.	%	24.8	33.0	0.9	25.7	15.6	3.26	1.47
	No.	27	36	1	28	17		
Total	**%**	**22.4**	**27.3**	**2.8**	**26.6**	**92**	**3.03**	**1.01**
	No.	**97**	**119**	**12**	**116**	**92**		

On the discussion of demonstrate professional development support for nurses, some points were assured in favor of the existing reality, and those are: the availability and support of educational opportunities, specialty and advanced credential are encouraged, and finally some cooperation exists between organization and higher education institution to open opportunity doors for nurses. However, the resource support for these activities was criticized, the availability of incentive programs is negated, leadership training and involvement were not among the thanked points and finally special type of orientation programs and refresher programs are also negated, in the sense that they are not available. See table 10.

Table 10: (Theme 6) Nurses' opinions towards" Demonstrate professional development support for nurses"

Characteristics of the Professional Nursing Practice Environment		Strongly agree	Agree	Neutral	Disagree	Strongly Disagree	Mean	St. Deviation
Professional continuing education opportunities available and supported.	%	12.8	52.3	1.8	19.3	13.8	3.31	1.3
	No	14	57	2	21	15		
Resource support for advanced education in nursing, including RN-to-BSN completion programs and graduate degree programs.	%	27.5	28.4	9.2	18.3	16.5	3.32	1.47
	No	30	31	10	20	18		
Preceptor ships, organized orientation programs, re-tooling or refresher programs, residency programs, internships, or other educational programs available and encouraged.	%	11.0	29.4	17.4	22.0	20.2	2.89	1.33
	No	12	32	19	24	22		
Incentive programs for registered nursing education for interested licensed practical nurses and non-nurse health care personnel.	%	13.8	22.0	8.3	44.0	11.9	2.82	1.29
	No	15	24	9	48	13		
Long-term career support program targeted to specific populations of nurses, such as older individuals, home care or operating room nurses, or nurses from diverse ethnic backgrounds.	%	3.7	13.8	16.5	42.2	23.9	2.31	1.10
	No	4	15	18	46	26		
Specialty certification and advanced credentials are encouraged, promoted, and recognized.	%	24.8	37.6	0.0	21.1	16.5	3.3	1.47
	No	27	41	0	23	18		
APNs, nurse researchers, and nurse educators are employed and utilized in leadership roles to support clinical nursing practice.	%	11.9	30.3	4.6	38.5	14.7	2.86	1.32
	No	13	33	5	42	16		
Linkages are developed between health care institutions and baccalaureate/graduate schools of nursing to provide support for continuing education, collaborative research, and clinical educational affiliations.	%	25.7	38.5	0.0	24.8	11.0	.3.43	1.39
	No	28	42	0	27	12		
Total	**%**	**16.4**	**31.5**	**7.2**	**28.8**	**16.1**	**3.03**	**0.91**
	No	**143**	**275**	**63**	**251**	**140**		

Table 11, shows responses details on the creation of collaborative relationships among members of the health care provider team, the population were generally positive, with the exception in various committees work and governmental links, the population was divided between an agreement of the existence of such collaboration or not.

Table 11: (Theme 7) Nurses' opinions towards "Creating collaborative relationships among members of the health care provider team"

Characteristics of the Professional Nursing Practice Environment		Strongly agree	Agree	Neutral	Disagree	Strongly Disagree	Mean	St. Deviation
Professional nurses, physicians, and other health care professionals practice collaboratively and participate in standing organizational committees, bioethics committees, the governing structure, and the institutional review processes.	%	11.0	23.9	22.9	27.5	14.7	2.89	1.24
	No.	12	26	25	30	16		
Professional nurses have appropriate oversight and supervisory authority of unlicensed members of the nursing care team.	%	19.3	38.5	3.7	31.2	7.3	3.31	130
	No.	21	42	4	34	8		
Interdisciplinary team peer review process is used, especially in the review of patient care errors.	%	28.4	36.7	7.3	15.6	11.9	3.54	1.36
	No.	31	40	8	17	13		
Total	**%**	**19.6**	**30**	**11.3**	**24.8**	**11.3**	**3.2**	**0.94**
	No.	**64**	**108**	**37**	**81**	**37**		

The last point of the themes was the technology utilization in clinical care and information systems. The population asserted that documentation is supported through appropriate application of

technology to the patient care process, but they did not support the amount of equipment, supplies, and technology to optimize the efficient delivery of quality nursing care or the monitoring and control of appropriate resource allocation. See table 12.

Table 12: (Theme 8) Nurses' opinions towards "Utilizing technological advances in clinical care and information systems"

Characteristics of the Professional Nursing Practice Environment		Strongly agree	Agree	Neutral	Disagree	Strongly Disagree	Mean	St. deviation
Documentation is supported through appropriate application of technology to the patient care process.	%	33.9	40.4	2.8	18.3	4.6	3.81	1.22
	No.	37	44	3	20	5		
Appropriate equipment, supplies, and technology are available to optimize the efficient delivery of quality nursing care.	%	19.3	25.7	0.0	44.0	11.0	2.98	1.39
	No.	21	8	0	48	12		
Resource requirements are quantified and monitored to ensure appropriate resource allocation.	%	8.3	33.0	0.0	42.2	16.5	2.74	1.3
	No.	9	36	0	46	18		
Total	**%**	**20.5**	**33.0**	**0.9**	**34.9**	**10.7**	**3.2**	**0.83**
	No.	**67**	**108**	**3**	**114**	**35**		

Discussion

As reported by AACN (2012), the hallmarks may be useful to new graduates, practicing nurses, students, faculty, nurse executives and managers, and employers across all nursing practice settings. Hallmarks are characteristics of the practice setting that best support professional nursing practice and allow baccalaureate and higher degree nurses to practice to their full potential. These Hallmarks are present in health care systems, hospitals, organizations, or practice environments and which were discussed in this study; (Philosophy and professional accountability, Recognition to contributions of nurses' knowledge and expertise to clinical care quality and patient outcomes, Promoting executive level nursing leadership, Empower nurses' participation in clinical decision-making and organization of clinical care systems, Maintaining clinical advancement programs based on education, certification, and advanced preparation, Creating collaborative relationships among members of the health care provider team, and Utilizing technological advances in clinical care and information systems).

The graduate from baccalaureate programs understands and respects more the discrepancies of care provided; the increased use of different healthcare resources available for patients' care and the complexity of healthcare. To ensure the baccalaureate graduates attain sufficient learning opportunities, including direct clinical experiences, it is important to focus on outcomes and integrate the described knowledge and skills into the graduates' professional nursing practice. Additionally, as part of an inter-professional team, it is important to attain proper clinical learning based on the knowledge and skills that are necessary to manage care (Allan *et al.* 2005 & AACN 2002).

Furthermore, ANA in their reports (2005), (2007) & (2008) reported that baccalaureate graduate nurses are the direct and the indirect patient care providers. They advocate and educate the patients in different healthcare settings. In order to ensure the active participation of the patients in healthcare decisions, the nurse emphasizes partnerships with patients, their families and communities. The advocacy of the patient is a hallmark of the professional nursing role which requires the delivery of high quality care, outcome evaluation and improving care by providing quality leadership.

Based on the results of this study and on the above mentioned eight themes or hallmarks, it can be concluded that the majority of the nurses believed that the organization has a philosophy and mission statement in place and the nursing staff have input into policy development and operational management of issues related to clinical quality, safety, and clinical outcomes evaluation. Also, there is recognition for the contributions of nurses' knowledge and expertise to clinical care quality and patient outcomes. The respondents also believed that nurses' executives have an important contact with highest level management, and that they have authority over financial resources, personnel, and patient care delivery. In addition, there were general agreements of the importance and need for nurses involvement in the decision making process. It was well recognized the availability and support of educational opportunities, specialty and advanced credential and the cooperation exists between organization and ministry of higher education.

An important reality of healthcare practice is the ongoing advances in science and technology and

demographic changes which enhance nurses to providing evidence based care to patients within this changing environment. The nurse also should be prepared for the ethical dilemmas that arise in practice and will be able to make and assist others in making decisions within a professional ethical framework. Therefore, it is very essential and important for the nurses to understanding advances in science and technology, the influence these advances have on health care and individual wellbeing, patients and the values they bring to the healthcare relationship (Cronenwett *et al.* 2007).

However, the results of the present study was noted that there is a severe shortage of qualified nursing staff to meet patients' needs which have negative implication on the quality of care provided. Moreover, the participation of nurses in various events related to knowledge transfer and nursing care practices based on research evidence is an issue. There is also a lack of clinical advancement programs, poor financial rewards, lack of opportunities related to educational and clinical expertise in their organizations and no satisfaction toward the evaluation of performance. Furthermore, leadership training and involvement, orientation programs and refresher programs are also in need for attention. The documentation in the organization is supported through appropriate application of technology to the patient care process; however, there are insufficient amount of equipment, supplies, and technology to optimize the efficient delivery of quality nursing care.

Strong nursing leadership is essential for quality patient care in any health care environment. As a leader in the health care system, nurses have the opportunity to expand their range of patient care beyond of nursing into other vital areas. However, health care administrators must be prepared to deal with the changing of health care delivery systems, technological innovations, an increasingly complex regulatory environment, restructuring of work, and a greater focus on preventive care. Leadership professionals in health care system can work as specialists, or as generalists who manage a hospital or work as an executive in a health care system (Fang *et al*. 2008)

Conclusion and Recommendations

Based on the findings, from quality assurance perspective would recommend that in the increasing health care workforce shortages especially nurses, there is a crucial need for high-quality professional nursing care due to changes in the socio-demographics of the population and in the health care system. There is a critical need also to fully utilize the knowledge and skills of professional nurses available and to ensure their retention in the profession as well as attract an increased number of individuals into the discipline.

A need for detailed analysis on some competencies which are covered within the nursing curricula, to give the students a more insight on the existing health organizations and the management structure and opportunities exist for further integration of these into curricula. These are some examples of competencies; Critical Thinking, Health Care Systems and Policy, Communication skills, Illness and Disease Management, Ethics, Information and healthcare Technologies.

Adding some workshops and/or subjects of study program where decision making process and involvement of nurses in that process are explained and trained.

It is also recommended that attention be given to publicising and making the nursing profession more attractive. For example, salaries for Saudi nursing staff should be increased.

As a future further extension of this work, the researcher would suggest taking each theme as a questionnaire topic and extending the geographical to cover the whole area of Kingdome of Saudi Arabia.

Limitation of the study

The study was conducted within a limited population in both senses; geographical and background sense most of the population come from one college and mostly work in the Eastern Region of Saudi Arabia, for more generalized results the study can be conducted on a larger scale geographical and in terms of numbers.

Implication for HRD

This paper shows that Saudi Arabia case in shortage of nurses and difficulty in minimizing nurses' dropout rates and maintaining the professionals in the workplace is purely human resource planning, management and development issue. Saudi Arabia is not a unique case. Globally, the nursing needs have far exceeded the supply. Nursing is considered a very precious, rare and very much needed profession as 80% of the care to patients is provided by nurses. It is very hard to maintain nurses in the job with all other attractive jobs, challenges and opportunities available nowadays.

References

1. Allan,J., Stanley, J., Crabtree, M., Wemer, K., & Swenson, M. (2005). Clinical Prevention and Population Health Curriculum Framework: The Nursing

Perspective. *Journal of Professional Nursing,* 21(5), 259267.
2. American Association of Colleges of Nursing (2001). 2000-2001 Enrollment and Graduations in Baccalaureate and Graduate Programs in Nursing. Washington, DC: Author.
3. American Association of Colleges of Nursing (2002). Hallmarks of the Professional Nursing Practice Environment. Washington, DC: Author.
4. American Association of Colleges of Nursing (2012). Hallmarks of the Profession of Nursing Practice Environment. Washington, DC: Author.
5. American Organization of Nurse Executives (AONE, 2000). Nurse recruitment and retention study. Chicago, IL: AONE Institute for Patient Care Research and Education.
6. American Nurse Association. (ANA, 2005) Code of Ethics for Nurses with Interpretative Statements. Silver Spring, MD: Author.
7. American Nurse Association. (ANA, 2007) Public Health Nursing: Scope and Standards of Practice. Silver Spring, MD: Author.
8. American Nurse Association. (ANA, 2008) Draft Position Statement: Mandatory Implementation of an ANA Recognized Nursing Terminology Set in all Professional Nursing Education Programs. Silver Spring, MD: Author.
9. Al-Mahmoud, S. Mullen, P., & Spurgeon, P. (2012). Saudisation of the nursing workforce: Reality and Myths about planning Nurse Training in Saudi Arabia. *Journal of American Science*, 8(4). (ISSN: 1545-1003)
10. Cronenwett, L., Sherwood, G., Barnsteiner, J., Johnson, J., Mitchell, P., Taylor, S., & Warren, J. (2007). Quality and Safety Education for Nurses. *Nurses Outlook*, 55(3), 122131
11. Fang, D., Htut, A., & Bandash, G. (2008). 2007/2008 Enrolment and Graduations in Baccalaureate and Graduate Programs in Nursing. Washington, DC: American Association of Colleges of Nursing.
12. Miller-Rosser K. Chapman Y., & Francis K. (2006). Historical, Cultural, and Contemporary Influences on the Status of Women in Nursing in Saudi Arabia, *J Issues Nurse*. **11**(3).

2/2/2013

Serum Antibody Detection in Ecchinococcosis: Specificity of *Hydatidosis* enzyme-linked immunosorbent assay (ELISA) IgG

Metwally D M[1&2] and Al-Olayan E M[2]

[1]Parasitology Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt.
[2]Zoology Department ,Faculty of Science ,King Saud University, Riyadh, KSA.
mdbody7@yahoo.com; eolayan@ ksu.edu.sa

Abstract: Hydatidosis is diagnosed by serological together with radiological and ultrasound examinations .but cross -reactivity with other parasites, over evaluating the real prevalence, represents a big limitation. The aim of the present study was to develop a specific and simple antibody detection (IgG) -based ELISA method .The samples in this study included 35 patients in the following groups: IHAT(Indirect Haem Agglutination Test) and abdominal ultrasonography confirmed hydatidosis patients (20 cases), control with other parasitic diseases (5cases Toxoplasmosis) and healthy controls (10cases). Antibody detection by indirect ELISA using *Ecchinococcus granulosus* antigen showed that 65% of hydatic patients (13cases) have anti-hydatid cyst antibodies in their serum while no cross reaction was detected. A sensitivity of 72.22% and specificity of 75% were found for the antibody detection assay. Findings of this study indicated that antibody detection assay is a highly sensitive and specific approach for diagnosis of hydatid cyst.

[Metwally D M and Al-Olayan E M. **Serum Antibody Detection in Ecchinococcosis: Specificity of *Hydatidosis* enzyme-linked immunosorbent assay (ELISA) IgG**. *Life Sci J* 2013;10(1):2107-2110]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 298

Keywords: hydatidosis, ELISA IgG diagnosis, sensitivity, specificity.

1. Introduction:

Echinococcus granulosus is the causal agent of human hydatidosis . Hydatid cysts are mainly located in liver(right lobe).The intensity of the immune response depends on the location and integrity of the cyst. Cysts in lung ,brain or spleen are less reactive than those in liver or bone.

This parasite has a worldwide distribution and is one of the most important zoonotic diseases prevalent in different parts of the world including the Middle East (Eckert and others, 2001; Sadjjadi and others, 2006) The infection may be asymptomatic ,especially in its early stages, many cases go unreported within endemic countries (Craig and Nelson,1984). Diagnosis of hydatidosis is based on immunodiagnostic methods along with radiological and ultrasound

Examinations (Parija,1998; Sadjjadi and others,2001) Large number of immunological assays have been developed for detection of anti-hydatid cyst antibodies (IgG).and recently, hydatid antigens in the serum(Ortona and others,2003) . These include indirect hemagglutination (IHA), indirect immunofluorescence (IFA), immunoelectrophoresis, counter-current immunoelectrophoresis (CIEP), radioimmunoassay (RIA), and ELISA (Siavashi et al., 2005 ; Sarkari et al.,2007). Moreover, enzyme-linked immunoelectrotransfer blots (EITB), enzyme-linked immunoelectrodiffusion assay (ELIEDA), time-resolved fluoroimmunoassay (TR-FLA), and immunoblot assay have been developed for detection of anti-hydatid cyst antibodies (Aceti and others, 1991; Ortona et al., 000). Antibody detection assays cannot distinguish between past and present infections and cannot be used for assessment of the efficacy of treatments but Antigen detection assay may circumvent this problem (Doiz et al., 2001). It has been shown that hydatid cyst antigen can be detected in the serum or urine of hydatidosis patients. Circulating hydatid antigens are present in the serum only during active infection, and the levels of these antigens continue to decrease after surgical removal of the hydatid cyst or successful chemotherapy (Devi and Parija, 2003). ELISA has been used for the detection of specific *E. granulosus* antibodies in serum with variable results(Kaddah and others,1992 ; Hernandez and others,2008). The ELISA, with hydatidic fluid of sheep origin as antigen, is widely used for the diagnosis of the disease, showing a high sensitivity for hepatic cysts, although lower for pulmonary locations. Cross-reactions may appear in patients infected by other parasites. The carbohydrate epitopes in hydatid cyst fluid antigen (Alyam and Knob loch,1989)cross-react with antigens shared with other pathogens leading to reduced specificity and sensitivity of diagnostic assays.

The present study aimed to test Specificity of the enzyme-linked immunosorbent assay (ELISA) for *Hydtidosis* IgG antibody

2.MaterialsAnd Methods

Hydatid sera

Serum samples from patients were collected from Almattaria teaching hospital, Cairo between June 2012 and September 2012 for the diagnosis of hydatidosis. Twenty serum samples of E. granulosus patients confirmed by abdominal ultrasonography, computerized tomography and IHAT (Indirect Heam Agglutination Test). Five serum samples from patients with toxoplasmosis were used as control for hydatic patients(to test the specificity of technique for hydatidosis). Sera were also collected from healthy controls (10 cases)

Enzyme linked immunosorbent assay

Enzyme-linked immunosorbent assay was performed using a specific kit Hydatidosis Elisa IgG(G1006) supplied by the Vircell microbiologist(GRANADA* SPAIN)

The principle of the assay is based upon the reaction of antibodies in the sample tested with the antigen adsorbed on the polystyrene surface. Unbound immunoglobulins are washed off. An enzyme-labelled anti-human globulin binds the antigen-antibody complex in a second step. After a new washing step, bound conjugate is developed with the aid of a substrate solution (TMB) to render a blue coloured soluble product which turns into yellow after adding the acid stopping solution. .The absorbance was measured at450 nm using a microplate ELISA reader (Model IRE96.CompanySFRI).

Calculations

Sensitivity was calculated by dividing the number of true positive cases to total number of positive cases and false negative cases . Specificity was also calculated by dividing the number of true negative cases to total number of negative cases and false positive cases.

3. Results

The positive predictive value of antibody IgG was detected in 13 out of 20 (35%) of sera among hydatidosis patients, 2 out of 20(10%) were equivocal and 5 of 20 (25%) were not having IgG specific antibody against hydatidosis(Table.1). Therefore, a sensitivity of 72.22% and specificity of 75% was calculated for the antibody detection assay. No antibody was detected in patients with toxoplasmosis (Table.2) so the sensitivity in case of toxoplasmosis is 0% while the specificity is 100%. .Antibody IgG was detected in 2 out of 10 healthy control group(Table.3).The equivocal samples were excluded from calculations. The results of Antibody detection in the serum by indirect ELISA (IgG)was illustrated in(Fig.1)

Table1.hydati group

RESULTS	-Ve	Equivocal	+Ve	Total
NO. oF Samples	5	2	13	20

Table2.toxoplasmosis group

RESULTS	-Ve	Equivocal	+Ve	Total
No. of Samples	5	0	0	5

Table3.control group

RESULTS	-Ve	Equivocal	+Ve	Total
No. of Samples	6	2	2	10

Fig.1 Antibody detection in the serum by indirect ELISA using plate coated with ecchinococcus granulosus antigen.

Antibody index > 11 positive

Antibody index 9-11 equivocal

Antibody index < 9 negative

4. Discussion

Large number of serological tests such as ELISA excuted on patients' sera to detect specific antibodies leads to variable results of sensitivity and specificity. False negative results in human hydatidosis make a serious puzzle to get a conclusion, as it ranges between 3-5% of hydatid patients and may up to 35-40% in hyper-endemic areas(Craig and Nelson, 1984; Gottstein, 1984).

Some of the hydatidosis patients not raised the antibody or the titer is low especially in infants and old people. Also the antibody titer is low and cannot be easily detected in cerebral, ocular, and calcified cysts, (Gottstein et al.,1984 ; Ravinder et al.,1997) From another aspect, the long persistence of anti- *E. granulosus* antibodies after surgical removal of the cysts gives rise to unreliable diagnosis of relapse in patients (Todorov and Stojanov,1979).

The antibody detection in this study was found that 65% of patients (13 out of 20 cases) have IgG specific antibodies against *Ecchinococcus granulosus* in their serum. The sensitivity of the assay to detect antibody was high(72.22%), its specificity was high too (75%) and no additional cross reactions were found in patients with toxoplasmosis but in 2 healthy control. These results are in agreement with (Sadjjadi et al.,2009) in which he found that 94.2% of patients (33 cases) have anti-CE antibodies in their serum(high sensitivity) and he recorded that no cross reaction in patient with toxoplasmosis too , but in his detection assay , specificity was rather low since cross reaction was noted in sera of patients with ascariasis and strongyloidiasis and one healthy control. In addition, (Younis et al.,2008) reported 90% sensitivity and 75% specificity by the conventional elisa, no cross reaction recorded with toxoplasmosis but with amoebiasis, fascioliasis, schistosomiasis and one healthy control.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper

The source(s) of financial support for the study.
Zoology Department ,Faculty of Science,King Saud University.

Corresponding author
Dina Mahmoud Ahmed Metwally
[1]Parasitology Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt.
[2]Zoology Department ,Faculty of Science ,King Saud University, Riyadh, KSA.
mdbody7@yahoo.com

Acknowledgments
This research project was supported by a grant from the"Research Center of the Center for Female Scientific and Medical Colleges", Deanship of Scientific Research, King Saud University.

References

1. Aceti A,Pennica A,Teggi A, Grilli A, Caferro M, Celestino D, Leri O,Sebastiani A,DeRosa F, 1991,The serological diagnosis of human hydatid disease by time-resolved fluoroimmunoass: J Infect,22,135–141.
2.AI-Yam Ffvl, Knob loch J,1989 isolation and partial characterization of species-specific and cross-reactive antigens of *Echinococcus granulosus* cyst fluid: Mol Biochern Parasitol,3 7, 101-7.
3.Craig PS, Nelson GS,1984, The detection of circulating antigen in human hydatid disease: Ann Trop Med Parasitol,78,219–227.
4. Devi CS, Parija SC,2003, A new serum hydatid antigen detection test for diagnosis of cystic echinococcosis: Am J Trop Med Hyg,69,525–528.
5. Doiz O, Benito R, Sbihi Y, Osuna A, Clavel A, Gomez-Lus R,2001, Western blot applied to the diagnosis and post-treatment monitoring of human hydatidosis: Diagn Microbiol Infect Dis,41,139–142.
6. Eckert J, Deplazes P, Craig PS, Gemmell MA, Gottstein B, Heath D, Jenkins DJ, Kamiya M, Lightowlers M,2001 Echinococcosis in animals: clinical aspects, diagnosis and treatment. In: Eckert J, Gemmell MA, Meslin FX, Pawlowski ZS, editors. WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern. Paris, France: World Organization for Animal Health, pp. 72–79.
7. Gottstein B,1984, An immunoassay for the detection of circulating antigens in human echinococcosis: Am J Trop Med Hyg,33,1185–1191.
8. Hernandez A, Muro A, Barrera I *et al,2008,* Usefulness for different *Ecchinococcus granulosu* recombinant antigens for serodiagnosis of uni locular hydatid disease (UHD) and post surgical follow-up of patients treated for UHD :ClinVaccine Immunol,15(1),147 - 53 .
9 .Kaddah MH, Maher KM. Hassanein HI *et al.,1992,* Eva luationof different immunodiagnostic technique s for diagnosis of hydatidosis in Egypt: J Egypt Soc Parasitol , 22(3),653 -65.
10. Ortona E, Riganò R, Buttari B, Delunardo F, Ioppolo S, Margutti P, Profumo E, Teggi A, Vaccari S, Siracusano A,2003, An update on

immunodiagnosis of cystic echinococcosis: Acta Trop,85,165–171.
11. Ortona E, Rigano R, Margutti P, Noralgiacomo S, Ioppolo S, Vaccari S, Barca S. Buttari B, Profuma, Teggi A, Siracusano A,2000, Native and recombinant antigens in the immunodiagnosis of human cystic Echinococcosis: Parasite Immunol,22,553–559.
12. Parija SC,1998, A review of some simple immunoassays in the serodiagnosis of cystic hydatid disease.:Acta Trop,70,17–24.
13. Ravinder PT, Parija SC, Rao KS,1997, Evaluation of human hydatid disease before and after surgery and chemotherapy by demonstration of hydatid antigens and antibodies in serum: J Med Microbiol,47,859–864.
14. Sadjjadi SM, Ardehali S, Noman-Pour B, Kumar V, Izadpanah A,2001,Diagnosis of cystic echinococcosis: imaging or countercurrent immuno-electrophoresis?: East Mediterr Health J,7,907–910.
15. Sadjjadi SM,2006, Present situation of echinococcosis in the Middle East and Arabic North Africa: Parasitol Int,S197–S202.
16. Sadjjadi SM ,Sedaghat F,Hosseini VH and Sarkari B,2009, Serum Antigen and Antibody Detection in Echinococcosis: Application in Serodiagnosis of Human Hydatidosis:KJP,47(2), 153–157.
17. Sarkari B, Sadjjadi SM, Abidi H, Izadpanah A, Kazemian S, Rafati A,2007, Application of western blotting using native antigen B for serodiagnosis of human cystic echinococcosis: Iran J Parasitol,2,7–12.
18. Siavashi MR, Taherkhani H, Rezaei KR, Razavi Deligani MR, Assmar M,2005, Comparison of dot-ELISA and sandwich ELISA diagnostic tests in detection of human hydatidosis: Iran Biomed J,9,91–94.
19. Todorov T, Stojanov G,1979, Circulating antibodies in human echinococcosis before and after surgical treatment.: Bull World Health Org,57,751–758.
20. Virginio V G, Hernandez A, Rott MB *et al.,*2003, A set of recombinant antigens from *Echinococcus granulosus* with potential for use in the immunodiagnosis of human cystic hydatid disease: Clin Exp lmmunol: ,132,309- 15.
21. Younis T,Fahmi I,Hala S E,Nihad M A,Ayman I,2008, Attempt to Improve the Diagnostic Efficacy of Enzyme-Linked lmmunosorbent Technique for the Serodiagnosis of Cystic Echinococcosis:PUJ,129-136.

2/2/2013

Screening of Some Antibiotics and Anabolic Steroids Residues in Broiler Fillet Marketed in El-Sharkia Governorate

Mohamed Abdallah Hussein[1] and Samah Khalil[2]

[1]Food Control Dept., [2]Forensic Medicine and Toxicology Dept., Faculty of Veterinary Medicine, Zagazig University, Egypt
Samah_vet2001@yahoo.com

Abstract: Antibiotics and anabolic steroid growth promoters usage have facilitated the efficient production of poultry, allowing the consumer to purchase, at a reasonable cost, high quality meat and eggs. Although these uses benefit all involved, unfortunately, the edible poultry tissues had harmful concentrations of drug residues. Therefore, this study was carried out on one hundred randomly collected fresh and frozen broiler fillet samples (50 of each) to evaluate the antibiotic residues level qualitatively by microbiological inhibition assay followed by quantitative detection for oxytetracycline and enrofloxacin by high performance liquid chromatography (HPLC). In addition to monitoring of anabolic steroids (Testosterone, Progesterone and Zeranol) quantitively by Enzyme- linked immunosorbent assay (ELISA). The obtained results revealed a detectable level of oxytetracycline residues which confirm widespread misuses of antibiotic especially oxytetracycline in farms and lack of application of recommended withdrawal times. The anabolic steroids residues level including testosterone and progesterone were within the permissible limit which refers to no illegal use of hormones as growth promoting agents in broiler production. None of the samples displayed the presence of zeranol residue as the level of it was below the detection limit of the used kits (<100 ng/kg). For monitoring the effect of cooking process on antibiotic residue levels in broiler fillet, ten broilers (40 days old) were classified into two groups, each group was dosed over 5 consecutive days with 15 mg/ kg day of oxytetracycline or enrofloxacin. Five slices of broiler fillet from each group subjected to cooking either via frying or grilling.The results showed that cooking had an effect in reducing the concentration of antibiotic residues as there are a signficant reduction percentages of oxytetracycline while enrofloxacin residues showed low reduction percentages. These findings recommended that restricted measures and harder regulations must be applied to prevent the misuse of drugs in poultry industry, application of withdrawal time as well as the inspection of chickens for drug residues prior to marketing.

[Mohamed Abdallah Hussein and Samah Khalil. **Screening of Some Antibiotics and Anabolic Steroids Residues in Broiler Fillet Marketed in El-Sharkia Governorate.** *Life Sci J* 2013;10(1):2111-2118] (ISSN:1097-8135). http://www.lifesciencesite.com. 299

Key words: Antibiotic, Anabolic steroid, Residue, Maximum residue limit, Microbiological inhibition test, HPLC, ELISA.

1. Introduction

Poultry products are important protein sources. For this purpose, birds are reared as broilers for meat and layers for eggs, under intensive or free range management. In Egypt, broiler meat production was 559,000 tones, representing 84% of total poultry meat production **(Maged and Hamdey 2006)**. Demands for high quality parts and further processed convenience foods have driven the poultry industry to change its marketing practices. Primarily poultry was sold in supermarkets as ready-to-cook whole carcasses. Today, boneless fillet have become critical to processors, yield of fillet (Boneless, skinless pectoralis major) ranged from 14.9 to 15.1% **(Young et al., 2001)**.

The administration of health-risk related substances such as growth promoting agents and veterinary drugs (antibiotics) is a recurring problem in animal production where these compounds are often used to increase the productivity and to reduce breeding costs **(Toffolatti et al., 2006)**. Uses of them in broiler chicken farms for therapeutic and performance-enhancing purposes may lead to deposit of residuals in their carcasses, particularly when the birds are slaughtered without the observance of withdrawal period of the drug **(Donoghue & Hairston, 2000 and Kan & Petz, 2000)**. Ignorance of observation of withdrawal period leads to a serious threat to human health upon exposure to these residues. Therefore, residues monitoring are required in detecting anabolic and veterinary drugs treatments for the safety of consumers.

Antibiotics are used extensively in poultry industry for the treatment and prevention of several diseases, as well as to improve feed efficiency and promote growth **(McEvoy 2002 and Di Corcia & Nazzari, 2002)**. In addition assist in converting stress due to environmental changes, vaccination, debeaking and other management practices. This wide spread use of antibiotic may cause residuals in foodstuffs, as well as the induction of allergic reactions in humans. In addition, resistance to pathogenic bacteria has been

constantly weakened as a result of antibiotic usage **(Schenck & Callery, 1998)**. Oxytetracycline is a natural tetracycline compound that is derived from the fungus, *Streptomyces rimosus*. It is a broad-spectrum antibiotic with bacteriostatic activity, It is poorly metabolized in target animals and excreted practically in its parent form, due to its high water solubility **(Slana & Dolenc, 2013)**. Enrofloxacin is a second generation fluoroquinolone with bactericidal activity. After oral application, is well absorbed and distributed at tissue level, metabolized in the liver, generating its major active metabolite, ciprofloxacin **(Unisol, 2010)**. Many endogenous steroids, including their semi-synthetic and synthetic analogues, have been produced and administered to animals to improve growth of animals for food production, as well as to regulate and enhance fertility **(Lone, 1997)**. Some scientific reports stated their possible carcinogenicity, genotoxicity and interfere with human and animal natural physiological function. Also, it has been found that the highest rates of hormone-related cancer, including cancer of breast, ovary, prostate, testes and colon were found where hormone-treated meat is consumed **(Andersson & Skakkebaek, 1999 and Sibbald, 1999)**. In light of the carcinogenic potential of their residues and obvious human health risks, the European Community forbade the use of steroids as growth-promoting agents in livestock breeding **(EC, 1996&2010)**. In some countries, a related synthetic estrogenic compound zeranol are officially registered for use as hormonal growth promoting compounds owing to their anabolic and/or partitioning effect because of the potential for toxicity at a very low level, the use of zeranol has been completely forbidden in the European Union. Hence the maximum residue limits (MRLs) of this compound in animal tissues is undetermined **(Fang et al., 2002)**.

The objective of this study is to through the light on safety of the broiler fillet which used in large quantities in homemade or in ready to eat food through residues monitoring of antibiotics (Oxytetracycline & Enrofloxacin) and anabolic steroid growth promoters either natural (Testosterone & Progesterone) or synthetic (the estrogen compound Zeranol), with special refrences to the effect of the most common cooking procedures of it (frying and grilling) on the antibiotic residues level.

2. Materials and methods

One hundred random broiler fillet samples (fresh from small scale production poultry processing shops and frozen from large scale production from the super market, 50 of each) were collected, wrapped in polyethylene bags and put in cool boxes with dry ice or freezer packs. The samples were subsequently rapidly transported under a complete aseptic condition to the laboratory of Food Control Department. Faculty of Veterinary Medicine, Zagazig University, then prepared for antibiotics and anabolic steroid growth promoters residues monitoring.

A. Evaluation of antibiotic residues

Two methods were used simultaneously for the determination of antibiotics residues in broiler fillet.

1. Qualitative evaluation: Microbiological inhibition assay using *Bacillus subtilis* (ATCC-6633) as indicator organism. The level of antibiotics can be evaluated by measuring the diameter of inhibition zone observed on an agar layer seeded with a test organism (with a caliper) according to **Levetzow and Wiese (1979)**. The indicator organism was obtained from Department of Bacteriology, Animal Health Research Institute in Doki, Giza.

2. Quantitative evaluation: HPLC analysis was used for determination of oxytetracycline and enrofloxacin residues level in positive samples resulted from the microbiological inhibition assay.

HPLC analytical procedures

Sample extraction

Fresh and thawed broiler fillet samples were finely sliced after trimming off external fat and fascia.

Oxytetracycline: Two grams of broiler fillet were cutted into very small pieces and subsequently ground into fine particles using Sartorius mincer, then homogenized for 2 min and then 0.1 g citric acid was added. To this mixture, 1 ml nitric acid (30%), 4 ml methanol (HPLC grade) and 1 ml deionized water were added, respectively. The suspension with solid particles was vortexed, kept in an ultrasonic bath for 15 min and centrifuged for 10 min at 4000 rpm. After filtering through a 0.45 µm nylon filter, 20 µl of solution was injected into HPLC for analysis according to **Senyuva et al. (2000)**.

Enrofloxacin: Five grams of broiler fillet were transferred to a 30-ml centrifuge tube, 20 ml of 1 M HCl was added and the mixture sonicated for 5 min. The tube was subsequently centrifuged for 5 min at 4000 rpm. The supernatant was taken after centrifugation and Sep-Pak C_{18} cartridges, previously conditioned, are then used for purification. The cartridges were washed with 10 ml of water and the elution of enerofloxacin was performed with 4 ml of mono potassium phosphate (1 mM), pH 2.5–methanol (1:1) mixture. The purification residues were evaporated in a nitrogen stream at 35º C, 20 µl of solution was injected into HPLC for analysis according to **Gigosos et al. (2000)**.

Chromatographic conditions

Oxytetracycline: The mobile phase consisted of methanol (HPLC grade) and formic acid 0.1% using a gradient method with a flow rate of 1.5 ml/min at 25^{o}C. The separation was done on hypersil gold C_{18} (10 µm, 100x4.6 mm) column with mobile phase. Detection was performed with photodiode array detector (PAD) set at 350 nm wave length. Quantification of residues in samples was obtained and calculated from areas under

curves extrapolated automatically by the software (Chromo Quest 5).

Enrofloxacin: The mobile phase consisted of a mixture of 0.1 M orthophosphoric acid, pH 3.5–acetonitrile (85:15, v/v). The eluent was filtered prior to use. Detection was performed with photodiode array detector (PAD) set at 280 nm wave length at a flow rate of 1 ml/min. Chromatographic separation was achieved on a C_{18} hypersil BDS (5 µm, 250×4.6 mm) column.

The concentration of antibiotics residue in the samples were calculated with reference to a calibration curves obtained from work solutions of oxytetracycline and enrofloxacin ranged from 0.01 to 50 µg/ ml and 0.5 to 10 µg/ ml respectively. For the preparation of the work solutions, Oxytetracycline hydrochloride (Sigma Aldrich, Inc., St. Louis, USA) and Enrofloxacin (Bayer Pharmaceuticals, West Haven, CT, USA) stock solutions (1 mg/ml in methanol) of the antibiotics were diluted to concentrations previously mentioned by using methanol as diluents.

B. Evaluation of anabolic steroid growth promoters

Anabolic steroids residue screening were carried out by using commercial ELISA kits (Art. No. DRG1561, DRG1559 and R3301) specific to progesterone, testesterone and zeranol respectively, obtained from r- Biopharm AG, Germany and stored at 4ºC. Kits were supplied with reagents for the enzyme immunoassay including standards and specific coated micro- titer plates. The sample extraction and estimation were performed based on the manufacturer procedure described by the ELISA kits.The senstivity range of progesterone and testesterone assays were 0.03-0.07 ng/ml, 0.05-0.09 ng/ml respectively and detection limit of the used zeranol kits was (100 ng/kg).

C. Effect of cooking procedures on oxytetracycline and enrofloxacin residues level

Animal treatment

Ten broiler chickens (40 days age with average weight 1.750- 2 kg), classified into two groups. Every 5 broiler housed in identified cage. Each group was dosed over 5 consecutive days with 15 mg/ kg day of oxytetracycline and enrofloxacin. through the intramuscular route. The pharmacological speciality Oxytetracycline 5% and Enroflox (Enrofloxacin 10 %) were obtained from El- Nasr Company for Pharmaceutical Industry.

The chickens were slaughtered after 24 hours from the last dose and dressed to obtain the fillet. Five representative slices of broiler fillet from each group weighting 50 ± 4 g subjected to cooking either via frying (fried for 10 min with 400 ml sunflower oil in a pan, turning occasionally. The cooked fillet had a "well done" appearance on the outside) or grilling (grilled for 10 min). The microbiological inhibition assay method was applied on cooked broiler fillet for monitoring the effect of cooking in antibiotics residues level.

Statistical analysis

Data of the current study was statistically analyzed using the computer program **SPSS/PC (2001)**. The statistical method was one way ANOVA test.

3. Results and Discussion

Antibiotic residues in fresh and frozen broiler fillet

Our results revealed that the 34% from small scale fresh broiler fillet samples were positive for antibiotic residues, with an inhibition zone of 1.82 ± 0.371 mm. While only 8 % from the samples of large scale frozen broiler fillet were positive for antibiotic residues, with an inhibition zone of 0.62 ± 0.24 mm. There are significant difference between the small scale and large scale at ($p<0.01$). The difference may be attributed to the selection of broiler flock after elapsing of the withdrawal time of antibiotic treatment in large scale production, while this selection not occur in small scale production. Not only selection process but also, freezing may be act as a factor in reduction of antibiotic residues in examined frozen samples as previously mentioned by many authors **Mansour (2000), Okerman et al. (2007) and Mahmoud and Mohsen (2008)**.

From the obtained data in Table (1): It was found that the sum of positive samples for antibiotic residues in both fresh and frozen fillet representing 21% of total examined samples. These results were nearly in accordance with the results obtained by **Shahid et al. (2007)** which represent 20.4% in examined muscle samples. While higher percentages were detected by **Mahmoud and Mohsen (2008)** and **Shareef et al. (2009)** as they recorded the antibiotic residues in 50 and 56% respectively from the analyzed breast muscles.

The positive samples resulted from the microbiological inhibition assay were analyzed by HPLC for quantification of oxytetracycline and enrofloxacin residues. six samples from the 21 analyzed samples (31.5%) revealed a detectable level for oxytetracycline residues in fresh samples. The residual level ranged from 0.156 µg/g to 0.900 µg/g as shown in Figures (1 and 2) with a mean value of 0.394 ± 0.111 µg/g. The results reported in this study were consistent with these previously reported by **Abd El. Monem et al. (2002)** and **Gad (2012)**. Our results were higher than that obtained by **Salehzadeh et al. (2006) and Shahid et al. (2007)**, who recorded a residue level ranged from 0.0066 to 0.2553 and from 0.030 to 0.085 µg/g respectively.

The high residual level of oxytetracycline may attribute to the production of oxytetracycline in different trade names and forms in many companies in Egypt. So the broiler stock holders use it as cheap effective antibiotic for control of infections and as feed additives at sub-therapeutic levels as a growth

promoting agent. Oxytetracycline residues detected in fresh broiler fillet in this study may be due to the destructive effect of freezing on oxytetracycline **(Okerman et al., 2007)**.

Regarding to enrofloxacin residues, there were two samples in the fresh fillet at a level of 0.04 µg/g (Figure 3) and 0.757 µg/g (Figure 4) and one sample in frozen fillet at a level of 0.218 µg/g (Figure 5). The obtained results were higher than those recorded by **Salehzadeh et al. (2007)** as they detect 0.018 µg/g in examined chicken muscle.

The MRLs permitted by the European agency for the evaluation of medicinal products, Committee for veterinary medicinal products, for enrofloxacin and its metabolite ciprofloxacine are 100- 300 µg/kg in muscle, liver and kidney of bovine, porcine, rabbit, ovine and poultry species **(EMEA, 1998)**. European Union commission recommended the MRLs of oxytetracycline and enrofloxacin in the edible tissues of food producing animals were 0.1µg/g **(EC, 2010)**. The results of this study showed indications of violation of these recommendations.

From the results achieved in Table (2) all oxytetracycline positive samples found to be higher than the MRLs, while only 66.7 % of enrofloxacin positive samples were exceed MRLs, this may attributed to the unpaid attention to the withdrawal period of the antibiotic and extra label use. The microbial resistance to antibiotics may arise as result of animal exposure to these agents and this resistance may be transferred to human pathogens **(Yorke and Froc, 2000)**. In addition human exposure to animal products containing significant level of antibiotic residues may prove immunological response in susceptible individuals and cause disorder of intestinal flora **(Zaki et al., 2000)**.

Anabolic steroids residues in fresh and frozen broiler fillet

The results in Figure (6) revealed that the testosterone level in fresh fillet ranged from 0.10 to 0.70 µg /kg with a mean value of 0.33 ± 0.026 µg /kg, while the level in frozen fillet ranged from 0.10 to 0.60 µg /kg with a mean value of 0.28 ± 0.017 µg /kg. Results obtained by **Kadimi et al. (2010)** and **Zeitoun and Ahmed (2011)** revealed higher detectable levels (25.531 µg /kg and 2.008 µg /kg respectively) in chicken meat in Sultanate of Oman and Saudi Arabia. Testosterone levels were not statistically different ($p>0.05$) in fresh than in frozen fillet.

In the present study, the mean progesterone level in fresh fillet were 0.414 ± 0.039 µg /kg, while in frozen fillet were 0.364 ± 0.026 µg /kg. Higher results were obtained by **Zeitoun and Ahmed (2011)**, they detect 4.065 µg /kg in chicken meat at Saudi Arabia. Progesterone levels were not statistically different ($p>0.05$) in fresh than in frozen fillet. The obtained results clear that the detectable testosterone and progesterone level were attributed to endogenous hormones and confirmed that the broiler stock holders in Egypt nowadays don't use hormones as growth promoters.

According to the US-FDA guideline, they allowed incremental increases above the normal levels of progesterone and testosterone in muscle up to 3 and 0.640 µg /kg **(Center for Veterinary Medicine, Food and Drug Administration, CVM-FDA, 1994 a&b)**. Moreover, acceptable daily intake (ADI) of progesterone, and testosterone were established by the Joint Expert Committee on Food Additives (JECFA) at 30 and 2 µg /kg body weight **(Joint FAO /WHO, 1999)**. This mean that human weighting 70 kg can tolerate about 2100 and 44.8 µg of progesterone and testosterone daily, so he can eat fillet in any quantity without complaining of steroid residues, but some *in vivo* studies indicated that even small differences in hormone levels and very low doses of steroid hormones may have significant adverse biological effects **(Caruso-Nicoletti et al., 1985 and Masamura et al., 1997).**

Effect of cooking procedures on antibiotic residues in broiler fillet

The most common methods in cooking of fillet were frying and grilling, so this study evaluate the effect of these methods on the concentration of antibiotic residues by microbiological inhibition method as shown in Figure (7). Frying significantly diminish the percentage of residues of oxytetracycline (95.7 %) more than grilling (91.4 %) while enrofloxacin residues reduction percentages were 25.6 % and 33.3 % for frying and grilling respectively. The effect of cooking method on oxytetracycline residues coincide with **Marouf and Bazalou (2005)**, who studied the effect of frying process and the reported reduction percentage was 85.71%. Also **Rose et al. (1996)** discussed the heat stability of oxytetracycline and found that the drug was unstable. The result of frying and grilling on enrofloxacin residues was nearly similar to that obtained by **Lolo et al. (2006)**. On contrary **Javadi et al. (2011)**, who revealed a significant decrease of enrofloxacin residues. What ever the reduction percentages of the antibiotic residues, not render the fillet safe for human as antibiotic may destructed to harmful metabolites. Only applications of strict measure for maintaining the flocks in the farm till elapsing of the withdrawal period could solve the problem of human exposure to antibiotic residues.

In Conclusion: The widespread misuse of antibiotics especially oxytetracycline in farm and lack of implementation of recommended withdrawal time was ensured. Also, this study stresses on the need for stricter regulation for the use of drugs in the poultry industry as well as the inspection of broilers for residues prior to marketing.

Table (1): Antibiotic Residues Level in Fresh and Frozen Broiler Fillet by Microbiological Inhibition Assay.

No of samples	No of positive samples	percentages of positive samples	Inhibition zone in mm		
			Minimum	Maximum	Mean ± SE
50 fresh broiler fillet	17	34%	zero mm	11 mm	1.82 ± 0.371
50 frozen broiler fillet	4	8 %	zero mm	5 mm	0.62 ± 0.24

Table (2): Residues Level of Oxytetracycline and Enrofloxacin in Positive Samples in Comparison with Maximum Residue Limits (MRLs) in µg/ g: according to EC (2010).

Antibiotic	Sample No	Residues level (µg/g)	MRLs (µg/g)	Judgment
Oxytetracycline	**1**	0.339 µg/ g	0.1µg/g	**Rejected**
	2	0.439µg/ g		**Rejected**
	3	0.350 µg/ g		**Rejected**
	4	0.156 µg/ g		**Rejected**
	5	0.900 µg/ g		**Rejected**
	6	0.178µg/ g		**Rejected**
Enrofloxacin	**1**	0.04 µg/ g	0.1µg/g	**Pass**
	2	0.757 µg/ g		**Rejected**
	3	0.218 µg/ g		**Rejected**

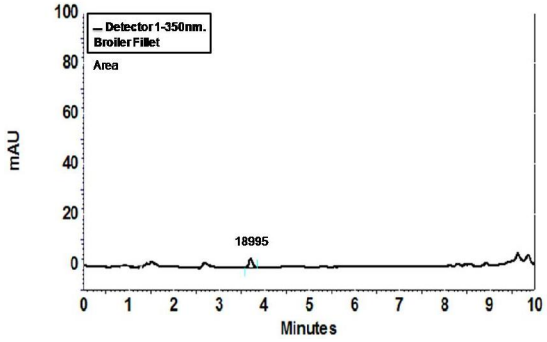


Figure (1): HPLC Chromatogram of Oxytetracycline Residue in Fresh Broiler Fillet.

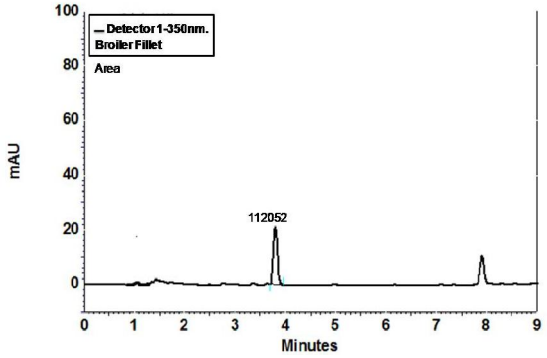


Figure (2): HPLC Chromatogram of Oxytetracycline Residue in Fresh Broiler Fillet.

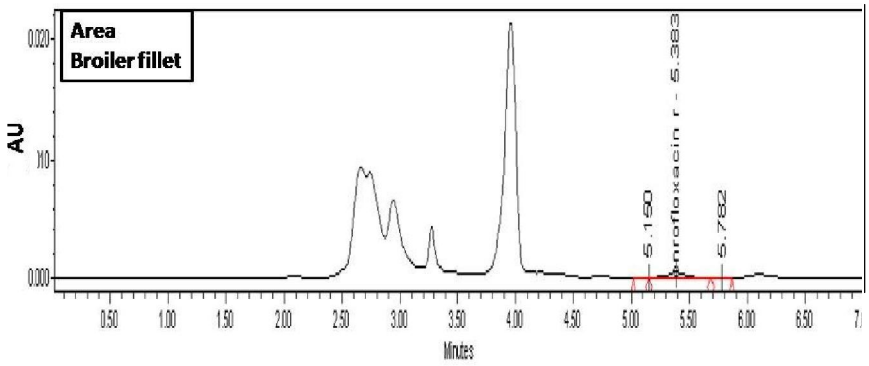


Figure (3): HPLC Chromatogram of Enrofloxacin Residue in Fresh Broiler Fillet.

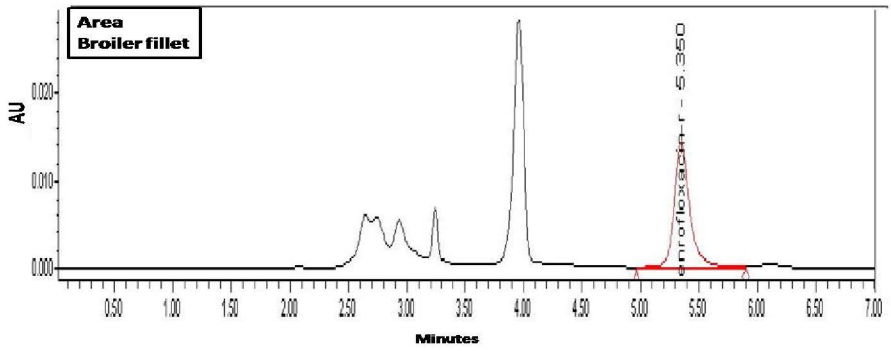


Figure (4): HPLC Chromatogram of Enrofloxacin Residue in Fresh Broiler Fillet.

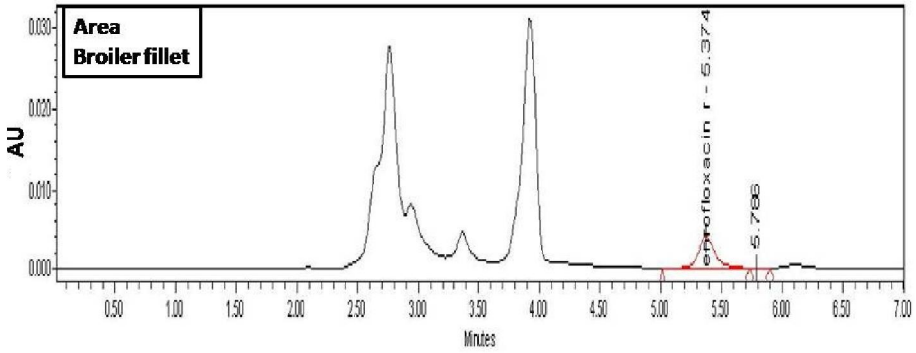


Figure (5): HPLC Chromatogram of Enrofloxacin Residue in Frozen Broiler Fillet.

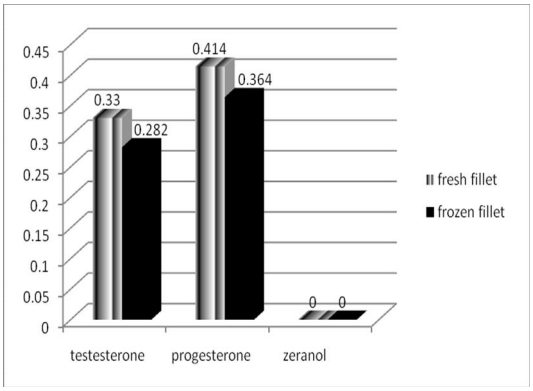


Figure (6): The Mean Detectable Residues Level of Testosterone, Progesterone and Zeranol in Fresh and Frozen Broiler Fillet.

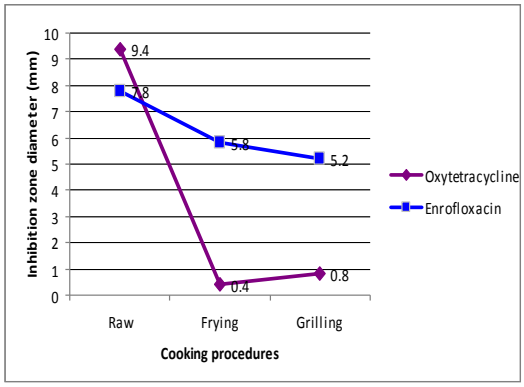


Figure (7): Effect of Different Cooking Procedures on the residual Level of Antibiotic Represented by Inhibition Zone Diameter (mm).

References

Abd El. Monem, K. M.; Soliman, M. R. and Saad, S. M. (2002): Oxytetracycline residues in Broiler carcasses produced by closed and open system. Journal of Egyptian Veterinary Medical Association, 62 (6a): 119-124.

Anderson, A. M. and Skakkebaek, N. E. (1999): Exposure to exogenous estrogens in food: possible impact on human development and health. Eur. J. Endocrinol. 140(6): 477-485.

Caruso-Nicoletti, M.; Cassorla, F.; Skerda, M.; Ross, J. L.; Loriaux, D. L. and Cutler, G.B. (1985): Short term, low dose estradiol accelerates ulnar growth in boys. J Clin Endocrinol Metab.61(5):896-8.

Center for Veterinary Medicine, Food and Drug Administration (CVM-FDA) (1994a):

Summary of NADA 009-576: Synovex® (estradiol benzoate and progesterone). http://www.fda.gov/cvm/efoi/section1/009576s81994.html

Center for Veterinary Medicine, Food and Drug Administration (CVM-FDA) (1994b): Summary of NADA 140-992: Revalor®-H (trenbolone acetate and estradiol), http://www.fda.gov/cvm/efoi/section2/140992.html

Di Corcia, A. and Nazzari, M. (2002): Liquid chromatographic–mass spectrometric methods for analyzing antibiotic and antibacterial agents in animal food products. Journal of Chromatography A, 974, 53–89.

Donoghue, D. J. and Hairston, H. (2000): Food safety implication: certain antibiotics may rapidly contaminate egg albumen during the process of its formation. British Poulty Science, 41, 174–177.

EC, European community (1996): Council directive 96/22/EC of 29 April 1996 concerning the prohibition on the use in stock farming of certain substances having a hormonal or thyrostatic action and of beta-agonists, and repealing directives 81/602/EEC, 88/146/EEC and 88/299/EEC. Official Journal of the European Communities, L125, 3–9.

EC, (2010): Commission regulation NO 37/2010 of 22, December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, off. J. Eur. Communities. L 15, pp.1-7.

EMEA, (The European Agency for the Evaluation of Medicinal products), Committee for veterinary medicinal products (1998): Enrofloxacin (modification for bovine, porcine and poultry), Summary report (2), EMEA/MRL/388/98, 1-6. http://www.eudra.org/emea.html.

Fang, X.; Chen, J. and Guo, D. (2002): Detection and Identification of Zeranol in Chicken or Rabbit Liver by Liquid Chromatography-Electrospray Tandem Mass Spectrometry. Journal of AOAC International. 85, NO. 4, 841-847.

Gad, T. M. M. (2012): Effect of age and sex of animal on the antibiotic residues in tissue of some slaughtered animals. Ph. D. Thesis, Faculty of Veterinary Medicine, Zagazig University.

Gigososa, P. G.; Revesadoa, P. R.; Cadah´ıaa, O.; Fenteb, C. A.; Vazquezb, B. I.; Francob, C. M. and Cepeda, A. (2000): Determination of quinolones in animal tissues and eggs by high performance liquid chromatography with photodiode-array detection. Journal of Chromatography A, 871 31–36.

Javadi, A.; Mirzaei, H. and Khatibi, S. A. (2011): Effect of roasting, boiling and microwaving cooking methods on Enrofloxacin residues inedible tissues of broiler. African Journal of Pharmacy and Pharmacology, 5(2): 214-218.

Joint FAO/WHO Expert Committee on Food Additives. (1999): Summary and Conclusions of the Fifty-second Meeting, Rome, 2–11 February 1999. http://www.fao.org/WAICENT/FAOINFO/ECONOMIC/ESN/Jecfa/jecfa52.pdf

Kadimi, I. T.; Mahgoub, O.; AL-Marzooqi, W.; AL-Maqbaly, R.; Annamali, K. and Khalaf, S. K. (2010): Enzyme-LS immunosorbent assay for screening antibiotic and hormone residues in broiler chicken meat in the Sultanate of Oman. Journal of Muscle Foods, 21: 243–254.

Kan, C. A. and Petz, M. (2000): Residues of veterinary drugs in eggs and their distribution between yolk and white. Journal of Agricultural and Food Chemistry, 48: 6397–6403.

Levetzow R. and Weise H. (1979): Method Zum Nachwies Von Ruckstanden Antibacteriell Wirksamer Substanzen in Frischen Fleish, Institute of Veterinary Medicine. (Meat Hygiene), Berlin.

Lolo, M.; Pedreira, S.; Vázquez Belda, B.; Miranda, J.; Franco, C.; Cepeda, A. and Fente, C. (2006): The effect of cooking on enrofloxacin residues in chicken muscle. J. Food Additives and Contaminants, 23, 10: 988-993.

Lone, K. P. (1997): Natural sex steroids and their xenobiotic analogs in animal production: Growth, carcass quality, pharmacokinetics, metabolism, mode of action, residues, methods, and epidemiology. Critical Reviews in Food Science and Nutrition, 37(2): 93–209.

Maged, O. and Hamdey, E. (2006): The analysis of livestock industry frame in Egypt: Proposal in the light of birds flu crisis, IDSC: Ministerial Cabinet Information and Designing Making Supporting Center: report 29/5/2006) http://www.idsc.gov.eg/Docs/DocsDetails.asp?rIssueCategory=2&MainIssues=9&DocID=294

Mahmoud, A. A. and Mohsen, A. M. (2008): Incidence of some antibiotic residues in broiler meat at North Sinai Governorate. Zagazig Veterinary Journal, 36(5):129-133.

Mansour, A. H. M. (2000): Studies on antibiotic residues in turkey meat and offal. Ph. D. Thesis, Department of food control (Meat Hygiene), Faculty of Veterinary Medicine, Moshtohor, Zagazig University, Benha Branch, Egypt.

Marouf H. A. and Bazalou M. S. (2005): Detection of antibiotic residues in meat sold in Damietta governorate. 4th Int. Sci. Conf., Mansoura, 5-6 April: 509– 519.

Masamura, S.; Santner, S. J.; Gimotty, P.; George, J. and Santen, R. J. (1997): Mechanism for maintenance of high breast tumor estradiol concentrations in the absence of ovarian function: role of very high affinity tissue uptake. Breast Cancer Res Treat.; 42(3):215-26.

McEvoy, J. D. G. (2002): Contamination of animal feedstuffs as a cause of residues in food: A review of regulatory aspect, incidence and control. Anal. Chim. Acta, 473, 3–26.

Okerman, L.; Hende, J. V. and De Zutter, L. (2007): Stability of frozen stock solutions of beta-lactam antibiotics, cephalosporins, tetracyclines and quinolones used in antibiotic residue screening and antibiotic susceptibility testing. Analytica Chimica Acta, 586: 284–288.

Rose, M. D.; Bygrave, J.; Farrington, W. H. and Shearer, G. (1996): The effect of cooking on veterinary drug residues in food: 4. Oxytetracycline. Food Additives and Contaminants, 13 (3): 275– 286.

Sadek, I. A.; Ismail, H.M.; Sallam, H. N. and Salem, M. (1998): Survey of hormonal level of meat and poultry sold in Alexandria, Egypt. Eastern Mediterranean Health Journal 4 (2):239-243.

Salehzadeh, F.; Salehzadeh, A.; Rokni, N.; Madani, R. and Golchinefar, F. (2007): Enrofloxacin Residue in Chicken Tissues from Tehran Slaughterhouses in Iran. Pakistan Journal of Nutrition 6 (4): 409-413.

Salehzadeh, F.; Madani, R.; Salehzadeh, A.; Rokni, N. and Golchinefar, F. (2006): Oxytetracycline Residue in Chicken Tissues from Tehran Slaughterhouses in Iran. Pakistan Journal of Nutrition 5 (4): 377-381.

Senyuva, H.; Ozden, T. and Sarica, D. Y. (2000): High- performance liquid chromatographic determination of oxytetracycline residue in cured meat products. Instrumental Analysis Center, Scientific and Technical Research Council of Turkey (TUBITAK) 06530, Ankara-Turkey. Turk. J. Chem. 24: 395-400.

Shahid, A. M.; Siddique, M.; Rehman, U. S.; Hameed, S. and Hussain, A. (2007): Evaluation of a microbiological growth inhibition assay as a screening test for the presence of antibiotic residues in poultry meat. American Journal of Food Technology, 2 (5): 457-461.

Shareef, A. M.; Jamel, Z. T. and Yonis, K. M. (2009): Detection of antibiotic residues in stored poultry products. Iraqi Journal of Veterinary Sciences, 23(I): 45-48.

Schenck, F. J. and Callery, P. S. (1998): Chromatographic methods of analysis of antibiotics in milk. Journal of Chromatography A, 812, 99–109.

Sibbald, B. (1999): " European ban on bovine growth hormones should continue: expert", Canadian Medical Association Journal.,September 21. http://www.cmaj.ca/cgi /content/full/161/6/677.

Slana, M. and Dolenc, M. S. (2013): Environmental Risk Assessment of antimicrobials applied in veterinary medicine—A field study and laboratory approach Environmental Toxicology and Pharmacology, 35: 131–141

SPSS (2001): SPSS/PC+ (2001), for the PC/XT. SPSS INC.

Toffolatti, L.; Rosa Gastaldo L.; Patarnello, T.; Romualdi C.; Merlanti, R.; Montesissa, C.; Poppi, L.; Castagnaro, M. and Bargelloni, L. (2006): Expression analysis of androgen-responsive genes in the prostate of veal calves treated with anabolic hormones. Domestic Animal Endocrinology 30: 38–55.

Unisol, (2010): UNISOL 2.5% Oral Solution for Calves. Summary of Product Characteristics. Revised: January 2011.AN:01275/, pp. 1–6.

Young, L. L.; Northcutt, J. K.; Buhr, R. J.; Lyon, C. E. and. Ware, G. O. (2001): Effects of Age, Sex, and Duration of Postmortem Aging on Percentage Yield of Parts from Broiler Chicken Carcasses. Poultry Science 80:376–379.

Yorke, J. C. and Froc, P. (2000): Quantitation of nine quinolones in chicken tissues by High-performance liquid chromatography with fluorescence detection. J. Chromatography A, 882: 63-77.

Zaki, H.; AL-Mustafa, S.; Mastour, A. and Al-Ghamdi, A. (2000): Use of norfloxacin in poultry production in the eastern province of Saudi Arabia and its possible impact on public health. Int. J. Environ. Health Res.,10: 291-299.

Zeitoun, M. M. and Ahmed, S. M. (2011): Effect of Cooking Method on the Residues of Natural Sex Steroid Hormones in Local and Imported Meats and Meat Products in Al-Qassim Region. Journal of Agricultural and Veterinary Sciences. Qassim University, 4 (2): 83-92.

2/2/2013

The Toxic Effect of Melamine on the Kidney of Male Rats as Revealed by Biochemical and Histopathological Investigations

Haddad A. El Rabey[*1], Abdulbasit I. Al- Sieni[1] and Abdullah A. Majami[2]

[1] Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.
[2] FDA, Jeddah, Saudi Arabia
elrabey@hotmail.com

Abstract: This study aimed to evaluate the toxic effect of four different melamine doses (5000, 10000, 15000 and 20000 ppm) supplemented orally in the diet for 28 days on male rats. The appearance, anatomy, serum electrolytes, kidney functions (creatinine, urea and uric acid), serum melamine concentration, total body weight, food intake, food efficiency ratio (FER), body weight gain (BWG), percentage of body weight gain (BWG %), water consumed and histopathological examinations of three organs (kidney, ureter and urinary bladder) were investigated. The melamine supplemented rats turned yellow and showed different degrees of toxicity, hypertrophy and congestion, particularly the kidneys and the ureters as a result of melamine toxicity. Serum Na and Cl levels were decreased, whereas serum K, P and Ca levels were increased compared to the negative control. Kidney functions showed elevation of the mean values of serum creatinine, urea and uric acid. The histopathological examination of the three organs under study showed adverse pathological signs according to the melamine dose.
[Haddad A. El Rabey, Abdulbasit I. Al- Sieni and Abdullah A. Majami. **The Toxic Effect of Melamine on the Kidney of Male Rats as Revealed by Biochemical and Histopathological Investigations**. *Life Sci J* 2013;10(1):2119-2130]. (ISSN: 1097-8135). http://www.lifesciencesite.com.

Keywords: electrolytes, melamine, creatinine, kidney, histopathology.

1. Introduction:

Melamine, or 1,3,5-triazine-2,4,6-triamine, is a small, nitrogen-rich molecule. It was considered non-toxic based on early laboratory animal studies until significant morbidity and mortality related to crystalluria, nephrolithiasis and nephrotoxicity have resulted from pet food contamination (Bischoff, 2011).

It is harmful if swallowed, inhaled or absorbed through the skin and chronic exposure may cause cancer or reproductive damage. It is eye, skin and respiratory irritant (Weise, 2007). Direct contact results in skin irritation and eye irritation, and inhalation causes respiratory tract irritation. Oral ingestion affects the digestive tract, presenting as nausea, vomiting, and diarrhea (Jeong *et al*., 2006). The mechanism of carcinogenesis was most likely secondary to epithelial hyperplasia caused by mechanical irritation (Hau *et al*., 2009). Melamine is minimally, if at all, metabolized in monogastrics, but could be partially metabolized in the rumen of cattle and small ruminants. Melamine does not accumulate over time in the animal body. Renal elimination of unchanged melamine is approximately 90% complete within 24 hours (Qin *et al.*, 2010).

The combination of melamine and cyanuric acid in diet does lead to acute renal failure in cats (Puschner *et al.*, 2007) and rats (Dobson *et al.*, 2008). Dalal and Goldfarb (2011) reviewed the toxicology, epidemiology, and pathology due to melamine contamination of foodstuffs due to melamine contamination only or in combination with cyanuric acid.

In 2008, melamine contamination of baby milk-based products was detected in China. Chinese authorities detected melamine concentrations between 2.5 and 2563 ppm in 13 commercial brands of milk powder and trace contamination in nine others (Bhalla *et al.*, 2009). Gonzalez *et al.* (2009) discovered renal failure in piglets in Spain between 2003 and 2006. They found that the kidneys contained melamine, cyanuric acid and relatively high concentrations of ammelide and ammeline.

Lv *et al.* (2010) found that melamine concentrations in the kidney were higher than concentrations in the skeletal muscle or liver of lambs, and concentrations decreased below 20 ppb 4 days after cessation of exposure. Addition of cyanuric acid to the diet did not affect melamine deposition. On the other hand, Shen *et al.* (2010) stated that melamine is excreted by dairy cattle into milk, particularly in high-producing cattle, though milk yield and composition are otherwise unaffected. Melamine can be detected in milk within 8 hours of exposure and remains detectable until 4 days after cessation of exposure. Approximately 0.3% of a melamine dose was excreted in milk in dairy goats, and milk melamine concentrations remained above the level of concern (1.0 µg/mL) until 3 days after cessation of dosing (Baynes *et al.*, 2010).

Kim *et al.* (2010) using rat models, investigated the renal crystal formation following the

ingestion of a melamine−cyanuric acid mixture (M+CA, 1:1) to gain insight into the M+CA-induced renal toxicity. They found that M+CA did not induce toxicity in precision-cut kidney slices, suggesting that M+CA does not have a direct nephrotoxicity. In addition, Puschner and Reimschuessel (2011) reported that both melamine and cyanuric acid were relatively nontoxic when given individually, but they caused crystal formation in renal tubules when given together. Moreover, Camacho *et al*. (2011) stated that co-exposure of rats to melamine and cyanuric acid leads to alterations in the expression of the genes encoding kidney injury molecule 1 (KIM-1), metallopeptidase inhibitor 1 (TIMP1), clusterin, osteopontin, and neutrophil gelatinase-associated lipocalin/lipocalin 2 (NGAL), which have been proposed as urinary biomarkers for nephrotoxicity.

Wen *et al*. (2010) reported that the basic treatment regimens for crystalluria and urolithiasis related to melamine ingestion include fluid therapy and supportive care in both veterinary and pediatric patients. Low urinary pH is associated with crystal formation in infants so, alkalization of the urine was used to maintain urine pH between 6.0 and 7.8 in affected children. Sodium bicarbonate or potassium citrate was added to intravenous fluids for this purpose (Gao *et al*., 2010 and Wen *et al*., 2010). Antispasmodic drugs such as anisodamine or atropine were given to facilitate excretion of uroliths in children, and pain management was instituted (Bhalla *et al*., 2009 and Wen *et al*., 2010). Most children recovered with this conservative management (Gao *et al*., 2010 and Wen *et al*., 2010). However, hemodialysis was required in some patients, as was surgical intervention (Wen *et al*., 2010). Most children recovered fully, but 12% were found to have renal abnormalities 6 months after treatment (Liu *et al*., 2010).

In the current study, the biochemical and histopathological changes resulted from the toxic effect of different melamine doses (for 28 days) on male rats were studied.

2. Materials and Methods

Male Albino Wister rats *(Rattus rattus)* of an average of 200-225 g were obtained from King Fahd Centre for Medical Research, King Abdulaziz University, KSA. Rats were housed five per cage and held for approximately two weeks before the study began for acclimatization. Cages, bedding, and glass water bottles (equipped with stainless steel sipper tubes) were replaced twice per week. Test diets, control diets, and tap water are available *ad libitum.* Conventional animal basal diet was obtained from Grain Silos & Flour Mills Organization–Makkah branch in Jeddah- Saudi Arabia and Melamine (99%) was purchased from Sigma-Aldrich Cat. No. M2659-5G.

The rats were distributed into five groups; rats of the first group were fed normal basic diet as a (-)ve control and four melamine supplemented groups as follows: i. G1 were supplemented with 5,000 ppm, ii. G2 were supplemented with 10,000 ppm, iii. G3 were supplemented with 15,000 ppm and iv. G4 supplemented with 20,000 ppm. Test diets were prepared by mixing the suitable melamine weight (according to the dose) to the basal diet. The rats were observed twice daily for mortality and morbidity and were weighed every week and at the end of the study that lasted 28 days. Necropsies were performed on all animals at the end of the study. All rats were dissected and the target organs for histopathological investigations were kept in 10% formalin.

Biochemical analysis

Serum electrolytes (Na, K, Cl, Ca and P) and kidney functions indices (creatinine, urea and uric acid) were measured also using Flex reagent cartridge, URCA method of Dimension Vista System, Siemens Health Care, Diagnostics Inc., Newark, DE 19714, USA, according to the instruction of the supplier.

Serum melamine concentration was estimated using GC-MSD 5975 series from Agilent Technologies, USA according to the instruction of the suppliers.

Body weight gain, food efficiency and consumed water

The water consumed was calculated for each group. Body weight per cage was recorded once per week. The mean body weight of each group was calculated by dividing the total weight of all surviving animals by the number of surviving animals in the group. Food intake per cage was recorded once per week. Weight gain (g), body weight gain ratio (BWG%) and food efficiency ratio (FER) were calculated as follows:

Weight Gain= Final weight (g) - Initial weight (g)

BWG%=Final weight-initial weight/initial weight X 100

FER= Weight (g)/food intake (g).

Histopathological investigations

The target organs (kidney, ureter, urinary bladder and testis) were dissected out and fixed in 10% formalin, dehydrated in gradual ethanol (50-99%), cleared in xylene, and embedded in paraffin. Sections were prepared and then stained with hematoxylin and eosin dye for microscopic investigation (Drury *et al.,* 1976). The stained sections were examined and photographed under a light microscope.

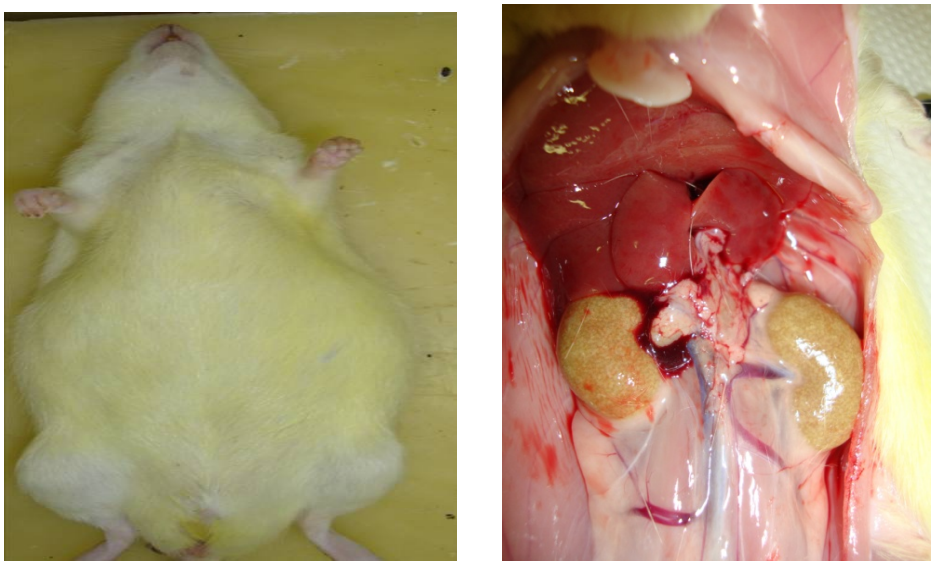
Data analysis

All data were analyzed using the SPSS (Statistical Program for Sociology Scientists) Statistics Version 17.0 for computing the mean values, the standard errors (SE), and the test of significance (t-test).

3. Results

Effect of melamine supplementation on the morphology and anatomy

Figure (1) shows the effect of melamine supplementation for 28 days on male rats under study. The melamine supplemented rats showed different degrees of toxicity according to the melamine dose. Figure (1 A), shows the melamine fed rat (dose 20,000 ppm) has turned yellow colored and swollen, while Figure (1 B), shows the same rat after dissection, with hypertrophic and congested organs (particularly, ureters both kidneys and liver. Kidneys were turned yellow and greatly enlarged.



A B

Figure (1): Effect of melamine supplementation for 28 days on the morphology and anatomy of rats; A; melamine fed rat for 28 days (yellow colored, dose 20,000 ppm), B; Dissected melamine fed rat as in A.

Serum electrolytes

Table (1), shows the effect of melamine supplementation for 28 days on serum electrolytes; Na, K, CA, CL, and P. The mean values of Na in all melamine supplemented groups (G1, G2, G3 and G4) were non significantly lower than that of the negative control (138.20±1.39, 136.60±1.88, 137.80±1.95, 136.40±1.43 and 141±0.82 mmol/L, respectively).

Table (1): Effect of melamine supplementation for 28 days on serum electrolytes (Na, K, CL, CA and P).

Parameter	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
Na mmol/L	Mean±SE	141±0.82	138.20±1.39	136.60±1.88	137.80±1.95	136.40±1.43
	T-test		1.15 NS	2.08 NS	0.91 NS	1.93 NS
K mmol/L	Mean±SE	4.84±0.12	5.56±0.21	5.12±0.17	5.26±0.25	5.54±0.34
	T-test		-2.29*	-2.74*	-1.36*	-1.73*
Cl mmol/L	Mean±SE	97.21±0.47	92.20±1.98	84.60±1.96	89.00±1.94	88.80±2.22
	T-test		2.21*	6.10***	3.80**	3.41**
Ca mmol/L	Mean±SE	2.57±0.03	2.63±0.04	2.70±0.15	2.88±0.09	2.93±0.16
	T-test		-0.95 NS	-2.03 *	-3.39**	-0.30 **
P mmol/L	Mean±SE	2.72±0.08	2.84±0.05	2.93±0.88	3.31±0.23	4.76±0.67
	T-test		-3.70*	-3.67*	-1.94**	-3.27**

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P<0.05), ** means high significant at 1% (P <0.01), *** means very high significant at 0.1% (P <0.001) and NS means non significant.

The mean values of K in all melamine supplemented groups (G1, G2, G3 and G4) were significantly higher at 1% (P <0.01), than that of the negative control (5.56±0.21, 5.12±0.17, 5.26±0.25, 5.54±0.34 and 4.84±0.12 mmol/L, respectively).

The mean values for Cl in all melamine supplemented groups (G1, G2, G3 and G4) were lower than that of the negative control (92.20±1.98, 84.60±1.96, 89.00±1.94, 88.80±2.22, 90.00±1.14 and 97.21±0.47 mmol/L, respectively). The differences were significant at 5% (P <0.05) in G1, high significant at 1% (P <0.01) in G3 and G4 and very high significant at 0.1% (P <0.001) in G2.

The mean values of serum Ca in all melamine supplemented groups (G1, G2, G3 and G4) were higher than that of the negative control (2.63±0.04, 2.70±0.11, 2.88±0.09, 2.93±0.16 and 2.57±0.03 mmol/L, respectively). Differences were significant at 5% (P <0.05) in G1 and G2 and highly significant at 1% (P <0.01) G3 and G4, when compared to the negative control.

The mean values of serum P in all melamine supplemented groups (G1, G2, G3 and G4) were higher than that of the negative control (2.84±0.05 and 2.93±0.88, 3.31±0.23, 4.76±0.67 and 2.72±0.08 mmol/L, respectively). Differences were non significant in G1, significant at 5% (P<0.05) in G2 and highly significant at 1% (P <0.01) in G3 and G4.

Kidney Functions

Table (2), shows the effect of melamine supplementation for 28 days on kidney function indices; creatinine, urea and uric acid. As shown, the mean values of serum creatinine in all melamine supplemented groups (G1, G2, G3 and G4) were higher than that of the negative control (141.80±11.57, 189.60±20.80, 221.80±22.33, 313.80±47.21 and 43.01±2.51 umol/L, respectively. Creatinine levels were proportionally increased with the increase in the melamine dose. However, differences were very high significant at 0.1% (P <0.001).

Table (2): Effect of melamine supplementation for 28 days on serum creatinine, urea and uric acid.

Parameters	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine G3	G4 20,000 ppm melamine
Creatinine umol/L	Mean±SE	43.01±2.51	141.80±11.57	189.60±20.80	221.80±22.33	313.80±47.21
	T-test		-9.16***	-7.46***	-8.36***	-5.53***
Urea umol/L	Mean±SE	43.32±1.55	45.80±9.97	48.20±1.93	51.80±3.24	56.00±1.51
	T-test		-0.84*	-5.51**	-2.21***	-11.25***
Uric acid mg/dl	Mean±SE	1.23±0.05	1.30±0.07	1.42±0.93	1.50±0.24	1.61±0.16
	T-test		-1.04 NS	-1.51*	-2.21**	-4.58**

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P <0.05), ** means high significant at 1% (P <0.01), *** means very high significant at 0.1% (P <0.001) and NS means non significant.

The same table shows that the mean values of serum urea in all melamine supplemented group (G1, G2, G3 and G4) were higher than that of the negative control (45.80±9.97, 48.20±1.93, 51.80±3.24, 56.00±1.51 and **43.32±1.55** umol/L, respectively). Differences were significant at 5% (P <0.05) in G1 and highly significant at 1% (P <0.01) in G2 and very high significant at 0.1% (P <0.001) in G3 and G4.

The mean values of serum uric acid in all melamine supplemented group (G1, G2, G3 and G4) were higher than that of the negative control (1.30±0.07, 1.42±0.93, 1.50±0.24, 1.61±0.16 and 1.23±0.05 mg/dl, respectively). Differences were significant at 5% (P <0.05) in G1 and G2 and highly significant at 1% (P <0.01) in G3 and G4, when compared to the negative control.

Serum melamine concentration

Table (3), show mean values of melamine content in serum after 28 days of melamine supplementation in all groups (G1, G2, G3 and G4). They were 6.70±1.08, 7.48±1.96, 13.60±6.35 and 19.13±8.21 mg/ml and the negative control was zero. However, differences were very high significant when compared to the negative control.

Table (3): Melamine concentration in the serum.

Parameters	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
Melamine mg/kg	Mean±SE	0.00±0.00	6.70±1.08	7.48±1.96	13.60±6.35	19.13±8.21
	T-test		-6.19***	-3.80***	-2.14***	-2.32***

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P <0.05), ** means high significant at 1% (P P <0.01), *** means very high significant at 0.1% (P <0.001) and NS means non significant.

Total body weight

Table (4), shows the effect of melamine supplementation for 28 days on the total body weight in rats. As shown, the mean values of the first weight (after one week) of all melamine supplemented groups (G1, G2, G3 and G4) were non significantly lower than that of the negative control. (215.56±12.03, 220.82±6.42, 227.02±7.81, 216.33±8.24 and 228.75±12.98 g, respectively).

Table (4): Effect of melamine supplementation for 28 days on total body weight.

Weight (g)	Treatments / Statistics	Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
1st weight	Mean±SE	228.75±12.98	215.56±12.03	220.82±6.42	227.02±7.81	216.33±8.24
	T-test		1.64 NS	0.76 NS	0.15 NS	1.03 NS
2nd weight	Mean±SE	263.06±13.59	249.93±10.82	253.88±6.09	255.51±6.81	250.62±8.99
	T-test		1.75 NS	0.76 NS	0.69 NS	0.95 NS
3rd weight	Mean±SE	288.87±11.58	273.56±10.35	253.88±6.09	286.85±8.39	275.33±5.76
	T-test		1.31 NS	3.16**	0.22 NS	1.17 NS
4th weight	Mean±SE	310.45±13.74	267.13±5.95	277.13±5.04	287.79±7.99	275.93±4.32
	T-test		3.39**	2.08 NS	1.75 NS	3.12**

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P <0.05), ** means high significant at 1% (P <0.01), *** means very high significant at 0.1% (P <0.001) and NS means non significant.

The mean values of the second weight (after two weeks) of G1, G2, G3 and G4 were non significantly lower than that of the negative control (249.93±10.82, 253.88±6.09, 255.51±6.81, 250.62±8.99 and 263.06±13.59 g, respectively).

The mean values of the third weight (after three weeks) G1, G3 and G4 were lower than that of the negative control (273.56±10.35, 253.88±6.09, 286.85±8.39, 275.33±5.76 and 288.87±11.58 g, respectively). However, the paired T-test did not show any significant differences in all groups except for G2 that was high significant at 1% (P <0.01) when compared to the negative control.

The mean values of the fourth weight (after four weeks and at the end of the experiment) of all melamine supplemented groups (G1, G2, G3 and G4) were lower than that of the negative control (267.13±5.95, 277.13±5.04, 287.79±7.99, 275.93±4.32 and 310.45±13.74 g, respectively). However, differences were not significant in G2 and G3 and highly significance at 1% (P <0.01) in G1 and G4 when compared to the negative control.

Food intake

Table (5), show the effect of melamine supplementation for 28 days on food intake in rats under study. As shown, the mean values of the food intake in the first week of G1 were non significantly lower than that of the negative control (147.80±13.42, and 151.24±7.95 g, respectively), whereas that of G2, G3 and G4 was higher than that of the negative control (160.70±11.55, 155.35±9.10, 155.90±9.06 and 151.26±7.93 g, respectively).

Table (5): Effect of melamine supplementation for 28 days on food intake.

Food Intake g	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
1st week	Mean±SE	151.26±7.93	147.80±13.42	160.70±11.55	155.35±9.10	155.90±9.06
	T-test		0.35	-0.90	-0.44	-0.53
2nd week	Mean±SE	116.15±10.62	160.88±3.51	172.07±5.62	187.18±2.98	197.85±12.00
	T-test		-4.54***	-6.27***	-8.31***	-4.65***
3rd week	Mean±SE	116.47±10.60	137.88±7.95	159.42±3.75	160.95±4.86	169.85±9.55
	T-test		-1.79***	-4.67***	-5.33***	-6.09 NS
4th week	Mean±SE	133.43±5.42	105.26±5.99	72.43±4.80	109.70±10.87	79.76±10.56
	T-test		3.30**	6.85***	1.78 NS	3.67**

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P <0.05), ** means high significant at 1% (P <0.01), *** means very high significant at 0.1% (P <0.001) and NS means non significant.

The mean values of food intake of all groups (G1, G2, G3 and G4) in the second week were higher than that of the negative control (160.88±3.51, 172.07±5.62, 187.18±2.98, 197.85±12.00 and 116.15±10.62 g, respectively). However, differences were very high significant at 0.1% (P <0.001) in all groups, when compared to the negative control.

The mean values of food intake of all groups (G1, G2, G3 and G4) in the third week were higher than that of the negative control (137.88±7.95, 159.42±3.75, 160.95±4.86, 169.85±9.55 and 116.47±10.60 g, respectively). However, differences were very high significant at 0.1% (P <0.001) in G1, G2 and G3 and non significant in G4.

The mean values of food intake of all groups (G1, G2, G3 and G4) in the fourth week were lower than that of the negative control (105.26±5.99, 72.43±4.80, 109.70±10.87, 79.76±10.56 and 133.43±5.42 g, respectively). However, differences were non significant in G3, highly significant at 1% (P <0.01) in G1 and G4 and very high significant at 0.1% (P <0.001) in G2, when compared to the negative control.

Food efficiency ratio, body weight gain and percentage of body weight gain

Table (6), show the effect of melamine supplementation for 28 days on food efficiency ratio (FER) and body weight gain body (g) weight gain % (BWG %) in rats under study. As shown, the mean value of weight gain of G1, G2, G3 and G4 were less than that of the negative control (49.77±9.23, 56.30±3.41, 60.77±1.79, 59.59±8.95 and 78.14±5.35 g, respectively), the differences were high significant at (P <0.01) in G1, G2, G3 and G4.

Table (6): Effect of melamine supplementation for 28 days on food efficiency ratio (FER) and body weight gain body (g) weight gain % (BWG %) in rats under study.

Parameters	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
Weight Gain (g)	Mean±SE	78.14±5.35	49.77±9.23	56.30±3.41	60.77±1.79	59.59±8.95
	T-test		3.25**	3.30**	4.22**	3.97**
BWG%	Mean±SE	34.85±3.57	24.17±5.51	25.68±2.04	26.88±1.20	28.18±4.82
	T-test		2.90**	3.12**	3.04**	1.76 NS
FER G	Mean±SE	0.106±.009	0.054±.012	0.079±.018	0.068±.006	0.079±.020
	T-test		3.90*	1.65 NS	5.77***	1.50 NS

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P <0.05), ** means high significant at 1% (P <0.01), *** means very high significant at 0.1% (P <0.001) and NS means non significant.

The same table shows the mean values of BWG% of all groups (G1, G2, G3, G4) were less than that of the negative control (24.17±5.51, 25.68±2.04, 26.88±1.20, 28.18±4.82, and 34.85±3.57%, respectively). The differences were non significant in G4, high significant at 1% (P <0.01) in G1, G2 and G3, when compared to negative control.

Concerning the FER, the mean FER values in all groups (G1, G2, G3 and G4) were less than that of the negative control (0.054±0.012, 0.079±.018, 0.068±.006, 0.079±.020 and 0.106±.009 g, respectively. However, differences were non significant in G2 and G4, significant in G1 at (P <0.05) and very high significant (P <0.001) in G3, when compared to negative control.

Water consumption

Table (7), shows the effect of melamine supplementation for 28 days on the amount of water consumed in rats under study. As shown, the mean values of water consumed in the 1st week of G1 were higher than that of the negative control (202.14±8.58 and 192.83±8.90 ml, respectively). Whereas the mean values of G2, G3 and G4 were lower than that of the negative control (184.28±6.49, 182.85±8.92, 174.28±7.51 and 192.83±8.90 ml, respectively). However, differences were non significant in G1, G2 and G3 when compared to the negative control, whereas differences in G4 were highly significant at1% (P <0.01).

The mean values of the water consumed in the second week in G2 and G4 were lower than that of the negative control (180.00±8.99, 174.28±11.92, and 185.14±9.46 ml, respectively), whereas that of G3 was equal to that of the negative control (187.14±8.370 and 185.14±9.46 ml, respectively). Moreover, the mean values of the water consumed in the second week in G1 were higher than that of the negative control (195.71±13.77 and 185.14±9.46 ml, respectively. However, the paired T-test did not show any significant differences between all groups and the negative control.

The mean values of the water consumed in the third week in G3 were lower than that of the negative control (194.28±7.19 and 196.71±2.95 ml, respectively, whereas that of all other groups (G1, G2 and G4) were higher than that of the negative control (208.57±9.36, 198.57±1.42, 210.00±2.97, 214.28±7.19 and 196.71±2.95 ml, respectively). However, differences were non significant in G1, G2

and G3 and highly significant at 1% (P <0.01) in G4, when compared to the negative control.

The mean values of water consumed in the fourth week of G2, G4 were lower than that of the negative control (166.66±7.76, 176.66±6.42 and 206.61±11.57 ml, respectively), whereas that of G1 and G3 were higher than that of the negative control (166.66±7.76, 210.00±6.17 and 206.61±11.57 ml, respectively). However, differences were non significant in G3, highly significant at 1% (P <0.01) in G2 and G4, and very high significant at 0.1% (P <0.001) in G1, when compared to the negative control.

Table (7): Effect of melamine supplementation for 28 days on the amount of water consumed.

water consumed ml	Treatments / Statistics	(-)ve Control (basal diet)	G1 5,000 ppm melamine	G2 10,000 ppm melamine	G3 15,000 ppm melamine	G4 20,000 ppm melamine
1st week	Mean±SE	192.83±8.90	202.14±8.58	184.28±6.49	182.85±8.92	174.28±7.51
	T-test		-1.11 NS	1.27 NS	0.95 NS	3.35**
2nd week	Mean±SE	185.14±9.46	195.71±13.77	180.00±8.99	187.14±8.370	174.28±11.92
	T-test		-0.82 NS	0.65 NS	0.00 NS	1.32 NS
3rd week	Mean±SE	196.71±2.95	208.57±9.36	198.57±1.42	194.28±7.19	210.00±2.97
	T-test		-1.59 NS	-1.00 NS	0.19 NS	-2.70**
4th week	Mean±SE	206.61±11.57	273.33±10.83	166.66±7.76	210.00±6.17	176.66±6.42
	T-test		-8.09***	3.69**	-0.16 NS	3.35**

Stars show the significant differences compared to the negative controls calculated by the paired sample t-test; * means significant at 5% (P <0.05), ** means high significant at 1% (P <0.01), *** means very high significant at 0.1% (P <0.001) and NS means non significant.

Histopathological studies

The target organs (kidney, ureter and urinary bladder) tissues were dissected out and fixed in 10% formalin, dehydrated in gradual ethanol (50-99%), cleared in xylene, and embedded in paraffin. Sections were prepared and then stained with hematoxylin and eosin dye for microscopic investigation.

Kidney

The histological examination of kidney sections of control animals (Figure 2, A) shows normal renal tissues and normal uriniferous tubules and glomeruli in untreated rat group.

The rat kidneys of groups which were regularly supplemented with 5,000 and 10,000 ppm melamine, respectively, showed tissues with tubular epithelial damage, capillary proliferation (Figure 2, B & C). Kidney of rats which were regularly supplied by 15,000 ppm shows certain degenerated uriniferous tubules and dilatation of Bowman's capsule (Figure 2, D). On the other hand, the histological examination of kidney sections of highly concentrated doses animal groups which were regularly supplied by 20,000 ppm melamine, show highly degenerated renal tissues and accumulation of salt particles in uriniferous and collecting tubules and glomeruli (Figure 2, E).

Histological diagnosis of melamine-associated renal failure based on renal crystal characteristics. (A) Dilated distal rat tubule contains clusters of round green melamine crystals with radiating spokes and concentric striations (arrow). Surrounding proximal tubules appear unaffected. (B) Dilated distal rat tubule contains fragmented or globular dense green melamine crystals (long arrows). Note attenuation of the lining epithelium with wide separation of nuclei (short arrow) and mitotic figure (arrowhead) indicative of tubular epithelial necrosis and regeneration as seen in Figure (2).

Ureter

The normal structure of the ureter of the rat is well represented in control group (Figure 3, A). The epithelium (E) is three to four cells deep with cuboidal or columnar basal cells, intermediate cells, and superficial squamous cells. The basal cells are attached by half desmosomes (D), or attachment plates, on their basal membranes to a basement membrane which separates the epithelium from the lamina propria (LP). Fine extracellular fibers, ca. 100 A□ in diameters, are to be found in the connective tissue layer (CT) immediately below the basement membrane of this epithelium. The rat ureters of in Figure (3, B & C) which were regularly supplied by 5,000 and 10,000 ppm melamine, respectively, showed tissues with tubular epithelial damage. The plasma membranes of the basal and intermediate cells and the lateral and basal membranes of the squamous cells are deeply interdigitated, and melamine toxicity is associated with them. All the cells have a dense feltwork of tonofilaments which ramify throughout the cytoplasm (Figure 3, B & C). The histological architecture of ureter sections of the rats supplemented with moderately concentrated material

of melamine (15,000 and 20,000 ppm) showed as in legated blood vessels, an increase in modified muscle cells with accompanying increase in connective tissue (particularly proximally to the ligature) occurs in the ureter (Figure 3, D & E).

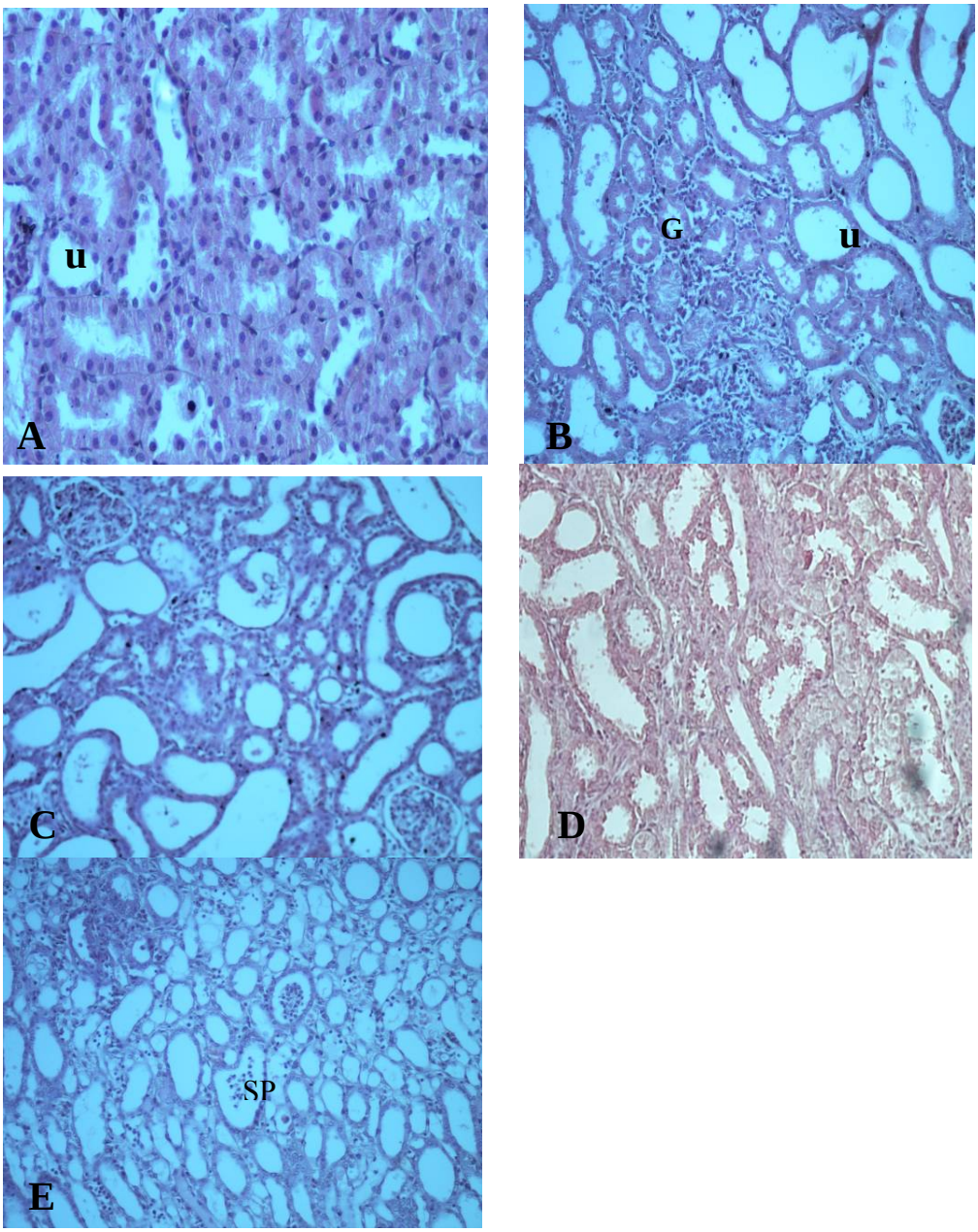


Figure (2): A; renal tissues of control group showing normal renal structure with regulated nuclear arrangement of urineferous tubules (u), **B:** Renal tissues of 5,000 ppm melamine fed group, showing convoluted urineferous tubules (u) and glomeruli (G), **C:** renal tissues of 10,000 ppm melamine fed group, showing certain degenerated urineferous tubules, **D:** renal tissues of 15,000 ppm melamine fed group, showing dilatation of Bowman's capsule, **E:** renal tissues of 20,000 ppm melamine fed group showing highly degenerated renal tissues and accumulation of salt particles in uriniferous (SP) X 200 (H&E stains).

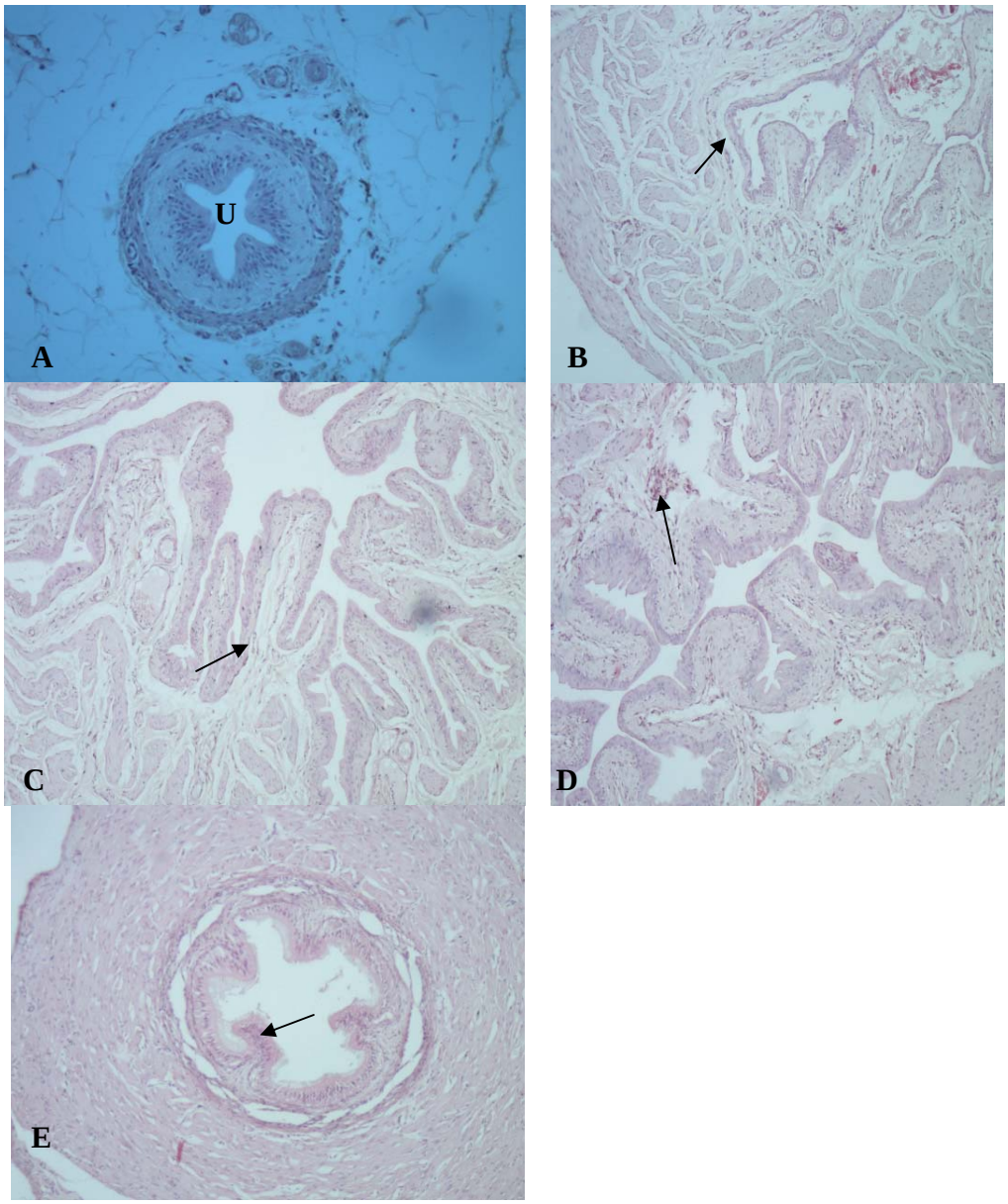


Figure (3): A; Ureter tissues of control group showing normal cuboidal or columnar basal cells, intermediate cells (U), and superficial squamous cells, **B;** ureter tissues of 5,000 ppm melamine fed group showing tissues with tubular epithelial damage (arrow), **C;** ureter tissues of 10,000 ppm melamine fed group showing plasma membranes of the basal and intermediate cells and the lateral and basal membranes of the squamous cells are deeply interdigitated (arrow), **D;** ureter tissues of 15,000 ppm melamine fed group, showing the cells have a dense feltwork of tonofilaments (arrow), **E;** ureter tissues of 20,000 ppm melamine fed group showing highly increase in modified muscle cells (arrow)**.** X 200 (H&E stains).

Urinary bladder

Bladder tissues of controlled rats showed epithelium (E) is three to four cells deep with columnar basal cells, intermediate cells, and superficial squamous cells (Figure 4, A). The rat groups treated with melamine in a two-generation dose-response study in which 5,000 and 10,000 ppm in the diet had been identified as the no- high effect level for primary bladder tissues with somewhat atretic tissues (Figure 4, B & C). Melamine crystals increase in the incidences of hyperplasia was found at the 15,000 and 20,000 ppm melamine doses (the high dietary levels tested). There were also compound-related increases in the incidence of transitional-cell papillomas (Figure 4, D & E).

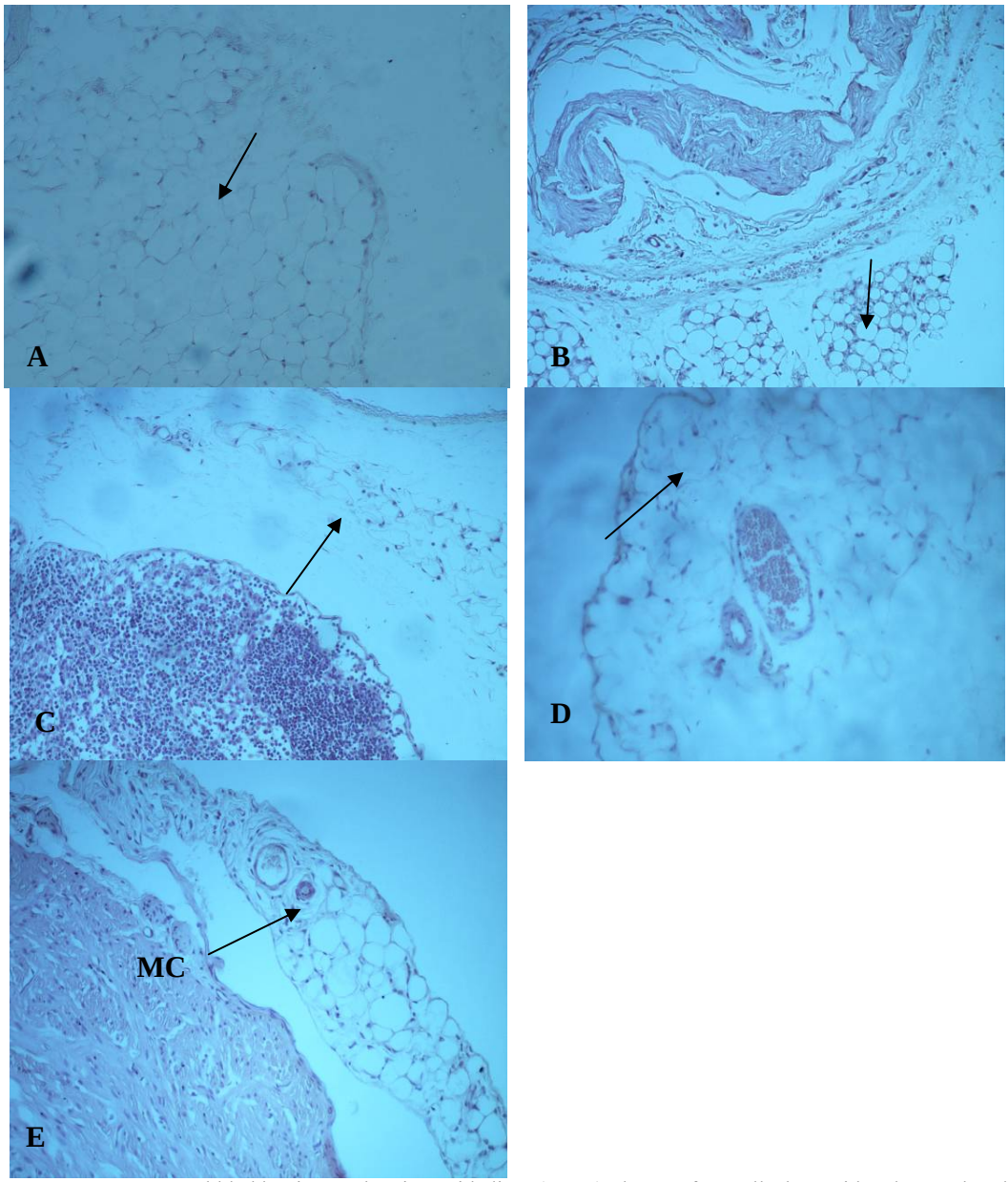


Figure (4): A; Normal bladder tissues showing epithelium (arrow); three to four cells deep with columnar basal cells, **B;** bladder tissues of 5,000 ppm melamine fed group showing somewhat atretic tissues (arrow), **C;** bladder tissues of 10,000 ppm melamine fed group showing mild atretic tissues (arrow), **D;** bladder tissues of 15,000 ppm melamine group showing increase in the incidences of hyperplasia (arrow), **E;** bladder tissues of 20,000 ppm melamine group showing highly degenerated bladder tissues and accumulation of granules in bladder tissues (arrow). X 200 (H&E stains).

4.Discussion

The toxic effect of oral administration of four melamine doses (5,000, 10,000, 15,000 and 20,000 ppm) for 28 days was investigated in male rats. Serum electrolytes, kidney functions indices, melamine concentration in the serum, as well as, the histopathology of kidney, ureters and urinary bladder were measured in order to investigate the toxic effect of melamine on the kidney. Some other nutritional parameters were also tested.

The color of melamine supplemented rats turned yellow by increasing the time of melamine supplementation, compared to the normal white colored. In addition, the dissected melamine supplemented rats showed congestion of organs and yellowish color of kidneys, as a result of melamine toxicity. This result is consistent with that of Chen *et*

al. (2009) who noted that high doses of melamine supplementation causes yellow color in kidney due to the numerous melamine crystals of various sizes found mixed with the necrotic cell debris in both proximal and distal renal tubules. Slight to severe inflammatory cell infiltration was accompanied by renal tubular dilation and epithelial cell regeneration with interstitial fibrosis.

The mean values of Na in the melamine treated rats were non significantly lower than that of the negative control. This result is consistent with that of Jeong *et al.* (2006) and Chen *et al.* (2009), whereas the mean values of K in the melamine treated group were non significantly higher than that of the negative control. This result is consistent with other results (Puschner *et al*., 2007) in cats. The mean values for Cl in the melamine supplemented group were significantly very low compared to the negative control. This result is consistent with that of Jeong *et al.* (2006) and Chen *et al.* (2009). The mean values of Ca in most treated groups were higher than that of the negative. Differences were non significant. This result is consistent with a similar one obtained by (Puschner *et al*., 2007) in cats. The mean values of P in the melamine supplemented group were very high significantly more than that of the negative control. This result is consistent with that of Jeong *et al.* (2006), Puschner *et al.* (2007) and Chen *et al.* (2009).

The present results could be correlated with cell membrane damage which leads to disturbances in Na+ and K+ pumping and disorders in membrane permeability (Ganong, 1999 and El-Missiry *et al.,* 2001). The decrease in the mean values of serum sodium levels in the melamine fed rats for 28 days compared to that of the control is consistent with other findings observed by Jeong *et al.* (2006). They observed decrease in sodium levels after feeding the dogs and cats with commercial melamine contaminated diets for about one month. In contrast, serum potassium levels were significantly increased after treatment with melamine for 28 days compared to the control. This result is in agreement with other study that showed elevated serum potassium in melamine contaminated infants (Sun *et al.,* 2010)**.**

The melamine toxicity resulted from supplementing the rats under experiment with melamine for 28 days, has greatly affected kidney function as revealed by the very high significant elevation of the mean values of serum creatinine, uric acid and urea in all treated groups than that of the negative control. The current results are consistent with that of Jeong *et al.* (2006), Tusing (2008), Kim *et al.* (2010) and Schnackenberg *et al.* (2012). Puschner *et al.* (2007) has got similar results in cats.

Melamine concentration in the serum was increased by the increase of the melamine dose. This result is consistent with that of Wu *et al.* (2009) and Ding *et al.* (2012).

The current results showed that the effect of melamine supplementation for the first 14 days, on the total body weight was non significant when compared the negative control. Whereas, the third weight (after three weeks) showed non significant decrease in the body weight compared to the negative control. This result is in agreement with Chen *et al*. (2009). On the other hand, the mean values of body weight was affected greatly after four weeks that showed high significant differences at 1% (P<0.01), when compared to the negative control.

In spite of the non significant increase in the mean values of food intake the first week of melamine supplementation, they were very significantly increased in the second week. The non significant increase in the mean values of food intake in the third week was faced with very high significant decrease in the fourth week. This result is in agreement with that of Dobson *et al.* (2008) and Cianciolo *et al.* (2008). The reduction in food intake in the fourth week is due to the toxicity of melamine resulted from the longer period of melamine supplementation.

The mean values of weight gain (g), BWG% and FER were less than that of the negative control. However, differences for weight gain were very high significant at (P<0.001). This result is consistent with that of Dobson *et al*. (2008).

The high significant increase in the consumed water in the first and the third weeks was faced with non significant increase in the second week. In the fourth week, the mean values of the consumed water were very high significantly lower than that of the negative control.

Ren *et al*. (2012) and Gao *et al.* (2012) reported that, renal tubules in melamine supplemented rats were extended and the lining epithelium cell was degenerated accompanied by testicular atrophy that was recovered three months after the end of administration, so did the reversibility of renal tubular epithelium.

In the current study, renal tissues of rats supplemented with melamine showed various dramatic pathological changes and accumulation of salt particles in most glomeruli and melamine crystals, in kidney, ureter and urinary bladder according to the melamine dose. This result agrees with the current elevated kidney functions results and with that of Tusing (2008), Hau *et al.* (2009), Chen *et al.* (2009), Kim *et al*. (2010) and Schnackenberg *et al*. (2012), who stated that histological examination revealed that renal crystals that could be observed in kidneys of animals showing signs of nephrotoxicity. Urinary bladder tissues showed dramatic pathological

changes. This result is consistent with Gao *et al.* (2012) who reported pathological changes in the heart, testes, spleen and urinary bladder of rats as a result of long term melamine supplementation. On the other hand, Chen *et al.* (2009) found that testes weight in male rats was increased as a result of melamine toxicity.

It could be said that the toxic effect of melamine increases with increasing of the melamine dose. In addition, melamine supplementation affected kidney, ureter and urinary bladder, as revealed by the very high significant differences of the kidney functions and the adverse histological changes of these tissues.

Acknowledgement

The authors thank King Abdulaziz City for Science and Technology for supporting and funding this project (Grant No. 0736 -11 - ط - ت).

Corresponding author:

Dr. Haddad A. El Rabey

Biochemistry Department, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia**.**

Permanent address: Bioinformatics Department, Genetic Engineering and Biotechnology Research Institute, Minufiya University, Sadat City PO Box 79, Egypt.

Email: elrabey@hotmail.com

References

1. Baynes RE, Barlow B, Mason SE, Riviere JE (2010). Disposition of melamine residues in blood and milk from dairy goats exposed to an oral bolus of melamine. Food Chem Toxicol 48: 2542–2546.
2. Bhalla V, Grimm P, Chertow GM, Pao AC (2009). Melamine nephrotoxicity: an emerging epidemic in an era of globalization. Kidney Internat 7: 774–779.
3. Bischoff K (2011). Melamine and cyanuric acid. RC Gupta (Ed.), Reproductive and Developmental Toxicology, Academic Press/Elsevier, Amsterdam.
4. Camacho L, Kelly KP, Beland FA, da Costa GG (2011). Gene expression of biomarkers of nephrotoxicity in F344 rats co-exposed to melamine and cyanuric acid for seven days. Toxicology Letters 206: 166-171.
5. Chen K, Liao C, Cheng F *et al.* (2009). Evaluation of Subchronic Toxicity of Pet Food Contaminated with Melamine and Cyanuric Acid in Rats. Toxicologic Pathology, 37: 959-968.
6. Cianciolo RE, Bischoff K, Ebel J, Van Winkle TJ, Goldstein RE, Serfilippi LM (2008). Clinicopathologic, histologic, and toxicologic findings in 70 cats inadvertently exposed to pet food contaminated with melamine and cyanuric acid. J Am. Vet. Med. Assoc. 333: 729–737.
7. Dalal RP, Goldfarb DS (2011). Melamine-related kidney stones and renal toxicity. Nature Reviews Nephrology 7: 267-274.
8. Ding X, Bai S, Zhang K, Wang L, Wu C, Chen D, Jia G, Bai J (2012). Tissue Deposition and Residue Depletion in Broiler Exposed to Melamine-Contaminated Diets. Journal of Integrative Agriculture 11(1): 109-115.
9. Dobson RL, Motlagh S, Quijano M, Cambron RT, Baker TR *et al.* (2008). "Identification and characterization of toxicity of contaminants in pet food leading to an outbreak of renal toxicity in cats and dogs.". Toxicological Sciences. http://toxsci.oxfordjournals.org/cgi/content/full/106/1/251. Retrieved 2009-08-13.
10. Drury RA, Wallington EA, Cancerson R (1976). Carlton's histopathological techniques, 4th edn. Oxford University Press, Oxford.
11. El-Missiry M, Othman A, Amer M, Abd El-Aziz M (2001). Attenuation of the acute adriamycin-induced cardiac and hepatic oxidative toxicity by N-(2-mercaptopropionyl) Glycine in rats, Free Radical Res. 35: 575-581.
12. Endreszl L (2008). "Safe Melamine Levels Named by World Health Organization". Health News. http://www.healthnews.com/alerts-outbreaks/safe-melamine-levels-named-world-health-organization-2252.html.
13. Ganong WF (1999). Review and Medical Physiology, Appelton, Lange, editors. Medical Publications, Calfornia, 19th Ed.
14. Gao H, Xu Y, He J, He X, Ma Y, Qin C, Gao J, Qin C (2012). Toxicity and prognosis of melamine and melamine analogues in rats after oral administration for eight weeks. International Journal of Automation and Computing, 26 (4): 555-562.
15. Gonzalez J, Puschner B, Pérez V, Ferreras MC, Delgado L, Muoz M, Pérez C, Reyes LE, Velasco J, Fernández V, Garcia-Marin JF (2009). Nephrotoxicosis in Iberian piglets subsequent to exposure to melamione and derivatives in Spain between 2003 and 2006. J Vet Diag Invest 21: 536–558.
16. Hau AK, Kwan TH, Kam-tao P (2009). Melamine toxicity in the kidney. J Am Soc Nephrol 20: 245–250.
17. Jeong WI, Do SH, Jeong H, Chung JY, Yang HJ, Yuan DW, Hong IH, Park JK, Goo MJ, Jeong KS (2006). Canine renal failure syndrome in three dogs. J Vet Sci. 7: 299–301.
18. Kim C, Yun J, Bae I, Lee J, Kang H, Joo K, Jeong H, Chung J, Park Y, Lim K (2010). Renal crystal formation after combined or sequential oral administration of melamine and cyanuric acid. Chem. Res. Toxicol. 23 (1): 220–227.
19. Liu JM, Ren A, Yang L, Gao J, Pei L, Ye R, Qu Q, Zheng X (2010). Urinary tract abnormalities in Chinese rural children who consumed melamine-contaminated dairy products: a population-based screening and follow-up study. Can Med Assoc J 182: pp. 439–443.
20. Lv, X, JWang J, Wu L, Qiu J, Li J, Wu Z, Qin Y (2010). Tissue deposition and residue depletion in lambs exposed to melamine and cyanuric acid-contaminated diets. J Agric Food Chem 58: 943–948.
21. Puschner B, Reimschuessel R (2011). Toxicosis caused by melamine and cyanuric acid in dogs and cats:uncovering the mystery and subsequent global implications. Clin Lab Med 31: 181–199.
22. Puschner B, Poppenga R, Lowenstine L, Filigenzi MS, Pesavento PA (2007). Assessment of melamine and cyanuric acid toxicity in cats. J Vet Diagn Invest 19: 616–624.
23. Qin Y, Lv X, Li J, Qi G, Diao Q, Liu G, Xue M, Wang J, Tong J, Zhang L, Zhang K (2010). Assessment of melamine contamination in crop, soil, and water in China and risks of melamine accumulation in animal tissues and products Environment Internat 36: 446–452.
24. Ren S, Xu C, Du Y, Gao X, Sun Y, Jiang Y (2012). The natural outcome of melamine-induced bladder stones with bladder epithelial hyperplasia after the withdrawal of melamine in mice. Food and Chemical Toxicology 50: 2318–2324.
25. Shen JS, Wang JQ, Wei HY, Bu DP, Sun P, Zhou LY (2010): Transfer efficiency of melamine from feed to milk in lactating dairy cows fed with different doses of melamine. J Dairy Sci 93, 2060–2066.
26. Schnackenberg L, Sun J, Pence L, Bhattacharyya S, da Costa G, Beger R (2012). Metabolomics evaluation of hydroxyproline as a potential marker of melamine and cyanuric acid nephrotoxicity in male and female Fischer F344 rats. Food and Chemical Toxicology 50: 3978–3983.
27. Sun N, Shen Y, He LJ (2010). Histopathological features of the kidney after acute renal failure from melamine. New England J Med 362: 662.
28. Tusing TW (2008). Chronic Feeding: Dogs, Hazleton Laboratories Inc., Princeton, New Jersey, Nov 27, 1953. Available at http://www.cdpr.ca.gov/docs/risk/toxsums/pdfs/1023.pdf. Accessed December 18, 2008.
29. Weise E (2007). "Poison pet food woes seem to hit cats harder". USA Today. http://www.usatoday.com/tech/science/2007-05-07-poison-pet-food-science_N.htm. Retrieved 2008-10-01.
30. Wen JG, Li ZZ, Zhang H, Wang Y, Rui FZ, Yang L, Chen Y, Wang JX, Zhang SJ (2010). Melamine related bilateral renal calculi in 50 children: single center experience in clinical diagnosis and treatment. J Urol 183: 1533–1538.
31. Wu Y, Huang C, Lin C, Ho W, Lin L, Chiu T, Tarng D, Lin C, Tsai T (2009). Determination of melamine in rat plasma, liver, kidney, spleen, bladder and brain by liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 1216: 7595–7601.

Evaluation of *Ginkgo biloba* as Alternative Medicine on Ova-Induced Eotaxin and Eosinophilia in Asthmatic Lung

Ghada Tabl [1] and Abd El-Hamid Mohamed Elwy[2]

[1] Zoology Department –Faculty of Science, Tanta University
[2] Forensic Medicine And Toxicology Department, Faculty of Medicine, Tanta University
Ghada_Tabl@Yahoo.Com

Abstract: *Ginkgo biloba* is an ancient plant; leaves of this plant have been used in asthma and bronchitis for many centuries. A model of lung eosinophilia based on the repeated exposure of mice to allergen ovalbumin (OVA). This model was used to investigate the effect of *Ginkgo biloba* leaf powder extract (GBE) on airway inflammation and asthmatic lung. Asthma is a chronic disease characterized by reversible airway obstruction. Airway inflammation is the key factor in the pathogenesis of asthma and current strategies for the management focus on suppressing airway inflammation. Eotaxin is an eosinophils specific chemo attractant that has been recently identified in rodent models of asthma. Cytokines IL-4 or IL-13, especially in combination with tumor necrosis factor alph (TNF-α), resulted in substantial release of the potent eosinophil chemoattactic factor, eotaxin. Eosinophilic leukocytes accumulate in high number in the lungs of asthmatic mouse and are, believed to be important in the pathogenesis of asthma. To observe the effects of GBE on asthmatic mice, leukocytes and eosinophils migration were counted in bronchoalveolar lavage fluid (BALF) . The production of IL-4, IL-13, and TNF-α were estimated in BALF. Moreover, the mRNA expression of eotaxin was analyzed in BALF by RT-PCR. The present study showed that, there was correlation between eotaxin level and eosinophils infiltration in the allergen OVA exposure group. The results revealed that, GBE markedly inhibit cells migration in BALF, in addition to, reduce the levels of IL-4, IL-13, and TNF-α . This reduction extends to the mRNA expression of eotaxin in BALF. The present results demonstrated that GBE can decrease the severity of asthma not only by suppressing eotaxin but also by inhibiting cytokines production In this research, we address the question of whether *Ginkgo biloba* leaf powder extract (GBE) can inhibit the eosinophils infiltration, eotaxin and cytokine production in asthmatic lung.
[Ghada Tabl and Abd El-Hamid Mohamed Elwy. **Evaluation of *Ginkgo biloba* as Alternative Medicine on Ova-Induced Eotaxin and Eosinophilia in Asthmatic Lung.** *Life Sci J* 2013;10(1):2131-2136] (ISSN:1097-8135). http://www.lifesciencesite.com. 301

Key words: *Ginkgo biloba* leaf powder extract (GBE)-Eotaxin mRNA expression-TNFα- Cytokines- Allergen OVA- induced eosinophilia

1. Introduction

Many patients harbour misgivings about conventional medical treatments for asthma, particularly inhaled corticosteroid treatment (Chan and DeBruyne, 2002). There is a need for development of additional effective treatments with fewer Side effects. Recently, there has been a surge in interest in herbal medicine, possibly because they have fewer side effects than current therapy (Bielory and Lupoli 1999 ;Hocaoglu *et al.*, 2012)

Ginkgo biloba extract (GBE) is an ancient plant, its standardized extract of leaves has been extensively used in diseases of cardio vascular system and cerebro-vascular system, *Ginkgo biloba* extract has been mentioned in the traditional Chinese pharmacopoeia, and Chinese has used ginkgo leaves for asthma and bronchitis for many centuries. (Hu *et al*., 2000 ; Tang, 2012). *Ginkgo biloba* extract (GBE), has been used therapeutically. It is a known inhibitor of platelet activating factor (PAF), which is important in the pathogenesis of asthma.(Chu *et al.,* 2011).This herbal medicine comes in the form of an herbal tea and capsule supplement form.

Asthma is a heterogeneous disease in which various cytokines orchestrate airway inflammation (Babu *et al*. , 2004). Eosinophils play an important role in allergic disorders such as Allergic asthma (Arm *et al*., 1997). Upon allergen challenge , eosinophils migrate from the peripheral blood into allergic inflammatory tissues and are subsequently activated in bronchoalveolar lavage fluid obtained from asthmatic patients , toxic granule products , such as eosinophils cationic protein ,can be detected (Woolley *et al*. , 1995). The release of these toxic eosinophil cationic proteins and major basic protein (degranulation) can result in damage of the respiratory ephithelium , leading to airway hyper-responsiveness (Bracke *et al*.,2000).

Eotaxin, a chemokine, likely plays an important role in the eosinophilia of asthma. Eotaxin was first identified as important chemo- attractant for eosinophils in antigen-sensitized and challenged guinea pig lungs (Jose *et al*. , 1994) . Subsequent experiments in murine models of airway inflammation confirmed these findings (Gonzalo *et*

al. , 1996). Furthermore, eotaxin is up regulated in bronchoalveolar lavage fluid and airways of asthmatic patients (Lamkhioued *et al*. , 1997). Expression of eotaxin mRNA was recently shown to be up-regulated after segmental allergen challenge in subjects with atopic asthma (Lilly *et al*. , 2001). Despite the importance of eotaxin recruitment, the regulation of eotaxin expression in the asthmatic airway remains to be established. *In vitro* studies on epithelial and endothelial cells have demonstrated that the proinflammatory cytokine TNF-α increases eotaxin expression (Gracia-Zepeda *et al*. , 1996). Many cell types in the lung appear to be capable of synthesizing eotaxin (e.g airway vascular endothelial cells and macrophages as well as eosinophils themselves) (Moore *et al*., 2001) . Eosinophilic leukocytes accumulate in high numbers in the lungs of asthmatic patients, and are believed to be important in the pathogenesis of asthma. A potent eosinophil chemo-attractant, eotaxin is produced in the asthmatic lung (Conroy and Williams, 2001) . This small protein, is synthesized by a number of different cell types, and is stimulated by IL-4 and IL-13, which are produced by T-helper (Th)2 lymphocytes. Low molecular weight compounds have been developed that can block the eotaxin receptor, and prevent stimulation by eotaxin. This provides the potential for orally available drugs that can prevent eosinophil recruitment into the lung and the associated damage and dysfunction (Conroy and Williams, 2001).

Cytokines IL-13 and IL-4 , which have been implicated in asthma, have been also shown to induce eotaxin expression in dermal and pulmonary fibroblasts and airway epithelial cells (Teran *et al*. , 1999). Animal models also support a role for IL-13 and IL-4 in eotaxin release and eosinophil recruitment. IL-13 has effect on immune cells that are similar to those of the closely related cytokine IL-4. However, IL-13 is suspected to be a more central mediator of the pathological changes induced by allergic inflammation in many tissues (Li *et al*., 1999 ; Zhu *et al*., 1999).

Tumor necrosis factor alpha (TNF-α) is a proinflammatory cytokine that has been implicated in the modulation of inflammation in various diseases, including asthma. TNF-α blocking strategies have been an effective therapeutic modality in diseases such as rheumatoid arthritis. Studies were demonstrated that the effect of blocking TNF-α as a possible therapeutic option in patients with severe corticosteroid-dependent asthma (Babu *et al*. ,2004). TNF-α was discovered more than a century ago, and its known roles have extended from within the immune system to include a neuro-inflammatory domain in the nervous system (Leung and Cahill,2010).

In this research, we address the question of whether *Ginkgo biloba* leaf powder extract (GBE) can inhibit the eosinophils infiltration, eotaxin and cytokine production in asthmatic lung.

2.Material and Methods

Study materials:

The IL-4, IL-13 and TNF-α kits were purchased from Biosource, Ovalbumin was purchased from sigma-Aldrich St. Louis, Mo, USA.

Gingkgo biloba capsule was purchased from Pharaonia Pharmaceutical (Pharo pharma).

Trizol reagent and RT-PCR reagents are from SuperScript Rnase H-RT Kit/Gibco / BR).

Experimental Animals

Pathogen free 6 to 9 weeks, male BALB/c mice, weighing 25-35g, were purchased from Theodor Bilharz Research Institute, Al-Giza Egypt, and maintained in a pathogen-free animal laboratory. They were kept in hygenic cages and at temperature rooms of 20-25°C ,12 hour light/12 hour dark cycle with food and water available *ad-libitum*.

Study Groups:

Twenty mice were divided into four groups:

- **Group I,** OVA- challenged.
- **Group II,** OVA- challenged Plus *Ginkgo biloba*.
- **Group III,** control group treated with GBE, mice received *Ginkgo biloba* 40mg/kg dissolved in saline .The extract was administered by intraperitoneal administration at 1h before the OVA challenge on days 25-27.
- **Group IV,** control group, considered as negative control.

Each group including five mice.

Antigen sensitization, challenge protocol and GBE administration as described by Chu *et al*. (2011)

- The mice were sensitized via two intraperitoneal injections, on day 0 and day 14 of the experiment, with 0.2ml saline containing 20 µg ovalbumin adsorbed in 0.4mg aluminum hydroxide (Alum.) as adjuvant .On days 25-27, mice were anesthetized with 0.2 ml i.p of ketamine (0.44mg/ml) before receiving an intranasal dose of 100 µg ovalbumin in a 50 µl volume of saline.

OVA-challenged group received 40 mg/kg *Ginkgo biloba* leaf powder extract as *Ginkgo biloba* capsule, dissolved in saline. The extract was given by intraperitoneal administration at 1h before OVA challenge on days 25-27. Mice without OVA exposure (control-GBE) group received the same dose of GBE on days 25-27 (Figure 1).

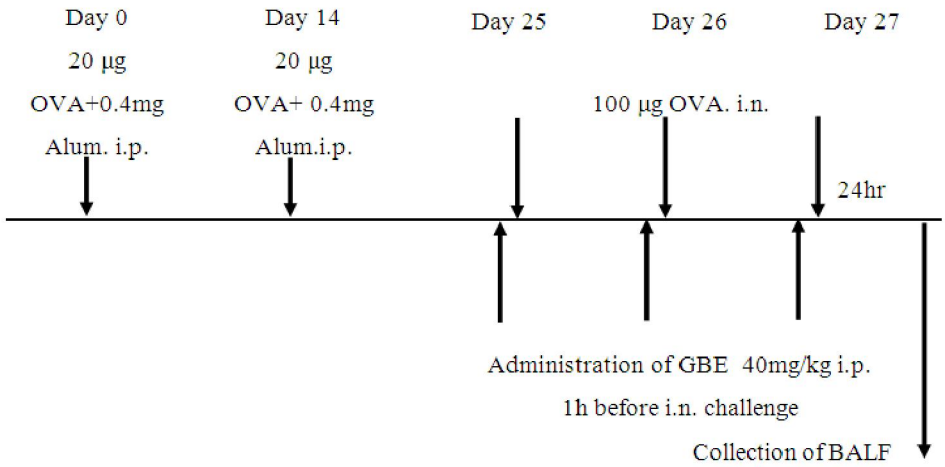


Figure 1. Sensitization, challenge protocol and treatment with *Ginkgo biloba extract.*

Determination of Bronchoralveolar lavage fluid (BALF) cells.

The mouse trachea was cannulated and the lungs were washed 3 times with 1ml of saline. The bronchoalvealar lavage was collected (BAL cells) . 0.05 ml of sample were counted using a hemocytometer and the remaining fluid was immediately centrifuged and the supernatant was collected for cytokine measurements, the pellets were resuspended in 20µl of 10% bovine serum albumin (BSA), then BALF smear were made on glass slides to calculate eosinophil number.

Determination the number of eosinophils,

The slides were stained with Wright-Giemsa, percentage of eosinophils were determined by counting in eight high power fields. Magnification, 40x; total area, 0.5 mm^2 per area selected and dividing this number by the total number of cells per high power field. This percentage of eosinophil in smear was multiplied by the total number of cells recovered in the lavage fluids according to Gonzalo *et al.* (1996) .

Enzyme linked immune sorbent assay (ELISA) for BALF cytokines analysis.

Cytokine protein levels in BALF; IL-4, IL-13, and TNF-α were measured by ELISA. According to the manufacturer's instructions.

Estimation of mouse eotaxin in BALF by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Twenty-four hour post the last intranasal challenge, bronchoalveolar lavage fluid cells were collected and centrifuged ,the total RNA was extracted using Trizol reagent, mRNA eotaxin was evaluated according to Szalay *et al.* (1994). cDNA quality was controlled by performing β-actin cDNA.

Statistical analysis

Data were fed to the computer using IBM SPSS software package version 20.0. Quantitative data were described using mean and standard error. Comparison between different groups was analyzed using F-test (ANOVA) and Post Hoc test (Scheffe) for pair wise comparison. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

3.Results

The number of cells migration was significantly higher ($p < 0.001$) in the OVA-challenged group than in the negative control group (Table 1). The OVA challenged GBE group was significantly less in cells number than the OVA-challenged mice group ($p < 0.001$). Contrary, there was no statistically significant difference ($p < 0.001$) between control-GBE group and negative control group (Table 1) .

Furthermore, significant increase ($p < 0.001$) were detected when comparing the levels of Cytokines; IL-4, IL-13, and TNF-α in OVA-challenged group compared to negative control group (Table 1). Concerning, the effect of GBE treatment, the results obtained showed significant decrease in the levels of IL-4, IL-13, and TNF-α compared to OVA-challenged mice group $p < 0.001$(Table 1).

Table (1):Effect of GBE (40 mg/ kg i.p.) on cell migration; (leukocytes, eosinophils) and cytokines production; IL4, IL3, TNF-α in BALF.

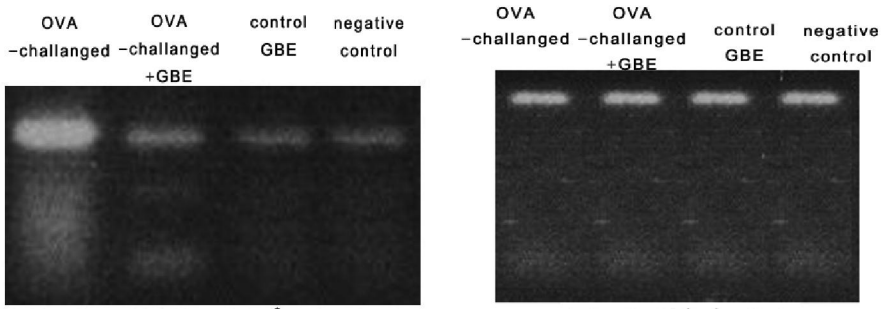
	OVA- challenged (n=5)	OVA- challenged GBE	control -GBE (n=5)	control(n=5)	*p*

		(n=5)			
Leukocytes×10^6/ml	$7.04^a \pm 0.14$	$2.58^b \pm 0.70$	0.30 ± 0.13	0.34 ± 0.09	<0.001*
Eosinophils×10^5/ml	$6.29^a \pm 0.60$	$2.10^b \pm 0.30$	0.15 ± 0.07	0.18 ± 0.07	<0.001*
IL-4 pg/ml	$90.78^a \pm 2.80$	$16.54^b \pm 3.55$	3.02 ± 1.41	4.14 ± 1.50	<0.001*
IL-13 pg/ml	$99.40^a \pm 5.39$	$17.05^b \pm 2.57$	5.15 ± 1.57	6.32 ± 1.15	<0.001*
TNF-α pg/ml	$969.12^a \pm 39.74$	$159.30^b \pm 26.99$	2.82 ± 0.99	3.82 ± 0.83	<0.001*

Data was expressed in mean ± SE
p: p value for F test (ANOVA)
pair wise between any two groups was assessed using Post Hoc test (Scheffe)
a: significant with control group
b: significant with OVA-challenged group
*: Statistically significant at $p \leq 0.05$

Effect of GBE on OVA-induced mouse eotaxin

As regards the expression of mRNA eotaxin, the results given in figure 1 showed remarkably increase in OVA-challenged group compared to OVA-challenged GBE group. On the other hand there was no differences in mRNA expressions eotaxin in both negative control group and control-GBE group (Figure 1).



Eotaxin **β-actin**

Figure 2. mRNA expression of eotaxin in BALF. The mRNA expression of eotaxin in OVA-challenged GBE group was less than OVA-challenged.

4.Discussion

Asthma is a complex disease characterized by acute and chronic air way inflammation which adversely affects normal lung function, airway hyper-responsiveness, eosinophilia and mucus hypersecretion by goblet cells. (Leonard and Sur, 2002).

Asthma is a heterogeneous disease in which various cytokines orchestrate airway inflammation (Babu *et al*.,2004)

Many cytokines (IL-4, IL-13) contribute to this inflammation mediated by T-helper cells, which play central roles in the pathogenesis of allergic asthma (Brightling *et al*., 2002; Bloeman *et al*., 2007). Airway inflammation is the key factor in the pathogenesis of asthma and current strategies for the management focus on suppressing airway inflammation (Kumar, 2001;Walsh , 2006) . The results of the present study come in contact with (Babu *et al*.,2004;Walsh, 2006;Bloeman *et al*., 2007) Moreover, the present data demonstrate that GBE significantly reduced leukocytes migration, consequently eosinophils infiltration in BALF of OVA-challenged group.

Eotaxin-induced eosinophil recruitment in asthma. Inhaled allergen activates mast cells and Th2 lymphocytes in the lung to generate the cytokines IL-4,IL-13 and TNF-α. These cytokines stimulate the generation of eotaxin by lung epithelial cells, fibroblast and smooth muscle cells. Eotaxin acting on receptor of eosinophils then stimulates the selective recruitment of these cells from the airway microvessels into the lung tissue (Conroy and Williams,2001).

Cytokines IL-13 and IL-4, which have been implicated in asthma induced eotaxin expression in dermal and pulmonary fibroblasts and in airway epithelial cells (Teran *et al*., 1999). Interleukin (Il-13) plays an important role in T-cell differentiation toward a Th2 phenotype and isotype switching of B cells to immunoglobulin IgE production (Horie *et al*., 1997). IL-13 and tumor necrosis factor-alpha synergistically induce eotaxin production in human nasal fibroblasts (Terada *et al*., 2000).

In the present study, pretreatment with GBE resulted in a significant reduction of the production of IL-13 and IL-4 in BALF. This result is in accordance with Jing *et al*. (2008) who demonstrated that extract of

Ginkgo biloba (EGb) blocks mucus hypersecretion of asthmatic rats by inhibiting IL-13.

The eotaxin levels correlated with the number of eosinophils infiltrating the lung tissue, on the other hand, the appearance of significant numbers of eosinophils in the BALF occurred later (12-24 hrs), which may be because the persistence of eotaxin in the airway lumen resulted in the direction of the chemoattractant gradient across the airway epithelium over this later period (Humbles *et al*.,1997). In the present investigation mRNA eotaxin expression appeared significant decrease in OVA- challenged group treated with GBE. This inhibition associated with the reduction of leukocytes migration and eosinophils infiltration in the BALF. Tumor necrosis factor alpha a protein manufactured by white blood cells to stimulate and activate the immune system in response to infection. Overproduction of this compound can lead to disease where the immune systems acts against healthy tissues. Some treatments for these diseases utilize drugs that bind and inactivate TNF-α, thereby reducing unhealthy inflammation (Berry *et al*., 2007).As well as, tumor necrosis alpha (TNF- α) is a pro-inflammatory cytokine that has been implicated in the modulation of inflammation in various diseases , including asthma(Babu *et al*.,2004). The data obtained showed significant increase in TNF-α in OVA-challenged group compared to control group, which is in the same line with Babu *et al*. (2004). Moreover, the treatment with GBE suppressed TNF-α in the BALF of OVA-challenged group, which may be attributed to the binding of GBE to TNF-α receptor (Berry *et al*., 2007) as a result, inactivate TNF-α reducing inflammation.

Ginkgo biloba has been used as an herb in traditional Chinese medicine for thousands of years. (Chu *et al*.,2011). The present results showed that GBE has a suppressing effect on airway inflammation by inhibiting the activity of cytokines and eotaxin, consequently inflammatory cell migration. That is in the line of our results which showed inhibitory effect on asthmatic lung by suppressing the levels of cytokines, eotaxin and cell infiltration.

Conclusion

The present results suggest that the cytokines levels, in addition to, mouse eotaxin reflect the intensity of eosinophilic airway inflammation as well as the disease activity, and may be useful as an inflammatory marker in asthma. Moreover, *Ginkgo biloba* leaf powder extract significantly decreased these inflammatory mediators and may serve as an alternative drug for patients with airway hyper-responsiveness.

Corresponding author

Ghada Tabl
Zoology Department –Faculty of Science, Tanta University
Ghada_Tabl@Yahoo.Com

References

1. Arm J. P., Nwankwo C., Austen K. F. **(1997):** Molecular identification of a novel family of human Ig superfamily members that possess immuno-receptor tyrosine-based inhibition motifs and homology to the mouse gp49 BI inhibitory receptor. J. Immunol. 159, 2342-9.
2. Babu K. S., Davies D. E., Holgate S. T. **(2004):** Role of tumor necrosis factor alpha in asthma. Immunol. Allergy Clin. North Am. 24 (4):583-97.
3. Berry M., Brightling C., Pavord I., Wardlaw A. **(2007):** TNF-α in asthma. Curr.Opin. Pharmacol. 7(3):279-82.
4. Bielory L, Lupoli K. **(1999):** Herbal interventions in asthma and allergy. J Asthma 36 (1); 1-65.
5. Bloeman K., Verstraelen S., Van Den Heuvel R., Witters H., Nelissen I., Schoeters G. **(2007):** The allergic cascade: Review of the most important molecules in the asthmatic lung. Immunol. Lett.,113:6-18.
6. Bracke M., Graaf E., Lammers J. J., Coffer P. J., Koenderman L. **(2000):** In-vivo primining of FcαR functioning on eosinophils of allergic asthmatics. J. of Leukocyte Biology. (68): 655-61.
7. Brightling C. E., Symon F. A., Birring S. S., Bradding P., Pavord I.D., Wardlaw A.J. **(2002):** Th2 cytokine expression in bronchoalveolar lavage fluid T lymphocytes and bronchial submucosa is a feature of asthma and eosinophilic bronchitis. J.Allergy Clin. Immunol. 110:899-905.
8. Chan P.W., DeBruyne J.A. **(2002):** Parental concern towards the use of inhaled therapy in children with chronic asthma. Pediatr Int. 42 (5):547-51.
9. Chu X., Ci X., He J., Wei M., Yang x., Cao.Q., Li H., Guan S., Deng Y., Pang D., and Deng X. **(2011):** A Novel anti-inflammatory role for Ginkgolides in asthma via inhibition of the ERK/ MAPK signaling pathway Molecules 16:7634-48.
10. Conroy D. M., Williams T. J. **(2001):** Eotaxin and the attraction of eosinophils to the asthmatic lung. Respiratory Research 2:150-6.
11. De Monchy J.G., Kauffman H. F., Venge P., Koeter G. H., Jansen H.M., Sluiter H.J., De Vries K. **(1985):** Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. Am. Rev. Respir. Dis. 131: 373-6.
12. Garcia-Zepeda E.a., Rothenberg M. E., Ownbey R. T., Celestin Leder P. D., Luster A. D. **(1996):** Human eotaxin is an eosinophil selective chemoattractant that provides a new mechanism to explain tissue eosinophilia. Nat. Med. 2:449-56.

13. Gonzalo J-A., Jia G. Q., Aguirre V., Friend D., Coyle A. J., Jenkins N. A., Lin G.S., Katz H., Lichtman A., Copeland N., Kopf M., Gutierrerez-Ramos J.P. **(1996):** Mouse eotaxin expression parallels eosinophil accumulation during lung allergic inflammation but is not restricted to a Th2 type response. Immunity 4:1-14.
14. Hocaoglu A.B., Karaman O., Erge D. O., Erbil G., Yilmaz O., Kivcak B., Bagriyanik H. A., Uzuner N. **(2012):** Effect of hedera helix on lung histopathology in chronic asthma. Iran J. Allergy Asthma Immunol. 11(4): 316-23.
15. Horie S., OkuboY. , Hossain M. , Sato E., Nomura H. , Koyama S., Suzuki J., lsobe M. , Sekiguchi M. **(1997):** Interleukin-13 but not interleukin-4 prolongs eosinophil survival and induces eosinophil chemotaxis. Inter. Med. 36: 179-85.
16. Hu L., Chen Z., Xie Y., Jiang Y., Zhen H. **(2000):** Alkyl and alkoxycarbonyl derivatives of Ginkgolide B: Synthesis and biological evaluation of PAF inhibitory activity. Bioorg. Med. Chem. 822: 1515-21.
17. Humbles A.A., Conroy D.M., Marleau S., Rankin S.M., Palframan R.T., Proudfoot A.E.I., Wells T.N.C., Li D., Jeffery P.K., Griffiths-Johnson D.A., Williams T.J., Jose P.J. **(1997):** Kinetics of eotaxin generation and its relationship to eosinophil accumulation in allergic airways disease: analysis in a guinea pig model in vivo.J. Exp. Med., 186: 601-12.
18. Jing H., Yulin F., Qing S., Fang H., Xia C., Li Y. **(2008):** Extract of *Ginkgo biloba* (E G b) blocks mucus hypersecretion of asthmatic rats by inhibiting IL-13. J. Pharmacology and Clinics of Chinese Materia Medica:-02.
19. Jose P.J., Griffiths-Johnson D.A., Collins P.D.**(1994):** Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. J Exp Med. 179:881-7.
20. Kumar R. K. **(2001):** Understanding airway wall remodeling in asthma: a basis for improvements in therapy?. Pharmacol. Ther. 91(2):93-104.
21. Lamkhioued B., Abi-Younes S., Garcia-Zepeda E.A., Allahkverdi Z., Ghaffar O., Rothenberg M. E., Luster A.D., Hamid Q. **(1997):** Increased expression of eotaxin in bronchoalveolar lavage and airways of asthmatic patients contributes to the chemotaxis of eosinophils to the site of inflammation. J. Immunol. 159:4593-601.
22. Leonard P., Sur S. **(2002):** Asthma: future directions .Med Clin. North Am. 86 (5):1131-56.
23. Leung L., Cahill C. M. **(2010):** TNF-α and neuropathic pain- a review. Journal of Neuroinflammation. 7: 27.
24. Li L., Xia Y., Nguyen A., Lai Y.H., Feng L., Mosmann T.R., Lo D. **(1999):** Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. J lmmunol. 162:2477-2487.
25. Lilly C. M., Nakamura H., Belstotsky O. I., Haley K. J., Gracia-Zepeda E. A., Luster A. D., Israel E. **(2001):** Eotaxin expression after segmental allergen challenge in subjects with atopic asthma. Am. J. Respir. Crit. Care. Med. 163:1669-75.
26. Moore P. E., Church T. L., Chism D.D., Reynold A. Panettieri Jr R. A., Shore S. A. **(2001):** IL-13 and IL-4 Cause eotaxin release in human air way smooth muscle cells: a role for ERK Am. J Physiol. Lung Cell Mol. Physiol 282:847-53
27. Szalay G., Hess J., Kaufmann S.H.E. **(1994):** Presentation of Listeria monocytogenes antigens by major histocompatibility complex class I molecule to CD8 cytotoxic T lymphocytes independent of listeriolysin secretion and virulence. Eur. J. Immunol. 24:1471- 7.
28. Tang C.Q.**(2012):** Evidence for the persistence of wild *Ginkgo biloba* (Ginkgoaceae) population in the Dalou Mountains, Southwestern China. American Journal of Botany 99 (8):1408-14.
29. Terada N., Hamano N., Nomura T. Numata T., Hirai K., Nakajima T. Yamada H., Yoshie O., Ikeda-Ito T., Konno A. **(2000):** Interleukin-13 and tumor necrosis factor-alpha synergistically induce eotaxin production in human nasal fibroblasts. Clin. Exp. Allergy. 30(3):348-55.
30. Teran L. M., Mochizuki M., Bartels J., Valencia E. L., Nakajima T., Hirai K., Schroder J. M. **(1999):** Th1- and Th2- type cytokines regulate the expression and production of eotaxin and RANTES by human lung fibroblasts. Am. J. Respir. Cell. Biol. 20:777-786.
31. Walsh G. M. **(2006):** Targeting airway inflammation: novel therapies for the treatment of asthma. Curr. Med. Chem. 13(25):3105-11.
32. Woolley K.L., Adelroth E., Woolley M. J., Ellis R., Jordana M. ÖByrne P.M.**(1995):** Effects of allergen challenge on eosinophils, eosinophil cationic protein, and granulocyte-macrophage colony-stimulating factor in mild asthma. Am. J. Respir. Crit. Care Med. 151,1915-24.
33. Zhu Z., Homer R.J., Wang Z., Chen Q., Geba G.P., Wang J., Zhang Y., Elias J.A. **(1999):** Pulmonary expression of interleukin-13 causes inflamrnation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and extaxin production. J Clin Invest. 103: 779-788.

Effect of Breast Milk versus Therapeutic Honey (Apicare) on Cracked Nipples' healing

Rasha Mohamed Essa[1] and Enas Mohamed Ebrahim[2]

[1] Obstetric and Gynecologic Nursing Dept, Faculty of Nursing, Damnhour University, Egypt.
[2] Community health Nursing Dept, Faculty of Nursing, Damnhour University, Egypt.
rashaessa111@yahoo.com , enas_moh2002@yahoo.com

Abstract: The aim of this study was to compare the effect of breast milk versus therapeutic honey on the healing of cracked nipples. A quasi-experimental research design was carried out on a sample of (60) lactating women who were randomly selected from **Salah abd-Rabo** obstetric and gynecological clinic **,** obstetric and gynecologic **Sahala** center in Alexandria and subjects homes . The selected subjects were equally divided into two study groups. Each group was instructed to apply one of the two treatment modalities for 4 weeks duration. **Four tools** were used to collect the necessary data. **The first tool** was a structured interview schedule to elicit the socio-demographic and biological characteristic. **The second tool** was WHO B-R-E-A-S-T Feed observation form. **The third tool** was the healing and pain assessment scale, which comprised two parts: part 1: modified Reeda Scale & part 2: Visual analogue Scale. **The fourth** tool was an observational follow up chick list to assess healing speed of cracked nipples among the studied groups. **The results** revealed that the entire respondent's (100.0%) were suffering from nipple's redness, fissure and pain. while more than one-tenth (13.7%) of them have bleeding. Complete recovery from signs and symptoms of cracked nipples was significantly faster among women who had used the therapeutic honey 'Apicare'. The study **concluded** that "therapeutic honey was a better treatment for cracked nipple compared to breast milk". Consequently, it is **recommended** that each lactating mother should be observed for mother's and infant's positioning and attachment at the onset of breastfeeding and if needed given counseling on correct positioning and attachment.

[Rasha Mohamed Essa and Enas Mohamed Ebrahim. **Effect of Breast Milk versus Therapeutic Honey (Apicare) on Cracked Nipples' healing.** *Life Sci J* 2013;10(1):2137-2147] (ISSN:1097-8135). http://www.lifesciencesite.com. 302

Key words: breastfeeding -cracked nipples- breast milk- therapeutic honey.

1. Introduction

Breastfeeding is the optimal method of infant feeding. Breast milk provides almost all the necessary nutrients, growth factors and immunological components a healthy term infant needs **(Leung and Sauve, 2005)**. Each year, new scientific and epidemiological evidence contributes to knowledge about breastfeeding's role in the survival, growth, and development of a child as well as the health and well-being of a mother. UNICEF baby friendly initiative was introduced in the UK in 1991 to improve breastfeeding rates. The World health organization recommends breastfeeding for at least the first 6 months of life (WHO, 2000). However, most babies are not exclusively breast fed; on the average, globally, only 39% of babies are breast fed exclusively for the first 6 months of life **(Gartner *et al.*, 2005; World Health Organization, 2011).**

Although breast feeding is a natural and spontaneous process from a mother‘s and baby’s side, there are certain simple problems that may lead to mother's apprehension and anxiety towards breast-feeding. This may results in discontinuation of breast-feeding or addition of supplements of animal milk/commercial infant formula. Examples of these problems are breast engorgement, flat, inverted and cracked or sore nipples **(Raising Children Network, 2009).**

Cracking / Sore nipples are a common complaint among breastfeeding women and it is one of the main reasons why some women decide to stop breastfeeding. It confronts nursing women 3-6 days after birth, especially primiparae. Nipples become painful and start to show small cracks, which may bleed. Furthermore, it is important to note that as with any other wound on the body, cracked nipples are a potential source of infection, either locally as within the wound itself or within the breast as seen with a mastitis infection **(World Health Organization, 2001).**

There are two common types of sore nipples; **the first** type is called transient soreness. It occurs during the first week after birth, usually beings between the third and sixth days. Most women develop some nipple tenderness or discomfort as they begin to feed their new babies, but this transient tenderness usually resolves after about one week and by 10 days mothers breastfeed normally. **The second** type is called prolonged abnormal soreness which lasts beyond the first week of breastfeeding. Where nipple pain becomes severe, and lasts beyond the

initial days of breastfeeding or there is a cracking and/or bleeding **(Ann and Caroline, 2003).**

The main causes of cracked nipples are: improper positioning of the baby at the breast, lack of nipple hygiene during lactation, dry skin or eczema as a result of soap, lotions, perfume, or the residue of clothes detergent. Other causes include short/ flat or inverted nipples, oral dysfunctions in the infant, prolonged nonnutritive sucking, and improper use of milk pumps as well as the improper technique of taking the infant off of the breast **(Buchanan *et al.*, 2002; Giugliani, 2004).**

Fortunately, most breast-feeding problems are preventable if proper care, support and counseling being right from antenatal period to prepare pregnant women for initiation and continuation of breast - feeding after delivery. Unfortunately, many women do not receive counseling during prenatal period regarding the benefits of breast milk, proper technique of breast-feeding, breast care, and breast-feeding initiation and maintenance **(Ganguli and Dhawan, 2000; Rani and Shaw, 2000).**

Women with cracked nipples need to know if there is anything to apply on the wound to speed up healing. In the case of nipple soreness or cracking, as with other types of skin fissures, there are two treatment options to hasten the healing of nipple trauma: dry wound healing and moist wound healing. The dry healing of cracked nipples includes: exposure to light, sunbathing and blow-drying. Such techniques were quite popular in the last few decades but have not been recommended anymore because scar healing is believed to be more efficient if the internal layers of the epidermis (exposed by the lesion) are kept moist. Currently, the moist treatment of nipple fissures which includes use of breast-milk, and appropriate creams and oils has been recommended, to increase the moisture content of the skin and reduce further drying. Some researchers concluded that wound in moist environments typically heal faster and with reduced scab and scar formation than those in dry environment. Moist wound healing allows the skin to regain the proper moisture content from within and rapid healing is facilitated without a hard crust or scab forming **(Biancuzzo, 2000; Page *et al.*, 2003).**

Breast milk is occasionally used to treat the cracked nipples. It has no risk of allergy, contains antibodies and epidermal growth factor which may potentially promote the growth and repair of skin cells. To promote healing, a drop or two of the hind milk is applied on the nipples after each feed and left to dry. Hind milk is the milk expressed from the breast towards the end of a feeding cycle. It is rich in fat and provides an emollient effect. In many cultures, human milk with its antibacterial properties is used to treat skin irritation without causing any problems **(Blumenthal *et al.*, 2000; Renfrew *et al.*, 2000).**

Natural remedies in conjunction with medical care may provide relief from breastfeeding problems. The use of therapeutic honey (Apicare®) in wound care is an ancient remedy that has been rediscovered. It is becoming of increasing interest as more reports of its effectiveness are published. History of apitherapy can be traced back to ancient Egypt, Greece, and India **(Allen *et al.*, 2000; Young, 2005).**

"Apicare" honey ointment is not a commercial honey but it is a sterilized honey. It is licensed under pharmaceutical and medical organization. It is pharmaceutically prepared in an ointment form. Each 100 gm of the ointment contains 85 gm of active pure honey "non- commercial" **(The report of Arafa and Ara zami chemical company, 2003)**. Api-care may be defined as the therapeutic use of bee products including bee venom, bee stings, honey and royal jelly. It is an established form of alternative therapy and is practiced by thousands of medical professionals and practitioners **(Jones, 2001).** Clinically, topical honey treatment has been shown by white and Molan 2005 to possesses anti-microbial properties, osmotic effect, promote debridement, stimulate anti-inflammatory activity that rapidly reduce pain, edema and exudates, and minimizes hypertrophic scarring and promote moist wound healing **(White and Molan, 2005; Melodee, 2011).**

Aim of the study

The study aims to compare the effect of breast milk versus therapeutic honey (Apicare) on cracked nipples' healing.

Research hypothesis

Lactating postpartum women who apply therapeutic honey exhibit faster recovery from signs and symptoms of cracked nipples than those who apply breast milk.

Operational definition

Signs and symptoms of cracked nipples in this study refer to : redness, approximation of the skin, pain and bleeding.

2. Material and methods

A quasi-experimental research design was followed.

Material

1- Setting:

The study was carried out at the following settings:

1- Salah abd-Rabo obstetric and gynecological clinic.
2- Center Sahala obstetric and gynecologic center.
3- Subjects ' homes.

Subjects:

A total study subject of (80) women were conveniently selected from the previously mentioned clinics according to the following criteria:

1- Currently lactating (breastfeeding)
2- Complaining from cracked nipples.
3- Did not start any treatment for cracked nipples.
4- Welling to participate in the study.

The selected subjects were equally divided into two study groups.

Study group (1) included 40 women who were advised to paint their nipples with their breast milk and air dry them after each feeding.

Study group (2) comprised 40 women who were instructed to apply APICARE OINTEMENT as local nipple treatment 3 times /day

Women in the two groups were instructed to continue the treatment for 4 week duration

3 -Tools:

Four tools were used to collect the necessary data.

Tool (1):

Basic data interview schedule. It was developed by the researchers. It entailed information related to:

- Socio-demographic data such as: name, age, residence, address, phone number, level of education, occupation.
- Reproductive history such as: gravidity, parity, type of the last delivery, number of living children. It also included question related to follow up of current pregnancy, as well as received antenatal care.
- Breast feeding history such as : initiation of breast feeding, duration of breast feeding, method of nipple withdrawal and number of breastfeeding/day.

Tool (2): WHO B-R-E-A-S-T Feed observation form:

It is a chick list used to observe the breastfeeding process for 5 minutes. It contains: mother's and infant's positions as well as the latter's attachment to the breast.

The following arbitrary scoring and grading system was adopted to grade positioning (mother and infant), infant's mouth attachment based on WHO criteria. Each criterion was assigned 1 point .

Correct body position:

1- Mother relaxed and comfortable
2- Mother sit straight and well supported back
3- Trunk facing forward and lap flat
4- Baby neck straight or bent slightly back and body straight
5- Baby body turned toward mother
6- Baby body close to mother body and facing breast with the newborn's nose opposite her nipple and chin touching the breast.
7- Baby whole body supported not just the neck and shoulders.

Criteria for grading the correct body position:	**Grad**	**Score**
One criterion from mother's position and one criterion from infant's position or both from mother's position	**Poor**	**0-2**
At least one criterion from mother's and two or three criterion from infant position	**Average**	**3-4**
At least two criteria from mother's position and three or fourth criteria from infant's position	**Good**	**5-7**

Correctness of attachment:

– Mouth wide and open
– Lower lip turned outwards
– Baby's chin touching breast
– More areola seen above baby mouth

Criteria for grading the correct attachment	**Grad**	**Score**
- Any one of four criteria	**Poor**	**1**
- Any two of four criteria	**Average**	**2**
- Any three or all the four criteria	**Good**	**3 – 4**

Tool (3): Healing and pain Assessment Scales (Appendix II)

It comprised two main parts:

Part (A) MODIFIED REEDA SCALE (RS).

The Modified Reeda Scale (RS) Was Developed by HILL .(1989) and used to measure 5 indicators for the perineal condition : namely, redness, oedema, ecchymosis, discharge and approximation(healing). In this study the scale was modified used to provide the most objective means for evaluating the condition of the cracked nipples after delivery in relation to two indicators redness (R), and approximation (a) of the skin to determine the healing process.

- Redness was assessed through four levels:
  a)- 0 = no redness,
  b)- 1=< .25 cm (mild),
  c)- 2 = .5 cm (moderate)
  d)- 3 = > .5 cm (severe redness).
- Healing process was determined through four levels:
  a)- 0 = closed,
  b)- 1= mild (skin separation) < 3 mm,

c)- 2 = moderate (skin or any subcutaneous fat separated)

d)- 3 = severe (skin or s.c.f, and facial layer separation**).**

Part (B): VISUAL ANALOGE SCALE (VAS). Developed by LAFOY AND GEDEN (1989) was used to identify the degree of pain intensity. Which is as follows

Mild pain is characterized by pinking and/or aching, whereas moderate pain is characterized by pressing, cramping, sharp and burning. While severe pain is characterized by, no tolerance to pain.

- Pain was determined through four levels:

a)- 0 = no pain,

b)- 1 = (mild pain),

c)- 2 = (moderate pain)

d)- 3 = (severe pain).

Tool (4): Follow-up observation checklist to assess the healing speed and pain of the cracked nipples among the two groups. The checklist included observation of signs and symptoms of cracking such as (redness, , approximation of the skin, pain and bleeding) throughout the four follow-up visits according to Reeda scale **(RS)** and Visual Analogue Scale **(VAS)**, and to identify the degree of improvement after applying the two suggested treating methods of cracked nipples : (Breast milk and Apicare ointment:

Methods

1- An official letter from the Faculty of Nursing, Damanhour University was obtained and forwarded to the responsible authorities of the study setting to take their permission to conduct the study after explaining its purpose.

2- Tool I was developed by the researchers after extensive review of recent and relevant literature. The content validity of the developed tool was tested by a jury of five experts in the field. Tools reliability was tested by **cronbach alpha** test. Its result was 0.721 which indicates an accepted reliability of the tool.

3- A pilot study was carried out on 8 women (who were excluded from the study sample) to ascertain the clarity and the applicability of the tool as well as to estimate the time needed to it.

4- The researcher selected the women who fulfilled the criteria from the aforementioned settings**.** The researcher explained the purpose of the study to every woman, and then her consent to participate in the study was obtained. Each subject was interviewed individually and in total privacy. Confidentiality of data and right to withdraw at any time will be assured.

5-Data were collected over a period of 10 months, starting from the beginning of October 2011 till the end of July 2012. The average number of interviewee per day was 1-2. The average time needed to complete the interview schedule ranged between 15 to 20 minutes depending upon the degree of understanding and response of the interviewee.

6-Tool (I) and Tool (II) were used with the whole sample (80 postpartum women with cracked nipple) at the previously mentioned clinics during third day postpartum (before intervention). The researcher also had observed the correctness of **body position** and correctness of **attachment**. She also instructed the study group (1) how to apply breast milk and train study group (2) how to apply Apicare ointment. The participants in the two study groups were advised to apply the intervention by themselves until the 4th week post partum.

7- The two methods of treatment **(BREAST MILK and APICARE OINTMENT)** were applied for 4 weeks as follows:

Group (I) was advised to paint their nipples with their **BREAST MILK** and air-dry them after each feeding.

Group (2) was instructed to apply **APICARE OINTMENT** on the nipples 3 time/day (it does not need to be removed before next feeding). The researcher was supply the participant with sufficient amount of ointment.

All women were instructed to follow proper breast feeding technique while applying the specific intervention of each group.

8- Using tool (3) Women in the two groups were followed- up every week for one month (4 times /month) the nipple was examined (observed for signs and symptoms of cracking: redness, broken skin, bleeding and pain). The first, second, third and fourth week's follow up was carried out at homes. During these visits, the researcher did ensure that the participants' were performing interventions correctly.

The healing process of the cracked was assessed and compared among the two study groups to find out the most effective method in dealing with cracking.

10-The collected data were categorized, tabulated and made ready for analysis.

Statistical Analysis:

The data collected were computerized, revised, categorized, tabulated, analyzed, and presented in descriptive and association form. The necessary tables were then prepared and statistical formulas were used as percentages, Chi square (Yates test X^2) and Z test at 5% level to find out the statistical significance difference of the results

3.Results:-

Table (I) shows that the study subject's mean age was 26.1± 5.21. Where almost two - thirds (65.0%) of them were in their twenties and only 13.7% of them were teenagers. They were almost

equally residing in either an urban (41.3%) or rural (58.7%) areas. One- half (50%) of them had either a primary or preparatory education while one – fourth (25%) of them were illiterate and only 6.3% were university graduates. The majority (82.5%) of them were housewives.

According to table (II) three - fourths (75.0%) of the study subjects were primigravida and primiparae. They almost equally delivered either vaginally (51.3%) or by cesarean section (48.7). More than three – fourths (78.7%) of them had one or two child. As much as 86.3% of them did receive antenatal care during their latest pregnancy. However 70% of them never received any knowledge about either breast care or breastfeeding before or after the child birth.

Table (III) exhibits that about one – half (53.7%) of the study subjects had initiated breastfeeding during the first 3 hours after child birth. Almost all (91.3%) of them were feeding their newborn's on demand. About three - fourths (78.7%) did introduce the nipple only in the baby's mouth. As much as 83.7% of them were pulling the nipple out of the newborn's mouth when he stops suckling. Interestingly enough, 70% of them were breast feeding either 8-10 times daily, (31.3%) or more than 10 times daily (38.7%). The duration of breast feeding session were either less than 10 minutes (31.3%) or just 10 minutes (43.7%) or more than 10 minutes (25.0%)

According to table (IV) about and more than two- thirds (73.7% & 63.7%) of respondent had poor body position and poor attachment grade respectively. While no one of them (0.0% & 0.0%) had either good body position or attachment grade.

Table (V) indicates that the entire respondents' (100.00%) suffer from nipple redness, fissures and pain. While more than one-tenth (13.7%) of them had bleeding. In relation to the severity of signs and symptoms of cracked nipples the table also clearly reveals also that the majority (88.7%) of the study subject's had severe pain, two-third of them (66.3%) had mild degree of fissured nipple, more than one-half (58.7) of them had moderate redness and less than one –tenth (7.5%) of respondent had moderate bleeding.

Table (VI) reveals that using breast-milk completely relieved nipple bleeding and redness (100.00%) by the third visit (after three week) , and pain by the fourth visit(after four week). While maximum relief of fissure (97.5% was achieved among the study group I (Breast milk) by the fourth visits.

A significant differences was observed between the follow up visits and complete or maximum recovery of signs and symptoms among the study group I (Breast milk) where P value were (0.001^{*}, 0.031^{*}, 0.015^{*} and 0.026^{*} for redness, fissure, pain and bleeding respectively.

Table (VII) shows that among the Therapeutic honey "Apicare group " complete recovery of fissure, pain and bleeding were achieved among all of them (100%) by the second visit (after 2 weeks) while only redness was relieved on the third visit.

The results reveal a statistically significant difference between complete recovery of symptoms among the study group II (Apicare group) and their follow up visits e.g. symptoms improved significantly by time until complete recovery occurs where *P* was 0.0148^{*}, 0.028^{*}, 0.026^{*}& 0.037^{*} for redness, fissure, pain and bleeding respectively.

Table VIII: Comparison between the group I(Breast milk) and group II (Therapeutic honey) regarding the complete or maximum recovery from signs and symptoms of cracked nipples throughout the follow up visits. The table reveals that complete/ or maximum recovery in group II (therapeutic honey Apicare) in the second visit was evident among all the women (100%, 100% &100%) in relation to fissure, pain, and bleeding. In addition, the most of them (97.5%) had complete or maximum recovery in relation to redness.

On the other hands, during the second visit, complete or maximum recovery in group I (Breast milk) represented to the majority of women (92.5%, 80.0%, 80.0% &72.5%) in relation to bleeding, ,redness, fissure and pain, respectively.

Group II (using therapeutic honey) showed better improvement when compared to group1 (using breast milk). A statistically significant difference was observed in relation to all items of cracked nipples.

(This means that group II achieved complete recovery earlier, faster than group 1) Therapeutic honey is a better treatment for cracked nipple compared to breast milk.

4.Discussion

Breastfeeding is one of the oldest practices, recommended in the ancient Hindu scriptures, Holy Quran and Biblical records. It confers short-term and long-term benefits on both child and mother, including helping to protect children against a variety of acute and chronic disorders. A review of some studies from developing countries shows that infants who are not breastfed are 6–10 times more likely to die in the first few months of life than infants who are breastfed **(Shembesh *et al.*,1997; World Health Organization, 2009; Shams, 2011).**

Table (I): Number and percent distribution of the study subjects according to their socio-demographic- characteristics.

socio-demographic- characteristics	No. (no=80)	%
Age		
< 20	11	13.7
20 –	52	65.0
30 or more	17	21.3
Mean±S.D.	26.11±5.21	
Residence		
Rural	33	41.3
Urban	47	58.7
Education		
Illiterate	20	25.0
Primary/preparatory	40	50.0
Secondary or more	15	18.7
University or higher	5	6.3
Occupation		
Working	14	17.5
Housewives	66	82.5

Table (II): Number and percent distribution of the study subjects according to their Reproductive history and follow up activities during the current pregnancy.

Reproductive history and follow up activities	No. (no=80)	%
Gravidity		
Primigravida	60	75.0
Multigravida	20	25.0
Parity		
Primipara	60	75.0
Multipara	20	25.0
Type of current delivery		
Vaginal	41	51.3
C.S.	39	48.7
Number of living children		
< 3	63	78.7
≥3	17	21.3
Mean ± S.D.	1.98±0.98	
Follow up during the present pregnancy	69	
Yes		6.3
No	11	13.7
Received Knowledge about breast care and Breast feeding during and after pregnancy	13	16.3
Yes		
No	56	70
Not applicable *	11	13.7

Not applicable * means women who did not do follow up during the present pregnancy

Table (III): Number and percent distribution of the study subjects according to their technique of breast feeding

Technique of breast-feeding (position and method of lactation)	No (no=80)	%
Initiation of breastfeeding after delivery		
-Immediately after delivery (half hour - one hour)	29	36.3
-1-3 h	43	53.7
-during the first day	8	10.0
Type of feeding		
On demand	73	91.3
Scheduled	7	8.7
Part of the breast introduced into the infant's mouth:	63	78.7
-The nipple only	17	21.3
-The nipple and areola		
Method of nipple withdrawal		
-Pulling the nipple from the infant's mouth	67	83.7
-The infant leaves the breast spontaneously	13	16.3
Number of breastfeeding times/day		
-<5	9	11.3
5-7	15	18.7
8-10	25	31.3
->10	31	38.7
Duration of breastfeeding (Bilateral):		
-<10 minutes	25	31.3
-10 minutes	35	43.7
-> 10 minutes	20	25.0

Table (IV) : Number and percent distribution of the study subjects according to the quality of their total position (mother and newborn) and attachment (latch on) grade.

Total position and attachment grade	No (no=80)	%
Body position		
Poor	59	73.7
Average	21	26.3
Good	0	0.0
Attachment		
Poor	51	63.7
Average	29	36.3
Good	0	0.0

Table (V):Number and percent distribution of the study subjects according to presence of signs, symptoms and nature of cracked nipple (before treatment).

Signs, symptoms and nature of cracked nipples before treatment	No. (no=80)	%
Suffering of pain		
Yes	80	100.0
No	0	0.0
Degree of pain		
Moderate pain	9	11.3
Severe pain	71	88.7
Presence of skin redness		
Yes	80	100.0
No	0	0.0
Degree of redness		
Moderate redness	47	58.7
Severe redness	33	41.3
Presence of skin fissure (approximation of the skin)	80	100.0
Yes	0	0.0
No		
Degree of skin fissure		
Mild	53	66.3
Moderate	16	20.0
Severe	11	13.7
Presence of bleeding		
Yes	11	13.7
No	69	86.3

Amount of bleeding		
Mild	4	5.0
Moderate	6	7.5
Severe	1	1.2
Not applicable.	69	86.3

Table (VI): Signs and symptoms of cracked nipples among the Breast milk throughout the follow up visits (in percent distribution)

Signs and symptoms	First visit (1st week)	Second visit (2nd week)	Third visit (3rd week)	Fourth visit (4th week)	X^2 *P*
Redness					
Mild	17.5	7.5	0.0	0.0	
Moderate	20.0	12.5	0.0	0.0	22.9
Severe	2.5	0.0	0.0	0.0	0.001*
Complete relief	60.0	80.0	100.0	100.0	
Fissure					
Mild	15.0	10.0	5.0	2.5	
Moderate	10.0	7.5	2.5	0.0	5.65
Severe	7.5	2.5	0.0	0.0	0.031*
Complete relief	67.5	80.0	92.5	97.5	
Pain					
Mild	20.0	12.5	7.5	0.0	
Moderate	17.5	7.5	2.5	0.0	7.01
Severe	10.0	7.5	0.0	0.0	0.015*
Complete relief	52.5	72.5	90.0	100.0	
Bleeding					
Mild	15	7.5	0.0	0.0	
Moderate	5	0.0	0.0	0.0	4.11
Severe	2.5	0.0	0.0	0.0	0.026*
Complete relief	77.5	92.5	100.0	100.0	

Table (VII): Signs and symptoms of cracked nipples among the honey group (Honey) throughout the follow up visits (in percent distribution)

Signs and symptoms	First visit (1st week)	Second visit (2nd week)	Third visit (3rd week)	Fourth visit (4th week)	X^2 *P*
Redness					
Mild	2.5	0.0	0.0	0.0	
Moderate	10.0	2.5	0.0	0.0	4.01
Severe	7.5	0.0	0.0	0.0	0.0148*
Complete relief	80.0	97.5	100.0	100.0	
Fissure					
Mild	7.5	0.0	0.0	0.0	4.37
Moderate	5.0	0.0	0.0	0.0	0.028*
Severe	2.5	0.0	0.0	0.0	
Complete relief	85.0	100.0	100.0	100.0	
Pain					
Mild	10.0	0.0	0.0	0.0	4.15
Moderate	7.5	0.0	0.0	0.0	0.026*
Severe	0.0	0.0	0.0	0.0	
Complete relief	82.5	100.0	100.0	100.0	
Bleeding					
Mild	10.0	0.0	0.0	0.0	4.68
Moderate	5.0	0.0	0.0	0.0	0.037*
Severe	0.0	0.0	0.0	0.0	
Complete relief	85.0	100.0	100.0	100.0	

Table (VIII): Comparison between the two studied groups regarding the complete recovery from cracked nipples thought the follow up visit.

Signs and symptoms	Group	First visit (1st week)	Second visit (2nd week)	Third visit (3rd week)	Fourth visit (4th week)
Redness	Group I	60.0	80.0	100.0	100.0
	Group II	80.0	97.5	100.0	100.0
	P	0.002*	0.044*	-	-
Fissure	Group I	67.5	80.0	92.5	97.5

	Group II *P*	85.0 0.0081*	100.0 0.039*	100.0 0.088	100.0 0.109
Pain	Group I Group II *P*	52.5 82.5 0.001*	72.5 100.0 0.013*	90.0 100.0 0.075	92.5 100.0 0.088
Bleeding	Group I Group II *P*	77.5 85.0 0.023*	92.5 100.0 0.088	100.0 100.0 -	100.0 100.0 -

Group I= "Breast milk" **Group II** = "Honey"

It is a dream for most mothers to have comfort in breastfeeding, but sore nipples are still a common problem, where pain or cracks occur frequently after breastfeeding. When the nipples are hurt, breastfeeding is in jeopardy. Up to one third of the mothers who experience these symptoms may change to alternate methods of infant nutrition within the first six postnatal weeks. Unfortunately, many women delay seeking treatment until substantial damage has already occurred and sore nipples remain a frustrating clinical dilemma **(Melli *et al.*, 2007).**

Therefore, the prevention of nipple pain and cracks is important. Proper nipple care, such as keeping nipples dry, and proper nursing techniques are the best preventative measures for sore nipples. Prenatal nipple preparation consisting of exposing nipples to the air and avoiding soaps and drying agents on the nipples can be beneficial. Recent studies have shown that additional prenatal preparation, such as "nipple rolls" and massage may actually cause sore nipples (not to mention possible causing premature contractions), although they have often been recommended as a means of preventing sore nipples in the past. Any nipple preparation that uses friction on the nipples (as opposed to gentle pressure) can actually destroy the protective keratin coating on the nipples and encourage soreness to develop **(Braund and Amir, 2001; Elsa, 2004).**

The results of the present study revealed that the majority of women did not receive any knowledge about breast care and breast feeding during and after pregnancy. This may reflect a deficiency in health institutions health education role. Where their interest focuses mainly on serious cases.

Evidence- based medical practice supports breastfeeding initiation within the first hour after delivery through direct uninterrupted continuous skin to skin contact as it is associated with reduction of mortality by 22% and increase in bonding and breast feeding rates in early months. Moreover, nipple stimulation from sucking releases oxytocin, which promotes uterine contraction and this reduce the chances of post partum hemorrhage **(Edmond *et al.*, 2006).**

On asking the participants about their breast feeding practice, the results clearly indicated that more than one- half of them had initiated breastfeeding within the first three hours after childbirth. Where, the majority of them did introduce nipple only into the infant's mouth during breastfeeding and usually pulling nipple out of infant mouth as well as breast feed their newborns 8-10 times or more. Where the breast feeding was mainly on demand. Similar findings were reported in two studies. The first was carried out in Egypt by **(Rashad *et al.*, 2006)** about the effect of early feeding practices and mode of feeding on neonatal jaundice. The second was carried out also in Egypt by (**El Bakery *et al.*, 2011)** about modulation of the intelligent quotient of pre-schoolers by improving early infant feeding practices.

However, these results are incongruent with the literature review which indicates that the areola and entire nipple should be in the mouth of the baby while breast feeding. Furthermore, the baby should let the nipple either by pressing on his chin or by inserting a little finger into the corner of baby mouth help him to detach. So, forcing the nipple out from the newborn's mouth while he is still sucking results in sore nipple **(Morland-Schultz and Hill, 2005).**

The present study further revealed an incorrect practice regarding both mother and baby position and attachment during breastfeeding. This is probably because the majority of participants were primigravidae with no previous experience. In addition, they did not receive any knowledge about breast care and breast feeding during or after pregnancy. This finding is supported by (**Sai *et al.*, 2009)** findings who mentioned that The baby's positioning and attachment to the breast during breastfeeding are fundamental toward the occurrence of different sorts of nipple trauma. Moreover, many studies had indicated a statistically significant association between position and holding variables for causing nipple lesions. Especially when newborns whose necks were bent, whose chins were held away from the breast and where lips were turned inward. Most difficulties can be avoided altogether through good attachment and positioning during the first early feeding **(Vinther and Helsing, 1997; Dongre *et al.*, 2010).**

The health benefits of breastfeeding for mothers and infant are well-documented. One of the

major problems in lactating women at the beginning of breastfeeding is nipple crack **(Tait, 2000; Akkuzu and Taskin,2000).** The results of the present study clearly revealed that all of the study subjects had suffered from signs and symptoms (redness, fissure nipple, pain) of cracked nipple. Several studies have shown that 80 to 90 percent of breastfeeding women experience some nipple soreness, with 26 percent progressing to cracking and extreme nipple pain. Nipple soreness' may vary from a slight tenderness while the baby is nursing to extreme pain both during and in between nursing **(Hagen and Brent, 1999).**
While there may be a limited evidence base specific to nipples, there is however, substantial evidence supporting moist wound healing. More and more nursing mothers are choosing to use natural and holistic remedies to relieve the pain and discomfort of breastfeeding problems naturally **(Shaw-Flach, 2004)**. In the present study the mother's who used their own breast milk (group1) to treat cracked nipples, their symptoms had significantly improved by time until complete recovery occurred. Complete recovery for redness, and bleeding was achieved by the third visit (after 3rd weeks) while fissure and pain were relived on the fourth visit (after 4 weeks). This result coincides with the findings of **(Smith and Tully, 2001)** who mentioned that expressing some milk after a feeding and air drying the nipples before replacing clothing may be another helpful strategy to encourage healing. Breast milk contains fat-soluble vitamins associated with healing, including A and E, and does not need to be washed off before the next feeding**.** The same result is also supported by **(Pugh *et al.*, 1996)** work who found that expressed breast milk has no risk of allergy of allergy contains antibodies and epidermal factor which potentially may promote the growth and repair of skin cell.

On the other hand, this result is not in accordance with **(Allen *et al.*, 2003)** findings. He stated that although expressed breast milk is considered helpful for preventing infection and promoting relief, a number of the articles had report that current dermatological thinking considers the environment produced by dried EBM to be conducive to cracking and scab formation. Moreover, **(Akkuzu and Taşkin, 2000)** had elaborated on the Impacts of breast-care techniques on prevention of possible postpartum nipple problems. They found that Applying warm compresses or expressed breast milk was found to be less effective in preventing cracked nipples.

In general, many local comfort measures and agents have been applied to the breast to relief the discomfort such as exposure to dry heat (sun light or electrical lamp) and exposure of nipple to air for some time every day would help in the healing process. application of an antiseptic cream. In addition, In United State of America some hospital began providing mother with hydrogel dressings to treat nipple soreness. The application of these dressings creates a moist environment for healing besides providing pain relief upon application. Moreover, the use of lanolin ointment and Anaflex cream as well as therapeutic honey (Apicare) was also recommended. **(Allison, 2002).**

Honey provides a moist healing environment, which gives high rate of tissue regeneration and prevents bacterial growth, even when wound are heavily infected. It is effective against antibiotic resistant strains of bacteria. Its antibacterial properties and its viscosity also provide a barrier to cross-infection of wound. In addition it reduces the edema and swelling of inflamed tissue with consequent pain relief, as well as promotes the healing process **(Andreas, 2012).**

A significant difference was observed in the complete or maximum relief of symptoms over time, among the Apicare ointment user. Complete relieved nipple fissure, pain and bleeding were achieved by second week (2^{nd} visits) as well as redness at the third week (3^{rd} visits). This result is in line with **(Abd alaziz's, 2005)** results. She did a study about the comparison between three wound dressing techniques (Apicare ointment, zinic saline and diluted iodine) for patients with diabetic foot ulceration. She reported that healing rate in group who managed by Apicare ointment was higher than two other agent. She added that, it reduces inflammation edema and exudation**.**

The same result is also in accordance with **(El-Agamy's 2004)** results she did a study about the effect of topical honey dressing on infected wounds after gynecological and obstetrical abdominal surgery. She reported that honey dressed group showed 100% significant improvement than her control group.
The present study further revealed that Apicare ointment users had achieved a faster recovery than breast milk users (Table VIII). This result is supported by **(Moore *et al.*,** 2001; **Andreas,** 2012) findings. They had indicated that honey has been used successfully for various wounds and the action varies widely.

Conclusion

According to the results yielded by the present study, its hypothesis is accepted. Where the cracked nipples healing was faster among the honey users than among breast milk users.

Recommendations:

1- Apicare® application could be suggested as a treatment of nipple crack alongside proper instruction at the initiation of breastfeeding.
2- Primiparae mothers need more support and guidance for appropriate breastfeeding techniques.
3- It is recommended that each mother should be observed for mother's and infant's positioning and attachment at the onset of breastfeeding and if needed given counseling on correct positioning and attachment.
4- All Maternal and Child Health (MCH) care agencies should highlight and formulate a policy for successful and effective initiation of breastfeeding as a part of an integrated neonatal care.

Acknowledgements
The authors thank and fully acknowledge the participants for making this study possible and Sahla center staff for their help. Finally thanks dr **Salah abd-Rabo** professor of obstetrics and gynecology University of Alexandria for supporting this work.

Corresponding author:
Dr. Rasha Mohamed Essa
Lecturer , Obstetric and Gynecological Nursing Dept Alex University , Egypt.
E-mail: rashaessa111@yahoo.com

References:-

1. Abd-Alaziz A. A comparative between three wound dressing techniques (Apicare ointment, zinic saline and diluted iodine) for patients with diabetic foot ulceration. 2005; Thesis BSc. Faculty of nursing, University of Tanta.
2. Akkuzu G, Taskin L: Impacts of breast-care techniques on prevention of possible postpartum nipple problems. Professional Care of Mother & Child, 2000; 10(2): 38–41.
3. Allen KL, Hutchinson G, Molan PC. The potential for using honey to treat wounds infected with MRSA and VRE. First world healing congress, Melbourne, Australia: 2000; 87-89.
4. Allen J, Allen A. The nature of evidence: Treating tender nipples in breastfeeding: The Journal of the Health Visitors' Association, 2003; 423-27.
5. Allison F. Moistness: The secret of healing core and cracked nipples. available at http:// www.breastfeedimg.org. Last accessed on 13/5/2002.
6. Andreas m. Honey - The World's Best Wound Healer . available at http://www.ener-chi.com/honey-the-worlds-best-wound-healer/ last accessed on 18/1/ 2012.
7. Ann H., Caroline L. The management of nipple pain and/ or trauma associated with breastfeeding. Center for evidence-based nursing South Australia (CENA) 2003; 7(3):1.
8. Biancuzzo M. Sore nipples: prevention and problem solving. Herndon, USA: WMC Worldwide 2000;43-46
9. Blumenthal M, Goldberg A, Brinckmann J. Herbal Medicine: Expanded Commission E Monographs. Newton, MA Integrative Medicine Communications 2000; 297–303.
10. Braund D, Amir LH: Review of the management of nipple pain and damage. Topics in breastfeeding. Melbourne, Lactation Resource Centre 2001.
11. Buchanan P, Hands A, Jones W. Assessing the evidence: Cracked Nipples and Moist Wound Healing. Paisley, Scotland: The Breastfeeding Network, March 2002;1-8.
12. Dongre AR, Deshmukh PR, Rawool AP, Garg BS. Where and How Breastfeeding Promotion Initiatives Should Focus Its Attention? A Study from Rural Wardha. Indian J Community Med 2010;35:226–29.
13. Edmond KM., Zandoh C., Quigleyc MA., Amengra-Etego S., Owusu-Agyei S., Kirkwood B. Delayed breastfeeding initiation increases risk of neonatal mortality. Pediatrics 2006; 117 (3): 380-86.
14. El-Agamy M. the effects of topical honey dressing on infected wounds after gynecological and obstetrical abdominal surgery.2004; Thesis BSc. Faculty of nursing, University of Tanta.
15. El-Bakry S T., Abul-Fadl AM., El- Mahdy MH., Attia MI. Modulation of the intelligent quotient of pre-schoolers by improving early infant feeding practices MCFC-Egyptian Journal of Breastfeeding(EJB) 2011; vol 4: 49-56.
16. Elsa R. Common problems during lactation and their management. Pediatric Journal 2004; 80 (5).43-47
17. Ganguli G, Dhawan NI. Antenatal breast complication and their management. Journal of Obstetric and Gynecology, Medical College 2000; 50(1):38.
18. Gartner LM, Morton J, and Lawrence RA. Breastfeeding and the use of human milk. American academy of pediatrics 2005; 115:496-501.
19. Giugliani E R .Common problems during lactation and their management. Journal of Pediatric 2004; 80 (5):167-69.
20. Hagen RL, Brent NB: Lanolin for sore nipples. Archives of Pediatrics & Adolescent Medicine. Chicago, 1999; 153(6): 658–59.
21. Jones R. Honey and healing through the ages. In: Honey and healing. International Bee Research Association, Cariff, UK 2001;24.

22. Leung AK, Sauve RS. Breast is best for babies: Journal of the national medical association 2005; 97(7):516-18.
23. Melli MS, Rashidi MR, Nokhoodchi A, Sadaghat K, Tahmasebi Z and Sheshvan MK. A randomized trial of peppermint gel, lanolin ointment, and placebo gel to prevent nipple crack in primiparous breastfeeding women. available at http:// www.medscimonit.com. last accessed on 3/9/2007. 406-11.
24. .Melodee H. Honey: An old or new remedy?: Long Term Management Care . Long-Term Living 60(5) (May 2011): 24-25.
25. Moore OA, Smith LA, Campbell F, Seers K, McQuay HJ, Moore RA. Systematic review of the use of honey as a wound dressing. *BMC Comp Alt Med* **1(2):** Available at .http:// www.biomedcentral.com/1472-6882/1/2 **last accessed on 5/12/2001.**
26. Morland-Schultz K, Hill PD: Prevention of and therapies for nipple pain: a systematic review. *J Obstet Gynecol Neonatal Nurs* 2005; 34:428-37.
27. Page T, Lockwood C, Guest K. Management of nipple pain and/or trauma associated with breast-feeding. *JBI Reports* 2003; 1(4):127-47.
28. Pizzorno JE, Murray MT. Textbook of Natural Medicine. NewYork: Churchill Livingstone; 1999; 827-29. 1361–62, 1558.
29. Pugh LC, Buchko BL, Bishop BA, Cochran JF, Smith LR, Lerew DJ: **A** comparison of topical agents to relieve nipple pain and enhance breastfeeding. *Birth* 1996; **23:**88-93.
30. Raising Children Network. Child breast- feeding problems and solution. Available at: http://www.raisingchildren.net.au/articles/brestfeeding problems and solution.html. last accessed on 29/5/2009.
31. Rani P, Shaw N. Improving antenatal management in Asians. Journal of Pediatric Nursing 2000; 139:337-38.
32. Rashed M., Abul-Fadl AM., Yousef MS., Ahmed MG., Abul-soud W. Effect of early feeding practices and mode of feeding on neonatal jaundice. ELCA Scientific Journal, special issue dedicated to the proceedings of the Egyptian Lactation Consultant Assocation's first scientific meeting 2006; 23-36.
33. Renfrew, M. J., Woolridge, M. W., Ross McGill, H. Enabling women to breastfeed. A review of practices which promote or inhibit breastfeeding – with evidence based guidance for practice. Norwich: Stationery Office, 2000.
34. Sai M, Kishore S, Kumar P, Aggarwal AK. Breastfeeding Knowledge and Practices amongst Mothers in a Rural Population of North India: A community-based study. *J Trop Pediatr* 2009; 55:183–8.
35. Shams S. Breast feeding and motherhood. Pakistan journal of nutrition 2011; 10 (6):599-601
36. Shaw-Flach A. Cracked nipples: evidence and care: The Journal of the Health Visitors' Association, Community Practitioner 77(2) 2004; 75.
37. Shembesh NM, Balo NN, Singh R. Breast-feeding and weaning patterns in Benghazi, Libyan Arab Jamahiriya. *East Mediterr Health J* 1997; 3:251–7.
38. Smith JW., TullyMR. Midwifery management of breastfeeding: Using the Evidence. Journal of Midwifery & Women's Health 2001; 46(6): 423-30.
39. Tait P. Nipple pain in breastfeeding women: causes, treatment, and prevention strategies. J Midwifery Women's Health, 2000; 45: 197–201
40. The report of Arafa and Ara zami chemical company, The role of Apicare ointment for wound dressing. Alexandria, Egypt. 2003.
41. Vinther T, Helsing E. Breastfeeding- how to support success, A practical guide for health workers. Copenhagen: World Health Organization, Regional Office for Europe 1997; 17–19, 24-26.
42. White RJ, , Molan PC. A summary of published clinical research on honey in wound management. In: White RJ, Cooper RA, Molan PC Honey: A modern wound management product. Wounds UK, Aberdeen 2005;130-43.
43. World Health Organization. Exclusive breast-feeding for six months best for babies everywhere. Geneva: WHO. 2011.
44. World Health Organization. Infant and young child feeding: Model Chapter for textbooks for medical students and allied health professionals. Geneva: WHO; 2009.
45. World Health Organization. Postpartum care of the mother and newborn. Geneva: WHO. 2001.
46. Young T. Honey: rediscovering an ancient healer. *Practice Nursing* 2005; 16(11): 542–47.

Exploring the Environmental Knowledge of Urban and Rural Consumers and Its Impact on Green Purchase Behavior

Nalini Palaniswamy[1], Dr. Muruganandam Duraiswamy[2]

[1]Department of Management, CIMAT, Coimbatore, Tamil Nadu, India
[2]Department of Management,Kongu Engineering College, Erode, Tamilnadu, India
pnsgem@gmail.com

Abstract: The rapid economic growth has increased the demand for products and services in the market place. On the other hand there is a vice-versa effect on the environment. This unsustainable growth was taken due consideration and this research was done with an objective to explore the environmental knowledge of consumers and their impact on green purchase behavior in the context of urban and rural scenario. This study indicates the inter-correlation effect of environmental knowledge and its impact on selected green product purchase behaviour. The study result provides a reasonable support for the companies who come out with green product offering and to design their marketing campaign as well.

[Nalini P, Muruganandam D. **Exploring the Environmental Knowledge of Urban and Rural Consumers and Its Impact on Green Purchase Behavior.** *Life Sci J*2013; 10(1):2148-2153] (ISSN:1097-8135). http://www.lifesciencesite.com 303

Key words: Environmental knowledge, Ecology, Green products, Purchase behaviour.

Introduction

Modern business is an integral part of current day society. This business has far reaching impact on social & economic life of people. As a socio-economic institution, each marketing company is to deliver the goods and services , the standard of living or a life style as per the aspirations of the members of this society. It has a great social responsibility which means an intelligent and objective concern for the well-being of society. The marketing philosophy revolves around doing business profitably by identifying and meeting needs and wants of customers. However, the conventional marketing view that the environment as limits, it becomes clear that meeting the need of today's customers unsustainably will reduce the ability to meet the needs of future generation customer's (Davis D.P 1999). Consumer Environmentalism is defined as the level of environmental concern and responsibility a consumer brings to the product purchase decisions he or she makes. This value should be the most significant and accurate predictor of consumer behaviour in the environmentally friendly marketplace. An enduring definition of a value was put forth by Rokeach (1973), describing a Value as an "enduring belief that a specific mode of conduct or end-state existence is personally or socially preferable to an opposite or converse mode of conduct or end-state of existence. "The rapid economic growth of the city increased the demand for office space and apartment's. Builders acquired vacant spaces, demolished old houses, and built tall towers. All these residential and office blocks need water, power and sewage facilities thousands of trees were removed to make space for the city's development. As green spaces shrink water bodies and wetland disappears, power consumption in the city as well as rural has an enormous increase. India is, of course not unique in experiencing environmental destruction. There are similar stories in all parts of the world .Glaciers are melting rapidly in the polar regions and in many mountain ranges are cut down for human benefits. Every major city in the developing countries faces serious problems. Keeping this in the backdrop, this study was done with the following objectives:

Objectives of the Study

1) To study the consumer awareness on environmental issues and its impact on purchase decision of selected green products.
2) To investigate the demographic profiles and their differences in understanding the environmental issues and preference for green products

Literature Review

The word *environment* often refer to the ter m as
"all external conditions and factors, living and non-li ving (chemicals and energy), that affect an organism or other specified system during its lifetime"
(Depoe, 2007). From the seventies ecological green marketing had been flourishing in developed countries. In this early period attention was payed to specific environmental problems, so solutions were searched for them separately, that is why only few products, companies and industries were affected by this new trend. Ecological green marketing was the

sport of the minority, and caused changes in the lifestyle of only a few consumers. **(Peattie, 2001).**

The First Age: "Ecological" Green Marketing

Ecological marketing was defined by Henion and Kinnear (1976) as "concerned with all marketing activities

(a) That have served to help cause environmental problems and

(b) That may serve to provide a remedy for environmental problems."

The characteristics of this first age of concern were as follows:

- It was narrowly focused on specific "environmental problems" such as air pollution, depletion of oil reserves, oil spills and the ecological impacts of synthetic pesticides. The emphasis was on pollution and resource depletion (particularly energy resources) and on local or national concerns.
- It sought to identify particular products, companies or industries which were causing, or in a position to help solve, these particular problems.
- It was debated across a relatively narrow "front line" of industries including automobiles, oil and agro-chemicals.
- It was something of a "minority sport" with relatively few consumers and companies significantly changing their behaviour.

The Second Age: "Environmental" Green Marketing

Green Marketing's second age emerged during the latter part of the 1980s. The potential vulnerability of the environment, and human life within it, was highlighted by a series of incidents and discoveries. These included the Bhopal tragedy in 1984, the discovery of the Antarctic hole in the ozone layer in 1985, Chernobyl in 1986 and the Exxon-Valdez oil spill in 1989. Media coverage of these and other disasters stoked public concern about the environment so that it became a mainstream issue.

The Third Age: "Sustainable" Green Marketing

Since 2000, green marketing has evolved into a third stage. With the implementation of more advanced technology, stricter state enforcement on deceptive claims, government regulations and incentives as well as closer scrutiny from various environmental organisations and the media, many green products have greatly improved and regained consumer confidence in the 2000s (Gura u and Ranchhod, 2005)(Ottman, 2007). Together, with the continuous rise of growing global concern about the environmental quality, green marketing has gradually picked up momentum again. Some researchers postulate (Stafford, 2003) that green marketing is now "making a comeback" (Ottman et al., 2006).Once again, there is renewed sensitivity towards the environment and towards social consciousness. With "sustainable development" being pressed as the dominating theme in twenty-first century commerce, two trends are predicted as inevitable in the near future of green marketing.

Profile of the Respondents

The rural and urban area respondents were given an equal contribution and the majority of age group falls in age group of 20-30 years (44 percent). The results clearly showcase that the youth population has higher impact in exploring environmental factors followed by above 50 years (18 percent) who have greater response and concern towards a better India. In gender classification male (61.4 percent) takes a higher response, it shows their interest towards exploring the environmental issues and environmental safety followed by education qualification of the respondents (33.6 percent) are graduates and (32.5 percent) were post graduates. It clearly indicates the educated respondents showed interest in participating the research. Majority of the respondents belong to employed category (36.3percent) followed by (28.2 percent) of the students who have greater influence in today's family decision making and (12.3 percent) of the respondents are professionals. The income of the respondents were classified and the higher group falls under the category of dependent (31.2 percent) the youth population followed by (20.3 percent) are in the income group of Rs15000 to Rs 25000 and (12 percent) are in the income group of Rs 25000 to Rs 30000 .The majority of the respondents fall with a family size of four (38.2 percent) followed by three member (30.6 percent) this shows the growth of nuclear setup families. The two earners family is in growing trend (47.2 percent) followed by one earners family (37percent) as compared to other product ranges green products price is comparatively high thus the research have given importance for the family earners. This study results are based on the above mentioned demographic segments it still have scope for other segments also.

Table 1:Profile of the respondents

Characteristics	*Percentage n=625*
Residing region	
Rural	50.0
Urban	50.0
Age (in years)	
Less than 20	11.7
20-30	44.0
30-40	12.5
40-50	13.8
Above 50	18.1
Gender	

Male	61.4
Female	38.6
Education	
Illiterate	6.1
School level	19.5
Graduate	33.6
Post graduate	32.5
Professionals	8.3
Occupation	
Student	28.2
Professional	12.3
Business	9.6
Employed	36.3
Retired	1.9
Others	11.7
Monthly income(in Rs)	
Dependent	31.2
Less than Rs.5000	3.5
5000-15000	11.7
15000-25000	20.3
25000-30000	12.0
30000 & above	21.3
Family size	
Single	3.8
Two	9.1
Three	30.6
Four	38.2
Five	10.6
More than 5	6.1
No of earners	
One	37.0
Two	47.2
Three	12.5
Four	3.4

Survey Instrument

As noted above, the goal of this study is to advance the marketing discipline in investigations addressing the concept of green marketing and its impact in the environmental alarmism of individual consumers who resides in urban and rural places in Coimbatore district. To guide our investigation, we have done an exploratory research in two stages in the first stage we have analysed the various aspects of consumers awareness and knowledge on environmental disasters and the concern towards this disasters and in the second stage we have analysed their green purchase behaviour with relevant to three selected green product category namely., organic fruits and vegetables, green electronics and green automobiles. The questionnaire used in this study was an attractive six-page booklet with a cover page of brief instructions. Before conducting the comprehensive survey, three marketing professors were first invited to assess the foregoing measurement instrument. After one pre-test with 100 respondents, the final version of the questionnaire with two sections was modified. In the first stage the first section consisted 9 items of demographic data such as age, gender, education level, etc., The second section includes 7 items and it covered 62 items to capture the perceived environmental awareness, knowledge and their personal and social concern towards each factors. Much research on environmental consciousness has focussed on very specific environmental concerns, such as packaging, recycling etc., but this study comprehensively define other relevant variables related to cause (environmental disasters) and effect(green purchase behaviours) relationship of each variables included in the study with relevant to environment. Whereas the purpose of the second stage of this survey instrument was to measure a broad range of respondents environmental (green) purchase behaviours with respect to selected green product category included for the study. A list of 25 items were included, which includes 110 questions to capture the green purchase behaviours with relevant to organic food stuffs, green electronics and green automobiles. The questions were mostly placed in 5-Point Likert scale and rank order questions were also used to know the priority in preferences.

Samples design

With relevant to the objective of the study the Coimbatore district was selected as the study area, due to its heavy concentration of both extended rural and urban places around it. The stratified random sampling method was adopted for the purpose of data collection. The district were divided into two taluks namely south taluk and north taluk. The south taluk contains 17 towns (urban) and 28 villages (rural) whereas the north taluk consists of 14 towns (urban) and 40 villages (rural). Further all the towns and villages have been alphabetically arranged and from this list applying the Lottery method 6 urban and 6 rural areas were selected for the study further the house numbers of the respective villages were collected and it was given random numbers based on the random number tables and the individual samples were thus selected for the study .The number of respondents were 625 out of 700 distributed questionnaire 625 were valid with all relevant data's and in that 312 were rural and 313 were from urban again for the purpose of operativeness and convenience ,it was decided that the sample would be formed for the respondents who are familiar with the purchase of these green products. In case of organic food stuffs women were more interestingly participating whereas in case of green electronics men were more interested and their responses were given importance. Age quotas were also established, so as to limit the interviewer's prejudice when collecting the sample elements.

Discriminant Function Analysis

Respondent's opinionaboutexploring the environmental knowledge of urban and rural Consumers and its impact on green purchase behavior. In the study area out of six hundred and twenty five respondents were divided into two groups .i.e., low level of impact on purchase decision and the

high level of impact on purchase decision. The difference of opinion of the respondents in one group from the other is studied with the help of discriminant function analysis. For the purpose of the study, the following variables were selected.

1. Gender
2. Age
3. Educational Qualification
4. Occupational status
5. Monthly Income
6. Family size
7. Earning members in the family
8. Present Job position
9. Residing Region

The discriminant function analysis attempts to construct a function with these and other variables so that the respondents belonging to these two groups are differentiated at the maximum. The linear combination of variables is known as discriminant function and its parameters are called discriminant function coefficients. In constructing this discriminant function all the variables which contribute to differentiate these three groups are examined.Mahalanobis minimum D^2 method is based on the generalized squared uclidean distance that adjusts for unequal variances in the variables. The major advantage of this procedure is that it is computed in the original space of the predictor (independent) variables rather than as a collapsed version which is used in the other method. Generally, all the variables selected will not contribute to explain the maximum discriminatory power of the function. So a selection rule is applied based on certain criteria to include those variables which best discriminate.

Table – 2:
Group Means (Between low and high groups)

S. No.	Factor	Low		High		Total	
		Mean	SD	Mean	SD	Mean	SD
1	Gender	1.36	.480	1.43	.496	1.39	.487
2	Age	2.84	1.346	2.80	1.280	2.83	1.319
3	Educational Qualification	3.13	1.064	3.24	.982	3.17	1.033
4	Occupational status	3.14	1.655	2.95	1.653	3.07	1.656
5	Monthly Income	3.36	1.932	3.52	1.916	3.42	1.926
6	Family size	3.54	1.200	3.63	1.079	3.58	1.154
7	Earning members in the family	1.78	.808	1.89	.714	1.82	.773
8	Residing Region	1.85	.815	1.73	.588	1.80	.736

The overall stepwise D.F.A results, after all significant discriminators have been included in the estimation of discriminated function is given in the following table

Stepwise selection method was applied in constructing discriminant function which selects one variable at a time to include in the function. Before entering into the function the variables are examined for inclusion in the function. The variables which could have maximum D^2 value, if entered into the function is selected for inclusion in the function. Once entered any variable already in the equation is again considered for removal based on certain removal criteria. Likewise, at each step the next best discriminating variable is selected and included in the function and any variable already included in the function is considered for removal based on the selection and removal criteria respectively.

Discriminant Analysis for This Study

Discriminant function analysis involved classification problem and also to ascertain the efficiency of the discriminant function analysis all the variables which satisfy the entry and removal criteria were entered into the function. Normally the criteria used to select the variables for inclusion in the function is minimum F to enter into the equation (i.e.) F statistic calculated for the qualified variable

To enter into the function is fixed as ≥ 1. Similarly any variable entered in the equation will be removed from the function if F statistic for the variable calculated is < 1. The two groups are defined as

Group 1 - Low level
Group 2 - High level

The mean and standard deviation for these groups and for the entire samples are given for each variable considered in the analysis.

Table -3
Summary table between low level and high level groups

Step	Variables entered	Wilk'sLamda	F-value	Significance
1	Residing Region	.994	3.94	.048**

**Significant at 5% level

The summary table indicates that variable Residing Region entered in step two. The variables such as Residing Region are significant at one per cent significance level. All the variables are significant discriminators based on their Wilk's lambda and F-value. The multivariate aspect of this model is given in the following table

Table -4
Canonical discriminant function (Between low and high groups)

Canonical correlation	Wilks Lamda	Chi -square	D.F	p-value
0.158	0.975	15.718	8	.047**

**P<0.05

The canonical correlation in the discriminant group can be accounted for by this model, Wilkslamda and chi square value suggest that D.F is significant at one per cent level.

The variables given above are identified finally by the D.F.A as the eligible discriminating variables. Based on the selected variables the corresponding D.F coefficients are calculated. They are given in the following table.

Table -5
Discriminant function coefficient
(Between low level and high level)

Residing Region	-.776
Constant	-1.566

Unstandardized coefficients

Z = -1.566 -0.776 (Residing Region)

Using this D.F coefficients and variables discriminating scores for 2 groups are found out and are called group centroids or group means

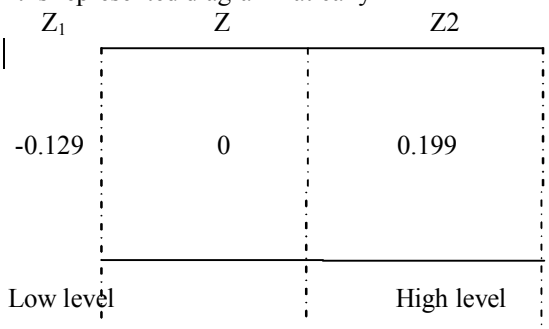
For low level user $(Z_1) = -.129$

For High level user $(Z_2) = .199$

Discriminating factor is the weighted average of Z_1, Z_2

$$(\text{i.e}) Z = \frac{(379x\ Z_1) + (246\ xZ_2)}{379+246}$$

It is represented diagrammatically



Thus to classify any respondent as to low or high user the Z score for the respondent is found out by using the equation. If the score found out for any respondent is Z_0 and if the value is > Z (i.e. Z_0> Z) then it is classified into high user and if Z_0<Z then (i.e. Z_0<Z) it is classified into low user.

Now the questions remain to be answered are

1. How efficient are the discriminating variables in the D.F.A?
2. How efficient the D.F itself is?

The first equation cannot be answered directly however the discriminating power or the contribution of each variable to the function can sufficiently answer the question. For this consider the following table

For each variable the respective D.F coefficient its mean for each group and R_j are given. R_j called relative discriminating index is calculated from the discriminant function coefficient and group means. R_j tells how much each variable is contributing (%) to the function. By looking at this column one education is the discriminating variable and the family income is the least discriminating variable.

Table – 6:Relative Discriminating Index
(Between low level group and high level group)

	Group I Mean X_1	Group II Mean X_2	Unstandardized coefficient	I_j=ABS (K_j) Mean (X_{j0}-X_{ji})	R_j=I_j/sum I_{jj}*100
Residing Region	1.85	1.73	-.776	0.954	100
TOTAL				0.954	100

Relative discriminating index

Table -7:Classification Results
(Between low level group and high level group)

Actual group	No. of cases	Predicted group membership	
		Group I	Group II
Group I	379	211 55.7 %	168 44.3%
Group II	246	117 47.6%	129 52.4%

The second question is answered by reclassifying the already grouped individuals into low or high level using the D.F (Z) defined in the equation. This classification is called predictor group membership. In short the efficiency of the D.F is called predictor group membership. In short the efficiency of the D.F. is how correctly it predicts the respondents into distinct groups. Per cent of grouped case correctly classified: 54.4 per centThe above table gives the results of the re classification. The function using the variables selected in the analysis classified 54.4 per cent of the cases correctly in the respective groups. It is found that the Discriminant function analysis was applied to the respondents on low user and high user. The following factors significantly discriminate the two users. They are the Current Gross Salary per month (1 per cent level).

Discussion and Implications

In the study area out of six hundred and twenty five respondents were divided into two groups .ie. Low level of impact on purchase decision and the high level of impact on purchase decision. The difference on opinion of the respondents in one group from the other is studied with the help of discriminant function analysis and the results indicates that the variable Residing Region entered in step two. The variables such as Residing Region are significant at one per cent significance level. The canonical correlation in the discriminant group can be

accounted for by this model, Wilkslamda and chi square value suggest that D.F is significant at one per cent level.

The function using the variables selected in the analysis classified 54.4 per cent of the cases correctly in the respective groups. It is found that the Discriminant function analysis was applied to the respondents on low level of impact and high level of impact. With all the demographic factors taken for the study current gross salary is discriminated both in high and low level and it is inferred that the salary plays a significant role even though the people are highly aware of the environmental issues .Thus the implications on green product portfolio manufacture should look for cost cutting technology as well the wide range of product offerings.

The relative comparison of the green products in the market are still for a particular niche segment ,thus this study can act as base to operate for new product offerings in green product ranges and also it can be explored to other samples for testing its reliability in odd samples. The environmental issues are the basic cause and whereas the effect is the green purchase behaviour of the customers this cause and effect model can be base for many attitude and behaviour studies.

Corresponding Authors:
P.Nalini
Assistant professor in Management
Coimbatore Institute of Management and technology
Coimbatore, TamilNadu,India
nalinimbammm@yahoo.com
Tel:+91--09843140154

Dr. D.Muruganandam
Associate professor, Department of management studies, Kongu Engineering College, Perundurai, Erode, Tamilnadu, India
muruganandan_mba@yahoo.co.in,
Tel: + 91-09842552729

Reference

1. Ayse Ozfer Ozcelik, A. U. (2008). Turkish academic staffs perception of organic foods. British Food Journal, Vol. 110, 948-960.
2. Bodo B. Schlegelmilch, G. M. (1996). The link between green purchasing decisions and measures of environmental consciousness. European Journal of Marketing, 35-55.
3. Chung, H. Y.-E. (2011). Consumer purchase intention for organic personal care products. Journal of Consumer Marketing, 40–47.
4. Clare D ' Souza, M. T. (2007). Examination of environmental beliefs and its impact on the infl uence of price, quality and demographiccharacteristics with respect to green purchase intention. Journal of Targeting, Measurement and Analysis for Marketing , 69 – 78.
5. Depoe, S. (2007). Environmental communication as nexus. Environmental, 1-4.
6. Devashish Pujari, G. W. (2003). Green and competitive Influences on environmental new product development performance. Journal of Business Research, 657– 671.
7. Davis ,D.P.,greening business :managing for sustainable development (oxford: Basil Blackwell.1991)
8. Rokeach, Milton, (1973). "Understanding Human Values", New York: Free Press,
9. Gura U, C. and Ranchhod, A. (2005), "International green marketing: a comparative study of British and Romanian firms", International Marketing Review, Vol. 22 No. 5, 547-61.
10. Hopkins, Michael S. (2009, summer). What the 'Green' Consumer Wants. Sustainability & Marketing, vol.50 No.4, 12-13
11. Henion, K.E. and Kinnear, T.C. (1976), Ecological Marketing, American Marketing Association, Chicago
12. Ken Peattie (2001) The Marketing Review, 2001, 2, 129-146.
13. Peattie, K. (1995), Environmental Marketing Management: Meeting the Green Challenge , Pitman, London
14. Ottman, J.A. (2007), "Next-generation green marketing: beyond billboards", J. Ottman Consulting, Inc.
15. Ottman, J.A., Stafford, E.R. and Hartman, C.L. (2006), "Avoiding green marketing myopia: ways to improve consumer appeal for environmentally preferable products", Environment, Vol. 48 No. 5, pp. 22-36.
16. Stone, C. M. (2009). Revisiting consumer environmental responsibility: a five nation cross-cultural analysis and comparison of consumer ecological opinions and behaviors. International journal of management and marketing research, 35-51.
17. Strengers, Y. (2011). Designing Eco-Feed back Systems for Everyday Life. CHI . 7–12.
18. Suplico, L. T. (2009). Impact of green marketing on the students' purchase decision. Journal of International Business Research, Vol. 8, No. 2,71-81.
19. Zainuddin, Y. B. (2011, march). The Impact of Media Exposure on Intention to Purchase Green Electronic Products amongst Lecturers. International Journal of Business and Management, Vol 6.
20. Zhang, S. M. (2009). Market for Green Signaling. The Business Review, Cambridge, Vol. 13 No. 2, 87-92.

02/03/2013

Sex Differences and the Ordering of Lengths of the Proximal phalanges in *Macaca Mulatta*

Xiaojin Zhao[1a], Fengchan Wang[1a], Xuan Zhao[1b], Jie Song[1a,2], Xiaojing Mao[1a,2]

1.a.College of Fisheries, b.College of Tourism, Henan Normal University, Xinxiang, 453007, Henan, P R. China.2.Faculty of Earth and Resources, China University of Geosciences, Beijing 100083, P R. China
zxj_6119@163. Com

Abstract: Background: In accord with past findings, the ordering of lengths of both metapodials for some nonhuman primates was highly similar on the two sides of the body, but the pattern of lengths for these primates was different for the two sexes. **Objective:** To examine sex differences and the ordering of lengths of proximal phalanges in *Macaca mulatta* from the Taihang Mountain in central China. **Materials and Methods:** The lengths of proximal phalanges of hands and feet were obtained from the 35 skeletons of adult *Macaca mulatta*, comprising 12 males and 23 females. SPSS (Version 19.0) was used for statistical analysis. **Results:** For *Macaca mulatta*, the lengths of proximal phalanges showed highly significant sex differences (P<0.001); however, no side differences for both hands and both feet were found in the lengths of proximal phalanges. The ordering of lengths of five proximal phalanges in hands, from the longest to shortest, was 3,4>2>5>1, and the ordering of lengths of the proximal phalanges in feet was 3>4>2>5>1. **Conclusions:** There were obvious sex differences among the lengths of all proximal phalanges (P<0.001), whereas the sex differences of the lengths of proximal phalanges of hands were greater than those of feet in *Macaca mulatta*.

[Xiaojin Zhao, Fengchan Wang, Xuan Zhao, Jie Song, Xiaojing Mao**. Sex Differences and the Ordering of Lengths of the Proximal phalanges in *Macaca Mulatta.*** *Life Sci J* 2013;10(1):2154-2159] (ISSN:1097-8135). http://www.lifesciencesite.com. 304

Keyword: *Macaca mulatta*；proximal phalanges；length；sexual dimorphism；ordering

1. Introduction

Some reports have displayed that there were sex differences in the lengths of both the metapodials in baboons, rhesus monkeys, gorillas and chimpanzees [1,2,3,4], and the sex differences of proximal phalanges were greater than those of the metapodials [1, 2]. These differences may be related to certain disease and the secretion of sex hormones in early embryonic development [3,4]. For example, sex differences in the relative lengths of metacarpals in gorillas and chimpanzees were affected by developmental mechanisms which involve androgens operating prenatally or postnatally[5,6].

The wild *Macaca mulatta* in Taihang Mountains is the largest and the northernmost population which exists in the area of Macaques distributing in conjunction of Shanxi and Henan province, in central China [7]. It is meaningful to study the morphological features, such as sex difference, asymmetry, the ordering of lengths for hand bones and population affinity distributing in different geography.

To date, sex differences in length ratios have been reported for few other species in non-human primates. At this time, we have some knowledge about sex differences in the absolute length for metapodials in rhesus monkeys, gorillas and chimpanzees, but not for their fingers and toes [7,8,9]. And we have knowledge about sex differences in the length for human fingers and toes, including the proximal, middle and distal phalanges, but not for rhesus monkey phalanges. The purpose of this paper was to extend the study of length to the proximal phalanges of *Macaca mulatta*.

Because the study was motivated by literature on the sex differences of lengths in other non-human primate's fingers, the initial desire was to investigate the possibility of sex differences in lengths for fingers in *Macaca mulatta*. In the end, we were forced to concentrate on the proximal phalanges because our prediction was that sex differences would exist in the lengths for proximal phalanges and the ordering of lengths would be different from human hands and feet [9].

2. Materials and methods

The wild *Macaca mulatta* are situated from 34°54′N to 35°16′N where belong to warm temperate climate. The population has their specificity in ecology, physiology, morphology, heritability and diet compared with the others [10,11]. Measurements were made on 32 specimens (9 males, 23 females) of hand bones and 34 specimens (12 males, 22 females) of foot bones. The skeleton specimens for this study are part of the population in Henan provinces, and are stored in College of Fisheries, Henan Normal University.

Some specimens were obtained from the wild *Macaca mulatta* which distributed from Taihang Mountains; and others were collected from the monkey farm in Henan Normal University; some of them died natrually and others died of injury. Pathological or damaged specimens were excluded. Paired bones were also excluded if postmortem damage influenced the

periosteal surface of the diaphysis or precluded accurate measurement of bone length. In addition, all of the skeletons were complete for the five proximal phalanges, and all were adults according to the bone healing seam in skull.

Measurements taken on each proximal phalanges are illustrated in Figure 1.

The length of proximal phalanges was measured as the distance between the midpoint of the basis and distal tip point of all the proximal phalanges with a sliding caliper according to standard measuring techniques [12,13,14] and approximated to the nearest 0.01 mm.

This measurement was used rather than the more conventional maximum length because it allowed for the sampling of partially disarticulated hands. As showed in figure 1, the first proximal phalanx of fingers was marked as 1Pf, and the second proximal phalanx of fingers marked as 2Pf, others were marked analogically. In the same way, the first proximal phalanx of toes was marked as 1Pt, and the second proximal phalanx marked as 2Pt, and others were marked analogically. In order to reduce measurement error, all of samples were measured by trained or professional staff.

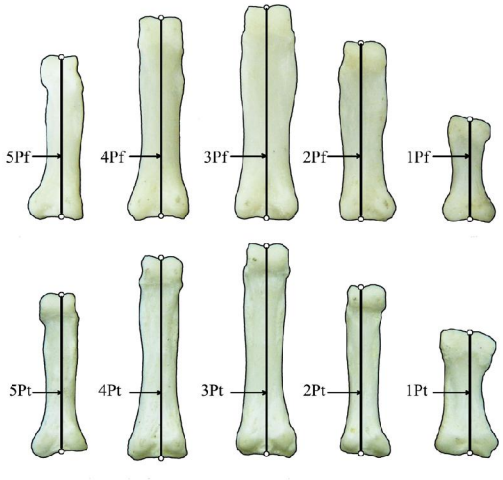


Figure 1. The measurements of proximal phalanges of *Macaca mulatta* (female, right).
Pf= proximal phalanges of fingers; Pt=: proximal phalanges of toes.

All data were then subjected to statistical analysis using the SPSS for Windows (version. 19.0). The t-test was employed in comparing the sex differences and the bilateral differences, and one-way ANOVA was used to study the differences of length among five proximal phalanges. Because homogeneity of the regression slopes for each group was not rejected in ANOVA, we then checked intergroup difference via Tukey's HSD test of post-hoc multiple comparisons using adjusted means. For both hands and both feet of both sexes, the ordering of proximal phalanges by length was obtained from longest to shortest.

3. Results

Descriptive statistics of the length of all five proximal phalanges were summed up in Table 1.

The sex comparison and the ordering of lengths of all five proximal phalanges, whatever the fingers and toes, were showed in Table 2 and Figure 2.

Table 1. Average lengths and *t*-test for the proximal phalanges of fingers and toesin *Macaca mulatta* (mm)

Bones	Male		Female		Male+Female		t-value	Discriminat rate (%)
	Mean±SD	N	Mean±SD	N	Mean±SD	N		
1Pf	13.55±0.75	18	12.51±0.81	46	12.81±0.92	64	4.692*	71.9
2Pf	22.93±0.49	18	20.81±0.83	46	21.40±1.22	64	10.18*	95.3
3Pf	27.60±0.94	18	24.89±0.92	46	25.65±1.53	64	10.49*	95.3
4Pf	26.91±0.77	18	24.39±0.95	46	25.10±1.46	64	10.04*	92.2
5Pf	21.66±0.70	18	19.70±0.68	46	20.26±1.12	64	10.26*	96.9
1Pt	16.44±0.88	24	15.33±0.83	44	15.72±1.00	68	5.11*	73.5
2Pt	23.23±1.16	24	21.74±1.13	44	22.26±1.34	68	5.13*	67.6
3Pt	28.57±0.93	24	26.36±1.00	44	27.14±1.44	68	8.91*	88.2
4Pt	27.45±0.93	24	25.33±0.96	44	26.08±1.40	68	8.78*	82.4
5Pt	22.52±0.83	24	20.87±0.81	44	21.45±1.14	68	7.93*	88.2

Abbreviations: Pf= proximal phalanges of fingers; Pt= proximal phalanges of toes; Data analyses were carried out using both sides; t-value: from independent-samples *t*-test between sexes; * *t*-values significant at $P<0.001$; Discriminat rate: The correctly classified percentages between the sexes for the single variable.

Table 2. Tukey's HSD test of the length of five proximal phalanges for the fingers and toes in *Macaca mulatta*

Order	Males	Females	Total	Order	Males	Females	Total
1Pf	13.55aA	12.51aA	12.81aA	1Pt	16.43aA	15.33aA	15.72aA
5Pf	21.66bB	19.70bB	20.26bB	5Pt	22.52bB	20.87bB	21.45bB
2Pf	22.93cC	20.81cC	21.40cC	2Pt	23.23bB	21.73cC	22.26cC
4Pf	26.92dD	24.39dD	25.10dD	4Pt	27.45cC	25.33dD	26.08dD
3Pf	27.60dD	24.89eE	25.65dD	3Pt	28.57dD	26.36eE	27.14eE

Note: The different letters followed mean values represent the significant differences among those proximal phalanges (P<0.05 or P<0.01), the same letters followed mean values represent no differences among those proximal phalanges (P>0.05). Capital letter indicate statistical significance at P<0.01, and small letter indicate statistical significance at 0.05≤P≤0.01.

As expected, the lengths of all proximal phalanges were uniformly greater for the males than for the females. For both sexes, the average lengths of proximal phalanges of both fingers and toes were remarkably similar.

The results obtained here imply that there were obvious sex differences among the lengths of proximal phalanges for both hands and both feet (P<0.001), whereas the sex differences of the lengths of proximal phalanges for both hands were greater than those for both feet in *Macaca mulatta*.

The ordering of the average lengths, from the longest to shortest, was 3>4>2>5>1 for both hands. Namely, there were no significant differences for the two sides of body (P>0.05). By One-way ANOVA, the total ordering of the average length of proximal phalanges was 3>4>2>5>1, and that every ordering of the five proximal phalanges showed was the same for the two sexes, the two hands and the two feet for *Macaca mulatta*.

However, there were statistically significant differences between sexes at the different level. Summary statistics of the five fingers were shown in the left half of Table 2.

At the P<0.01 or P<0.05 level, the ordering of five Pfs was 3, 4, 2, 5, 1 for both sexes and the total group (males+females), but the differences between 3Pf and 4 Pf were not statistically significant for male and total groups. By contrast, the pattern of lengths for proximal phalanges was different for the two sexes. For the females, the ordering from longest to shortest was 3>4>2>5>1 for both hands, but for the males, the ordering was 3,4>2>5>1 for both hands. Furthermore, the ordering of lengths in the five Pfs of *Macaca mulatta*, as showed in Table 2, may be different between sexes at the certain degree, but the ordering of length of the Pfs in males may be closer to the total ordering.

Similar results were also obtained for the ordering of lengths in the five Pts for both feet by Tukey's HSD test of post-hoc multiple comparisons. For the males, the ordering from longest to shortest was 3>4>2,5>1, but for females and total group the ordering were 3>4>2>5>1 for both feet at the P<0.05 or P<0.01 level (see Table 2).

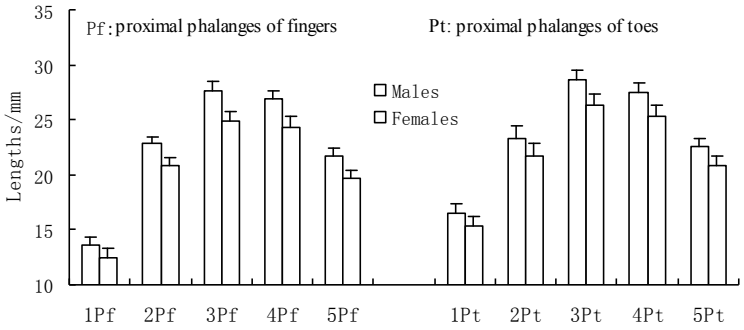


Figure 2. The sex comparison and the orderings of the length of thefive proximal phalanges of the finger and toe in *Macaca mulatta*

4. Discussion

Interspecies similarities and differences in the metapodials and phalanges in nonhuman primates long have been topics of considerable interest to physical anthropologists, biologists and behaviorists [15]. Comparisons across species have been especially informative about interspecies differences in such aspects of behavior as locomotion, use of tools, grip in hand and manual work [16]. For example, some physical anthropology studies had reported sex differences in the relative size of the proximal phalanges [13,14], and they had reported that the finger

length ratio of human and non-human primates may reveal some important information during early development [18]. The orderings of length of gorillas, chimpanzee, and human proximal phalanges reported by McFadden et al. also were 3>4>2>5>1 [2]. As expected, for *Macaca mulatta*, the lengths of male proximal phalanges were longer than those of the female. For both sexes, both hands and both feet, the ordering of proximal phalanges by length was 3>4>2>5>1 from longest to shortest.

In this paper, sex differences of lengths of the proximal phalanges were significant ($P \leq 0.001$). Compared with the previous studies on sex differences of metacarpals and metatarsals[9], the lengths of the metapodials for both hands and both feet may be likely to exhibit larger sex difference.

In the direct discriminant analysis included all lengths of metacarpals, the average accuracies (74.2%-96.9%) obtained in this study for the proximal phalanx were higher than those (84.6%-91.7%) reported in previous studies for the metacarpals and metatarsals of *Macaca mulatta*[8,9], particularly as regards to the lengths of proximal phalanx for both hands.

At present, it is interesting to contemplate the origins of sex differences for the metapodials and phalanges in the nonhuman primates. Perhaps the most convincing evidence, which comes from two studies on people with congenital adrenal hyperplasia, was that prenatal sex hormones exposure may affect the changes of relative finger length in human [19,20]. It is a malady in which the adrenal gland produces abnormally high levels of androgens during prenatal development. McFadden et al. [5] reported that both gorillas and chimpanzees were affected by developmental mechanisms, possibly androgenic mechanisms, similar to those in humans. There is some important evidence for sex differences in locomotion, foraging, and other behaviors in primates [20]. In addition, there are some facts to consider when contemplating the various logically possible interpretations about the origins of sex differences. Cillo et al. [21] and Goodman et al. [22] reported that the early developments of finger length are under the control of the highly conserved homeobox genes, and in all mammals, androgens are responsible for producing males from the default state of females.

Sex differences in length of the phalanges may be related to the following factors:

(1) Previously, the results showed that sex differences in length ratios of metapodials in primates were almost related to sex dimorphism in body size [5,8]. This problem can be verified by a simple experiment. Sometimes, as the body size of individuals can not be directly measured, according to previous literature, the cranial length may replace the body size [23,24]. At first, the two larger male specimens and four smaller female specimens were removed on the basis of cranial size. As result, the male and female specimens which were retained were roughly equal in body size, and then the changes of the differences between the sexes were observed in this study. When the larger female specimens were compared with the small male specimens, it also showed that the sex differences of lengths of the phalanges were not significantly reduced except 1Pf. So, the results further confirmed that the sex differences in length of the phalanges can not simply attribute to the body size between the sexes. In addition, body size, as measured by height, was not highly correlated with the metapodial length ratios for different species in humans and non-human primates [25].

(2) Another question is why these sex differences were so different on metacarpal and finger lengths. One possible explanation for these outcomes is that the auditory and finger-length measures are affected by mechanisms operating at different times in ear development and that the growth of the metapodials and phalanges are not synchronization [26]. Perhaps the metapodials achieve their essential development prior to the existence of large sex differences in androgen concentrations, but the phalanges develop while a substantial sex difference does exist [14]. This may be one of the reasons which sexual difference of phalanges are larger than that of metapodials. Another possible type of explanation appeals to the prospect that the dimensions of the extremities (and thus the length ratios) may be affected by the uses to which those extremities are put [25,26].

(3) According to previous studies, there was reason to believe that prenatal androgen exposure in the embryo influenced the growth of ring finger, while the estrogen influences the growth of index finger [5, 26] . That is to say, a reasonable working hypothesis to adopt is that the sex differences reported here for measurements of proximal phalanges are attributable, at least in part, to sex differences in exposure to androgens during early development. Confirming these outcomes was very important because from after about 6 months of age to puberty, hormone levels are not different in boys and girls [2,5].

(4) From the perspective of the etiology, sex hormone levels during the embryo may be related to the adult's disease. A human disease named adrenal hyperplasia produces high levels of androgen during prenatal development [27]. The disease will significantly affect the changes of length of fingers and some characters after birth (such as autism and hyperactivity, etc.). Adults suffering from the diseases, such as myocardial infarction and breast cancer, were all related to the level of sex hormones in the early embryo environment [28,29,30].

For *Macaca mulatta*, the ordering of lengths of all

hands and feet proximal phalanges from biggest to smallest was 3>4>2>5>1, which was consistent with the ordering of proximal phalanges in gorillas, chimpanzees and humans, it showed that non-human primates and humans would be homologous in the evolution. However, partial results were inconsistent with past findings [5].

Statistical analysis showed slight differences between the sexes in the ordering of lengths of all hands and feet. By post hoc comparisons, the ordering of lengths in the males did not show significant difference between the 3Pf and the 4Pf (P>0.05), but those in the females showed significant difference between the 3Pf and the 4Pf (P<0.05).

Note that the degree of the sex determination or the ordering of lengths by the phalanges across populations varies, but inter-study comparison is difficult because of methodological differences [13]. Alıcıoğlu et al. investigated the use of length of phalanges for sex determination in 65 individuals from Turkish people [13]. They measured the interarticular distance of the phalanges through the use of radiographic imaging, whereas we directly measured here the length from the proximal phalanges by means of osteometric in rhesus monkeys.

Note again, that the results for the ordering of phalanges length for both males and females in this study would be different in terms of statistical approaches. For example, in *Macaca mulatta* males, the ordering of lengths of hand proximal phalanges from biggest to smallest was 3,4>2>5>1 by employing Tukey test, whereas the ordering of length was 3>4>2>5>1 by employing SNK test of SPSS at the P<0.05 level. It is clear that the ordering of lengths by employing SNK test in males are similar to those by Tukey's HSD test in females, as are the ordering of lengths of foot proximal phalanges. Another reason may be related to a small number of the male specimens in this study, but this was less likely. The ordering of lengths of phalanges was remarkably similar on both sides.

For the proximal phalanges of feet, the result showed no significant difference between 2Pt and 5Pt in males (P>0.05), while it was significant difference between 2Pt and 5Pt in females (P<0.05). These differences were also found in metatarsal length and weight in *Macaca mulatta* [31]. The relative lengths of the proximal phalanges may be likely affected by the absolute length of the proximal phalanges between sexes, and may be different for two sexes and for two levels.

7. Conclusion

There were obvious sex differences among the lengths of proximal phalanges (P<0.001), whereas the sex differences of the lengths of proximal phalanges of hands were greater than those of feet in *Macaca mulatta*. For both sexes, both hands, and both feet, the ordering of proximal phalanges by length was 3>4>2>5>1 from longest to shortest.

Acknowledgements

We are very grateful to Prof. Zhang Hong-xu, College of Life Science, Henan Normal University, China, for kindly providing skeletal collection.

Corresponding author:
Dr. Xiao-jin Zhao
College of Fisheries
Henan Normal University,
Xinxiang, Henan P R.China
E-mail:zxj_6119@163.com

Reference

1. McFadden D, Bracht MS. (2002): Sex differences in length ratios from the extremities of humans, gorillas, and chimpanzees. Hormones and Behavior, 41(4): 479-480.
2. McFadden D, Bracht MS. (2005): Sex difference in the relative lengths of metacarpals and metatarsals in gorillas and chimpanzees. Hormones and Behavior, 47(1): 99-111.
3. McFadden D, Bracht MS. (2003): The relative lengths and weights of metacarpals and metatarsals in baboons (*Papio hamadryas*). Hormones and Behavior, 43(2): 347-355.
4. Zhao XJ, Wang XL, Dang XY, Liu Y, Zhao JJ. (2009): Sex differences in the length ratios of metapodials in *Macaca mulatta* from the Taihang mountains. Acta Anatomica Sinica, 40(6): 993-996.
5. McFadden D, Bracht MS. (2009): Sex and race differences in the relative lengths of metacarpals and metatarsals in human skeletons. Early Human Development, 85(2): 117-124.
6. Smail PJ, Reyes FI, Winter JSD, Faiman C. (1981): The fetal hormonal environment and its effect on the morphogenesis of the genital system. In: Kogan SJ, Hafez ESE (eds) Pediatric andrology, Martinus Nijhoff, The Hague, 9-19.
7. Xiao-jin Zhao, Feng-chan Wang, Li-guo Li. (2012): Comparison of whorl types on the palms of *Macaca mulatta* from the Taihang Mountains (central China). Life Science Journal, 9(3):2185-2189.
8. Zhao XJ, Zhang Y, An N, Wang G. (2008): Morphology of metacarpals and metatarsals of *Macaca mulatta* in the Taihang Muntains. Chinese Journal of Anatomy, 31(3): 412-415.
9. Zhao XJ, Zhao JJ, Wang G, Ma J, Liu Y. (2009): Sex assessment in the lengths of metacarpals and metatarsals of *Macaca mulatta* living in the Taihang Mountains. Acta Anthropologica Sinica, 28(1): 88-89.
10. Green DJ, Gordon AD. (2008): Metacarpal proportions in australopithecus africanus. Journal of Human Evolution, 54 (5) : 705-719.

11. Zhao XJ, Zhang HL, Lü XT, Hou JH, Zhang HX, Yang GZ. (1989): Survey and research of morphological characters of monkeys (*Macaca mulatta*) in the Taihang Mountains. Journal of Henan Normal University, 62 (2): 120-125.
12. Susman RL. (1979): Comparative and functional morphology of hominoid fingers. American journal of physical anthropology, 50:215-296.
13.Alıcıoĝlu B, Yilmaz A, Karakaş HM, Cigalı BS, Çıkmaz S, UluçAM. (2009): Sex determination by the interarticular distance of metacarpals and phalanges: a digital radiologic study in contemporary Turkish people. Anatomy, 3:14-20.
14. Smith SL. (1990): Attribution of Hand Bones to Sex and Population Groups. Journal of Forensic Science, 41(3):469-477.
15.Shreuer JL, Elkington NM. (1993); Sex determination from metacarpals and first proxinal phalanx. Journal of. Forensic Science, 38(4): 769-778.
16. Jin QB, Du WS. (1989): Karyotype analysis of Taihang mountain *Macaca mulatta* (rhesus monkey). Journal of Henan Normal University, 62 (2): 102-105.
17. Sarringhaus LA, Stock JT, Marchant LF, McGrew WC. (2005): Bilateral asymmetry in the limb bones of the chimpanzee (*Pan troglodytes*). American journal of physical anthropology, 128: 840-845.
18. McFadden D, Bracht MS. (2008): Sex and race differences in the relative lengths of metacarpals and metatarsals in human skeletons. Early Human Development, 85(2): 117-124.
19. Williams JT, Leinster S, Christensen SE, Cooke BM, Huberman AD, Breedlove NJ, Breedlove TJ, Jordan CL, Breedlove SM. (2000): Finger-length ratios and sexual orientation. Nature, 404: 455-456.
20.Tague RG. (2002): Variability of metapodials in primates with rudimentary digits: Ateles geoffroyi, Colobus guereza, and Perodicticus potto. American journal of physical anthropology, 117: 195-206.
21. Cillo C, Faiella A, Cantile M, Boncinelli E. (1999): Homeobox gene and cancer. Experiment Cell Research, 248(1): 1-9.
22. Goodman FR, Scambler PJ. (2001): Human hox gene mutations. Clinical Genetics, 59(1): 1-11.
23. Lavelle CLB. (1974): Relationship between tooth and skull size in a population rhesus monkey (*Macaca mulatta*). American journal of physical anthropology, 43: 336-340.
24. Zhao XJ, Duan WC, Liang ZD, Chen XG, Wang FY. (2009): Correlation between metapodials length and cranial length of *Macaca mulatta*. Journal of Henan Normal University, 37(3): 186-188.
25. Huo SJ, Fan SQ, Zhao CY. (2003): Measurement of fingers width and length of every segment in human. Progress of Anatomical Sciences, 9(4): 326-329.
26. Becker JB, Breedlove SM, Crews D, McCarthy MM. (2002): Behavioral endocrinology. MIT Press, Cambridge, 1-50.
27. Sanderson M, Williams MA, Malone KE, Stanford JL, Emanuel I, White E, Daling JR. (1996): Perinatal factors and risk of breast cancer. Epidemiology, 7(1): 34-37.
28. Peter M, Manning JT, Reimers S. (2007): The effects of sex, sexual orientation, and digit ratio (2D:4D) on mental rotation performance. Archives Sex Behavior, 36: 251-260.
29. Phillips GB, Pinkernell BH. (1994): The association of hypotesteronemia with conronary artery diseases in men. Ateriocler Thromb, 14(5): 701-706.
30. Trichopoulos D. (1990): Hypothesis: dose breast cancer originate in utero? Lancet, 335(8695): 939-940.
31. Dang XY, Liang ZD, Zhao XJ, Duan WC, Ding JZ. (2009): Sex differences in the weight ratios of metapodials in *Macaca mulatta* from the Taihang Mountains. Journal of Henan Normal University, 37(5): 108-111.

2/15/2013

Nurses' Perceptions of Safety Climate and Barriers to Report Medication Errors

Ebtsam Aly Abou Hashish and Gehan Galal El-Bialy

Nursing Administration Department, Faculty of Nursing, Alexandria University. Alexandria, Egypt
ebtsam_ss@hotmail.com

Abstract: Patient safety issues, including safety climate and medication safety, are central concerns for the nursing profession and nurses' job responsibility. Creating an environment conducive to reporting errors requires and related to a systems approach to patient safety and safety climate. Therefore, this study aimed to assess nurses' perceptions of safety climate and barriers to report medication errors. The study conducted at all in-patient medical and surgical care units at Alexandria Main University Hospital. A random sample of (50%) staff nurses (N = 204) who working in the previous units were included. Safety Climate Scale (SCS) was used to measure nurses' perceptions of safety climate. Barriers to Reporting Medication Administration Errors Questionnaire (BRMAE-Q) was used to measure nurses' perceptions of barriers to report medication errors. Nurses perceived high safety climate in their units and perceived that the most barriers that hinder them to report medication errors are "Disagreement over what is medication error and its definition, and power distance". While, reporting effort is the least barrier to report medication errors. Also, there was a positive significant correlation between nurses' perception of overall safety climate and perceived barriers to report medication errors. Nurses might perceive that safe work climate could be related to their unreporting of medication errors. Continuous in-service educational programs on quality and safety including safe work environment and safe climate as well as a blame-free culture for reporting errors are recommended.

[Ebtsam Aly Abou Hashish and Gehan Galal El-Bialy. **Nurses' Perceptions of Safety Climate and Barriers to Report Medication Errors**. *Life Sci J* 2013;10(1):2160-2168] (ISSN:1097-8135). http://www.lifesciencesite.com. 305

Keywords: Safety Climate, Medication Errors, Reporting Medication Errors.

1. Introduction

Generally speaking, creating a culture of safety is thought to be an important prerequisite in ensuring that patients receive the safest possible care. Healthcare organizations are therefore encouraged to improve safety culture and climate as part of an overall drive to improve patient safety. [1] For nurses, safety climate is defined as the shared perception of nurses regarding the protection of patients from medication errors and injuries resulting from health care intervention and environment. [2, 3]

Blegen *et al,*. (2005) clarified components of safety climate such as; ***unit manager's role*** emphasizing management commitment, support for safety programs and safety-related feedback from managers, ***safety emphasis*** revealing patient safety as top priority throughout the organization, ***socialization and training*** emphasizing communication among staff members , safety–related training and training on technology, ***blame system*** and non punitive reporting of errors, ***pharmacists role*** toward safety, ***use data for improvement*** emphasizing learning from errors through information and reported data, and, ***worker safety*** issues including provision of personal protective equipments, safety procedures and precaution programs and safe work place design.[3]

The American Nurses Association (ANA, 2001) reported that nurses' perceptions of the safety climate and nurses' perspectives in health care are critical information that must be available when analysis, discussion and changes are considered in relation to patient safety efforts and medical errors management.[4] As a matter of fact, nurses comprise the largest component of the health care workforce, and they are involved in provision, management, research and education related to patient safety and error reduction in health care. [5,6] Therefore, nurses are responsible for the safety of their patients. One area of patient safety to consider is medication use, specifically medication management processes and medication administration safety. [7]

Medication management processes involves five main stages namely: prescribing, ordering, dispensing, distributing and administration.[8]. Medication administration, include giving medication, management and reporting of medication errors, identified as a priority area for quality improvement. [9] In fact, medication administration is an integral part of nurses' duties, consuming up to 40% of their time.[10] This role is considered a high risk procedure, as it requires high level of concentration and skills.[8,10]

Medication Administration Errors (MAE's) are often used as indicators of patient safety in hospitals because of their common occurrence and potential risk

to patients. A study done by Bar-Oz *et al.* (2008) had reported that approximately one third of adverse drug events is associated with medication errors that are viewed as preventable. [11] A Medication error is defined as "any preventable event that may cause or lead to inappropriate medication use or patient harm while the medication is in the control of the healthcare professional, patient, or consumer". Such event may be related to professional practice, healthcare products, procedures, and systems.[12, 13]

Obviously reporting is fundamental to the broad goal of medication error reduction. Underreporting of medication errors is a significant problem among nurses. Many barriers were identified that could weaken nurses' willingness to report errors and diminishes the opportunity to learn from these errors.[6] For these reasons, barriers to report medication errors must be explored from nurses' perceptions in their units.[2,3]

Wakefield *et al.,* (2004,1996) categorized barriers to report medication errors as ***fear*** of punishment of causing and reporting medication errors, ***disagreement over what is medication*** error and its clear definition, ***administrative responses*** to errors, ***reporting effort*** including time and procedure to report error, ***face-saving*** and maintaining a positive image of coworkers to avoid interpersonal conflict and ***power distance*** between managers and subordinates. [14, 15]

Creating an environment conducive to reporting errors requires a system approach to promote patient safety. An optimal error reporting system with voluntary and blame–free features can provide abundant information for learning about medication errors and managing medical errors comprehensively.[2] Thus, a heath care organization featuring safety commitment and decrease punishment should encourage nurses to report errors and mitigate barriers to report medication administration errors. [2, 4, 15]

In Egypt, up to the knowledge of current researchers, there is no statistical data regarding the incidence of medication errors. However, few studies have focused only on the assessment of the occurrence and reasons of nursing medication errors , assessment of safety culture and developing manual for safety measures. [16-21] These studies recommended further studies to determine and investigate the causes of medication errors and barrier of reporting these errors in order to help in their prevention in the future and improve patient safety and safety climate in work place.

.Different studies were conducted in U.S.A and European countries investigated nurses' perception of safety climate and medication errors[2,3,8,10-15,22-24] These studies found a relationship between incidence of medication errors and safety climate in hospital setting and recommended further studies to identify the causes that hinder nurses to report the medication errors which could affect patients' safety.

Yet, little is known about how safety climate can contribute to the elimination of barriers to medication administration errors reporting from nurses' perspectives.[2,3,15] Analysis of medication errors and barriers to reporting these errors can lead to system improvement and reduce risk only if the errors are detected, reported, and used to design better patient-care practices and systems.

Likewise, the way in which safety climate is linked to nurses' perceptions of medication administration errors reporting requires more research. It is hoped that the findings of such study will emphasis the role of nurses in promoting safety climate for patients in relation to preventing medication errors through the reporting approach.

Aim of the study: this study aims to assess nurses' perception of safety climate and barriers to report medication errors at Alexandria Main University Hospital.

Research questions:

- How do nurses perceive safety climate in their units?
- What are the barriers nurses perceive to report medication errors in their working units?
- What is the relationship between nurses' perception of safety climate in their working units and barriers to report medication errors?

2.- Materials and Method:

Materials:

a- Research design: A descriptive correlational design was used to conduct this study.

b-Setting:

The study was conducted at all in-patient medical and surgical care units at Alexandria Main University Hospital (N=37).

c-Subjects:

A random sample of 50% staff nurses (N = 204) working at in-patient care units and willing to participate in the study was included.

d- Tools:

Two tools were used for this study namely;

1- Safety Climate Scale **(SCS)** was developed by Blegen *et al.,*[3] to measure nurses' perceptions of safety climate regarding medication and patient safety. This scale consists of 33 items covering seven safety dimensions namely; unit manager role (5 items), safety emphasis (5 items), socialization and training (6 items), blame system (5 items), pharmacists role (3 items), use data for improvement and reporting (4 items), and worker safety (5 items). Responses were rated on 5-point likert scale ranging from "5" strongly agree to "1" strongly disagree.

***2- Barriers to Reporting Medication Administration Errors Questionnaire* (BRMAE),** it was developed by Wakefield *et al.,*[14] to measure nurses' perceptions of barriers to reporting medication errors. This questionnaire consists of 25 items categorized in six subscales namely; Fear (5 items), disagreement over medication error (4 items), administrative responses (4items), reporting effort (2items), face-saving (4 items), and power distance (5 items). Responses were rated on 5-point likert scale ranging from "5" strongly agree to "1" strongly disagree. In addition, one open ended question was used to identify other reporting barriers from nurses' point of view.

A Data sheet was developed for nurses to elicit information related to; (unit, age, education, years of experience, marital status and working shift). In addition to two questions about previous experience and reporting of medication errors.

Method:

1-A written approval was obtained from the administrative authority in the identified setting to collect the necessary data.

2- Tools were translated into Arabic and tested for content validity by 5 experts in the field of study. Accordingly, some items were modified.

3- Tools were tested for reliability using the Cronbach's alpha coefficient to measure the internal consistency of items. The two tools were reliable **(SCS= 0.79)** and **(BRMAE= 0.832)**.

4- A pilot study for the questionnaires was conducted on 20 nurses (10%) who were excluded from the study subjects. In the light of the findings of the pilot study, no changes occurred in the tools.

5- Data were collected from nurses through the study's tools after obtaining their approval to participate in the study and maintaining the confidentiality of data. Actually data were collected over three months.

Statistical analysis:

1- Data were coded by the researchers and statistically analyzed using SPSS version 16.

2- Descriptive statistics:

- Frequency and percentages were used for describing and summarizing qualitative and categorical data.
- Mean (X) and Standard Deviation (SD): were used as measures of central tendency and dispersion respectively for quantitative data.

For safety climate If the mean is

= <2.5 this indicate poor safety climate

= 2.5 – 3.75 this indicate moderate safety climate

= > 3.75 this indicate high safety climate

For Barriers to Report Medication Errors If the mean is

= <2.5 this indicate low barriers to Report Medication Errors

= 2.5 – 3.75 this indicate moderate barriers to Report Medication Errors

= > 3.75 this indicate high barriers to Report Medication Errors

3- Inferential statistics:

- *ANOVA test (f), Student t test (t)* and *Pearson Correlation Coefficient (r) were* used. All tests of significance were done at the 5% level.

3. Results:

Table 1 shows that 52.9% of nurses work in surgical care units, while 47.1% of them work in medical care unit. About thirty eight (37.7)% of nurses were in age group ranging 30-40 years old, while 12.3% of them were over 50 years old. The majority of nurses (83.8%) had a diploma of Secondary Technical Nursing School, while, 8.3% of them had a Bachelor Degree of Nursing Science. Moreover, the highest percentage of nurses had over ten years of experience in nursing, working in all shifts and were married represented by 67.7%, 49.5% and 73.0%, respectively. Only, 17.2% of nurses had previous experience of medication error and 39.2% of nurses reported medication errors made by themselves or by their colleagues.

Table 1: General demographic characteristics of nurses working at in-patient care units at the Main University Hospital (N=204).

Variable	No	%
Working unit		
– Medical	96	47.1
– Surgery	108	52.9
Age (years)		
– <30	57	27.9
– 30 – 40	77	37.7
– 41 – 50	45	22.1
– >50	25	12.3
Educational qualification		
– Bachelor of Nursing Science	17	8.3
– Diploma of Technical Health Institute	16	7.8
– Diploma of Secondary Technical Nursing School	171	83.8
Experience in Nursing		
– <5	20	9.8
– 5 – 10	46	22.5
– > 10	138	67.7
Marital status		
– Single	50	24.5
– Married	149	73.0
– Divorced	5	2.5
Working shift		
– Morning	96	47.1
– Night	7	3.4
– All shift	101	49.5
Previous experience of causing medication errors		
– Yes	35	17.2
– No	169	82.8
Reporting of medication errors done by self or colleagues		
– Yes	80	39.2
– No	124	60.8

Figure (1) indicates that the total mean of nurses' perception of overall safety climate in their working units was (3.96). The highest mean score of nurses' perception regarding the different components of safety climate was related to *safety emphasis, using data for improvement, and unit manger's role* (4.10, 4.05 and 4.00), respectively. On the other hand, the lowest mean score of nurses' perception was related to *blame system* (3.71).

Figure (2) illustrates that the total mean of nurses' perceptions of barriers to report medication administration errors in their working units was (3.93). However, the highest means score of nurses' perception regarding the different barriers were related to *Disagreement over what is medication error and its definition* and *power distance* where mean (4.24 and 4.07), respectively. Conversely, the lowest mean of nurses' perception was related to the barrier *reporting effort* represented by (3.65). In contrast, nurses' responses on the open ended question about other barriers to reporting medication errors from their points of view did not add new barriers.

Regarding nurses' experience with medication errors, **Table 2** reveals that there is a significant difference between nurses who had previous experience with medication error and those who did not and their perceptions of safety climate where (t=3.281,*p*=0.001). Furthermore, there is a significant difference between nurses who report the medication errors to their head nurses and who did not and their perception of safety climate where (t=2.358,*p*=0.019). Nurses who experienced previous medication errors and reporting these errors perceived safety climate less than those who neither had experience nor reported errors.

Table 3 shows significant relationships between nurses' perceptions of safety climate and each of the nurses' educational qualification, marital status as well as the type of working shift where *p*=(0.005,0.001 and 0.006) respectively. On the other hand, there is no significant relationship between nurses' socio-demographic characteristics and their perceptions of barriers of reporting medication errors except for working unit where *p*= (0.015).

Table 4 illustrates that there is a positive significant correlation between nurses' perceptions of overall safety climate and perceived barriers to reporting medication errors where (r= 0.137, *p*= 0.049).

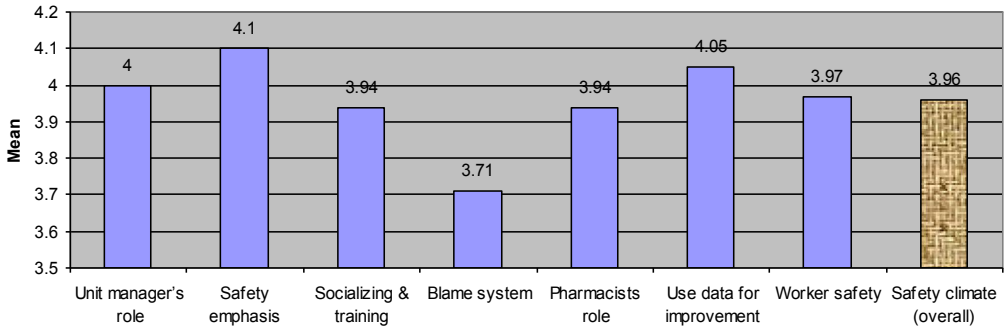


Figure (1): Nurses' perception of safety climate at in-patient care units at the Main University Hospital

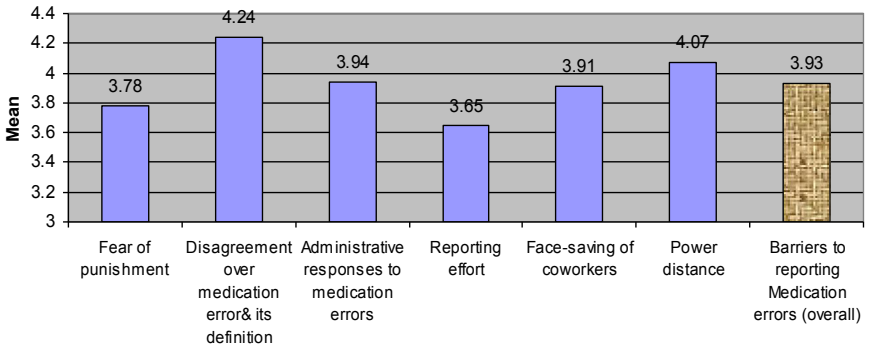


Figure (2): Nurses' perception of barriers to report medication errors at in-patient care units at the Main University Hospital

Table (2): Relationship between nurses' experience of medication errors and their perception of safety climate and barriers to report medication errors.

Variable	Response	Safety climate	Barriers to medication errors reporting
Previous experience of medication errors	**Yes**	3.92 + 0.48	3.92 ± 0.50
	No	4.20 + 0.41	3.95 ± 0.41
t(p)		**3.281* (0.001)**	**0.313 (0.755)**
Reporting of medication errors	**Yes**	3.87 ± 0.47	3.92 ± 0.50
	No	4.03 ± 0.47	3.95 ± 0.46
t(p)		**2.358* (0.019)**	**0.431 (0.667)**

(t): Student t test *p<0.05.

Table (3): Relationship between nurses' demographic characteristics and their perceptions of safety climate and barriers to report medication errors.

Variable	Safety climate	Barriers to reporting medication errors
Working unit		
- Medical	3.97 ± 0.53	4.02 ± 0.47
- Surgery	3.96 ± 0.43	3.85 ± 0.48
t(*p*)	**0.140 (0.889)**	**2.450* (0.015)**
Age (years)		
- <30	3.93 ± 0.53	3.84 ± 0.53
- 30 – 40	3.99 ± 0.51	3.97 ± 0.44
- 41 – 50	4.02 ± 0.33	3.99 ± 0.45
- >50	3.89 ± 0.47	3.89 ± 0.57
F(*p*)	**0.590 (0.622)**	**1.051 (0.371)**
Educational qualifications		
- Bachelor of Nursing Science	4.23 ± 0.65	4.04 ± 0.51
- Diploma of Technical Health Institute	4.19 ± 0.37	4.01 ± 0.43
- Diploma of Secondary Technical Nursing School	3.92 ± 0.46	3.91 ± 0.48
F(*p*)	**5.514* (0.005)**	**0.827 (0.439)**
Experience in nursing		
- <5	3.92 ± 0.47	3.92 ± 0.48
- 5 – 10	3.83 ± 0.42	3.88 ± 0.51
- > 10	4.01 ± 0.49	3.95 ± 0.48
F(*p*)	**2.534 (0.082)**	**0.332 (0.718)**
Marital status		
- Single	3.70 ± 0.50	3.93 ± 0.51
- Married	4.05 ± 0.43	3.94 ± 0.46
- Divorced	3.89 ± 0.58	3.60 ± 0.79
F(*p*)	**11.076* (<0.001)**	**1.183 (0.308)**
Working shift		
- Morning	4.06 ± 0.44	3.97 ± 0.46
- Night	4.12 ± 0.32	4.22 ± 0.28
- All shifts	3.86 ± 0.50	3.87 ± 0.50
F(*p*)	**5.250* (0.006)**	**2.357 (0.097)**

(t): Student t test (f):ANOVA test * p<0.05.

Table (4): Correlation between nurses' perceptions of safety climate and barriers to report medication errors.

Variable	r	*p*
Safety climate	**0.137**	**0.049***
Barriers to report Medication errors		

(r) Pearson Correlation Coefficient * p <0.05.

4.Discussion:

The findings of the present study revealed that the mean score of safety climate indicated that nurses perceived high safety climate in their units particularly with regard to the components/ dimensions such as ; safety emphasis, using data for improvement, and unit manger's role. This result could be attributed to the new and strong trend in health care setting and educational hospital for quality improvement and the issue of patient safety. Various quality improvement programs at Alexandria Main University Hospital have focused on safety especially infection control measure. Moreover, there were many initiatives calling for teaching nurses about these issues. This goes in the same line with Chiang (2005) who found that nurses had a positive regard toward safety climate in their hospital and gave the highest agreement to education on quality and safety, and the head nurse role with major emphasis on safety for patient and workers.[2] In this respect, Hus and Lin (2002) stated that it is important to provide continuous in-service educational programs on quality and safety which are required for nurses in order to maintain safe work environment as well as for their clinical ladder. Improving knowledge about medications through

orientation and training program especially for newly hired nurses is essential for emphasizing the safety climate in work place. [25]

On the other hand, nurses gave the lowest mean score to the "blame system" in the safety climate components. This could be attributed to the fact that it is possible that a blameless reporting culture is still underdeveloped in the hospital of the study. Health care professionals especially physicians as well as nurse managers tend to judge nurses' performance depending on reporting any error to them. Nurses could fear from and avoid being blamed for making any error for the patient. This goes in the same line with Duthie (2005) who stated that reporting errors reflected so badly on nurses that nurses did not like to report any error especially to their mangers and physician to avoid blame. [26] In this respect, Hartnell *et al.,* (2006) recommended that hospital and nurse managers must demonstrate positive responses to staff members for reporting medication errors and adhere to a quality management program that is perceived by nurses as designed to improve patient safety rather than discover mistakes. Nurses must be involved and believe in this process. [23]

The finding of the present study revealed that nurses perceived high barriers to report medication errors particularly with regard the barriers of "disagreement over what is medication error and its definition and power distance" as the most perceived barriers faced them to report medication error. This could be explained as nurses may not clearly recognize the actual meaning of what is medication error and they fear to ask about it as well as fear of reporting any error because the unexpected responses of their administrators. Fear of being blamed and fear of administrators' reactions hinder them to report any error. This result is consistent with Coyle (2005) who found that, lack of recognition and understanding of medication errors as well as fear of administrative decision toward who caused medication error were considered among the most barriers that nurses perceived to report medication errors.[27] in addition, Mohamed and Gaber (2010) stated that, nurses have limited knowledge and work experience about medication safety and may not recognize high-risk situations of medications.[28] For this issue, Elliot (2010) highlighted that, nurses and nurse managers need to be educated on medication errors and clearly defined working expectations. A strong culture of patient safety needs to be in place to allow nurses to learn from errors to focus on changing systems and not place blame on individuals for making errors.[29]

Moreover, nurses perceived "reporting effort" among the barriers to report medication errors but the least one. This could be attributed to the complicated reporting process in filling documents about medication errors and lack of nurses' time to report medication errors in a written form. Nurses at the Main University Hospital still do not apply the use of Incident Report in communication of errors. This could be supported by Chiang (2005) who stated that, nurses may not report medication errors if too much time is needed in reporting and they lacked a well-defined tool or a form for reporting which should be available such as an incident report.[2] Furthermore, Ulanimo *et al.,* (2006) found that lack of knowledge about policies, procedures, and unit routine in reporting errors; busy units and insufficient time to report a medication error; nurses' negligence to report; and nurses' attitude, personality, and little compliance were the barriers to reporting medication errors.[30] In this respect, Elliot (2010) clarified that, increasing medication error reporting by nurses at the bedside involves a change in culture within organization in terms of learning from medication error and policies and procedures should be specific regarding medication errors reporting.[29]

What is more, the present study showed that nurses who had previous experience with medication errors and reporting these errors perceived safety climate less than those who had not. This could be explained as nurses perceived that safety climate and their safe behaviors could be affected by each other. Nurses might perceive that a positive perception of safety climate could promote safe performance of tasks and decrease errors in work place and because they caused previous errors, their perception of safety climate could be negatively affected. This could be supported by Clark *et al*., (2002) who indicated that, nurses' perception of safety climate correlate with and is affected by error incidence and reporting.[31] The finding of this study is consistent with Chiang (2005) who found that nurses who made and recognized medication errors perceived safety climate negative in comparison with those who had no experience and recognition of medication errors.[2] Moreover, Duthie (2005) found that nurses in units with high number of years of experience and reporting medication errors lacked a positive perception of safety climate in work.[26] In this respect, Flin *et al.,* (2004) stated that, safety climate at hospital and unit level influence employees' safe behaviors and there is a strong correlation between safety climate and medication safety. Unsafe behaviors include medication errors, rule violations and none reporting of medication errors incidents and these behaviors which affect negatively on patient safety and safety climate. [32]

In addition, the present study revealed that nurses with Bachelor degree, and who are married and working in night shifts perceived safety climate higher than those with a Diploma degree, single and working in morning shifts. This could be attributed to nurses'

educational level and degree of responsibility that could affect their attitude toward safety climate. Nurses with a Bachelor degree could learn about safety culture in their curricula. Furthermore, married nurses tend to be more mature in the way of acting and dealing with the greater responsibility on their social and clinical status. This could be supported by Blegon *et al.,* (2001) *who clarified that, Bachelor degr*ee nurses and nurses with greater responsibility could be well prepared through their educational and clinical career with different activities that equipped them with competencies in different care situations and promote safe quality care. In addition, nurses working in night shifts may not encounter safety problems and error incidence compared with nurses of day shifts who are burdened by high workload.[33] This result goes in the same line with Carrico (2001) who found a significant relationship between safety practice and the type of shift. He found that errors are significantly low in night shifts than those in morning shifts and safe work behaviors tend to be higher among bachelor nurses and in night shift nurses than working in morning shift.[34] However, Chiang (2005) found that, there is no significant relationship between nurses' perceptions of safety climate and their socio-demographic characteristics.[2]

Moreover, the present study revealed that there is no significant relationship between nurses' demographic characteristics and perceived barriers of reporting medication errors except for the working unit. Nurses in medical units perceived greater barriers for reporting medication errors than nurses in surgical units. This could be attributed to the nature of work in these units where medical care units are characterized by large number of patients especially at the hospital of the study and multiple medications that could require high number of medication administration which causes more liability and opportunity to the risk and incidence of medication errors. thus, nurses in medical units might perceive greater barriers for reporting errors. This could be supported by Cullien *et al.,* (1997) who stated that reporting medication errors is attributed to the high number of medications should be administered. [35] In addition, Duthie (2006) indicated that, the higher workload and acuity demands create an environment that carries a greater risk of errors and high rate of reporting reflecting these errors. [26]

The present study found that there is a positive significant relationship between the overall safety climate and the perceived barriers to report medication errors in the study's units. Nurses might perceive that safe work climate could be related to their unreporting of medication errors. This could be attributed to the previous clarification in this study that the culture of reporting errors still underdeveloped in these units and that safety climate could be related to the lower incidence of reporting medication errors. In addition, nurses' fear of reporting medication errors made the perception of reporting errors barriers correlated to safety emphasis. This could be supported by Duthie (2005) who stated that, if nurses believe that fewer medication errors or fewer adverse events represent a safety climate, this may create an incentive not to report these errors and this is consistent with the traditional perception that medication errors reflect badly on nurses and would explain the positive relationship between the perceived barriers and low rating of reporting medication errors and unit's safety climate.[26] In this respect, Parsons (2000) recommended, creating incentives to reporting errors, indemnity from punishment, root cause analysis of errors with feedback and educating staff about the outcomes of error reporting as among the cultural changes that required to achieve safety climate.[36]

However, the finding of this study is inconsistent with Chiang (2005) who found a significant but negative correlation between nurses' perception of the strength of safety climate and the degree of barriers of reporting medication errors. Nurses who have more agreement about safety climate perceived fewer barriers in error reporting. [2] Moreover, Gershon *et al.,* (2004) found that units with a positive safety climate are more compliant with reporting medication errors. [37]

Conclusion and Recommendation:

The findings of this study concluded that nurses slightly perceived high safety climate in their units. Furthermore, they perceived "disagreement over what is medication error, power distance" as the most barriers to reporting medication errors. However, reporting effort is the least barrier to report medication errors. In addition, there was a positive significant correlation between nurses' perceptions of overall safety climate and the perceived barriers to report medication errors. Nurses might perceive that safe work climate could be related to their unreporting of medication errors.

Based on the previous findings the following recommendation are geared toward:

1- Hospital administrators should:

- Put a great emphasis on a strong culture of safety to focus on changing systems toward provision of safe work climate and allowing nurses to learn from errors reporting.
- Provide continuous in-service educational programs on quality and safety which are required for nurses to maintain safe work environment and safety climate.

- Review policies and procedures and methods of documentation for error reporting to encourage staff of all healthcare providers to report medication errors and to support a blame-free culture in the organization.

2-Nurse Managers should:

- Provide educational programs for improving and updating nurses' knowledge of medication safety and medication errors to monitor the effectiveness of treatment, and report adverse events.
- Create incentives for nurses to report errors, and demonstrate positive responses for them when reporting medication errors.
- Encourage active involvement of nurses in determining root causes of medication errors and how promptly reporting them and give feedback about the outcomes of error reporting that required for achieving safety climate.

3-Further research study to:

- Assess nurses' perceptions of causes of medication errors and barriers to reporting at intensive care units.
- Assess perceptions of patients and health care professionals about factors contributing to medication errors and potential areas for improvement.

Strengths of the study:

From researchers points of views the strength points of this study are:

- Using standardized measures that could be adapted to Egyptian culture, and the two tools were reliable.
- All study's subject responded on the questionnaires with no refusal rate.
- There is a paucity of research in the area of investigating barriers for reporting medication errors among nurses in Egypt. Thus, this research could make a unique contribution to the literature by providing insight into Egyptian nursing staff perceptions of safety climate and perceived barriers for reporting medication errors.

Limitations of the study:

- Researchers surveyed nursing staff only and did not include other healthcare professionals, such as pharmacists or physicians, who could also provide valuable perspectives on patients and medications safety.
- This research used nurses' self-report method which might introduce some bias.

Corresponding author:
Ebtsam Aly Abou Hashish
Nursing Administration Department, Faculty of Nursing, Alexandria University. Alexandria, Egypt.
ebtsam_ss@hotmail.com

References:

1- Hutchinson A, Dean E, Cooper L, Patterson M, McIntosh A, Stride C, Smith C, Laurence B. Assessing the usefulness of a safety climate questionnaire in UK healthcare, University of Sheffield , UK,2004.
2- Chaing H. Nurses' demographics and perceptions of safety climate, work environment and barriers to medication administration errors in southern Taiwan. Published Doctorate Dissertation, College of Nursing, University of Utah,2005.
3- Blegen A, Pepper A , Joseph R. Safety climate on hospital units: A new measure, advances in patient safety. From Research to Implementation 2005; 4: 429-33.
4- ANA 2001. In: Carrico C. Nurses' perception of safety climate in work place. MSCs. Published Thesis, Faculty of Wilmington College, 2001.
5- Sexton J, Helmreich R , Pronovost P, Thomas E. Safety Climate Survey. The Center of Excellence for Patient Safety Research and Practice, University of Texas, 2010.
6- Maddox J, Wakefield M, Bull J. Patient safety and the need for professional and educational change. Nursing outlook 2001; 49(1):8-13.
7- Schull D. "Five rights still resound." Nurse Week (South Central) 2005; 12(20): 9 -18.
8- Mansour M. Critical care nurses' views on medication administration: An organizational perspective. Doctorate Dissertation, School of Nursing, University of Nottingham, 2009.
9- Armitage G, Knapman, H. Adverse events in drug administration: A literature review. JONA 2003; 11(2): 130-40.
10- Koohestani H, Baghcheghi N. Barriers to the reporting of medication administration errors among nursing students. Aust JAN 2009; 27 (1):66-74.
11- Bar-Oz B, Goldman M , Lahat E , Greenberg R. Medication Errors and Response Bias: The Tip of the Iceberg. IMAJ 2008; 10:771–4.
12- NCCMERP. In: Committee of experts on pharmaceutical questions. Survey on medication errors. 2008. Available at: *www.coe.int/t/e/social_cohesion/soc.../Survey%20med%20errors.pdf.* Retrieved on. March 2011.
13- Stratton K , Blegen A , Pepper G, Vaughn T. Reporting of Medication Errors by Pediatric Nurses. J Ped Ng 2004; 19(6): 385-92.
14- Wakefield S, Wakefield J , Uden-Holman T , Blegen A. Perceived barriers in reporting medication administration errors. Best Practices

and Benchmarking in Health Care 1996; 1(4):191-7.
15- Wakefield J, Uden-Holman T, Wakefield S. Development and validation of the medication administration error reporting survey. Advances in Patient Safety 2004; 4:475-89.
16- Kamel S. Studying medication administration errors in Ain –Shams University Hospital. MSCs. Thesis. Faculty of Medicine , Ain Shams University, Egypt ,2008.
17- Abo El-Maged N, Gaber E, El-Maghraby M. Relationship between work setting and the occurrence of medication errors among nurses of Assiut University Hospital, Egypt, 2002. Assuit Med.J 2002; 26(3):55- 66.
18- Mousa S. Assessment of nursing medication errors factors causing them in the critical care unit At El Manial University Hospital, Egypt, 2000.
19- Mohamed N. Drug administration errors and their determinants in intensive care units of El-Shatby Pediatric University Hospital in Alexandria, Unpublished Doctorate Dissertation in public health sciences, High Institute of Public Health, Alexandria, Egypt,2009.
20- Abdou H, Saber K. A baseline assessment of patient safety culture among nurses at Student University Hospital. World Journal of Medical Sciences 2011;6 (1): 17-26,
21- Khattab M. Development of manual for safety measures in general critical care units. Unpublished Doctoral Dissertation. Faculty of Nursing, Alexandria University.Egypt 2005.
22- Sarvadikar A, Prescott G, Williams D . Attitudes to reporting medication error among differing healthcare professionals. Eur J Clin Pharmacol 2010; 66:843–53.
23- Hartnell N, MacKinnon N, Jones E, Genge R, Nestel M. Perceptions of patients and health care professionals about factors contributing to medication errors and potential areas for improvement. Can J Hosp Pharm 2006; 59:177-83.
24- Garbutt J, Brownstein D, Mdklein E, Waterman A, Krauss M, Marcuse E, Hazel E, *et al.* Reporting and disclosing medical errors: Pediatricians' attitudes and behaviors. Arch Ped Adolesc Med 2007; 161:179-85.
25- Hus N, Lin C. Yange P. The comparison and influence factors in implementing clinical ladder system pre and post nursing competence .2002.
26- Duthie E. The relationship between nurses' attitudes towards safety and reported medication errors rates. Doctorate Dissertation, College of Nursing, New-York University, 2006.
27- Coyle A. Designing and implementing a close call reporting system. J Nursing Administration Quarterly 2005;29(1):57-62.
28- Mohamed N, Gabr H. Quality improvement techniques to control medication errors in surgical intensive care units at emergency hospital, Al-Mansoura University, Egypt. Journal of Medicine and Biomedical Sciences, 2010; 5: 2073-78.
29- Elliot J. Nursing-related medication errors: A review of factors that facilitate and hinder error reporting and recommendations for improvement. Published Master thesis of Public Health. School of Public Health, University of Texas, 2010.
30- Ulanimo V, Leary-Kelley C, Connolly P. Nurses' perceptions of causes of medication errors and barriers to reporting. J Nurs Care Qual 2007; 22(1): 28–33.
31- Clark S. Rokett J, Sloane D, Aiken L. Organizational climate, staffing, and safety equipments as predictors of needle stick injuries and near-misses in hospital nurses. Americ J Infect Cont 2002; 30:207-16.
32- Flin R, Burn C, Mearns K, Yule S. Safety climate in health care: A review of measurement instruments.2004. In: Duthie E. The relationship between nurses' attitudes towards safety reported medication errors rates. Published Doctorate Dissertation, College of Nursing, New-York University, 2006.
33- Blegon M, Vaughn T, Goode C. Nurse experience and education. Effect on quality of care. JONA 2001; 31 (1): 33-9.
34- Carrico C. Nurses' perception of safety climate in work place. MSCs. Published Thesis, Faculty of Wilmington College, 2001.
35- Cullen D, Sweitzer B, Bates B, Burdick E, Emonddson A, Leap L. Preventable adverse drug events in hospitalized patients: A comparative study of intensive care and general units. Critical Care Medicine 1997; 25:1289-97.
36- Parsons W. Federal legislation efforts to improve patient safety. Effective clinical practice 2000; 3:309-12.
37- Gershon M, Stone W, Bakken S, Larson E. Measurement of hospital culture and climate in health care. JONA 2004;34:33–40.

2/15/2013

Life Science Journal

Acta Zhengzhou University Overseas Edition

(Life Sci J)

ISSN: 1097-8135

Call for Papers

The academic journal "***Life Science Journal***" (ISSN: 1097-8135) is inviting you to publish your papers.

Life Science Journal, the Acta Zhengzhou University Oversea Version registered in the United States, is an international journal with the purpose to enhance our natural and scientific knowledge dissemination in the world under the free publication principle. The journal is calling for papers from all of the world. Any valuable papers or reports that are related to life science - in their broadest sense - are welcome. Other academic articles that are less relevant but are of high quality will also be considered and published. Papers submitted could be reviews, objective descriptions, research reports, opinions/debates, news, letters, and other types of writings. Let's work together to disseminate our research results and our opinions.

Please send your manuscript to editor@sciencepub.net; lifesciencej@gmail.com

Address:
Life Science Journal - Acta Zhengzhou University Overseas Edition
Marsland Press
PO Box 180432, Richmond Hill, New York 11418, USA
Telephone: (347) 321-7172
Emails: editor@sciencepub.net; sciencepub@gmail.com; lifesciencej@gmail.com
Websites: http://www.lifesciencesite.com; http://www.sciencepub.net

Volume 10, Number 1 (Cumulative No.32) Part 14 March 25, 2013 ISSN:1097-8135

Life Science Journal

Acta Zhengzhou University Overseas Edition

Marsland Press
PO Box 180432
Richmond Hill, New York 11418, USA

Website:
http://www.lifesciencesite.com

Emails:
editor@sciencepub.net
lifesciencej@gmail.com

Phone: (347) 321-7172

Cover design: CAO, Zhaolong
Photograph: YANG, Yizhong; YOUNG, Mary

Copyright © 2013 Marsland Press / Zhengzhou University

ISSN 1097-8135