CONTENTS

91	**Headache in School Age Children and Its Possible Related Expected Predisposing Factors: An Assessment Study** Safaa Salah Ismail and Afaf Salah Abed El-Mohsen.	**617-627**
92	**Transport and Mechanical Properties of Silica Fume Lightweight Aggregate Concrete** Majid Matouq Assas	**628-635**
93	**Dose Phenol Toxicity Affected Endocrine Status in African Catfish (*Clarias gariepinus*)** Mona S. Zaki, Nabila El-Batrawy and Nadia M. Taha	**636-639**
94	**Synergistic Effect of combined antioxidants on Noise-Induced Acoustic Trauma in Adult Guinea Pigs. Audiological and Histological Study** Nagwa Kostandy Kalleny, Nevine Bahaa E. Soliman and Rasha Elkabarity	**640-653**
95	**Effective Use of Technology to Convert Waste into Renewable Energy Source** Neeha Farouqe, Shafqat Hameed	**654-661**
96	**Molecular Study on Relatedness between Clinical and Tap Water Isolatesof*Pseudomonas aeruginosa*in Two Burn Units** Mohammed S. Salama Hala M. Abu Shady, Mohammed M. B. El-Gameal,Mervat G. El Anany and Ghada M.Abd-El-Rehem	**662-666**
97	**Detection of Serum Kl-6 as a Tumor Marker in Hepatocellular Carcinoma** Soha Z. El-Shenawy,Maha M El-Sabawy,Eman Abd El-Razik,Maha M Allam	**667-673**
98	**The mechanisms by which Oxidative Stress and Free Radical Damage produces Male infertility** Magda M El-Tohamy	**674-688**
99	**Design and Optimisation of Low Noise Amplifier in Superhetrodyne GPS Receiver Front End** Shafqat Hameed Umar F. Khan	**689-694**
100	**Reactions of 5-Bromo-4,6-Dimethyl-2-Thioxo-1,2- Dihydropyridine -3- Carbonitrile with Organophosphorus Reagents** Hoda A. Abdel – Malek, Marwa. S. Salem, and Leila S. Boulos	**695-700**
101	**Forecasting Gamma radiation levels using Digital image processing** Abou-Bakr M.Ramadan, Ahmed M. El-Garhy, Fathy Z.Amer, and Mazhar M. Hefnawi	**701-710**
102	**Microwave-Assisted Synthesis of Novel Pyrazole, Pyrimidine and Pyrazolo[1,5-a]pyrimidines Containing Aryl Sulfone Moiety** Ahmed A. El-Kateb, Naglaa M. Abd El-Rahman, Tamer S. Saleh, Ibrahim F. Zeid, Mohamed F. Mady	**711-718**
103	**Lipid Profile in Tuberculous Patients: A Preliminary Report** Mohamed M. Metwally and Hesham Abdel Raheem	**719-722**

104	**Dynamic Characteristics of a Hollow Femur** B.W. Huang, C.H. Chang, F.-S. Wang, A.D. Lin, Y.C. Tsai,M.Y. Huang, J.-G. Tseng	**723-726**
105	**Assessment of Ischemia in Asymptomatic Type II Diabetics versus Asymptomatic patients with other Risk Factors of Coronary Artery Disease. Preliminary Data from GSPECT Tc^{99m}sestaMIBI Myocardial Perfusion Imaging Study in Egyptian Patients.** Akram Abd Elbary; Mohamed Khaled,Wael Sami; Mohga Said, Hala Zakaria, Lamiaa Hamed, Shaban Mohamed and Alia Abd El-Fattah	**727-732**
106	**Transplantation of bone marrowderived stromal cells to chronic cerebral ischemia rats on the influence ofCognitive functionand proteins Nogo-A and NgR expression in the hippocampus**. Yanlin Wang, Songbo Yang, Huili Zhang, Guangming Gong, Qin Jie,Yuming Xu	**733-739**
107	**Nurses' Performance, isolation policy and HCV Sero-conversion among Hemodialysis Patients in Egyptian Hospitals** Sahar Yassin, Manal El Dib and Dalia Roshd	**740-749**
108	**Study on the molecular mechanisms of the inflammation induced by β-amyloid in vivo** Jun Wu, Zhe Min, Yongjie Xiong, Qiuyue Yan, Yuming Xu, Suming Zhang	**750-755**
109	**Construction and identification of a recombinant adenovirus vector expressing His-tag-ICP47 fusion gene** WANG Peng, KAN Quan-cheng, YU Zu-jiang, LI Ling, ZHANG Zhenxiangand PAN Xue	**756-763**
110	**The Theoretical Framework and Applicationon the Relationships between Family Functioning, Alexithymia and Emotional Intelligence among Early Adolescents** Fataneh Naghavi,Ma'rof Redzuan	**764-770**

Headache in School Age Children and Its Possible Related Expected Predisposing Factors: An Assessment Study

[1]Safaa Salah Ismail and [2]Afaf Salah Abed El-Mohsen.

[1]Pediatric Nursing, Faculty of Nursing, Helwan University.
[2]Community Health Nursing, Faculty of Nursing, Helwan University.
smmaa@hotmail.com

Abstract: **Background:** Headache is a common symptom with a high prevalence in most epidemiological studies. It is one of the 10 most common reasons for outpatient physician visits. Headaches are common in children and the prevalence increases with increasing age. **The aim of the study:** was to find out the prevalence of headache among school age children and study different attributes associated with headaches in Ain Helwan district. **Method:** Descriptive research design was carried out at primary, preparatory and secondary public schools in Ain Helwan district. **Subjects**: included 378 school age children 10-17 years, from both gender. **Sample:** A simple random sample was recruited, utilizing the non-probability sampling technique. **Tool:** The researchers utilized self-administered interview sheet to collect data in relation to personal information of the studied sample, their eating habits, and life style pattern. Data were collected in the period from beginning of March 2011 till the end of April 2011. **Results:** revealed that, out of 378, students 221 were complaining from headache (58.5%). No statistically significant differences were detected between middle childhood and late childhood students regarding dizziness during car riding, sensitivity to certain smile, extreme noise, in addition to intensity of headache that increases with climbing stairs, sport, cough, change body posture, activity, teeth or gums' pain, ear problems, nervousness, vision problems and taking vitamins or drugs. Less than three quarters of sample had inadequate or irregular sleep (72.4%) and there were highly statistically significant differences between middle childhood and late childhood students regarding inadequate or irregular sleep and eating chocolate or cheese, skipping meals, drinking caffeine, staying long time in front of TV or computer, ingestion of cold drink or ice cream, smoking, and complying of any unhealthy condition. More than three quarters of the studied sample showed clinical manifestations of headache in stabbing pain followed by pressing headache (83.7% & 77.8 % respectively). Results revealed adverse effects of headache through scholastic achievement, school attendance and daily activity with no significant differences. **Conclusion:** Headache represents a common disabling health problem among school age children in Ain Helwan district with a prevalence rate of 58.4%. In general, the prevalence of headache increases with age and it is higher in males than females. Stress following staying long time in front of TV or computer was the most common precipitating factor for headache in the study. **Recommendation:** Health education of the parents and children about the precipitating factors which triggers for headache, and increasing awareness about healthy life style, and early diagnosis and treatment of headache are recommended to avoid its drawbacks on child health and school performance.

[Safaa Salah Ismail and Afaf Salah Abed El-Mohsen **Headache in School Age Children and Its Possible Related Expected Predisposing Factors: An Assessment Study**] Life Science Journal 2012; 9(1):617-627]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 91

Key words: Headache, school age children

1. Introduction

Headache is the most frequently reported pain in children followed by abdominal and musculoskeletal pain and it is a common symptom and complaint in pediatric practice, but consultation rates do not reveal the true prevalence **(Alawneh & Bataineh, 2006).** The prevalence of headache increases with age and reaches adult population prevalence in the early teens **(Lipton, 1997).**

Headaches are common in children and the prevalence increases with increasing age. In the researchers' practice, almost half of referrals from primary care are because of headache. Unfortunately, most parents think that headache is an uncommon symptom in children, hence their understandable concern. As well, hoping to relieve the pain, parents are often seeking reassurance that their child's headache is not a sign of serious intracranial disease, such as brain tumors. If this is understood, then no need to explain every headache, but nurses must be able to reassure the child and family that it is not a sign of serious illness **(Mukhopadhyay & White, 2008).**

Headache is one of humanity most common afflictions. It has been estimated that one person in three experiences sever headache at some stage of life. Most people with a mild recurrent or isolated headache do not consult a physician, and therefore

the true prevalence is unknown. The lifetime prevalence for any type of headache as estimated from population based studies is more than 90 % for men and 95 % for women **(Boes et al., 2004).** A classification was also primarily developed for headache disorders in adults. There are no specific criteria for children, which makes difficult to classify their headaches **(Koenig et al., 2002).**

Headache is very common in children and its prevalence increases in adolescence. Data from multiple studies have shown the prevalence of headache to be in the range of 37-51% in 7 years old, growing steadily to 57-82 % in 15 years old. Pre-pubertal boys are more often afflicted than girls whereas after puberty headaches occur more often in girls **(Lewis et al., 2002).**

The epidemiology of headaches in children and adolescents has been well studied in Western countries, but very little similar information has been recorded in developing countries **(Ayatollahi & Ayatollahi, 2002).** In addition, results from the different studies have varied according to the diagnostic criteria, methods of data collection, specific population and regions. In addition, variations in prevalence rates have been attributed to case definition and the age and sex of population surveyed **(Ayatollahi & Khosravi, 2006).**

" Frequent" headache was reported in 2.5% of children who were at least seven years of age and 15 % of those who were at least 15 years of age. The prevalence of headache ranged from 37- 51% in those who were at least seven years of age and gradually rose to 57-82 % by age 15. Before puberty, boys are affected more frequently than girls, but after the onset of puberty, headaches occur more frequently in girls **(Donald & Lewis, 2002).**

Recurrent headaches adversely affect academic performance, memory, school attendance, personality and peer relations **(Shivpuri et al., 2003)**. Headaches have a significant impact on the lives of children and adolescents, resulting in school absence decreased extracurricular activities and poor academic achievement **(Abu-Arefeh & Russel, 1994).**

The term "headache" was defined as a pain in the area of the head, which handicapped the pupil's school attendance, homework, leisure activities, or daily living. Recurrent headache, such as migraine and tension headaches, is a common problem which often begins in childhood and extends into adulthood. Until recently, little attention has been devoted to this common chronic pain problem in children and adolescents **(Abu-Arafeh & Macleod, 2005).**

About 5% of school-age children and at least 10% of teens get headaches, recurrent headaches with additional symptoms. Often triggered by things like stress, sleep deprivation, and menstruation, it can cause the following symptoms: pounding, throbbing pain or dull, steady pain on one or both sides of the head, dizziness, stomachaches, nausea and/or vomiting, seeing spots or halos sensitivity to light, noise, and/or smells, which in addition, last anywhere from 30 minutes to several hours, and some can last as long as a couple of days **(Lipton, 1997).**

In general, children get the same types of headaches as adults, and headaches often are hereditary, so if a parent gets them, their children might too. In addition, a family member with headaches may give a clue to the cause but may also be acting as a role model for the headache behavior **(Mukhopadyay & White, 2008).**

Once the headache diagnosis is established, management must be based on the frequency and severity of headache and the impact on the patient's lifestyle. Experienced pediatric nurses can play key roles in pediatric care of children with headache. The nurse has a key role in obtaining the patient history, assisting with diagnosis and management, and providing counseling to prevent further headaches. Certain modifications may prevent many of the headaches. These changes include improving sleep habits, meal schedules, fluid intake, and stress reduction and eliminating food and environmental triggers. Nursing expertise can be used to develop improved collaborative care plans, which, when shared with the child, family, and primary care provider, will lead to successful continuity of long-term care **(Johnson et al., 2009).**

On the same context, **Lewis (2007),** identified that management of headache by teaching process must be the incorporation of life style change, such as regulation of sleep and eating habits, avoidance of skipping meals and caffeine, adequate fluid intake, regular exercise, identification of triggering factors, and stress management.

Aim of the study:

To find out the prevalence of headache among school age children and study different attributes associated with headaches in Ain Helwan district.

Research questions:

1. What is the prevalence of headache among school age children in Ain Helwan district?
2. What are the different attributes associated with headaches?

2. Subjects and Methods

Research design: This was a descriptive analytical research design through cross-sectional survey, based study on school children aged, between 10- 17 years.

Research setting: This study was carried out at schools at Ain Helwan district.

Subjects: Children fulfilling the following criteria were included in the study sample such as; students from both genders, and aged 10-17 years. They were recruited from primary, preparatory and secondary public schools in Ain Helwan district in the period from beginning of Marsh 2011 till end of April 2011.

At the beginning of the study, all children from the students who fulfill the sampling criteria were present on the day of data collection. They agreed to be interviewed and were included as subjects for the study.

Sample: A simple random sample of 378 students was recruited, utilizing the non-probability sampling technique. The students were chosen from six schools: Tarek Ben Ziad and Belal Ben Rabah primary schools, Omer Ben Abd El-Aziez Preparatory school for boys, Om El-Abtal Preparatory school for girls, Helwan Preparatory school for girls, Helwan Secondary school for girls.

Tools: A self-administered interview sheet was designed and utilized by the researchers to collect the necessary data. It included to four parts and entailed the following items:

- Personal information of students such as: age, gender, parents' education and occupation etc.
- Headache characteristics included questions about starting headache, numbers of attacks, duration, and site etc.
- Precipitating factors included questions about dizziness during the care ride, sensitivity to certain smile, extreme noise, vision problems, sinusitis etc.
- Life style pattern of students included questions related to inadequate sleep, skipping meals, drinking caffeine, staying long time in front of TV or computer, visits to health care units, seeking health advice from school nurse etc.

Field work:

- Tools were reviewed by experts in different fields of nursing and medicine.
- Official letters from the Faculty of Nursing, Helwan University were forwarded to the Ministry of Education to obtain their permission to visit the schools and collect data.
- Official letters were available with the approval of the Ministry of Education, addressed to the directors of the schools. Each director was informed about the time and date of data collection .
- Before conducting the main study, a pilot study was done with the assistance of the children's teachers' involving the application of the questionnaire, to ten children in a class chosen randomly. This was done to assess whether the students could comprehend it easily and estimated the time needed to fill in the questionnaire.
- Students who shared in the pilot study were excluded from the main study sample.
- Based on the results obtained from the pilot study, some modifications were done on the questionnaire and then, it was administered to the students.
- Each student was interviewed individually after explaining the purpose and method of the study and obtaining his/her approval to participate in the study and they were assured about confidentiality of data collected.
- Each interview took approximately 15-20 minutes to complete filling in the study tool, depending upon the understanding and response of the students. Data were collected during the period from beginning of March to the end of April 2011.
- Instructional handouts were designed by the researchers in simple Arabic language to explain the meaning of headache, causes, and risk factors from headache and healthy life style pattern to decrease headache. They were distributed to the participants.

Statistical Analysis:-

Upon completion of data collection, each answer sheet was coded and scored. The researchers coded the data into a coding sheet so that data could be prepared for computer entry. Data were statistically analyzed by using the Statistical Package for Social Science (SPSS) version 15, and test of significance. The level of significance was considered at $p<0.05$.

Limitations of the study:

There were no students in 2nd and 3rd sacondary schools beside few students were in schools due to revolution.

3. Results

Figure (1) illustrated that out of 378 students under study 221 were complaining from headache (58.5%), while, 157 (41.5%) were not. In addition, 104 children were in middle childhood from 10 < 14 years, and 117 children in late childhood from 14-17 years.

Table 1: displayed the socio-demographic characteristics of students. Age for less than two thirds of the studied sample (65.6%) ranged from 14-17 years (late childhood). The same table indicated that the majority of the studied sample (80.7%) was

males. In addition, 65.3 % of them were in 2nd and 3rd preparatory, school and 1st secondary school.

Table 2: showed the socio-demographic characteristics of students' parents. The father occupations for more than third of the studied sample (34.9 %) were workers, followed by employees (22.7%). The same table indicated that the highest percentage of the studied sample (36.5%) their fathers' educational level was secondary education, followed by more than one quarter (26.4%) were illiterate. The table also showed that two thirds of the studied sample of mothers were housewives (66.7%), and secondary educated mothers constituted the highest percentage accounting for slightly more than one third (34.4%).

Table 3: demonstrated that an equal percentage of more slightly more than three fifths (61.5 %) of middle childhood (10- < 14 years) under study have onset of pain as acute and repeated headache. The same table showed significant relations between middle childhood and late childhood students as regards having onset of pain as acute , repeated headaches , number of attacks per month, pain headache in unilateral location, continuous pain all the time, and gender. In addition the same table showed no statistically significant differences between middle childhood and late childhood students as regards headache pain fixed and slow, overhead as heaviness, pulsating quality headache, and duration of headache (P=0.50, 0.45, 0.54 & 0.038 respectively).

Table 4: showed no statistically significant differences between middle childhood and late childhood students regarding dizziness during car riding, sensitivity to certain smile, extreme noise. In addition, to intensity of headache increase with climbing stairs, sport, cough, change body posture, activity ,teething or gums pain, ear problems, nervousness, vision problems and taking vitamins or drugs .

In addition, the table showed statistically significant differences between middle childhood and late childhood students regarding family history of headache, time of menses in girls, headache increase when carrying heavy thing and walking adding to children complaining from sinusitis.

Table 5: revealed that less than three quarters of sample had inadequate or irregular sleep and there was a highly statistically significant difference between middle childhood and late childhood students regarding inadequate or irregular sleep and eating chocolate or cheese.

In addition, the same table showed statistically significant differences between middle childhood and late childhood students regarding their life style pattern such as; skipping meals, drinking caffeine, staying long time in front of TV or computer, ingestion of cold drink or ice cream, smoking , and complain of any unhealthy condition (P= 0.64, 0.13,0.33,0.172, 0.08 & 0.20, respectively).

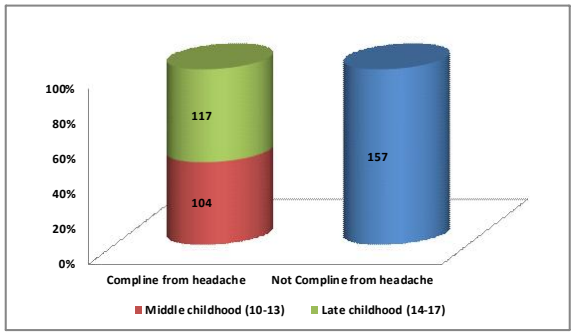


Figure (1): Prevalence of headache among studied sample.

Table (1): Socio demographic characteristics of the studied sample (n= 378).

Socio demographic characteristics	No	%
Age (in years):		
10<14	130	34.4
14-17	248	65.6
Gender:		
Male	305	80.7
Female	73	19.3
Grade :		
5th & 6th primary school, and 1st preparatory school	131	34.7
2nd & 3rd preparatory school, and 1st secondary school	247	65.3

Table 6: highlighted that the majority of studied sample showed clinical manifestations of headache in stabbing pain followed by pressing/ tightening headache. In addition, the table showed that the lowest percentage regarding clinical manifestations of headache as sense of nauseating and/or vomiting in starting headache and elevation of body temperature .

In addition, table (6) showed no statistically significant differences between middle childhood and late childhood students regarding their clinical manifestations of headache as pressing headache, facial redness, leakage from nose, elevation of body temperature, sense of nauseating or vomiting , blurred vision and numbness in the face .

Table 7: revealed that a minority of studied samples representing only one tenth were making referral to hospital for follow up, and less than fifth were going to doctor for advice, followed by near one third seeking advice from school nurse. In addition, the table showed significant differences between middle childhood and late childhood students regarding to prefer to stay in dark and quite place, going to doctor advice and satisfied from advice of school nurse.

Table 8: showed frequently of adverse effects of headache through scholastic achievement, school attendance and daily activity and there were no statistically significant differences between middle childhood and late childhood students regarding to scholastic achievement, school attendance and daily activity (P=0.28, 0.09 &0.053 respectively).

Table (2): Socio-demographic characteristics for parent of the studied sample (n= 378).

Items	No	%
Father occupation:		
Worker	132	34.9
Private work	66	17.5
Driver	46	12.2
Retirement	18	4.8
Employee	86	22.7
Died	30	7.9
Father education:		
Illiterate	100	26.4
Read & write	66	17.5
Secondary education	138	36.5
University education	74	19.6
Mother occupation:		
House wife	252	66.7
Employee	54	14.3
Teacher	42	11.1
Worker	30	7.9
Mother education :		
Illiterate	110	29.1
Read & write	84	22.2
Secondary education	130	34.4
University education	54	14.3

Table (3): Distribution of Common Characteristics Headache of the studied sample (no= 221).

Variable	Middle childhood 10<14 year		Late childhood 14-17 year		Total		P –value
	No= 104	%	No=117	%	No=221	%	
Onset of pain is acute							
	64	61.5	82	70.1	146	66.1	0.000
Repeated headache							
	64	61.5	85	72.6	149	67.4	0.004
Number of attacks of headache / month							
1-3	76	73.1	75	64.1	151	68.3	0.001
4-6	13	12.5	28	23.9	41	18.6	
≥ 6	15	14.4	14	12	29	13.1	
Headache pain in unilateral location							
	23	22.1	52	44.4	75	33.9	0.001
Headache pain is fixed & slow							
	42	40.4	55	47	97	44	0.50
Headache pain over head as heaviness							
	38	36.5	45	38.5	83	37.6	0.45
Continue headache all time							
	43	41.3	17	14.5	60	27.1	0.000
Pulsating quality headache							
	49	47.1	55	47	104	47.1	0.54
Duration of headache attacks							
30 min-< 2hour	90	86.5	109	93.2	199	90	0.038
2-4 hours	4	3.8	6	5.1	10	4.5	
≥ 4+ hours	35	9.6	2	1.7	12	5.4	
Tie head							
	47	45.2	60	51.3	107	48.4	0.259
Gender							
Male	69	66.3	80	68.4	149	67.4	0.000
Female	35	33.7	37	31.6	72	32.6	

Table (4): Distribution the Precipitating Factors for Headache of the Studied Sample (no= 221).

Variable	Middle childhood 10<14 year		Late childhood 14-17 year		Total 221		P -value
	No	%	No	%	No	%	
Dizziness during the car riding							
	34	32.7	29	24.8	63	28.5	0.12
Sensitive to certain smile							
	27	26	30	25.6	57	25.8	0.53
Extreme noise increase headache							
	37	35.6	86	73.5	123	55.7	0.15
Intensity increase with walking							
	50	48.1	60	51.3	110	49.8	0.003
Intensity increase with climbing stairs							
	41	39.4	40	34.2	81	36.7	0.144
Intensity increase with sport							
	35	33.7	38	32.5	73	33	0.40
Family history of headache							
	77	74	96	82	173	78.3	0.00
Increase with time of menses							
	12	11.5	26	22.2	38	17.2	0.003
Pain of headache increase with cough							
	52	50	39	33.3	91	41.2	0.006
Pain of headache increase when carrying heavy thing							
	51	49	22	18.8	73	33	0.000
Pain of headache start when changing body posture							
	28	26.9	41	35	69	31.2	0.143
Intensity increases by activity							
	74	71.1	117	100	191	86.4	0.053
A companied with taking vitamins or drugs							
	43	41.3	72	61.5	115	52	0.36
Increased with teeth or gums pain							
	48	46.2	66	56.4	114	51.6	0.022
Increased with ear inflammation							
	46	44.2	75	64.1	121	54.8	0.07
A companied with nervousness or stress							
	58	55.8	95	81.2	153	69.2	0.23
A companied with change in atmosphere							
	56	53.8	83	70.9	139	62.9	0.03
Increased with sinusitis							
	59	56.7	27	23.1	86	38.9	0.00
A companied from vision problem							
	26	25	28	23.9	54	24.4	0.46

Table (5): Distribution of Components of life style among the studied sample (no= 221).

Variable	Middle childhood 10<14 year		Late childhood 14-17 year		Total		P –value
	No	%	No	%	No	%	
Inadequate or irregular sleep							
	58	55.8	102	87.2	160	72.4	0.000
Skipping meals							
	40	38.5	29	24.8	69	31.2	0.064
Drinking caffeine							
	29	27.9	24	20.5	53	24	0.13
Eating chocolate or cheese							
	24	23.1	22	18.8	46	20.8	0.02
Staying long time in front of TV or computer							
	59	56.7	100	85.5	159	71.9	0.33
Ingestion of cold drink or ice cream							
	31	29.8	48	41.1	79	35.7	0.172
Complain of any unhealthy condition							
	39	37.5	64	54.7	103	46.6	0.20
Smoking							
	28	26.9	63	53.8	91	41.2	0.08

Table (6): Distribution of Clinical manifestation of headache among the studied sample (no= 221).

Variable	Middle childhood 10<14 year		Late childhood 14-17 year		Total		P –value
	No	%	No	%	No	%	
Pressing / tightening headache							
	60	57.7	112	95.7	172	77.8	0.425
Stabbing pain							
	83	79.8	102	87.2	185	83.7	0.003
Facial redness occur							
	35	33.6	85	72.6	120	54.3	0.10
Decent of eye tears							
	54	51.9	64	54.7	118	53.4	0.004
Leakage from nose (running)							
	38	39.4	61	52.1	99	44.8	0.36
Elevation of body temperature							
	41	39.4	30	25.6	71	32.1	0.012
Sense of nauseating and /or vomiting in starting headache							
	32	30.8	32	27.4	64	29	0.34
Blurred vision							
	55	52.9	62	53	117	52.9	0.43
Numbness' in the face							
	36	34.6	76	65	112	50.7	0.34

Table (7): Frequency of practice to relive headache among the studied sample (no= 221).

Variable	Middle childhood 10<14 year		Late childhood 14-17 year		Total		P –value
	No	%	No	%	No	%	
Prefer to stay in dark and quite place							
	78	75	83	70.3	161	72.9	0.000
Sleeping							
	83	79.8	108	92.3	191	86.4	0.055
Taking period of rest							
	79	76	95	81.2	174	78.7	0.006
Go to seeking advice from school nurse							
	31	29.8	41	35	72	32.6	0.054
Go to doctor for advice							
	29	27.9	12	10.3	41	18.6	0.000
Taking medication							
	70	67.3	111	94.9	181	81.9	0.21
Making referral to hospital							
	10	9.6	12	10.3	22	10	0.177
Satisfy from advice of school nurse							
	49	47.1	47	40.2	96	43.4	0.000

Table (8): Frequency of adverse effects of headache among the studied sample (no= 221).

Variable	Middle childhood 10<14 year		Late childhood 14-17 year		Total		P –value
	No	%	No	%	No	%	
Scholastics achievement							
	36	34.6	61	52.1	97	43.9	0.28
Daily activity							
	74	71.2	110	94	168	76	0.053
School attendance							
	18	17.3	22	18.8	40	18.1	0.09

4. Discussion

Headache is one of the most common complaints in childhood and adolescence. Despite this fact, relatively little was known about the variations of headache prevalence by age, sex and race **(Wang et al., 2005).**

The current study showed that the total prevalence of headache among 378 school children aged 10-17 years in Ain Helwan district represented almost three fifths distributed between mild childhood less than half and late childhood more than half. These results were in agreement with **Abdou et al. (2005),** who reported a prevalence of 72.3 % of headache among 1200 child, age ranged between 6-11 years in Alexandria. Other investigators reported lower prevalence of 39% and 45.4 % among school age children in England and Sweden, respectively **(Isik et al., 2006).**

In some Arabic countries, studies estimating the prevalence of headache among school age children reported a prevalence of 37% in the Emirates **(Bener et al., 1998)** and 44 % in Saudi Arabia **(Jumah et al., 2002).** On the other hand, **Alawneh & Bataineh, (2006)** reported a lower prevalence of 24 % of headache among school age children in Jordan.

In addition, headaches are common during childhood and become more common and more frequent during adolescence. In epidemiological survey of 9000 schoolchildren, one-third of the children who were at least seven of age had had headaches and half of those were at least 15 years.

In the present study, male students suffered from headache more frequently than females accounting for almost two thirds versus slightly less than one third and this was statistically significant. However, headache was more common in male students below the age of 14. In a similar study, **Lewis et al. (2002)** found that pre-pubertal boys were more often afflicted by headache than girls whereas after puberty headaches occur more often in girls. In accordance with the previous results **Martin & Behbehani (2006) and Kroner - Herwig & Vath (2009)**

suggested that pubertal hormone imbalance may be a risk factor for developing headache in girls at puberty. Similarly **Laurell et al., (2004) and Gassmann et al., (2008)** confirmed that teenaged females suffer from headache more frequently than males.

In the present study, the prevalence of headache among middle childhood age from 10 < 14 years was lower than late childhood age from 14-17 years .This finding contradicted with the study carried out by **El-Tallawy et al., (2006),** who found a prevalence of headache of 21.1 % in 2088 school age children in Assiut Governorate, of whom 734 children chosen from the 4th and 5th grade of primary schools aged 9-11 years, 834 children from the 1st and 2nd grades of preparatory school with age ranged from 11-13 years and 520 students from secondary schools representing 1st grade whose age ranged from 14-15 years.

This discrepancy between the current study results and prevalence in the other studies may be attributed to the difference in age grouping and its impact on the ability of young children to express their symptoms, to the different constitutional factors of the studied populations, to the difference in criteria used for diagnosis or environmental risk factors.

The current study finding clarified that regarding socio-demographic characteristics in relation to parents, education, the illiterate fathers represented approximately one quarter and for the worker fathers accounted for almost one third. Other studies found that headache were more common when the students' family had a low income as that of **Unp et al., (2005).**

The present study result showed in relation to family history of headache, that more than three quarters of the studied sample have a positive family history with statistically significant differences between mild childhood and late childhood. This is in agreement with **Laura et al., (2011),** and **Isik et al., (2006),** who found that 60.9 % and 87.3% respectively of children with primary headache have a positive family history for headache.

Studying different precipitating factors for headache, the current study clarified factors such as; sensitive to certain smile, noise, skipping meals, and inadequate or irregular sleep, and stress. This is in agreement with **Isik et al., (2006),** who reported that stress is the most common precipitating factors for headache in 69%, 83.6% and 72.6% respectively. According to **a National Headache Foundation survey (2011),** nearly 30% of children miss school because of headaches. For many children, the start of the school season can be a particularly stressful time.

The previous finding are also is in accordance with a study carried out by **El-Tallawy et al., (2006),** whose findings clarified significant high rate of different precipitating factors such as, sleep disturbances whether hypersomnia or insomnia this may be attributed to the fact that students especially in secondary school may have sleep disturbance due to bad methods of studying in addition to extra school courses (private lessons). Other reported precipitating factors include bright sunlight, noise as most of schools were present in noisy areas which may increase the headache attacks, as well as hunger or missed meals where most of students delay breakfast to 11.00 -12.00 a.m.

In accordance with previous results, those mentioned by **Blau (2004),** who reported that too much or too little sleep provokes headache in 5% of normal children and in a proportion of children who complain from headache. These environmental triggers, light, sound and smell, are transmitted directly to the central nervous system (CNS) by the special senses; and cause direct excitation of the neural pathways, then cause headache attack but light is a more common precipitant than others, also the study recorded that hunger or missed meal represents a precipitating factor in 60.6% of children.

Additionally, This study result clarified that headache increased with teething or gums pain, activity, ear inflammation, and sinusitis .This finding contradict with that of a study by **Stovner et al. (2007),** which reported that 48% of children with headache explained that headache is a common symptom common in many pain in children, such as; ear infections, sinusitis infections, colds and flu are often accompanied by headache.

Studying different precipitating factors in common life style for headache, the current study clarified factors such as; stress , staying long time in front of TV or computer, ingestion of cold drink or ice cream and eating chocolate or cheese . This is in agreement with a study carried out by **Stovner et al. (2007),** who mentioned that; additives in foods monosodium glutamate (MSG), found in many foods such as bacon, bologna and hot dogs, which are known to trigger headaches. Caffeine, soda, chocolate, coffee and tea may cause headaches.

In the present study results revealed that, less than half of the studied sample described a pulsating headache, more than two fifths described fixed and slow headache, more than third described heaviness and less than half described tie in the head. Similarly, other previous studies reported pulsating headache to be the most common type of headache in school age children constituting 41.2-89.5% **(Unp et al., 2005; and Ayatollahi & Khosravi (2006),** which means that ability of children to express and remember their symptoms make the possibility that the quality of

pain may not be usually easily differentiated in the population.

Regarding to the site of headache, the present study revealed that the unilateral location was the commonest site. **Unp et al. (2005)** found that most of students who suffered from headache defined more than one location. This difference might be explained by the fact that most students forgot the site of headache.

Regarding to duration of headache, the present study findings revealed that the duration of headache attacks lasts from 30 minutes to 2 hours in 90%, from 2-4 hours in 4.5%, and more than 4 hours in 5.4 % of students. This finding contradicted with study by **Andersen et al. (2006),** who reported that 51.4% of children with headache had attacks that lasted less than 3 hours, while 41.2% had attacks that lasted 4-24 hours and 3.7% had attacks that lasted more than 24 hours.

In the current study, the majority of the studied sample used medication or analgesics to relieve headache but none of them were on maintenance treatment. Previous studies reported that 20 - 77.5% of students with headache use pain relieving medicine **(Isik et al., 2006 and Holstein et al., 2008).** This suggests that self-treatment is a relevant problem in population.

It is noted that while nearly more than two fifths of children with a headache had a severe disorder affecting their scholastic achievement in productive life, and more than three quarter had effect on daily activity but, none was on maintenance treatment. This results was with in accordance with a study carried out by **Rhee (2001),** who found that; headache affect social competency in a form that can be measured in terms of days of school missed, inability to participate in after-school activities, loss of friendships, and isolation. For the preteen and adolescent, inability to grow up and be independent can be a consequence of headache.

Conclusion:

Headache represents a common disabling health problem among school age children in Ain Helwan district with a prevalence rate of 58.4%. In general the prevalence of headache increased with age and it was higher in males than females. Stress followed by staying long time in front of TV or computer were the most common precipitating factors for headache in the study.

Recommendation:

Health education of the parents and children about the precipitating factors triggers for headache, and increase awareness about healthy life style. In addition, early diagnosis and treatment of headache to avoid its drawbacks on child health and school performance.

Corresponding author
Safaa Salah Ismail
Pediatric Nursing, Faculty of Nursing, Helwan University.
smmaa@hotmail.com

References:

Abdou, A., Hafez, N., Deif, A. & El Sharkawi, M. (2005): Prevalence of headache among school age children in Alexandria, Egypt. Journal of the Neurological Sciences; 238-482.

Abu-Arafeh, I. & Macleod, S. (2005): Neurological disorders in children with chronic headache. Arch Dis Child, 90: 937-940.

Abu-Arafeh, I., & Russel, G. (1994): Prevalence of headache and migraine in school children. BMJ, 309:765-769.

Alawneh, H.F., & Bataineh, H. A. (2006): Prevalence of headache among school children in Jordan. Sudanese Journal of Public Health, 1(4): 289-292.

Andersen, A., Holstein, B.E., & Hansen, E.H. (2006): Is medicine use in adolescence risk behavior? Cross sectional survey of school aged children from 11 to 15.Journal Adolescent Health; 39: 362-366.

Ayatollahi, S.M., & Ayatollahi, S.A. (2002): Prevalence of migraine and tension-type headache in adolescent girls of Shiraz. Headache, 42:287– 90.

Ayatollahi, S.M., & Khosravi, A. (2006): Prevalence of migraine and tension type headache in primary school children in Shiraz Eastern Mediterranean Health Journal; 12(6): 809- 817.

Bener, A. (1998): Prevalence of headache and migraine in school children in the United Arab Emirates. Annals of Saudi medicine; 18: 522- 524.

Blau, J.N. (2004): Migraine pathogenesis: The neural hypothesis re-examined. J. Neurol. Neurosurg. Psychiatry, 47: 437-442.

Boes, C.J., Capobianco, D.J., & Cutrer, F.M. (2004): Headache and other craniofacial pain. Philadelphia, 3: 2055- 2104 .

Donald, W., & Lewis, M.D. (2002): Headaches in children and adolescents. Journal of The American Academy of Family Physicians, 65(4); 625 – 633.

Gassmann, J., Morris, L., Heinrich, M., & Kroner- Herwig, B. (2008): One year course of pediatric headache in children and adolescents aged 5-15 years. Cephalalgia, 28; 1154- 1162.

El-Tallawy, H . N., Farweez, H. M., Fargha1y, W. M., & Ahmed, M. A. (2006): Prevalence of migraine among school children (Primary, preparatory and secondary) in Assiut City-Egypt.

Egypt J. Neurol. Psychiat. Neurosurg, 43(1); 131-140.

Holstein, B., Andersen, A., Krolner, R., Due, P., & Hansen, E.H. (2008): Young Adolescents' use of medicine for headache: Sources of supply; availability and accessibility at home. Pharmacoepidemiol Drug Saf, 17; 406-410.

Isik, U., Refika, H. E., & Pinar, A.y. (2006): Prevalence of headache and its association with sleep disorders in children. Pediatric Neurology, 36; 146-151.

Johnson, A., Cathy, J., & Claire, M. (2009): Off-therapy headaches in pediatric patients: a retrospective review. Journal of Pediatric Oncology Nursing, 26(6); 354–361.

Jumah, A.M., Awada, A., & Azzam, A.S. (2002): Headache syndromes amongst schoolchildren in Riyadh, Saudi Arabia. Headache, 42; 281-286.

Koenig, A., Gladstein, J., McCarter, J., & Hershey, A.D. (2002): Chronic daily headache in children and adolescents presenting to tertiary headache clinics. Headache, 42; 491-500.

Kroner-Herwig, B., & Vath, N. (2009): Menarche in girls and headache – a longitudinal analysis. Headache, 49; 860-867.

Laura, P., Daniele, S., & Gian, V. Z. (2011): Headache in children and adolescents aged 6-18 years in Northern Italy: Prevalence and risk factors. European Journal of Pediatric Neurology, 3; 234-240.

Laurell, K., Larsson, B., & Eeg-Olofsson, O. (2004): Prevalence of headache in Swedish school children with a focus on tension type headache. Cephalalgia, 24; 1-9.

Lewis, D., Kellstein, D., & Burke, B. (2002): Children Ibuprofen suspension for the acute treatment of migraine headache. Headache, 42; 780-786.

Lewis, D.W. (2007): Headache in children and adolescents. Current problems in pediatric and adolescent health care, 37; 1-207.

Lipton, R.B. (1997): Diagnosis and epidemiology of pediatric migraine. Curr Opin Neurol, 10; 231-236.

Martin, V.T., & Behbehani, M. (2006): Ovarian hormones and migraine headache: Understanding mechanisms and pathogenesis part II. Headache, 46; 365-386**.**

Mukhopadhyay, S., & White, C. (2008): Evaluation of headache in children, Pediatrics and child Health, 18; 1-6.

National Headache Foundation Survey (2011): Available at : http://www.umm.edu/patiented/articles/who_gets_tension type_chronic_daily_headaches_000011_4.htm#ixzz1mu2c6XWD www.headaches.org

Rhee, H. (2001): Risk factors for and sequelae of headaches in school children with clinical implications from a psychosocial perspective. J Pediatr Nurs, 16; 392–401.

Shivpuri, D., Rajesh, M.S., & Jain, D. (2003): Prevalence and characteristics of migraine among adolescents: a questionnaire survey. Indian pediatrics Journal, 40; 665–9.

Stovner, L.J., Hagen, K., Jensen, R., & Katsarava, Z. (2007): The global burden of headache: a documentation of headache prevalence and disability worldwide. Cephalalgia. Mar, 27(3); 193-210.

Unp, A., Eray, D., & Semra, K. (2005): Prevalence and characteristics of recurrent headache in Turkish adolescents. Pediatric Neurology, 34; 110-115.

Wang, S. J., Fuh, J.L., & Juang, K. D. (2005): Rising prevalence in Taiwanese adolescents aged 13-15 years. Cephalalgia, 25, 433-438.

2/12/2012

Transport and Mechanical Properties of Silica Fume Lightweight Aggregate Concrete

Majid Matouq Assas

Civil Engineering Department, College of Engineering & Islamic Architecture, Umm al Qura University, Makkah, KSA. mmassas@uqu.edu.sa

Abstract: This paper describes the use of scoria, a natural lightweight aggregate in concrete. The purpose of this paper is to study the performance of a structural Light weight aggregate concrete up to 28 days in terms of permeability and compressive strength concrete. The concrete mixes contained silica fume at 10% replaced by weight of cementitious materials. Variables, which were taken into consideration, were the percentage of sand as a partial replacement for lightweight fine aggregate (0, 25, 50 and 75% by weight). The effect of sand replacement is considerable in terms of densities, strength-to-density ratio and chloride ion permeability. It was found that when the sand content in increased in the mixes, both the density and compressive strength were increased in value up to 30% and 27%, respectively. The resistance of scoria lightweight aggregate concrete to chloride ion penetration was increased when the silica fume was utilized in the mix. As per the assessment parameters, all the lightweight concrete containing silica fume exhibited much lower corrosion rates when compared to normal weight aggregate concrete.

[Majid Matouq Assas **Transport and Mechanical Properties of Silica Fume Lightweight Aggregate Concrete**] Life Science Journal 2012; 9(1):628-635]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 92

Keywords: Compressive strength; Lightweight aggregate; Permeability; Concrete; Silica fume.

1. Introduction:

Permeability is defined as the ease with which a particular substance (liquids, gases, ions, etc.) can flow through a solid. Defects such as those caused by settlement in fresh concrete, plastic and drying shrinkage cracks, thermal cracks, structural cracking, segregation or honeycombing of the concrete will increase the permeability rates, which in turn leads to less durable concrete. Comparative studies on the water permeability and chloride penetration of lightweight aggregate concrete (LWAC) and normal weight aggregate concrete (NWAC) were conducted [1- 3]. In these studies, W/C ratios of 0.35 and 0.55, and silica fume content of 0% and 10% were used as a partial replacement of cement by weight to obtain concrete with different compressive strength. The results indicate that the water permeability for the LWAC with a W/C ratio of 0.55 was lower than that of the NWAC. However, at a W/C ratio of 0.35 the water permeability of LWAC and NWAC was similar. The resistance of the LWAC to chloride ion penetration was similar to that of the corresponding NWAC with the same W/C ratio. Finally, the results indicated that the LWAC could have lower water permeability and better resistance to chloride ion penetration than NWAC with an equivalent 28 day compressive strength. Data on the resistance of LWAC, NWAC and high strength lightweight concrete (50-100 MPa) to water permeability and chloride penetration is presented in references [4] and [5]. Test results show that the permeability appears to be very low, but it may be higher than that of normal strength concrete having a similar strength. A direct relationship between water permeability and accelerated rate of chloride penetration was observed. Other theoretical investigations have reported a more rapid increase in permeability occurring at lower levels of applied stress-to-strength ratio with NWAC than with LWAC [6,7].

Two LWAC specimens, with 28-day cube compressive strength of 35 and 50 MPa were cast. The concrete samples made with lightweight coarse aggregates and dune sand were continuously cured in water for 1 or 7 days, and then exposed to predominantly hot and humid coastal ambient conditions containing air-borne salts. After 7 days of initial curing, and on subsequent exposure to hot and humid air, both attained a similar strength to those continuously water cured cubes at 12 months. In contrast, the water penetrability of 35 and 50MPa after 7 days of initial curing and subsequent exposure to coastal conditions was about 2 and 1.8 times the water penetration of those slabs, which were water cured for the entire duration of 12 months.

The results suggest that compressive strength is comparatively less sensitive to the curing regimes investigated. Both the chloride and sulphate penetration after 12 months exposure were found to be within tolerable limits. Also the replacement of light weight fine aggregate with normal weight sand produces a concrete that is somewhat more durable as indicated by their water penetrability and the depth of carbonation when concretes are of equal strength [8].Using natural lightweight aggregates

instead of processed artificial aggregates can significantly reduce the cost of such concretes. In this research, selected samples of these lightweight rocks were used to produce high strength LWAC. The binding medium was made of Portland cement, silica fume and super plasticizing admixture. For each concrete mixture properties at various ages, as well as splitting tensile strength, modulus of elasticity and thermal conductivity values were determined to find the optimum quantities of materials to be used. Tests showed that it is possible to produce a natural LWAC with a 28-day compressive strength of 55 MPa, a dry unit weight in the range of 1700-2100 kg/m^3 and a thermal conductivity coefficient value of about 0.55 W/(m-K)[9].

Experimental results over one year showed the benefits of incorporating LWA in the concrete, with permeability and diffusion coefficients being reduced significantly. Concrete samples were fabricated with a blended silica fume cement at a W/C ratio of W/C = 0.40 or 0.30 and with combinations of aggregate as follows: (i) limestone coarse aggregate and river sand, (ii) expanded slate coarse aggregate and river sand, or (iii) expanded slate coarse and fine aggregate. There appeared to be improvement with the maturity of the concrete and, after three years continuous curing, the reduction in the apparent chloride diffusion coefficient was observed to be as much as 70%. Also, as expected, the addition of fly ash produced further reductions in permeability and diffusion [10,11].The results of experimental study to evaluate the influence of coarse and fine LWA, the quality of the paste matrix on water absorption, permeability, and resistance to chloride ion penetration in concrete, indicate that incorporation of pre-soaked coarse LWA in concrete increases water sorptivity and permeability slightly compared to normal weight concrete of a similar W/C ratio. Furthermore, the resistance of lightweight sand concrete (LWSC) to water permeability and chloride ion penetration is inversely proportionate to the porosity of coarse LWA. With a low W/C ratio and silica fume, low unit weight LWAC (1300 kg/m^3) was produced with a higher resistance to water and chloride ion penetration compared with NWC and LWC of higher unit weights [12]. The influence of different lightweight fly ash aggregates on the behavior of concrete mixtures was investigated. In order to investigate the aggregate–cement paste interfacial transition zone (ITZ), SEM observations were performed. Regression and graphical analysis of the experimental data obtained were also performed. An increase in compressive strength was observed with the increase in oven-dry density. The ratios of splitting tensile strength to compressive strength of LWAC were found to be similar to that of NWAC. All the 28 and 56 day concrete specimens had a durability factor of 85 and 90, respectively, which met the requirement for freezing and thawing durability [13].This paper studies permeability, the effect of sand replacing and silica fume on concrete permeability. The strength development and strength-to-density ratio were also studied up to 28 days. Pozzolanic natural lightweight aggregate (scoria) occurring in the western province of Saudi Arabia was utilized**.**

2. Research Significance

There is a growing awareness of the importance of permeability with regard to the long-term durability of concrete structures. If an aggressive substance (water, sulfates, chloride ions, etc) can be kept out of concrete by virtue of low permeability, then the associated problems, such as freeze-thaw deterioration, corrosion of steel reinforcement and formation of expansive components may be mitigated. Therefore, there has been interest both in determining the permeability of lightweight concrete made from locally produced LWA compared with conventional concrete and in the development of improved concretes with lower permeability.

3. Experimental Work

The effect of sand content at 0,25,50 and 75% as a partial replacement of fine LWA and an addition of silica fume of 10% by weight of cement content on chloride ion permeability and compressive strength of lightweight aggregate concrete were studied.

4.Material properties

A pozzolanic natural LWA (scoria), occurring in the western province of Saudi Arabia, was used in this investigation. Scoria is volcanic in origin and is geologically categorized as pozzolanic scoria and tuff material. This type of LWA is available in different sizes and was used as a coarse aggregate with size varying between 5-20mm. LWA has been tested extensively at the quality control laboratories of the Saudi Arabian Standards Organization (SASO). The results show that values for coarse and combined aggregate fall within the limit specified by ASTM C-332, both for grading and unit weight tests conducted by SASO on light weight concrete panels made from LWA. It yielded a thermal conductivity value of 0.140 w/m-k (<0.43 as required by ASTM C-332). The physical properties of LWA used in this research are given in Table 1.A locally produced

ordinary Portland cement (Type I) was used in this investigation. The density of cement was 3.15 and its fineness was 3315cm^2/gm. The cement content was kept constant at 400 kg/m^3. Normal weight fine, clean sand, free from any impurities such as silt, clay organic compound aggregate was used. The fine aggregate met the ASTM C-33 requirements: The specific gravity of the sand was 2.62åand fineness modulus was 2.84. Clean, fresh water, free from impurities was used for mixing and curing the samples. Silica fume as a fine powder consisting of over 90% silicon dioxide was used. The bulk density and surface area of the used silica fume were 600 kg/m^3 and 18000-22000m^2/kg. For the concrete mixtures, a suffocated naphthalene formaldehyde condensate high-range water reducer was used (super plasticizer). The super plasticizer is available as a dark-brown aqueous solution containing 40–42% solids, with a density of 1,210 kg/m^3 and was used to control the slump at 120 mm±25 mm.

Table 1 : Physical properties of LWA.

Color	Grayish/Black
Bulk density (kg/m^3) for coarse aggregate	615-800
Bulk density (kg/m^3) for fine aggregate	850-1075
Bulk specific gravity (SSD)	1.85
Oven dry specific gravity	1.66
L-A abrasion value	28-33
Thermal conductivity of insolating concrete from cicolite	0.140 W/mk
Fire rating	3 hours
Noise attenuation	3-6 dB

5. Mix proportions

The procedures adopted for mix proportioning are still experimental in spite of considerable work done on the theoretical aspects of mix proportioning of normal weight and lightweight concretes. This is partly because LWA has a high absorption capacity and thus the W/C ratio of LWA concrete cannot be fixed. Hence, mix proportioning of lightweight concrete is generally accomplished by an experimental method. For the experiments, 24 light weight concrete mixes representing the main variables were prepared. The water binder ratio (W/B) was kept constant at approximately 0.5for all mixes, as reported in Table 2.

A concrete mixer with a maximum capacity of 0.1m^3 was used to prepare the specimens. The aggregates were used after 24hour of immersion in water in order to avoid any change of water-to-cement ratio following water absorption of aggregate during mixing. The aggregates were then dried under sun light for 120 minutes until their surface moisture became constant. The mixing sequence was as follows: firstly, coarse and fine lightweight aggregate (scoria) and 1/3 of the water were loaded into the mixer for 1 minute. Then the cement (or cement and silica fume), remaining water, and super plasticizer were added. Finally, the ingredients were mixed for 3 minutes. The mixture was rested for 3 minutes then mixed again for a further 2 minutes.

Table 2 : Mix proportions by weight for mix A.*

Sand replacement (%)	Cement (Kg)	Water (Kg)	Light weight coarse aggregate (Kg)	Light weight fine aggregate (Kg)	Normal weight fine aggregate (Kg)
0%	6	2.9	6.142	12.272	0
25%	6	2.9	6.142	9.204	4.72
50%	6	2.9	6.142	6.136	9.44
75%	6	2.9	6.142	3.068	14.16

* Mix B: silica fume were added at 10 % by weight of cement (0.6 kg).

6. Sample preparation and test method

The compressive strength of lightweight concrete samples was determined in accordance to ASTM C-39 specifications. Concrete sample cubes measuring 150-x150x150 mm were used for the compressive strength test and cylindrical samples with a diameter of 100 mm and a height of 51mm were prepared to determine chloride ion permeability of LWAC. The test method consisted of monitoring the amount of electrical current passed

through 50 mm slices of 100 mm nominal diameter cores or cylinders during a 6-hour period. A potential difference of 60 V DC was maintained across the ends of the samples, one of which was immersed in a sodium chloride solution, the other in a sodium hydroxide solution. The total charge passed, in coulombs, was found to be related to the resistance of the sample in terms of chloride ion penetration. Figure 1 shows a schematic of rapid chloride permeability test apparatus.

7. Results and Discussions

The behavior of LWAC at 28 days was investigated in terms of compressive strength and chloride ion permeability. The effect of sand content (0, 25, 50, 75%) and silica fume as an addition (10% by weight of cement content) was considered. The results were shown in Table 3 and plotted in Figures 1 to 4.

Table 3: Test results

Mix Type	Sand Replacement%	Density kg/m³	Compressive Strength MPa	Percentage Increase of compressive strength (%)	Chloride Ion Penetration, Charged Passed (Coulombs)	Strength/density
A	0	1700	16.5	0	11,567	9.7
	25	1850	19.7	20	8,870	10.6
	50	1915	24.1	46	4,165	12.6
	75	1975	29.8	80	2,147	15.1
B	0	1770	20.5	0	9,800	11.6
	25	1887	26.5	30	6,890	14.0
	50	1920	33.7	64	3,346	17.5
	75	1960	39.4	95	1,784	20.1

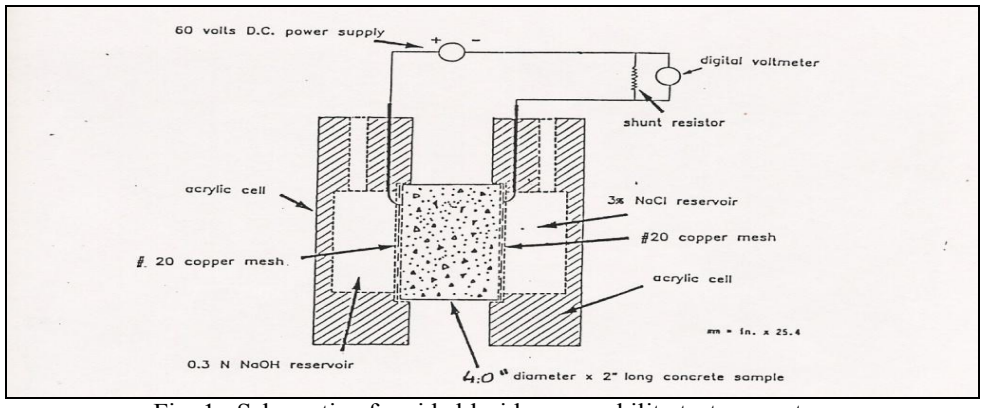


Fig. 1 : Schematic of rapid chloride permeability test apparatus.

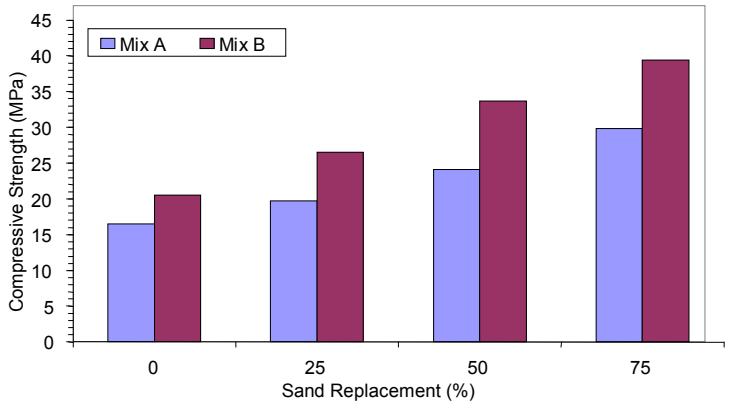


Fig. 2 Effect of Sand Content on the Compressive Strength of lightweight concrete.

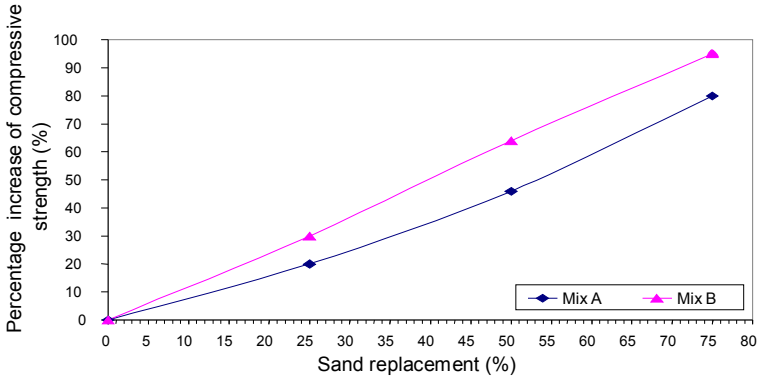


Fig. 3 Effect of Sand Content on the percentage increase of compressive strength of light concrete.

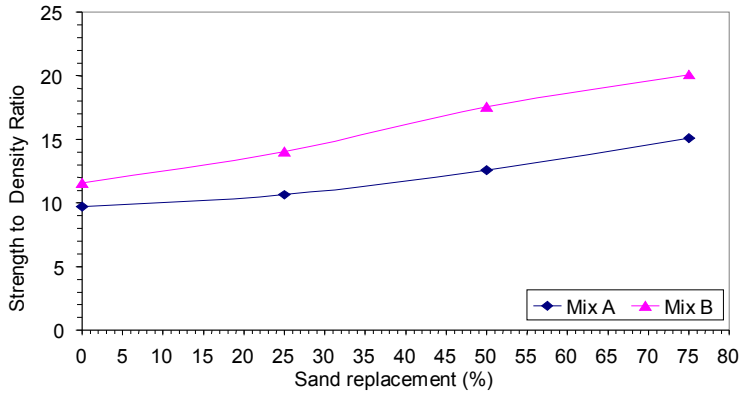


Fig. 4 Effect of Sand Content on the strength to density ratio of lightweight concrete.

8. Effect of sand content

The density, compressive strength, chloride ion permeability and strength-to-density at 28 days are given in Table 3. It can be observed from this table it was generally observed that the chloride ion permeability decreases and compressive strength increases gradually when the sand content increased up to 75%. Both compressive strength and the resistance of LWAC to chloride ion penetration were enhanced by using silica fume as an addition of 10% by weight of the cement content.

The effect of sand replacement on compressive strength is shown in Figures 1 and 2. The compressive strength increased by increasing the sand content by about 80% and 95% for mix A and mix B respectively at a level of sand replacement equal to 75% as shown in Fig.2. From the above figures, it can be observed in Figure 3 that there is a linear relationship between the percentage increase in compressive strength and the sand content percentage for all mixes. By increasing the percentage of sand from 0% to 75%, the density of concrete increased gradually in the range between 1700 and 2000 Kg/m^3. Moreover, as shown in Fig.4, the strength-to-density ratio was found to increase when increasing the sand content. These results may be attributed to the fact that concrete density was increasing when the sand content increased.

The resistance to chloride ion penetration is an important aspect that needs a better definition in relation to structural materials, which fill the pores between coarse aggregate and consequently increasing the compressive strength of concrete. It is generally accepted that mineral admixtures significantly improve this through the chloride binding and pore filling effects. The pore filling effect is expected to be the factor that helps in the case of silica fume. The charge passing through these concretes ranged from 1500 to 2000Coulombs, which is considered to be very low chloride permeability as shown in Table4. Also, the concrete made with sand showed lower chloride permeability for all types of mixes as reported in Table3. The results are similar to those observed for high-strength concrete made with expanded clay aggregates [4]. It was observed that the Coulombs charge passing through high-strength concretes containing silica fume varied from 1000 to 2000

Coulombs, which compares well with the present investigation at the corresponding w/(c + s) ratios.

Table 4: Chloride permeability classifications

Charged Passed (Coulombs)	Chloride Permeability	Typical of
> 4,000	High	High water-cement ratio (> 0.6), conventional PCC.
2,000-4,000	Moderate	Moderate water-cement ratio (0.4-0.5), conventional
1,000-2,000	Low	PCC.-
100-1,000	Very Low	Low water-cement ratio « 0.4), conventional PCC.
< 100	Negligible	Latex-modified concrete, internally sealed concrete. Polymer impregnated concrete, polymer concrete.

9. Effect of silica fumes addition

The effect of adding silica fume for different percentages of sand replacement is shown in Fig. 5. The effect of silica fume as a modification for aggregate structure or as a cementitous material was significant at 28 days. It was found that there is an interaction of both effects (physical and chemical), especially at 28 days, where silica fume had enough time to react and hydrate. It was reported that the penetration of silica fume particles into the open pores of the lightweight aggregate is deeper than that of cement, which is due to lower viscosity and the smaller particles of silica fume. Thus, silica fume will penetrate into the aggregate and result in a low dosage of cementation when compared to the mixture of Portland cement. For LWA without dense outer shells, the effect may be shown clearly, resulting in an increase in compressive strength and a decrease in chloride ion permeability.

The above-mentioned reasons may explain the behavior of lightweight concrete presented in Fig. 5 and Fig. 6. The resistance to chloride ion penetration was observed higher in lightweight concrete samples containing silica fume in mix B compared with mix A. The chloride ion permeability was nearly the same and the effect of silica fume can be negligible at higher values of sand replacement (75% sand replacement) as shown in Fig.5.

The effect of compressive strength of lightweight concrete, with and without silica fume, on the chloride permeability was plotted in Fig.6. It was found that the resistance to chloride ion penetration increased by increasing the compressive strength of lightweight concrete for all types of mixes. This may be attributed to the fact that the increase of compressive strength depends mainly on the enhancement of the matrix performance due the physical and chemical effect of silica fume and the increase of overall density of lightweight concrete by increasing the sand content.

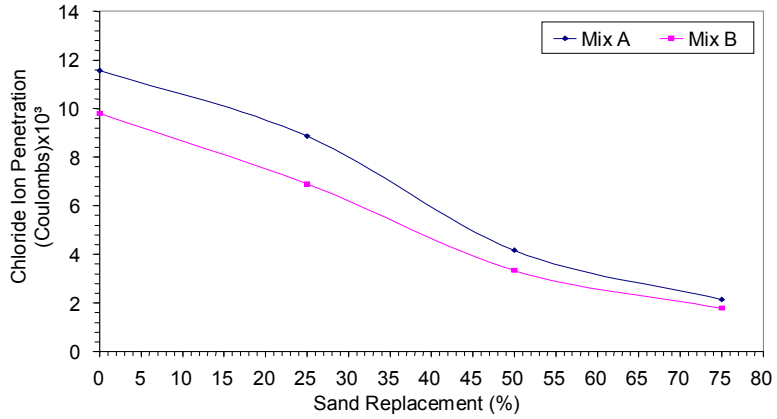


Fig. 5 Effect of silica fume content for different ratios of sand replacement on the Chloride Ion Permeability of Lightweight concrete.

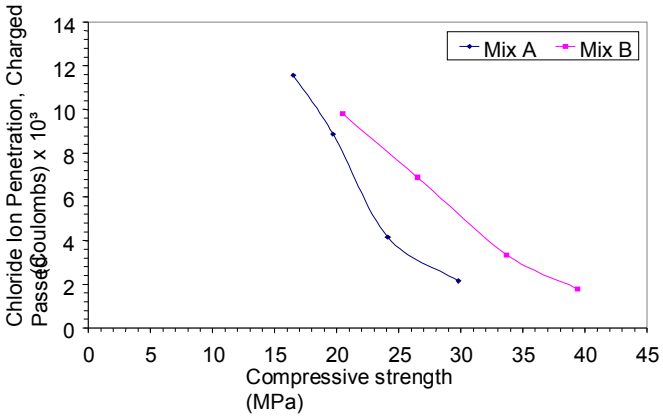


Fig. 6: Effect of Compressive strength on the Chloride Ion Permeability of Lightweight concrete.

10. Conclusions

The effect of sand replacement (0, 25, 50, and 75%) was studied in terms of density, compressive strength, strength to density ratio and chloride permeability of natural scoria lightweight aggregate concrete with and without silica fume.

The analysis of the experimental test results leads to the following conclusions:

- Socria lightweight aggregate can be used with silica fume to produce a lightweight concrete with cube compressive strength of about 33 MPa.
- The compressive strength of lightweight aggregate concretes increases when adding silica fume.
- The chloride ion permeability of concrete samples decreases when using silica fume in the mix.
- The effect of replacement LWA by natural sand gives acceptable results in terms of strength-to-density ratio, densities and chloride ion permeability of concrete.
- With the increase of sand content in the mixes, both density and compressive strength of concrete increases by 30 % and 27 % respectively.
- As per the assessment criteria, all the lightweight concrete containing silica fume showed a low chloride permeability of < 2000 C. Consequently these concretes also exhibited much lower corrosion rates compared to the normal concrete.

Corresponding author
Majid Matouq Assas
Civil Engineering Department, College of Engineering & Islamic Architecture, Umm al Qura University, Makkah, KSA
mmassas@uqu.edu.sa

References

1. Chia K.S. and M. H. Zhang (2006): Water Permeability and Chloride Penetration in Lightweight and Normal Weight Aggregate Concrete. ACI Materials Journal .,V.234, March 22, , pp:607-620.
2. Qiao X.C., B.R. Ng, M. Tyrer, C.S. Poon, C.R. Cheeseman (2008): Production of lightweight concrete using incinerator bottom ash " Construction and Building Materials, V. 22, (I. 4): April, pp. 473-480.
3. Jiang L., M.H. Zhang, and V. M. Malhotra (2004): Evaluation of Test Methods for Determining the Resistance of Concrete to Chloride- Ion Penetration. ACI Materials Journal., V.221, April 1, , pp.:1-28.
4. Song H.W., S.W. Pack, S.H.Nam (2010): Estimation of the permeability of silica fume cement concrete. Construction and Building Materials, V.24 (I.3): March, , pp: 315-321.
5. Tommy Y. Lo, H.Z. Cui, AbidNadeemand Z.G. Li (2006): The Effects of Air Content on Permeability of Lightweight Concrete" **Cement and Concrete Research,** V. 36(I.) 10, October, pp:1874-1878.
6. KokSeng Chia and Min-Hong Zhang (2002):Water Permeability and Chloride Penetrability of High-Strength Lightweight

Aggregate Concrete**. Cement and Concrete Research,** V. 32(I. 4), April, pp:639-645.
7. Min-Hong Zhang and Odd E. Gjorv (1991):Permeability of High- Strength Light weight concrete.ACI Materials Journal., V.88: (I5 September, pp.:463-469.
8. Haque M.N , H Al-Khaiat, O Kayali(2004): Strength and durability of lightweight concrete. Cement and Concrete Composites, V. 26 (I): 4, May, pp: 307-314.
9. Yeginobali A., K.G. Sobolev, S.V. Soboleva and M. Tokyay (1998): High Strength Natural Lightweight Aggregate Concrete with Silica Fume. ACI Materials Journal V.178, June 1, , pp.: 739-758.
10. Odd E. Giorv, Kefeng Tan, and Min- Hong Zhang (1994): Diffusivity of Chlorides From Sea Water into High-Strength Lightweight Concrete. ACI Materials Journal V.91, (I5) September, pp.:447-452.
11. Thomas M. D. A. (2006): Chloride Diffusion in High-Performance Lightweight Aggregate Concrete. ACI Materials Journal, V.234, March 22, , pp.: 797-812.
12. Xuemei Liu , KokSeng Chia, Min-Hong Zhang (2010): Development of lightweight concrete with high resistance to water and chloride-ion penetration. Cement and Concrete Composites, V. 32(I.10), November, pp.: 757-766.
13. Niyazi kockai , Turan Ozturan "Effects of lightweight fly ash aggregate properties on the behavior of lightweight concretes (2010): Journal of Hazardous Materials, V. 179 (I. 1-3) , 15 July, pp.: 954-965.

2/12/2012

Dose Phenol Toxicity Affected Endocrine Status in African Catfish (*Clarias gariepinus*)

Mona S. Zaki[1], Nabila El-Batrawy[2] and Nadia M. Taha[3]

[1]Department of Hydrobiology, National Research Center Dokki, Cairo, Egypt
[2]Department of Microbiology, Veterinary, Zagazig University, Zagazig, Egypt
[3]Department of Physiology, Faculty of Veterinary, Cairo University, Cairo, Egypt
dr_mona_zaki@yahoo.co.uk

Abstract: The influence of dietary phenol on immunity, and hormonal profile was studied in catfish. The results revealed that, treatment of Catfish (*Clarias gariepinus*) with 12mg/l phenol for 3 months decreased IgM, Insulin, Thyroxin, however there was elevation in cortisol hormone level. Phenol may induce an immunosuppressive effect on humoral immune response of African Catfish which was suggested by reduction of immunoglobulin.
[Mona S. Zaki, Nabila El-Batrawy and Nadia M. Taha **Dose Phenol Toxicity Affected Endocrine Status in African Catfish (*Clarias gariepinus*)].** Life Science Journal 2012; 9(1):636-639] (ISSN: 1097-8135). http://www.lifesciencesite.com. 93

Key words: Phenol; Endocrine; African Catfish **(***Clarias gariepinus***)**

1. Introduction:

Phenol and phenolic compounds are examples of toxic chemicals acts as endocrine disruptors; which mimic or antagonize hormones and disrupt the endocrine system. It is also has great potential for compromising the immune system and increases susceptibility of fish to secondary infections (Writer *et al*., 2010).

Phenols are discharged into water from the effluents of a variety of industries such as coal refineries, phenol manufacturing, pharmaceuticals, industries of resin, paint, dyeing, textile, leather, petrochemical, and pulp mill. Natural processes such as the decomposition of plant matter also contribute to phenol accumulations in the aquatic environment (BuBuikema *et al*., 1979 and Ali *et al*., 2011). Phenols are of growing concern due to their high persistent and toxicity in the aquatic environment in addition to the difficulty in detecting them given their lack of taste and odor (Tilak *et al*., 2007).Unfortunately, there is a lack of information regarding phenol pollution and its effect in the Egyptian aquatic environment.

The record level of phenol in Egyptian waste water was 0.05 ppm (Nazih *et al.,* 2008). *C. gariepinus* was extensively used as fish model by many scientists to monitor microbial, pathological or environmental studies (Ibrahem *et al.,* 2011). Unfortunately, there is a lack of information about the toxicity and pathological consequences in *C. gariepinus* exposed to phenol (Ibrahem, 2011)

2. Material and Methods

One hundred and twenty African Catfish **(***Clarias gariepinus***)** were used in the present study. Their live body weight was averaged 37.5 grams. The fish were healthy and clinically free from external and internal parasites. They were maintained in tanks containing well aerated water at atmospheric temperature for two weeks before the experiments began. Fish were randomly distributed into two groups;, each of 60 fish. Group one not given any treatment and considered a control group, the second group treated with sublethal dose of phenol at a dose level of 12mg/L (Verma, *et al*., 1980).

Analytical grade phenol, C_6H_5OH (purity 99%; E. Merck, made in Germany) was used as test chemical. Test fish were not fed from 2 d prior to the end of the experiments. The test medium was replaced in both control and experimental tanks.

The experimental fish were fed on ration composed of 16.3% crude protein, 2.5% crude fat and 14% crude fiber, the digestible energy was 26% cal/kg. The diet contained feed additives which included minerals, vitamins and amino acids. Body weight measured every month for four month.

Samples:

Serum samples were collected 4 times at one month interval and Sera were frozen at -20^{0}C for later analysis. Serum cortisol, IgM, T4, and insulin were determined using kits.

1 - IgM determination:

The serum IgM was measured according to Fuda *et al*. (1991).

1-a. Preparation of antisera:

Antisera for catfish were prepared by immunizing rabbits with catfish antigen as described by Hara (1976).

Catfish IgM antibody:

The procedure for labeling antibody fragment with enzyme was performed according to the method of Bagee *et al*. (1993).

EIISA assay procedure:

Assays were carried out in 96-well polystyrene ELISA microtiter plates (Titertex, Horsham, PA). The micortiter plates were coated with rabbit antitilapia IgM which was fractionated by DE-52 according to the method described by Bagee *et al*. (1993).

Incubation of samples and standards

After washing as described above 100 µL of sample and standard were placed into the appropriate wells in the microtiter plates and incubated at room temperature.

Incubation with peroxidase labeled antibody. After washings as described above, each well received 150µ1 of peroxidase labeled antibody 1: 1600 in PBS-BSA, followed by incubation for 12 hrs at room temperature.

Enzymatic color reaction

The plates were washed as described above and O-phenylenediamine (3mg/ml O.1M citric acid-phosphate buffer (pH 5.0) containing 0.02% H_20_2) were added to each well for enzymatic color reaction. The reaction was stopped after 30min at room temperature by adding 100ul of 4N HCL. The results were recorded at absorbance of 492nm.

Double antibody sandwich Elisa according to the method of Matsubara *et al*. (1985) was used for determination of IgM.

Cortisol was estimated using radio immunoassay technique according to the method of Wedemyer (1970) and Pickering and Potinger (1983).

Serum thyroxin was estimated using radioimmunoassay (RIA) using coat (A) count provide by diagnostic product corporation Los Angelos U.S.A. (Defetoff, 1979).

Insulin was determined by RIA according to the method described by Sundly (1991).

Statistical analysis:

The difference between the groups were calculated according to Snedecor and Cochran (1967) by t-test

3. Results

Table (1) showed the influence of phenol on IgM. Highly significant decrease of IgM levels was detected in treated group with phenol.

Table (2) showed the serum hormonal changes in infected fish treated with phenol. The results revealed decrease level of insulin, and thyroxin while a highly significant elevation of cortisol level was observed

Table (1): Effect of Phenol toxicity (12mg/l) on IgM level of Catfish (*Clarias gariepinus*)

Groups/Duration (months)	1 Month	2 Months	3 Months
Control	1.84± 0.72	1.80± 0.45	1.23± 0.40**
Phenol (12mg/l)	1.20± 0.14	1.14± 0.19**	0.98**± 0.84

**P<0.01 M Month Number of catfish each group 60

Table (2): Effect of phenol toxicity(12mg/l) on some Hormonal profile in Catfish (*Clarias gariepinus*)

	Insulin µg/dl			Thyroxin			Cortisol µg/dl		
	1M	2M	3M	1M	2M	3M	1M	2M	3M
Control	10.1 ± 0.40	10.2 ± 1.2	10.00 ± 1.50	0.0780 ± 0.065	0. 750 ± 0.059	0.740 ± 0.054	0.764 ± 0.1286	0.752 ± 0.113	0.740 ±0.103
Phenol (12mg/l)	10.00* ± 0.16	15.00 ± 0.24	18.40 ± 4.8	0.0654 ± 0.072	0.0554* ± 0.081	0.0500* ±0.044	0.870 ± 0.65	0.945* ± 0.70	0.982** ± 0.84

*P<0.05 **P<0.01 M Month

4. Discussion

IgM level was determined to find out information about catfish immune system, which was previously investigated in different species by many authors as Matsubara *et al.* (1985) and Fuda *et al.* (1991).

In this work the purified IgM revealed a single preciption in this work reacted against specific polyvalent antiserum to catfish IgM a similar result was obtained by Bagee *et al*. (1993). They found that chum salmon (IgM) was detected by specific anti (IgM) antibodies.

While the lower limit was 5 mg/ml reported, by Fuda (1991). There is a significant decrease in IgM level in fish treated with phenol in comparison with control group. Anderson *et al*. (1982) found a relation between cortisol and IgM as when cortisol increased IgM decreased.

The significant increase of cortisol level in intoxicated group with phenol could be attributed to stress factors and the intoxication has examined response of fish to stress factors e.g. crowding, continuous handling infection. Wedemyer (1970); Strange (1978); Barton *et al*. (1980) and John *et al*.

(1994),reported that the elevation of cortisol in phenol treated fish may be attributed to intoxication, and continuous handling of fish. These observations emphasizes the extreme care needed during design and analysis of experiments, involving the (HPI) axis of test fish due to extremely sensitive HPI axis. Similar results were reported by Pickering and_Pottinger (1983).

Serum thyroxin (T4) concentrations in the serum of Cat fish species decreased in the intoxicated group. It has been shown that intoxication, and chronic stress in a marked long lasting depression of serum T_4 levels in catfish (Osborn *et al*., 1978 and Milne and Leatherland, 1980). The response of thyroid gland of tested catfish needs further investigation with particular attention to possible relationship between the HPI axis and pituitary thyroid axis. Mooreoud *et al*. (1977); Osborn *et al.* (1978) and Milne and Leatherland (1980) using histological approach concluded that cortisol reduced thyroidal activity in sock eye salmon. The significant decrease of insulin values may be attributed to phenol which may somehow reduce the metabolic activities in the phenol treated catfishes. The decrease in body weight was observed, while detectable agrees with Ostrowski (1984), Hilton *et al.* (1987) and Sundly *et al*. (1991) as they observed a detectable decrease in body weight of duck infected with phenol.

The perfuse skin mucous secretion was prominent in phenol intoxicated catfish. This can be explained by the fact that skin in among the first to be in close contact with the dissolved pollutants. Hence, reactions in the skin cells are spontaneous as a protection mechanism through increasing level of mucous secretion over the body surface, forming a barrier between the body and the toxic medium, minimizing its irritation effect, thus, scavenge or even eliminates toxicants through the epidermal mucous (Chebbi and David, 2010) .

Nervous manifestation; skin expressed perfuse mucous, black patches with skin erosion and ulceration in the later stages. All observation were correlated to the time and dose exposure ((Ibrahem, 2011)

In conclusions phenol reduces of the hormonal immune response as detected by decrease of IgM level and cortisol elevation. Suppress IgM, Thyroxin *(T4)* hormone and insulin levels.

Corresponding author
Mona S. Zaki
Department of Hydrobiology, National Research Center Dokki, Cairo, Egypt
dr_mona_zaki@yahoo.co.uk

References

1. Ali, S. M., Sabac, S. Z., Fayez, M, Monib, M. and Hegazi, N. A. (2011): The influence of agro-industrial effluents on River Nile pollution. J. Adv Res.; 2: 850-895
2. Anderson, D.P.; Roberson, B.S. and Dixon, O. W (1982): Immunosuppression induced by corticosteroid or an alkylating agent in Rainbow trout. Dev, Comp. Immunol. Suppl., 2:197-204.
3. Bagee, M.; Fuda, HI; .Mara, H.; Kawamura, H. and Yamauchi (1993): Changes in serum immunoglobulin M (IgM) concentrations during early development of chum salmon as determined by sensitive Elisa technique, Comp. Biochem. Physiology, 106A:69-74
4. Barton, B.A.; Peter, R.E. and Paulence C.R. (1980): Plasma cortisol level of fingerling rainbow trout at rest and subjected to handling continent transport and stocking fish . Aqua Sci., 37: 805-811.
5. BuBuikema Jr. Al, McGinniss M. J. and Carirns Jr J. (1979): Phenolics in aquatic ecosystems: A selected review of recent literature. Mar. Environ. Res.; 2: 8181.
6. Chebbi, SG, and David M. (2010): Respiratory responses and behavioural anomalies of the carp *Cyprinus carpio* under quinalphos intoxication in sublethal doses. Sci Asia, 36: 12-7
7. Defetoff, S. (1979): Thyroid function tests endocrinology. Degvoated Philadelphia Crume and Spratton. , 1:387-428.
8. Fuda, H.; Sayano, K; Yamaji, F. and Haraj (1991) : Serum immunoglobulin M (IgM) during early development of Masu salmon on Corhyrchus masu. Comp. Biochem. Physiol., 99A: 637-643.
9. Hara, A. (1976): Iron binding activity of female specific serum proteins rainbow trout salm'o and chum salman Oncorchynchus. Journal of Biochem. Physiology, 427: 549-557.
10. Hilton, J. W.; Plisetskeya, E.M. and Leatheland, J.F. (1987): Dose oral 3, 5, 3 triiododthyroxine affect dietary glucose utilization and plasma insulin levels in rainbow trout. Fish Physiol. Biochem., 4:113-120
11. Ibrahem MD.(2011): Experimental exposure of African catfish *Clarias gariepinus* (Burchell, 1822) to phenol: clinical evalution, tissue alterations and residue assessment. Journal of Advanced Research (in press)
12. Ibrahem, M. D., Shaheed, I. B. , Abo El Yazeed H, Korani H. (2011): Assessment of the susceptibility of polyculture reared African Catfish and Nile tilapia to Edwardsiella. J. Am Sci., 7: 779-786.

13. John, F.; Carragler, and Christine, M.R (1994) : Primary and secondary stress responses in golden pereh Macquoria ambigua (J. comp. Biochem. Physiol., 107A (1): 40-56 .
14. Matsubara, A.; Mihara, S. and Kusuda, R. (1985): Quantitation of Yellow tail immunoglobulin by enzyme-linked immunosorbent assay (Flisa) Bull. Japan Sac, Sci. Fish, 51: 921-925.
15. Milne R.S. and Leatherland J. F. (1980): Changes in plasma thyroid hormones following administration of exogenous pituitary hormones and steroids hormones to rainbow trout, Comp. Biochem. Physiol., 66A: 679-686 .
16. Mooreoud, M.M, Mazeaud F & Donaldson E.M (1977) : Primary and secondary effects of stress fish some new data with a general review trans Am Fish Soc., 106:201-212 .
17. Nazih, M. Adel Haliam W, Halim H. A. S, and Abo Elaa S. (2008): Pollution control and waste minimization of chemical products industry: a case study of polymers production industry. In: Twelfth International water technology conference (IWTC12), Alexandria, Egypt, p. 415-436
18. Osborn, R.H.; Sinpson, T.H. and Yaungson, A.F. (1978): Seasonol and diurnal rhythms of thyroidal status in the rainbow traut J. Fish Biol., 12:531-540.
19. Ostrowski, M. (1984): Biochemical and physiological responses of growing chickens and ducklings of dietary aflatoxins. Comp. Biochem. Physio., 79:1, 193-204.
20. Pickering, A.D. and Pottinger, P. (1983): Seasonal and diet changes in plasma cortisol levels of the brown trout, Salmo trutta L. Gen. Corn. Endocrinol., 49; 232-239 .
21. Snedecor, G.W. and Cochran, W.G. (1967): Statistical methods Iawa State University press, Ames USA. pp 327-329.
22. Strange, R.J. (1978): Changes in plasma cortisol concentrations of juvemile salmonids during stress. Ph.D Thesis Oregan state University U.S.A.
23. Sundly, A.; Fliassen, K A. Blom, A.K. and Asyard, T. (1991): Plasma insulin, glucogen like peptide and glucose levels in response to feeding, starvation, life long restricted fed starvation in salmonids, Fish Journal of Physiol. & Biochem., 9 (3): 253-259 .
24. Tilak, K. S., Veeaiah K, and Butchiram M. S. (2007): Effect of phenol on haematological components of Indian major carps *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*. J. Environ Biol,. 28: 177-179.
25. Verma, SR. Rani, S., Tyagi AK., Dalela, RC. (1980): Evaluation of acute toxicity of phenol and its chloro-and nitro-derivatives to certain teleosts. Water Air Soil Poll., 14: 95-202
26. Wedemyer, G.A (1970):The role of stress in the disease resistance of fishes spec. Publs Am. Fish Soc., 5:30-35.
27. Writer, J. H. Barber, L. B.; Brown G. K., Taylor H. E. Kiesling, R. L., Ferrey, M. L. *et al.* (2010): Anthropogenic tracers, endocrine disruption in Minnesota lakes. Sci. Total Environ., 409: 100-111.

2/1/2012

Synergistic Effect of combined antioxidants on Noise-Induced Acoustic Trauma in Adult Guinea Pigs. Audiological and Histological Study

Nagwa Kostandy Kalleny[1], Nevine Bahaa E. Soliman[1] and Rasha Elkabarity[2]

Histology [1] and Audiology [2] Departments, Faculty of Medicine, Ain Shams University, Cairo. Egypt
nbahaasoliman@gmail.com

Abstract: Introduction: Cochlear noise injury is considered one of the most debilitating diseases worldwide. Numerous drug trials have been made for complete protection from this acoustic trauma, unfortunately with little success. Recently, drug combination has showed promising effects in treating this trauma; however, this has to be further documented by in-depth researches. **Aim of the work:** To estimate the effect of combination of the antioxidants; vitamins A, C and E, plus magnesium (A, C, E+ Mg) in either protection or treatment of noise-induced cochlear injury in adult guinea pigs. **Materials and methods:** Twenty five guinea pigs were used in this study and were divided equally into five groups. Group I served as a control group. Group II administered the drug combination for 5 successive days. Group III exposed once to 120 dBSPL octave band noise for 5 successive hours. Group IV pre-treated with vitamins A, C, E+ Mg for 5 successive days prior to noise exposure. Group V first exposed to same noise injury, and then same drug combination was administered for 5 successive days, starting one day after noise exposure. **Results:** Noise exposure resulted in profound cochlear damage. Prophylactic administration of the drug combination showed partial protection of the cochlea as detected audiologically and histologically. In contrast, significant improvement of both function and structure of the cochlea was revealed with post-treatment 1 day after noise- induced cochlear damage. **Conclusion:** Delayed treatment by this combination of drugs (vitamins A, C, E+ Mg) proved to be effective even if started one day after noise exposure. However, drug combination used as prophylactic treatment was not as effective.

[Nagwa Kostandy Kalleny, Nevine Bahaa E. Soliman and Rasha Elkabarity **Synergistic Effect of combined antioxidants on Noise-Induced Acoustic Trauma in Adult Guinea Pigs. Audiological and Histological Study**] Life Science Journal 2012; 9(1):640-653]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 94

Key words: noise- cochlea- antioxidants- magnesium- guinea pigs.

1. Introduction:

With rapid industrialization in modern society, noise pollution is an ever-increasing problem. The resulting noise induced hearing loss (NIHL) is considered one of the most common causes of hearing disabilities. Millions of workers in many industrial environments are exposed to noise induced acoustic trauma despite their usage of mechanical noise protection **(1)**. Others, as orchestra musicians **(2)** and military personnel **(3)** are also exposed to similar trauma. Furthermore, NIHL definitely has a negative effect on the quality of life **(4)** so that finding an effective protection or therapy for the noise induced cochlear damage would be extremely beneficial.

Modern researches had provided new insights for the possible mechanisms of NIHL. Oxidative stress has been widely implicated in neuronal cell degeneration **(5)** and has been well characterized after noise-induced cochlear trauma **(6)**. Therefore, antioxidant drug administration, as vitamins, has been widely proposed as a potential therapeutic intervention in acoustic trauma **(7)**. Additionally, magnesium supplements (Mg) have also been used as a potential treatment for noise trauma owing to its positive action on noise-induced micro-circularity impairment **(8)**.

Unfortunately, any single protective agent to be effective, it must be provided for long periods of time prior to noise exposure. High-dose vitamin C, as an example, did not completely prevent NIHL even with 35 days pre-treatment. Its serum level has been found to be stabilized in humans after a minimum of 3 weeks of daily intake **(9)**. Vitamin E serum levels have also been found to stabilize after over a month of daily intake in human subjects **(10)**. In addition, authors recommended using Mg with other agents to improve its therapeutic efficiency **(8)**. Emerging from the previous point, several studies showed that using combination of several agents had fast promising effects and were more effective than single agent **(6, 11, 12, 13)**. This was accompanied by confirmation of the safety of combined use of antioxidants in several studies in humans or experimental animals **(5, 11, 12)**.

Timing of intervention obviously has a key role in success of either protection or therapeutic regimen. A previous study reported that initiation of combined treatment as shortly as one hour before noise exposure failed to prevent hair cell death **(11)**. On the other hand, delayed treatment initiated 5 days post-

noise exposure has not met any mentionable success **(13)**.

So, the aim of the present study was to verify and provide further insight into the potential efficiency of combined use of vitamins A, C, E + magnesium as otoprotectants as well as a delayed therapy for noise-induced cochlear damage.

2. Materials and Methods

Twenty five adult male guinea pigs were used in this study. They were purchased and housed at the Medical Research Center, Faculty of Medicine, Ain Shams University. The animals were put in wired mesh cages with food and water *adlibitum.* They were divided equally into 5 groups as follows:

Group I: comprised 5 animals that served as a control group.

Group II: comprised 5 animals that received combined vitamin A, C, E and magnesium (A, C, E + Mg) once daily for 5 days. Details of doses and routes of administration are described later in the drug regimen.

Group III: comprised 5 animals that were exposed to noise trauma once for 5 continuous hours.

Group IV: comprised 5 animals that received vitamin A, C, E + Mg in same routes and doses as group II once daily, beginning 5 days before the day of noise exposure.

Group V: comprised 5 animals that received vitamin A, C, E + Mg in same routes and doses as group II, once daily for 5 successive days, starting one day after noise exposure.

Drug regimen (11):

-Vitamin A was given in a dose of 2.1 mg/kg/day orally by an intragastric tube (β carotene forte®, 15 mg capsules, equivalent natural vitamin A 25000 IU, Medizen Pharmaceutical Industries for Arab Co. for Pharm. & medicinal plants "Mepaco-Medifood", Enshas, Sharkeya, Egypt).

-Vitamin C was given in a dose of 71.4 mg/kg/day intraperitoneally. (Cevarol® 1000 mg ampoules, Memphis Co. for Pharmaceutical & Chemical Industries, Cairo, Egypt).

-Vitamin E was given in a dose of 26 mg/kg/day orally by an intragastric tube (Vitamin E- antioxidant 400 mg capsules, Pharco Pharmaceuticals, Alexandria. Egypt).

-Magnesium (Mg) was given in a dose of 343 mg/kg/day intramuscularly (10% magnesium sulphate, Egypt Otsuka Pharm. Co).

Audiological study:

A) Auditory Brainstem Response (ABR):

This was used to measure hearing threshold in all guinea pigs before administration of drugs or noise exposure. All recording was conducted under anesthesia by ketamine hydrochloride (Ketalar®, Sigma), 40 mg/kg **(14)** in a soundproof chamber.

Stimulus parameters:

The ABRs were generated in response to 100 μs alternated clicks at a range of 2-4 KHz. The stimulus was presented at a rate of 21 pulses / second. Monaural thresholds were obtained via headphone at 10 dB steps between 100 dBSPL down to threshold.

Recording parameters:

The ABRs were recorded by means of three platinum-iridium needle electrodes, placed sub-dermally over the vertex (positive), the mastoid (negative) and the contra-lateral mastoid (ground). The recording window included a 10-millisecond post-stimulus times. ABRs were amplified 20000-fold and filtered from 30 Hz to 3000 Hz. At least two repeatable traces with approximately 1000 response sweeps for each trace were collected for each subject. The test session including electrode application and evoked response recording for each subject lasted for about 30 minutes.

Response analysis:

The ABRs was defined by three positive peaks (I, III, V) at supra-threshold intensity (100 dB SPL). Three recording parameters were analyzed. Absolute and inter-peak latencies for wave I, III and V measured. Threshold was defined as the lowest intensity capable of producing a visually detectable, reproducible wave V.

B) Noise exposure:

All animals were housed in wired mesh cages anaesthetized, and unrestrained with free access to food and water. They were exposed to noise simultaneously at the same session to ensure the same testing environment and the same level of noise exposure. They were exposed to 120 dBSPL**,** continuous octave band noise (centered at high frequency of 4 KHZ) in a ventilated sound treated chamber via loud speakers. Sound was delivered from Single channeled audiometer (Maico). Noise was calibrated prior to testing and at the end of the experiment using sound level meter to ensure uniformity of the stimulus through the entire test that lasted for 5 continuous hours.

On day 14 after noise exposure, ABR in all guinea pigs in groups II, III, IV and V were re-measured by the same procedure. Hearing loss induced by single noise exposure in guinea pig has been found to be stabilized by 10-14 days **(10, 15)**.

Histological study:

After the final ABR measurements on day 14, all animals were anesthetized with ketamine hydrochloride, 40 mg/kg **(14)**. Transcardial perfusion was done by cold 2.0% paraformaldehyde /2.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) **(16)**. The animals were then sacrificed by decapitation. The temporal bone was taken, and the cochleae were dissected carefully. The right cochleae of all groups were processed for light microscopic examination (LM), and the left cochleae of all groups were processed for transmission electron microscopic examination (TEM). Complete infiltration of the cochlea by the proper fixative was ensured by making a tiny hole at the apex of the cochlear capsule by a curved stapes pick, and gently forcing the fixative using a fine needle fitted onto a tuberculin syringe.

A) Light microscopic study (LM):

The right cochleae were further fixed in 10% formalin for 2 days. Decalcification was then done for 4 weeks using Di-sodium EDTA. Specimens were processed to form paraffin blocks and five µm-thick serial mid-modiolar longitudinal sections were cut and subjected to Haematoxylin and Eosin (H&E) staining **(17)**.

B)Transmission electron microscopic study (TEM):

The left cochleae were further fixed in Phosphate buffered gluteraldehyde and processed to form capsules. Semi-thin sections of 1 µm were cut and stained by toluidine blue. Ultra-thin sections (50-60nm in thickness) were then cut using ultra-microtome. Sections were then mounted on copper grids and stained with saturated solution of uranyl acetate followed by lead citrate **(17)**. Stained ultra-thin sections were examined and photographed by JEOL-1010 JEM transmission electron microscope in The Regional Center for Mycology and Biotechnology, Al Azhar University.

Morphometric and Statistical study:

1- Auditory brainstem response (ABR) thresholds and threshold shifts were measured.
2- Measurements of the spiral ligament thickness. The central parts of the ligament were examined in serial H&E-stained sections from all animals.

Histological measurements were done in five high power fields /section. The measurements were performed using Image Analyzer (Olympus Image J, NIH, 1.41b, USA) in the Oral Pathology Department, Faculty of Dentistry, Ain Shams University. The standard error of means (SEM) of the audiological and histological data was calculated and statistical analysis was carried out using SPSS statistical program version 17; IBM Corporation, NY 10589. One-way analysis of variance test (ANOVA) was used to evaluate the data. Post hoc least significant difference (LSD) was used for comparison of measurements between all groups. All data were expressed as (mean±SEM). The P value considered significant when less than 0.05.

3. Results

I) Audiological results:

Twenty five adult guinea pigs were enrolled in the present study (groups I, II, III, IV and V). Prior testing, all animals showed normal mobile tympanic membranes together with normal ABR morphology and thresholds (Table 1).

Table (1): Showing the Mean ± SEM of different ABR parameters and comparison between all groups: (Before noise exposure or administration of drugs)

	Group I	Group II	Group III	Group IV	Group V
Threshold	10.00 ± 0.00 (5)	10.00 ± 0.00 (5)	10.00 ± 0.00 (5)	10.00 ± 0.00 (5)	10.00 ± 0.00 (5)
I Lat.	1.07 ± 0.03 (5)	1.18 ± 0.09 (5)	1.19 ± 0.05 (5)	1.13 ± 0.06 (5)	1.17 ± 0.04 (5)
III Lat.	2.41 ± 0.13 (5)	2.56 ± 0.05 (5)	2.47 ± 0.07 (5)	2.38 ± 0.12 (5)	2.42 ± 0.11 (5)
V Lat.	3.69 ± 0.05 (5)	3.75 ± 0.03 (5)	3.63 ± 0.06 (5)	3.68 ± 0.04 (5)	3.67 ± 0.03 (5)
V Lat Ths	4.35 ± 0.10 (5)	4.51 ± 0.08 (5)	4.35 ± 0.15 (5)	4.41 ± 0.11 (5)	4.37 ± 0.12 (5)
I-III	1.34 ± 0.14 (5)	1.38 ± 0.05 (5)	1.28 ± 0.04 (5)	1.25 ± 0.09 (5)	1.31 ± 0.06 (5)
III-V	1.28 ± 0.10 (5)	1.28 ± 0.06 (5)	1.09 ± 0.06 (5)	1.25 ± 0.06 (5)	1.27 ± 0.09 (5)
I-V	2.62 ± 0.05 (5)	2.62 ± 0.08 (5)	2.56 ± 0.05 (5)	2.51 ± 0.07 (5)	2.59 ± 0.06 (5)

-Values are mean ± SEM. - Number in parenthesis indicates the number of guinea pigs.

As shown in table (1), all Animals of all groups showed normal mean hearing thresholds with normal mean absolute latencies of waves I, III and V & normal inter-peak latencies (I-III, III-V and I-V). There was non-significant statistical difference ($p>0.05$) in all groups of the study prior to noise exposure and prior to the administration of any drug. This emphasized that all animals were normal hearers before any intervention.

Table (2): Showing the Mean ± SEM of different ABR parameters and comparison between all groups: (After noise exposure and administration of drugs).

	Group I (Control)	Group II (drug combination)	Group III (Noise)	Group IV (pre-noise treated)	GroupV (post-noise treated)
Threshold	10.00 ± 0.00 (5)	10.00 ± 0.00 (5)	63.33 ± 3.33 [a] (3)	50.00 ± 3.16 [ab] (5)	18.00 ± 3.74 [bc] (5)
I Lat.	1.07 ± 0.03 (5)	1.13 ± 0.06 (5)	1.09 ± 0.16 (3)	1.24 ± 0.12 (5)	1.14 ± 0.08 (5)
III Lat.	2.41 ± 0.13 (5)	2.44 ± 0.07 (5)	2.45 ± 0.09 (3)	2.70 ± 0.10 (5)	2.65 ± 0.17 (5)
V Lat.	3.69 ± 0.05 (5)	3.79 ± 0.07 (5)	3.88 ± 0.08 (3)	3.80 ± 0.08 (5)	3.68 ± 0.04 (5)
V Lat Ths	4.35 ± 0.10 (5)	4.49 ± 0.08 (5)	4.43 ± 0.20 (3)	4.43 ± 0.08 (5)	4.35 ± 0.05 (5)
I-III	1.34 ± 0.14 (5)	1.39 ± 0.08 (5)	1.48 ± 0.06 (3)	1.36 ± 0.07 (5)	1.34 ± 0.09 (5)
III-V	1.28 ± 0.10 (5)	1.29 ± 0.07 (5)	1.49 ± 0.11 (3)	1.31 ± 0.08 (5)	1.30 ± 0.08 (5)
I-V	2.62 ± 0.05 (5)	2.64 ± 0.05 (5)	2.78 ± 0.06 (3)	2.70 ± 0.09 (5)	2.63 ± 0.03 (5)

-Values are mean ± SEM. - Number in parenthesis indicates the number of guinea pigs.
- **a:** significance of difference by LSD from Group I (Control) at least $P<0.05$.
- **b**: significance of differences by LSD from Group III (Noise) at least $P<0.05$.
- **c**: significance of differences by LSD from Group IV (pre -treated) at least $P<0.05$.

As shown in table (2) and diagram (1), guinea pigs of group II (drug combination), showed non-significant statistical difference ($P>0.05$) in mean threshold and all other parameters means of ABR testing compared with group I (control). This documented that this drug combination is not ototoxic.

Noise exposure in group III resulted in "No Response" in 2 animals in ABR parameters, at highest threshold (70 dB). The remaining 3 guinea pigs of group III (noise-exposed) were included in the statistical different ABR parameters. They showed significant statistical difference in mean threshold parameter only ($P<0.05$) with non-significant statistical difference as regards the other parameters means of ABR testing ($P>0.05$) compared with group I (control). This documented the ototoxic effect of noise in both ears.

In-addition, guinea pigs of group IV (pre-noise treated group) showed significant statistical difference in mean threshold parameter only ($P<0.05$) with non-significant statistical difference as regards the other parameters means of ABR testing ($P>0.05$) compared with group I (control) and group III (noise-exposed). This documented the limited protective effect of combined drugs when administered prior to noise exposure on ototoxicity of noise in both ears.

On the other hand, guinea pigs of group V (post-noise treated group) showed significant statistical difference in mean threshold parameter only ($P<0.05$) with non-significant statistical difference as regards the other parameters means of ABR testing ($P>0.05$) compared with group II (noise-exposed) and group IV (pre-noise treated group). However, group V (post-noise treated group) showed non-significant statistical difference ($P>0.05$) in mean threshold and all other parameters means of ABR testing compared with group I (control). This documented the marked anti-ototoxic effect of combined drugs when administered one day after noise exposure on ototoxicity of noise in both ears.

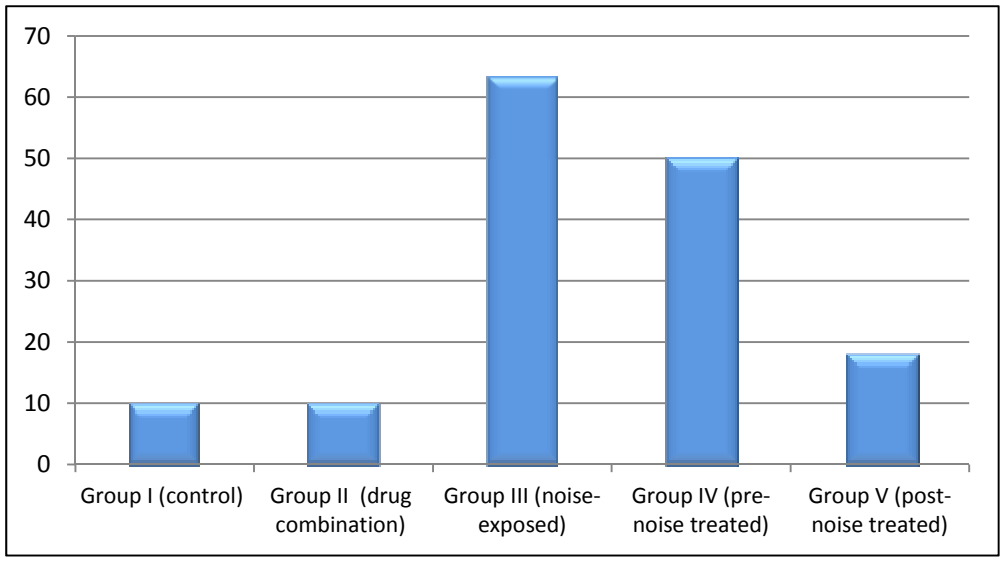


Diagram 1: Showing mean ABR thresholds after noise exposure and drug administration.

II-Histological results:

Group I *(Control animals)***:**

Examination of H&E-stained sections of this group showed the wedge-shaped cochlear duct roofed by Reissner's membrane separating it from the scala vestibuli, while the basilar membrane made its floor separating it from the scala tympani. The basilar membrane extended from the spiral lamina medially to the spiral ligament on the lateral wall, supporting the organ of Corti (Figure 1). Neuroepithelial cells of the organ of Corti were seen as three outer hair cells (OHCs) with acidophilic cytoplasm and basal rounded vesicular nuclei and one inner hair cell (IHC) with central rounded vesicular nucleus. Outer phalyngeal cells supported the OHCs and the inner phalyngeal cell was seen supporting the IHC. Outer and inner pillars surrounded the tunnel of Corti. Other supporting cells as Hensen, Claudius, Böttcher cells laterally and border cells medially could be recognized. The tectorial membrane was seen hanging as homogenous acidophilic structure (Figure 2). Stria vascularis covered the spiral ligament at the lateral wall of the cochlear duct. The three layers of strial epithelium were seen; marginal, intermediate and basal layers. The fibrous connective tissue meshwork of the spiral ligament was seen underneath (Figure 3). Mean thickness of the spiral ligament measured 23.64 ± 0.52 (mean± SEM) (Table 3 and Histogram 1).

Toluidine blue-stained sections showed the spiral ganglion neurons studded with Nissl's granules (Figure 4).

Ultrastructural examination by TEM showed the OHCs containing multiple mitochondria with apparent cristae and their nuclei showed regular chromatin pattern. Stereocilia were seen projecting from the apical surface of the cells (Figure 5). Nerve fibers of the spiral ganglion neurons showed regular myelin wrapping (Figure 6).

Group II *(animals that received drug combination)***:**

No structural and statistical differences were found in the animals' cochleae of this group compared with the control group.

Group III (*animals exposed to noise*)**:**

Examined H&E-stained sections showed that the OHCs were the most affected after noise exposure especially in the basal turn. Loss of OHCs was noticed in many sections mostly in the third row, with degeneration of the remaining OHCs (Figure 7). Other sections showed OHCs with cytoplasmic vacuolization and karyolytic nuclei (Figure 8). The IHCs were seen slightly affected showing few cytoplasmic vacuolization (Figures 7, 8). Supporting cells were seen degenerated, vacuolated. Disrupted pillar cells were frequently encountered (Figures 7, 8). Vacuolated cytoplasm of strial cells was noticed with apparent increase in melanin pigment as compared to the control. Congested capillaries could be easily noticed. The fibrocytes of the spiral ligament were seen highly vacuolated, degenerated with pyknotic nuclei (Figure 9). Statistically significant decrease in mean thickness of the spiral ligament was obvious ($P< 0.05$) as compared with the control sections, with mean thickness measuring 12.60 ± 0.70 (mean± SEM) (Table 3 and Histogram 1).

Toluidine blue-stained sections showed apparent decrease in Nissl's granules content of the spiral

ganglion neurons. The neurons were widely spaced, showed paler cytoplasm and apparent decrease in size compared with control sections (Figure 10).

Examination by TEM showed irregular, disrupted OHCs with degenerated mitochondria. Disarranged, bent or lost OHCs' stereocilia, together with shrunken electron dense nuclei were also observed (Figure 11). The IHC exhibited only few cytoplasmic vacuolization (Figure 12). Supporting cells with vacuolated cytoplasm and irregular electron dense nuclei. The heterochromatin was seen accumulated under the nuclear envelope (Figure 13). Few nerve fibers showed swelling with very thin and disrupted myelin sheath compared with the control sections (Figure 14).

Group IV (*pre-noise treated animals*)**:**

Examined H&E-stained sections of the cochleae revealed that treatment by the drug combination five days before the day of noise exposure had partial protective effect. Degenerated OHCs with pyknotic nuclei were seen in some sections (Figure 15). Other sections showed OHCs with vacuolated cytoplasm and loss of some OHCs' nuclei particularly in the third row (Figure 16). The IHC was seen apparently normal. Some supporting cells were extremely thin and degenerated with pyknotic nuclei (Figure 15), while others showed vacuolated cytoplasm (Figure 16). Cytoplasmic vacuolization could be noticed in the strial epithelial cells. The melanin content of the stria was apparently increased compared with the control. The spiral ligament showed vacuolated cytoplasm of many fibrocytes (Figure 17). Its mean thickness measured 22.47 ± 0.53 (mean±SEM) showing statistically non-significant difference ($P>0.05$) compared with the control group. However, it was significantly increased ($P<0.05$) as compared with the noise-exposed group (Table 3 and Histogram 1).

Toluidine blue-stained sections showed apparently decreased Nissl's granules content in the spiral ganglion neurons that showed paler cytoplasm compared with control. Some widely spaced Spiral ganglion neurons were seen with apparent decrease in their size compared with the control sections (Figure 18)

Examination by TEM showed some OHCs with shrunken nuclei and slightly vacuolated cytoplasm with degenerated mitochondria. The supporting cells were degenerated, electron dense and showed cytoplasmic vacuolization (Figure 19). Myelin sheath of nerve fibers showed apparently normal appearance (Figure 20).

Group V (*post-noise treated animals*)**:**

Examination of the H&E-stained sections of the cochleae showed that treatment of animals by drug combination after one day of noise exposure efficiently improved the OHCs' structure. The OHCs were seen comparable to the control appearing organized, with homogenous acidophilic cytoplasm and vesicular nuclei. The supporting cells' cytoplasm was also comparable to the control sections (Figure 21). The structure and melanin content of the stria vascularis as well as structure of the spiral ligament were apparently similar to the control (Figure 22). The mean thickness of the spiral ligament measured 22.98 ± 0.35 (mean± SEM). It showed statistically non-significant difference ($P>0.05$) compared with the control group, and with the pre-noise treated group. However, it was significantly increased ($P<0.05$) as compared with the noise-exposed group (Table 3 and Histogram 1).

Toluidine blue-stained sections showed the structure and Nissl's granules content in the spiral ganglion neurons apparently similar to the control (Figure 23).

Examination by TEM showed that the OHCs comparable to the control however, few supporting cells appeared vacuolated (Figure 24). Myelin sheaths of the nerve fibers showed similar appearance as the control sections (Figure 25).

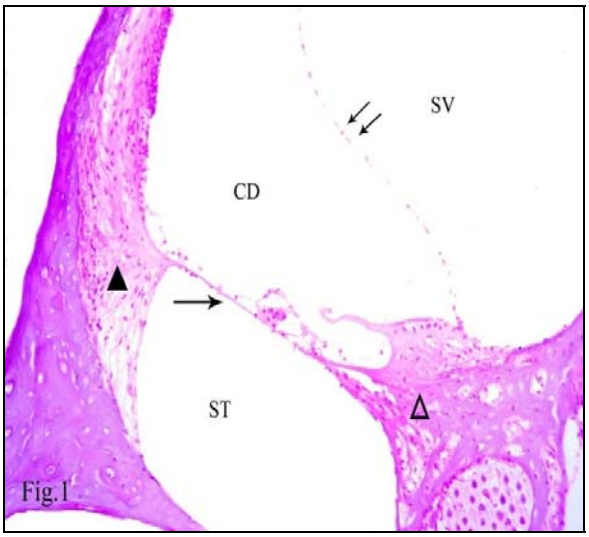


Fig. 1: Showing the wedge-shaped cochlear duct (CD) lying between the scala vestibuli (SV) above and scala tympani (ST) below it. Basilar membrane (↑) is seen extending between spiral ligament (▲) and the spiral limbus (Δ). Reissner's membrane is seen making the roof of the cochlear duct as 2 layers of simple squamous cells (↑↑). [Group I *(control)*: H&E × 250]

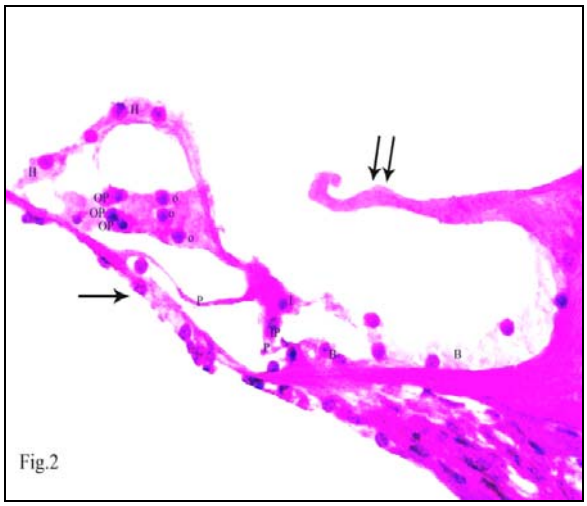


Fig. 2: Showing the organ of Corti resting on the basilar membrane (↑). OHCs (O), IHC (I), pillar cells (P) can be seen. Other supporting cells as outer phalyngeal (OP), inner phalyngeal (IP), border cells (B), Hensen cells (H) can be noticed. Notice the homogenous acidophilic tectorial membrane hanging over the hair cells (↑↑). [Group I *(control)*: H&E × 640]

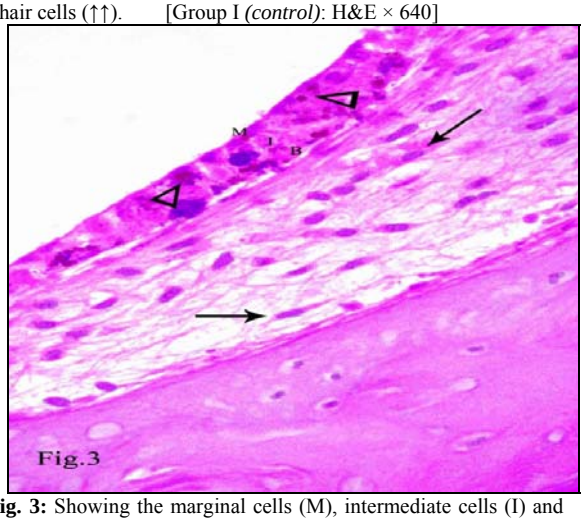


Fig. 3: Showing the marginal cells (M), intermediate cells (I) and basal cells (B) of stria vascularis epithelium. Notice the strial melanin granules (Δ) and fibrocytes of the spiral ligament (↑). [Group I *(control)*: H&E × 640]

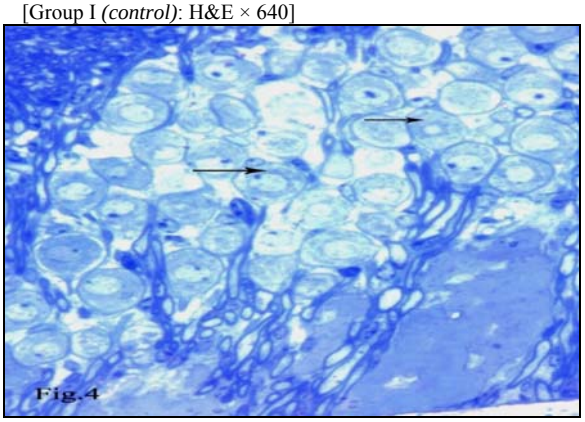


Fig. 4: Showing the spiral ganglion neurons and their Nissl's granules content (↑). [Group I *(control)*: Toluidine blue × 640]

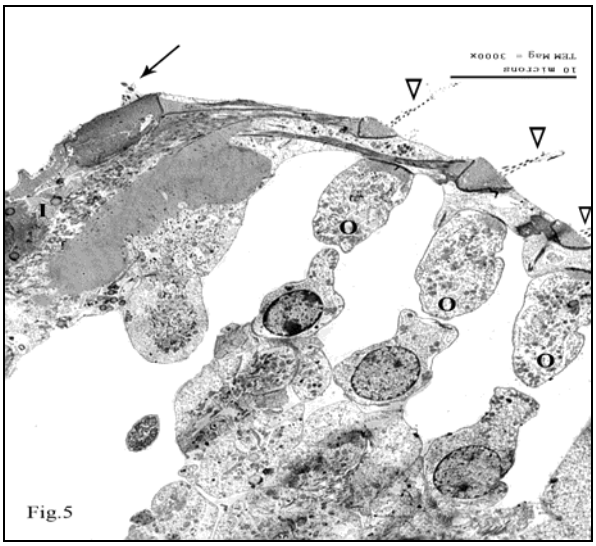


Fig. 5: Showing OHCs (O) with multiple mitochondria and apical projecting stereocilia (Δ). The IHC (I) can be noticed with its stereocilia (↑). [Group I *(control)*: TEM × 3000]

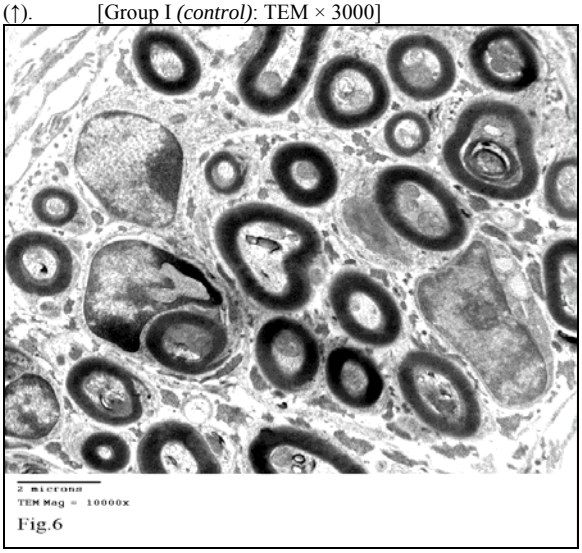


Fig. 6: Showing regular myelin wrapping of the nerve fibers. [Group I *(control)*: TEM × 10000]

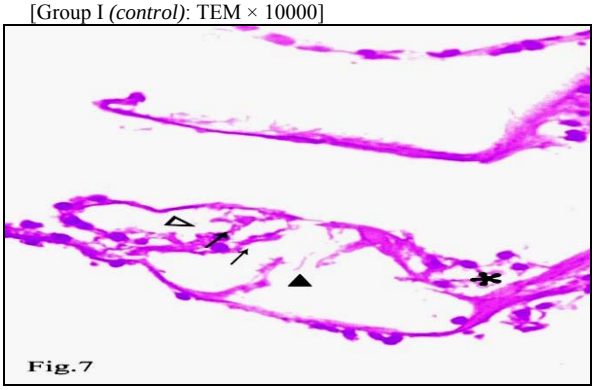


Fig. 7: Showing lost OHC in the third row (Δ) and degeneration of the other OHCs (↑). Few vacuolization of IHC can be observed (♦). Vacuolated border cells (*) and disrupted outer pillar cell (▲) can be noticed. [Group III *(noise exposed)*: H&E × 640]

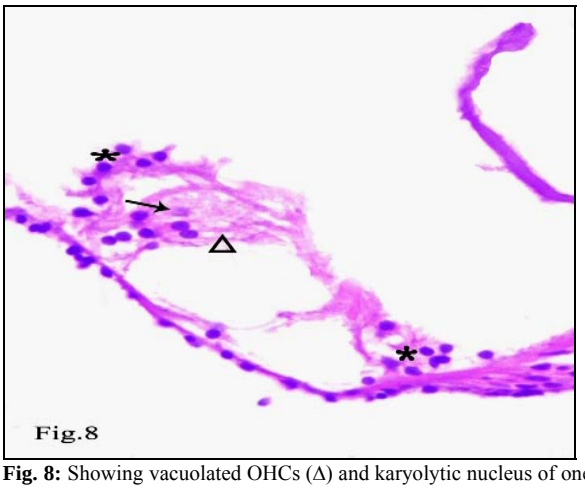


Fig. 8: Showing vacuolated OHCs (Δ) and karyolytic nucleus of one of them (↑). Notice the slightly vacuolated IHC (♦) and the degenerated supporting cells (*). [Group III *(noise exposed)*: H&E × 640]

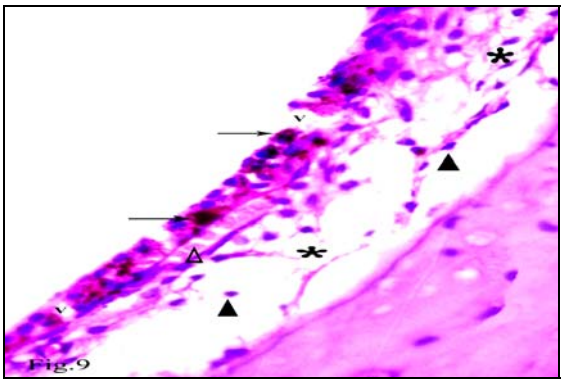


Fig. 9: Showing vacuolated strial cells (V), with apparently increased melanin content compared with the control (↑). Congested blood vessel can be seen (Δ). Apparently decreased thickness of the spiral ligament compared with control can be observed. Notice highly vacuolated (*) and degenerated fibrocytes (▲) [Group III *(noise exposed)*: H&E ×640]

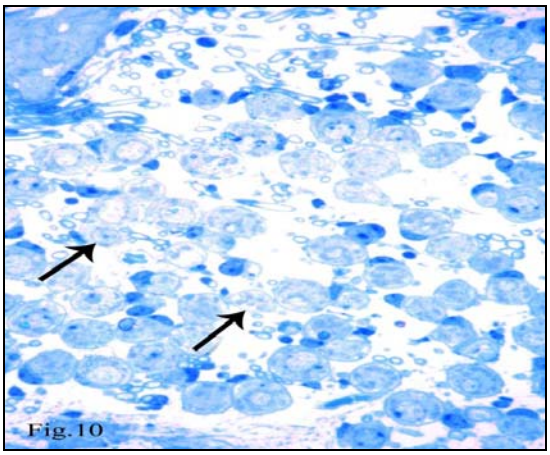


Fig. 10: Showing apparently decreased Nissl's granules content of the spiral ganglion neurons compared with the control. Notice the widely separated neurons and the apparent decrease in size of some neurons (↑). [Group III *(noise exposed)*: Toluidine blue × 640]

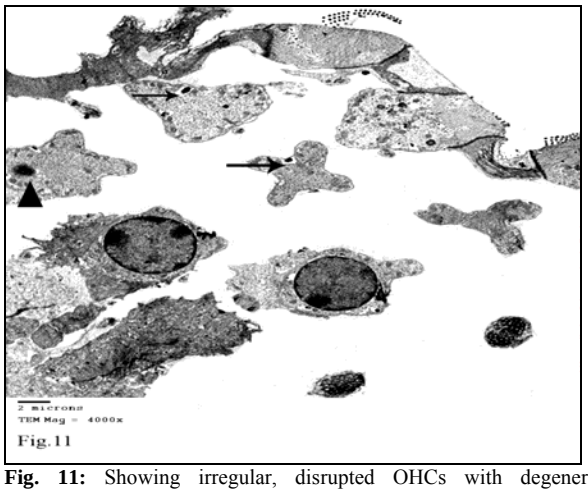


Fig. 11: Showing irregular, disrupted OHCs with degenerated mitochondria (↑) and a shrunken electron dense nucleus (▲). Disarranged, bent or lost OHCs' stereocilia can be observed. [Group III *(noise exposed)*: TEM × 4000]

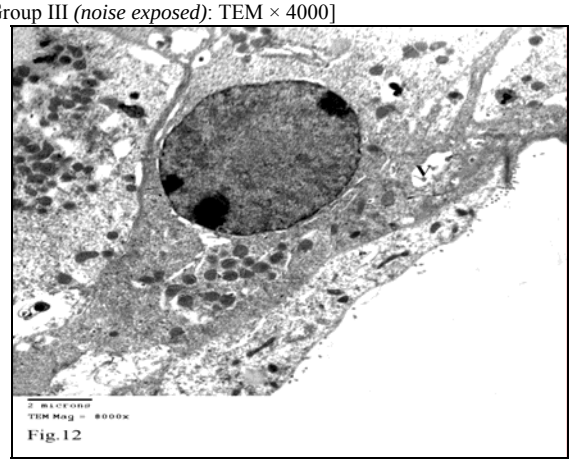


Fig. 12: Showing the IHC with few cytoplasmic vacuolization (V). [Group III *(noise exposed)*: TEM × 8000]

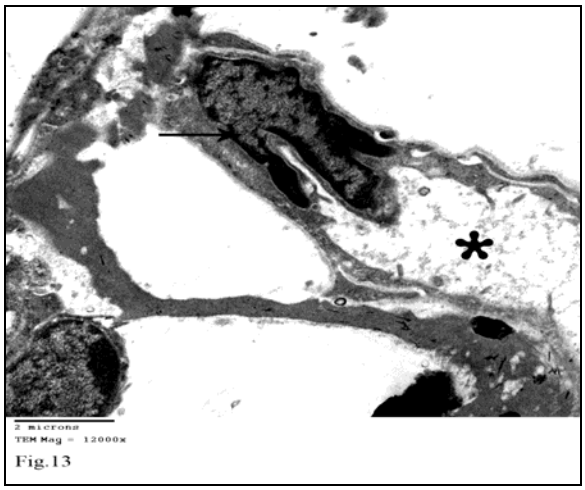


Fig. 13: Showing a supporting cell with an electron dense irregular nucleus (↑). The heterochromatin is seen accumulating under the nuclear envelope. Notice the vacuolated cytoplasm (*). [Group III *(noise exposed)*: TEM × 12000]

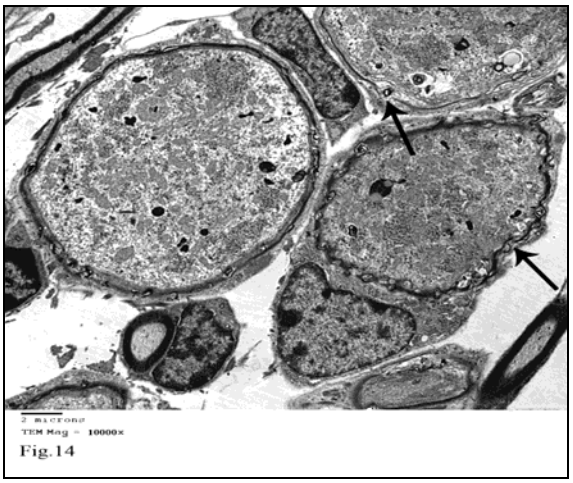


Fig. 14: Showing very thin disrupted myelin sheath (↑) wrapping the apparently swollen nerve fibers.[Group III *(noise exposed)*: TEM × 10000]

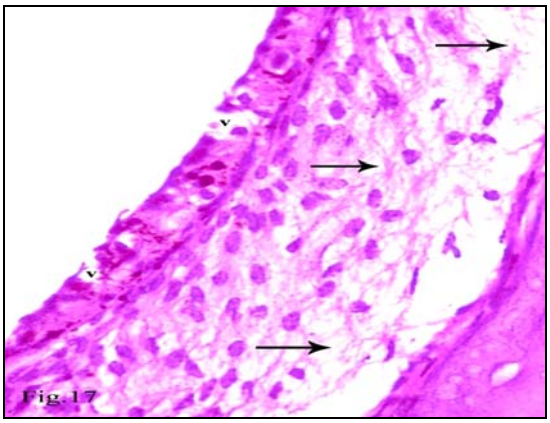


Fig. 17: Showing cytoplasmic vacuolization in the strial epithelial cells (V). The melanin content of the stria is apparently increased compared with the control. Many fibrocytes are noticed with vacuolated cytoplasm (↑). Thickness of the spiral ligament is seen comparable to the control. [Group IV *(pre-noise treatment)*: H&E × 640]

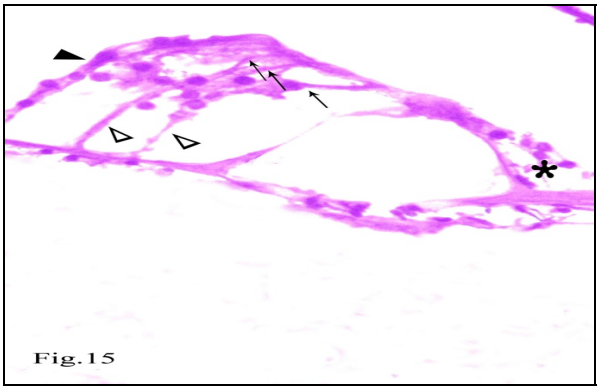


Fig. 15: Showing degenerated OHCs (↑) with pyknotic nuclei. Degenerated outer phalyngeal (Δ), Hensen's (▲) and border cells (*) can be noticed. [Group IV *(pre-noise treatment)*: H&E × 640]

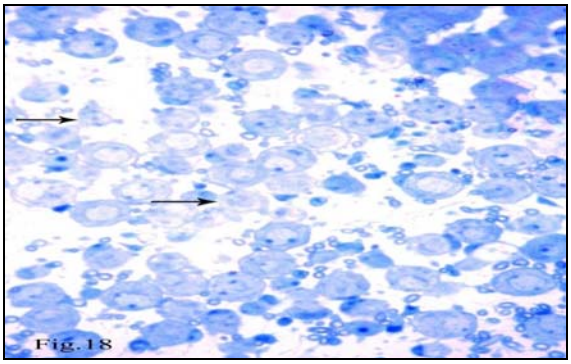


Fig. 18: Showing widely spaced spiral ganglion neuron. Notice the apparently decreased Nissl's granules content and decreased size of some neurons (↑) compared with the control. [Group IV *(pre-noise treatment)*: Toluidine blue × 640]

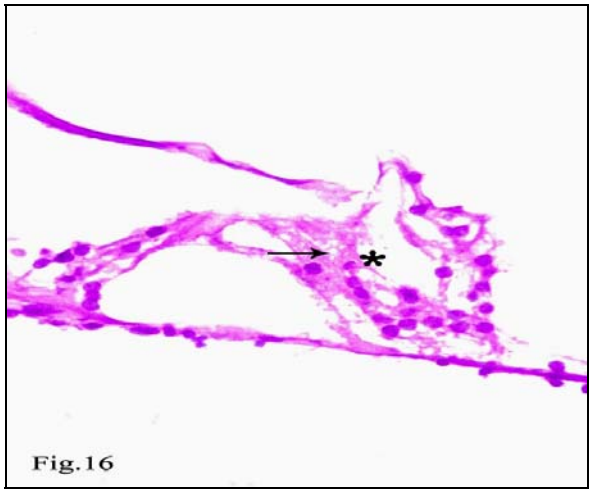


Fig. 16: Showing vacuolated cytoplasm of the OHCs (↑) with loss of OHC in the third row (*). Degeneration of supporting cells can be seen. [Group IV *(pre-noise treatment)*: H&E × 640]

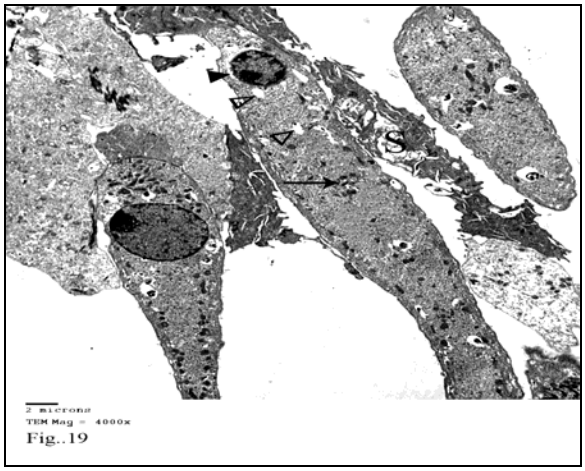


Fig. 19: Showing OHCs with slightly vacuolated cytoplasm (Δ), shrunken nuclei (▲) and degenerated mitochondria (↑). Notice the supporting cell with degenerated electron dense and cytoplasmic vacuolization (S). [Group IV *(pre-noise treatment)*: TEM × 4000]

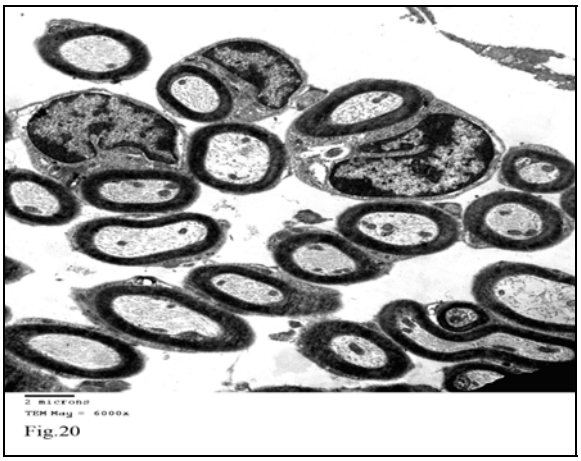


Fig.20: Showing apparently normal myelin sheath appearance. [Group IV *(pre-noise treatment)*: TEM × 6000]

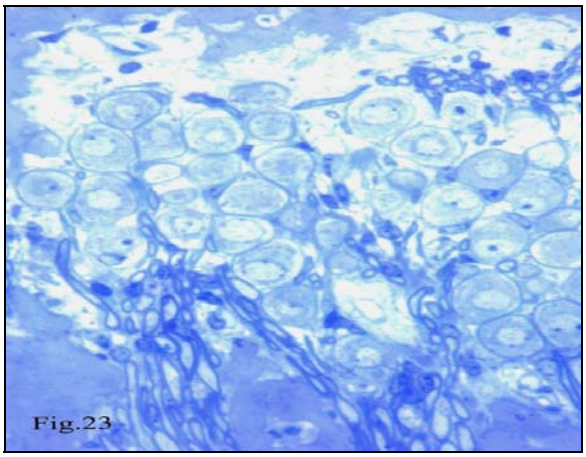


Fig. 23: Showing structure and Nissl's granules' content in the spiral ganglion neurons apparently similar to the control. [Group V *(post-noise treatment)*: Toluidine blue × 640]

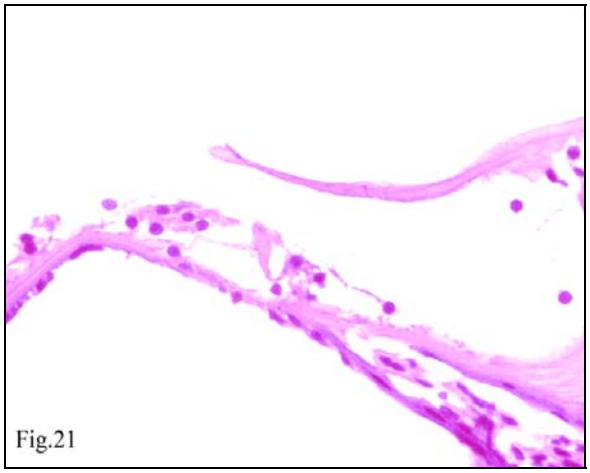


Fig. 21: Showing the hair and supporting cells comparable to the control sections. [Group V *(post-noise treatment)*: H&E × 640]

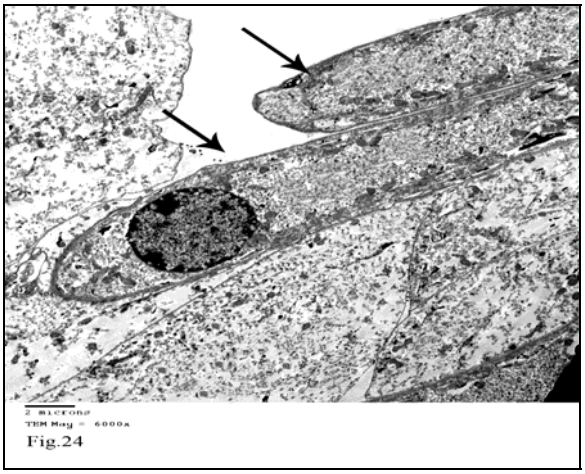


Fig. 24: Showing OHCs (↑) comparable to the control. [Group V *(post-noise treatment)*: TEM × 6000]

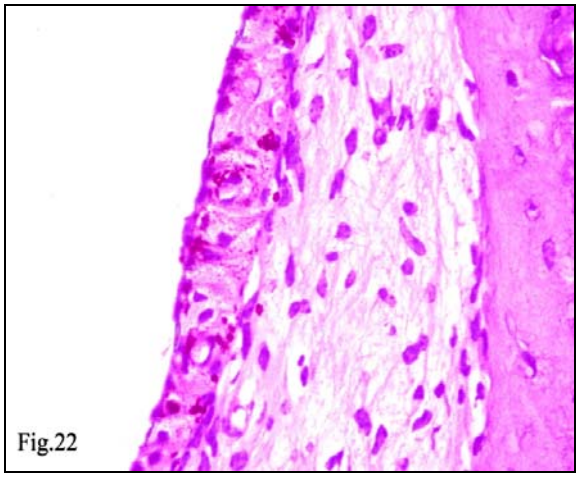


Fig. 22: Showing the stria vascularis and the spiral ligament apparently similar to the control. [Group V *(post-noise treatment)*: H&E × 640]

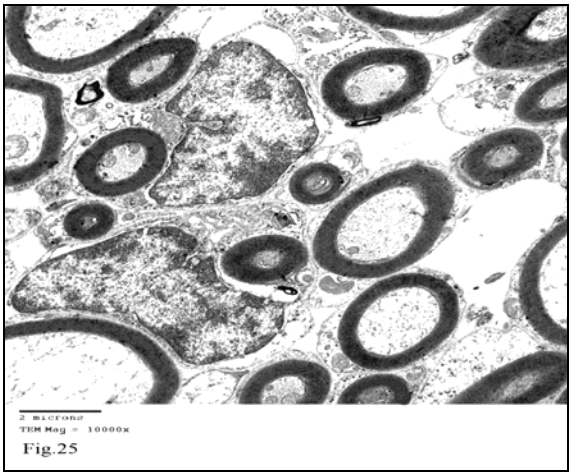
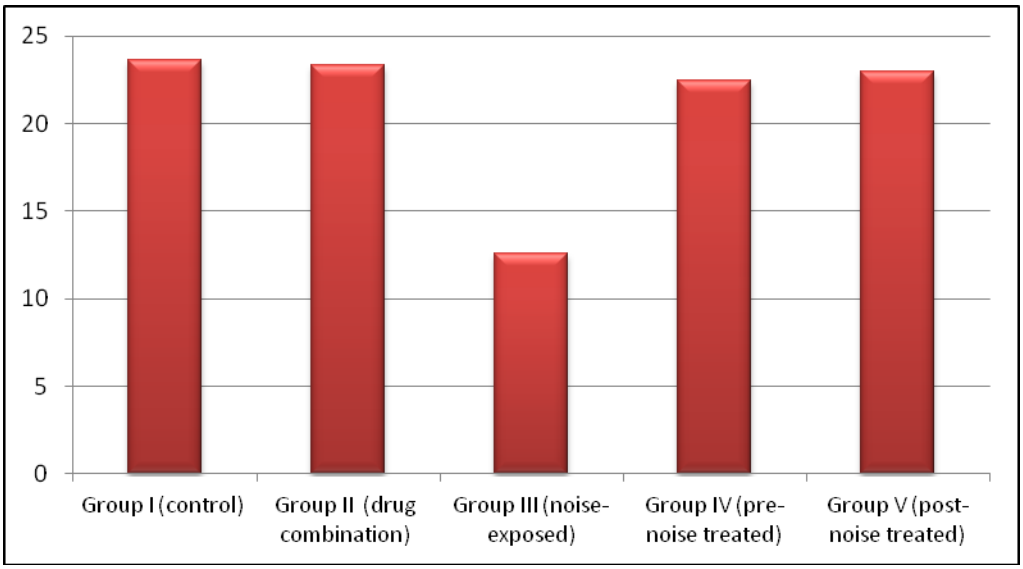


Fig. 25: Showing myelin sheath comparable to the control. [Group V *(post-noise treatment)*: TEM × 10000]

Table (3): Showing the Mean ± SEM of the thickness of the central part of the spiral ligament in μm:

	Group I (control)	Group II (drug combination)	Group III (Noise)	Group IV (pre-noise treated)	GroupV (post-noise treated)
Thickness of spiral ligament	23.64 ± 0.52 (5)	23.36 ± 0.53 (5)	12.60 ± 0.70 [a] (5)	22.47 ± 0.53 [b] (5)	22.98 ± 0.35 [b] (5)

Values are mean ± SEM. - Number in parenthesis indicates the number of guinea pigs.
- **a:** significance of difference by LSD from Group I (Control) at least $p<0.05$.
- **b**: significance of differences by LSD from Group III (Noise) at least $p<0.05$.



Histogram 1: Showing the mean thickness of the spiral ligament.

4. Discussion:

In the present study, combined drug administration had no negative effect on the cochlear function or structure. This coincides with previous studies that confirmed that this drug combination was not ototoxic **(4, 11).**

Acoustic trauma caused by noise exposure in the present study exhibited profound cochlear damage as documented audiologically and histologically. Exposure to high intense noise led to hearing loss in guinea pigs (Table 2).These findings extend the results of previous studies reporting elevated ABR thresholds and threshold shifts after noise exposure **(1)**. In agreement, other researchers reported missing OHCs **(18, 19)** and vacuolization of the sensory and supporting cells of the organ of Corti **(20)** after noise exposure. Minimal affection of the IHC was noticed in the present study coinciding with other researches reporting no loss of IHC's stereocilia with non significant loss of IHCs **(18, 21)**.

Degradation of the filamentous actin (F-actin) -a cytoskeleton protein of OHCs- has been reported after noise exposure in murine cochleae **(19).** This F-actin is primarily distributed in the stereocilia of the hair cells and found to be involved in their motility and maintenance of cell shape **(22)**. Therefore degradation of F-actin might pave the way to the disarrangement of hair cells' stereocilia noticed in the present study. Moreover, lethal alteration of cytoskeletal organization has been found to be triggered by increased Ca^{++} influx **(23)**. This might also explain the irregularly disrupted shapes of OHCs seen in the present study by TEM.

Noise-induced lateral wall histopathology found in the present study in the form of atrophic spiral ligament with vacuolated strial cells. This was in agreement with what described in previous studies which confirmed its significant contribution to noise-induced hearing loss **(16, 24).** Vacuolization of strial basal cells and type II fibrocytes was reported in a recent study. They added that degeneration of type I fibrocytes was also observed 2 weeks after noise exposure**.** It was suggested that noise exposure might lead to interruption of the cochlear ion homeostasis at multiple points along normal route of ion transfer **(16)**.

Melanocytes have been known for their action in maintaining ion homeostasis **(25)** and for their antioxidant properties by inhibition of reactive oxygen species (ROS) formation **(26)**. Other studies reported increased strial pigmentation after acoustic trauma **(27, 28).** An earlier study stated that melanin

migrated from the intermediate to the marginal layer possibly to be involved in mechanisms underlying prevention of acoustic trauma **(29)**. Therefore, increased strial pigmentation as noticed in the present study in group III could be considered a protective way against noise exposure.

Myelin sheath degradation in apparently swollen nerve fibers was observed after noise exposure in the present study. These were in accordance with other studies reporting degeneration and swelling of afferent nerves **(30, 31)**. Some authors suggested that after degeneration of hair cells, neurons lack either stimulation or neurotrophic growth factors normally provided by these cells **(32)**. Other investigators stated that noise-induced excessive release of glutamate from the damaged hair cells might be responsible for these effects **(33)**. Moreover, decreased Nissl's granules in some neurons of the spiral ganglia were also detected in semi-thin toluidine blue-stained sections in the present study. Some authors suggested that this might be a reflection of cell stress **(34)**. Moreover, it has been found to be associated with increased Ca^{++} in noise-induced damage of hippocampal cells of rats **(35)**. Other authors attributed it to decreased neuronal function by noise-induced exitotoxicity **(36)**.

Noise-induced hearing loss (NIHL) was assumed to be primarily caused by mechanical destruction of organ of Corti **(37)**. Later on, intense metabolic activity has been claimed to be the major cause of this cochlear damage. In turn, this would increase mitochondrial activity with subsequent increase in free radical formation **(38).** Both oxidative stress and mitochondrial destruction have been found to increase intracellular Ca^{++} that activates apoptosis **(39, 40)**. This might occur through an apoptotic inducer known as *BCL-2 associated death promoter* (BAD) that works via the Ca^{++}-dependant calcineurin. Next, translocation of BAD into the mitochondria might lead to the release of apoptosis-inducing factors initiating DNA fragmentation and apoptosis **(41)**. Additionally, this Ca^{++} overload could be higher in the basally-located OHCs, thereby might have intrinsically higher susceptibility to the reactive oxygen species (ROS) that was found to be increased following noise exposure **(42)**. This might explain that the findings of the present study were seen mostly in the basal turn. Oxidative stress has also been implicated in lipid peroxidation (**43)** that has been involved in the damage of proteins embedded in the cell membranes with subsequent cell death **(9)**.

Decreased blood flow of the inner ear was also implicated in NIHL **(7, 37)**. Some researchers found that noise decreased red blood cell velocity with blood stagnation in strial capillaries **(44-46)**. This coincides with the finding of congested strial capillaries observed in noise-exposed group in the present study. In addition, noise has been found to decrease blood vessel diameter due to formation of 8-isoprostaglandin $f_2 \alpha$; a vasoactive byproduct of free radicals **(7)** or due to Noise-induced hypoxia **(47).**

Most of the recent studies have focused on antioxidants in treatment of NIHL. The antioxidants properties of each of vitamins A, E and C have been well known and documented by many studies **(48, 49)**. B carotene -which is metabolized into vitamin A in vivo- has been found to scavenge singlet oxygen radicals. These free oxygen radicals are known to react with lipid to form lipid hydroxyperoxides, thus scavenging them by vitamin A prevents lipid peroxidation**.** Vitamin E as well is a well known fat-soluble antioxidant that reacts with and decrease peroxyl radicals within the cell membrane inhibiting the propagation of lipid peroxidation cycle **(49)**. In contrast to vitamins A and E, vitamin C is water-soluble antioxidant detoxifing free radicals by reducing and scavenging them in the aqueous phase **(48)**. Furthermore, the two lipid soluble vitamins E and A are complementary to each other since the antioxidant properties of vitamin E is effective at high oxygen concentrations, however that of vitamin A is effective at low oxygen concentrations. Moreover, it was reported that vitamin C could reduce vitamin E radical, thus restore back vitamin E after its possible oxidation into vitamin E radical in the oxidative attack **(50)**. In agreement the present study showed that these vitamins acted in synergism since they significantly inhibited the noise-induced acoustic trauma both audiologically and histologically particularly when administered one day post noise.

Beneficial effect of magnesium (Mg) on noise trauma have been well documented, however, the specific molecular mechanisms underlying this effect have not yet been explained. It seems to be a complex process, including protection against impairment of cochlear microcirculation and oxygenation **(8)**. Previous studies reported decrease in noise-induced vasoconstriction increasing blood flow to the cochlea **(51, 52).** In addition, Mg has a calcium antagonistic action by modulating Ca^{++} channels permeability, decreasing Ca^{++} influx into the cochlear cells, reducing NIHL **(52)**. Magnesium also may have an otoneuroprotective effect via N-methyl-D-aspartate receptor antagonism. Hence, in this way Mg reduces intracellular glutamate release that is responsible for noise-induced neuronal damage **(53)**. Furthermore, Mg may also act as an antioxidant protecting the cochlea against free radicals which

have been shown to be partly responsible for noise-induced hearing loss **(54)**.

The mechanisms of noise injury include parallel pathways that are not limited to oxidative stress. Thus, therapies combining multiple antioxidants may have advantages over single-agent approaches. Several trials using combination therapies have demonstrated clear additive and beneficial interactions against different damaging mechanisms in NIHL **(4, 6, 11)**.

In the present study, the combination of drugs taken one day after noise exposure were more effective than pre-treatment 5 days pre-noise. These findings were matched audiologically and histologically. In agreement, previous studies reported that administration of drugs shortly before noise exposure failed to prevent hair cell death in mice **(4, 11)**. However, some authors reported that pre treatment with drug combination decreased the loss of fibrocytes in the lateral wall of the cochlea **(5)**. This coincides with the results of the present study reporting nearly similar thickness and structure of the spiral ligament in the pre-noise treated group as compared to the control sections. Better hearing outcomes were reported with treatment by drug combination initiated 1 and 3 days post-noise. This was evidenced by significant functional recovery and reduction of acute temporary threshold shifts in guinea pigs **(12)**. Previous investigations documented the presence of late-forming free radicals in NIHL. Several studies reported that noise-induced oxidative stress begins early and became substantial with time **(15, 55, 56)**. This would potentially explain observations of hair cell death that accelerates with time up to 14 days after noise exposure. Using 4-hydroxynonenal, it was confirmed that peak ROS production in cells in the organ of Corti occurs 7–10 days post-noise, and that noise-induced hair cell death is similarly delayed **(15)**. It could be assumed that pre-treatment with a variety of scavengers reduced the early formation of free radicals that has been well characterized by previous investigators **(38, 56)**. However, it would be more effective if these drugs continued for 5 more days after noise exposure. This also could justify why post-noise treatment was more effective than pre-noise administration of drugs in the present work.

Conclusion:

The results of the present study provide a window of opportunity for rescue from noise cochlear trauma. It also provides morphological and functional evidences that delayed treatment one day after noise exposure may have beneficial clinical outcomes especially after unexpected noise exposure.

Corresponding author:
Nevine Bahaa E. Soliman
Histology Department, Ain Shams University,
Cairo, Egypt. nbahaasoliman@gmail.com

References:

1- Lorito G, Giordano P, Prosser S, Martini A, Hatzopoulos S (2006). Noise-induced hearing loss: a study on the pharmacological protection in the Sprague dawley rat with n-acetyl-cysteine. Acta Otorhinolaryngol Ital.; 26: 133-139.

2- Raymond DM 3rd, Romeo HJ, Kumke Kv (2012). A pilot study of occupational injury and illness experienced by classical musicians workplace health saf;60(1):19-24.

3- Heupa AB, Gonçalves CG, Coifman H. (2011). Effects of impact noise on the hearing of military personnel. Braz J Otorhinolaryngol;77(6):747-753

4- Tamir S, Adelman C, Weinberger JM, Sohmer H (2010). Uniform comparison of several drugs which provide protection from noise induced hearingloss. J Occup Med Toxicol.;5:26

5- Le Prell CG, Gagnon PM, Bennett DC, Ohlemiller KK (2011). Nutrient-enhanced diet reduces noise-induced damage to the inner ear and hearing loss. Transl Res.; 158(1):38-53.

6- Le Prell CG, Yamashita D, Minami S, Yamasoba T, Miller JM (2007). Mechanisms of noise-induced hearing loss indicate multiple methods of prevention. Hear Res;226(1-2): 22–43.

7- Miller JM, Brown JN, Schacht J. (2003). 8-iso-prostaglandin F (2 alpha), a product of noise exposure, reduces inner ear blood flow. Audiol Neurootol.; 8:207–221.

8- Scheibe F, Haupt H, Mazurek B, Konig O (2001). Therapeutic effect of magnesium on noise-induced hearing loss. Noise & Health; 3(11): 79-84

9- Levine M, Conry-Cantilena C, Wang Y, Welch RW, Washko PW, Dhariwal KR, Park JB, Lazarev A, Graumlich JF, King J, Cantilena LR. (1996). Vitamin c pharmacokinetics in healthy volunteers: Evidence for a recommended dietary allowance. Proc Natl Acad Sci USA; 93:3704–3709.

10- Kappus H, Diplock AT. (1992). Tolerance and safety of vitamin e: A toxicological position report. Radic Biol Med.; 13:55–74.

11- Le Prell C G, Hughesb L F, Miller J M (2007). Free radical scavengers, vitamins A, C, and E, plus magnesium reduces noise trauma. Radic Biol Med;42(9): 1454–1463.

12- Le Prell CG, Dolan DF, Bennett DC, Boxer PA. (2011). Nutrient plasma levels achieved during treatment that reduces noise-induced hearing loss. Transl Res;158(1):54-70.

13- Yamashita D, Jiang HY, Le Prell CG, Schacht J, Miller JM (2005). Post-exposure treatment attenuates noise induced hearing loss. Neuroscience; 134:633 -642.

14- Dang V, Bao S, Ault A, Murray C, Mills JM, Chiedi C, Dillon M, Todd JP, DeTolla L, Rao S (2008). Efficacy and safety of five injectable anesthetic regimens for chronic blood collection from the anterior vena cava of guinea pigs. Journal of the American Association for Laboratory Animal Science; 47(6):56-60.

15- Yamashita D, Jiang H, Schacht J, Miller JM (2004). Delayed production of free radicals following noise exposure. Brain Res.; 1019:201–209.

16- Ohlemiller KK, Rosen A D, Gagnon PM (2010). A Major Effect QTL on Chromosome 18 for Noise Injury to the Mouse Cochlear Lateral Wall. Hear Res.; 260(1-2): 47–53

17- Bancroft JD and Gamble M (2008). Theory and Practice of Histological Techniques. 6th edition. Churchill Livingstone ElSevier;126: 601.

18- Fetoni Ar, Ralli M, Sergi B,.Parrilla C, Troiani D, Paludetti G (2009). Protective effects of N-acetylcysteine on noise-

induced hearing loss in guinea pigs. Acta Otorhinolaryngologica Italica;29:70-75.
19- Lim HW, Choi SH, Kang HH, Ahn JH, Chung JW (2008). Apoptotic Pattern of Cochlear Outer Hair Cells and Frequency-specific Hearing Threshold Shift in Noise-exposed BALB/c Mice. Clinical and Experimental Otorhinolaryngology; 1(2): 80-85.
20- Fridberger A, Flock A, Ulfendahl M, Flock B (1998). Acoustic overstimulation increases outer hair cell Ca2+ concentrations and causes dynamic contractions of the hearing organ. Proc Natl Acad Sci USA; 95(12):7127–7132.
21- Ye Q, Ren X, Tang J (1998). effects of exposure to noise in oil drilling well sites on cochlea of guinea pigs. Chung Hoa Yu Fungi H Such Tsa Chih; 32(2): 103-5 (abstract).
22- Asumendi A, Andollo N, Boyano MD, Hilario E, Perez-Yarza G, Atencia R, *et al.* (2000). The role of cleavage of cell structures during apoptosis. Cell Mol Biol (Noisy-le-grand); 46(1):1-11.
23- Hu BH, Henderson D, Nicotera TM (2002). F-actin cleavage in apoptotic outer hair cells in chinchilla cochleas exposed to intense noise. Hear Res.;172:1–9.
24- Hirose K**,** Liberman MC **(**2003). Lateral Wall Histopathology and Endocochlear Potential in the Noise-Damaged Mouse Cochlea. J Assoc Res Otolaryngol.; 4(3): 339–352.
25- Cullington HE (2001). Light eye colour linked to deafness after meningitis. British Medical Journal; 322:587.
26- Xiong M, He Q, Lai H, Wang J (2011). Oxidative stress in spiral ganglion cells of pigmented and albino guinea pigs exposed to impulse noise. Acta Otolaryngol.; 131(9):914-20.
27- Meyer zum Gottesberge AM (1988). Physiology and pathophysiology of inner ear melanin. Pigment Cell Res.;1: 238–249.
28- Da Costa EA, Castro JC, Macedo ME. (2008). Iris pigmentation and susceptibility to noise-induced hearing loss. International Journal of Audiology; 47:115–118.
29- Kawaguchi K (1992): Susceptibility of organ of Corti with or without melanin to acoustic overstimulation. Nihon Jibiinkoka Gakkai Kaiho;95(4):556-66. [abst.]
30- Eybalin M (1993). Neurotransmitters and neuromodulators in the mammalian cochlea. Physiol Rev.; 73:309 –373.
31- Ruan F, Wang H, Gao W, Ji H, Xiao J, Wang F, Liu C, Pan X, Zhang L (2000): Amelioration of nerve growth factor against noise-induced threshold shift: a transmission electron microscope observation. Zhonghua Er Bi Yan Hou Ke Za Zhi;35(4):267-70. [abst]
32- Hansen MR, Zha XM, Bok J, Green SH (2001). Multiple distinct signal pathways, including an autocrine neurotrophic mechanism, contribute to the survival-promoting effect of depolarization on spiral ganglion neurons *in vitro*. Journal of Neuroscience; 21:2256–2267.
33- Zhang YM, Ma B, Gao WY, Wen W, Liu HY (2007). Role of glutamate receptors in the spiral ganglion neuron damage induced by acoustic noise. Sheng Li Xue Bao; 59(1):103-10.
34- Gao X, Deng P, Xu Z C, Chen J (2011). Moderate Traumatic Brain Injury Causes Acute Dendritic and Synaptic Degeneration in the Hippocampal Dentate Gyrus. Plos One; 6 (9): e24566
35- Cui B, Wu MQ, She XJ, Liu HT (2009). Effects of noise exposure on event-related potential P300 and mechanism in hippocampus of rats. Zhongguo Ying Yong Sheng Li Xue Za Zhi;25(3):404-7
36- Cui B, Wu M, She X (2009). Effects of chronic noise exposure on spatial learning and memory of rats in relation to neurotransmitters and NMDAR2B alteration in the hippocampus. J Occup Health; 51(2):152-8.
37- Mulroy MJ, Henry WR, McNeil PL (1998). Noise-induced transient microlesions in the cell membranes of auditory hair cells. Hear Res.; 115:93–100.
38- Henderson D, Bielefeld EC, Harris KC, Hu BH (2006). The role of oxidative stress in noise-induced hearing loss. Ear Hear; 27(1):1–19.
39- White BC, Sullivan JM, DeGracia DJ, O'Neil BJ, Neumar RW, Grossman LI, Rafols JA, Krause GS (2000). Brain ischemia and reperfusion: molecular mechanisms of neuronal injury. J Neurol Sci.; 179:1–33.
40- Stavrovskaya IG, Kristal BS (2005). The powerhouse takes control of the cell: Is the mitochondrial permeability transition a viable therapeutic target against neuronal dysfunction and death? Radic Biol Med.; 38:687–697.
41- Vicente-Torres MA, Schacht J (2006). A BAD link to mitochondrial cell death in the cochlea of mice with noiseinduced hearing loss. J. Neurosci. Res.; 83:1564–1572.
42- Sha S-H, Taylor R, Forge A, Schacht J. (2001): Differential vulnerability of basal and apical hair cells is based on intrinsic susceptibility to free radicals. Hear Res.; 155:1–8.
43- Ohinata Y, Miller JM, Altschuler RA, Schacht J (2000). Intense noise induces formation of vasoactive lipid peroxidation products in the cochlea. Brain Res.; 878:163–173.
44- Quirk WS, Avinash G, Nuttall AL, Miller JM (1992). The influence of loud sound on red blood cell velocity and blood vessel diameter in the cochlea. Hear Res.; 63:102–107.
45- Quirk WS, Seidman MD (1995). Cochlear vascular changes in response to loud noise. Am J Otol.; 16:322–325.
46- Miller JM, Yamashita D, Minami S, Yamasoba T, Le Prell CG (2006). Mechanisms and prevention of noise induced hearing loss. Otol Jpn; 16:139–153.
47- Nuttall AL (1999). Sound-induced cochlear ischemia/hypoxia as a mechanism of hearing loss. Noise Health, 2:17–31
48- Niki E (1991). Action of ascorbic acid as a scavenger of active and stable oxygen radicals. Am J Clin Nutr.; 54:1119S–1124S.
49- Schafer FQ, Wang HP, Kelley EE, Cueno KL, Martin SM, Buettner GR (2002). Comparing beta-carotene, vitamin E and nitric oxide as membrane antioxidants. Biol Chem.; 383:671–681.
50- Murray RK, Granner, DK, Mayes PA, Rodwell VW (1996): Harpers Biochemistry. 24th edition. Appleton and Lange, Norwalk, Connectuit/ Los Altos, California.
51- Haupt H, Scheibe F (2002). Preventive magnesium supplement protects the inner ear against noise-induced impairment of blood flow and oxygenation in the guinea pig. Magnes Res.; 15:17–25.
52- Cevette MJ, Vormann J, Franz K (2003). Magnesium and hearing. J Am Acad Audiol.; 14:202–212.
53- Ehrenberger F, Felix D (1995): Receptor pharmacological models for inner ear therapies with emphasis on glutamate receptors: a survey. Acta Otolaryngol (Stockh); 115: 236-240.
54- Hoane MR, Raad C, Barth TM (1997). Non-competitive NMDA antagonists and anti-oxidant drugs reduce striatal atrophy and facilitate recovery of function following lesions of the rat cortex. Restor Neurol Neurosci.; 11: 71-82
55- Bohne BA, Harding GW, Nordmann AS, Tseng CJ, Liang GE, Bahadori RS (1999). Survival-fixation of the cochlea: a technique for following time-dependent degeneration and repair in noise-exposed chinchillas. Hear Res.; 134:163–178.
56- Ohlemiller KK, Wright JS, Dugan LL (1999). Early elevation of cochlear reactive oxygen species following noise exposure. Audiol Neurootol.; 4:229–236.

Effective Use of Technology to Convert Waste into Renewable Energy Source

Neeha Farouqe, Shafqat Hameed

National University of Sciences and Technology (NUST), Islamabad, Pakistan.
Hameed.shafqat@yahoo.com, shafqat.hameed@ceme.nust.edu.pk

Abstract: As energy demand is increasing in the world, renewable energy sources are utilized. Biogas is a renewable energy source which helps us in conserving fossil fuel. There are favourable scenarios for using biogas energy in rural areas through an arrangement of community biogas plants. This technology is not only used in houses for lightening and cooking purposes but it also serves as a good fertilizer. It can also be used in various automotive applications. This study proposes the method to convert animal waste into biogas.
[Neeha Farouqe, Shafqat Hameed. **Effective Use of Technology to Convert Waste into Renewable Energy Source.** Life Science Journal. 2012;9(1):654-661] (ISSN:1097-8135). http://www.lifesciencesite.com. 95

Keywords: Biogas, Waste, Renewable energy, technology

1. Introduction

Energy is an absolute need of everyday life .It is used in the form of light, heat and electricity. Pakistan is facing serious energy crisis in gas and electricity, because no steps have been taken for a long time to increase the capacity of energy resources. Observation indicates that earth's average temperature is increased by 0.6°C during the last century (Wong, 2011). It has caused serious problems like sea-level rise, threats to food supplies and human health .Human activities like consumption of fossil fuels are the cause of global warming that occurred in last 40-50 years. As a result, levels of carbon dioxide are increasing rapidly. Therefore, government should look for some alternative energy sources. Alternative energy sources are now become important to cater for energy crisis. These alternative resources include sun, wind, water, biomass etc and are known as renewable energy sources.

1.1 Waste to Energy

Waste is any unwanted or undesirable material. Waste to energy conversion technologies have the potential to get renewable energy sources from waste including municipal solid waste, industrial waste or animal waste. It utilises the waste that would have been disposed off in the lands. There is an obvious need to stop generating this waste and to recycle it. Thus by converting waste into energy we can get rid of pollution from landfills and can make use of lands which would result in safe and secure environment. Moreover, the heat produced can be used to generate electricity.

1.2 Renewable Energy Sources and Biogas

Renewable energy is obtained from natural resources like wind, sun etc. It is a form of energy which is replaced by natural resources. As these resources are obtained from natural processes, thus are more reliable and cost effective. It offers excellent benefits to meet energy crisis. Renewable energy is clean and carbon dioxide free source. Globally, 16% of total energy consumption comes from renewable sources, with 10% comes from biomass, and 3.4%from hydroelectricity. The renewable power capacity globally has now reached 200 GW and by 2030, the overall demand is predicted to almost double the current levels (Energy Business Reports August 2008). It plays a vital role to fulfil energy demands. Biogas is a renewable energy source, produced by anaerobic digestion of recyclable materials like municipal solid waste, animal manure, Biomass etc. (NNFC Renewable fuels and energy Facts sheet, 2011). It is mainly composed of carbon monoxide and oxygen with few amounts of Hydrogen sulphide, moisture etc. The gases released after combusting with oxygen are used as a fuel. It may also serve as a source of electricity after proper treatment. After compression it can replace CNG and can be used as fuel in vehicles. It protects the environment from adverse affects of gases emission due to climate changes. Thus farmers can use animal waste as a source of energy and fertilizer as well.

1.3 Biogas Production Aspects in Pakistan

Renewable energy source is very helpful in Pakistan to meet the energy demand of people, special living in rural areas. People in these areas belongs to agriculture thus keep livestock with them. There are 62.9 million of animals (Buffaloes, Cows, Bullocks) in Pakistan (Arif, 2008-09) .If on average 50% waste is obtained from these livestock, then millions of cubic meters biogas can be produced. Thus the land which would be used for digging the waste could be used for agriculture purposes. Cost of producing the biogas is very low. If plants are also included in biogas production process then the residue can be recycled as good quality fertilizer in

the farming. Farmers do not need to buy any fertilizer. When Biogas is produced from the grass, it can help in setting the structure of landscapes.

1.4 Research Question

The main focus of this research is to address the following research question:

How the effective and efficient use of technology can help to meet the energy demand by converting livestock waste into Biogas?

1.5 Objective

With conversion of animal waste to biogas, the manure can not only be used as a source of energy but also to reduce the adverse effects of waste on environment. The objective of this paper is to design a biogas production process from animal wastes and to propose an environment friendly and safe method to treat waste. Thus the harmful effects of waste on environment can be mitigated and this waste can be best utilized to meet energy demand. It is an economical process as no extra labour is required for this purpose. During processing, waste going into the machine is equal to the waste that comes out after decomposition. But the out coming material has fewer odours, becomes a good fertilizer and causes no pollution to the atmosphere. The liquid part is used as a fertilizer and the solid part is recycled. Fresh waste is preferred as it contains more moisture. The source of waste could be human excreta, slaughter house waste, manure, fruit and vegetable waste etc. Cattle dung and manure is the most suitable for biogas production. Usually fresh cattle dung is collected and water is added in it, when carried to the system. Chicken droppings can also be used as a raw material for biogas. When this waste is converted to biogas, the remaining slurry or sludge can be used as excellent fertilizer. It reduces the odour comes out of waste because the gas is burnt in the machine before coming to the environment. The environmental impacts as well as the benefits of biogas will also be studied in this research Biogas. Fruitful results have been observed in Pakistan because 50% of Pakistan's population living in rural areas and have cattle(Tirmizi.A, 2008). Biogas is not only a fuel for green energy but it has many other benefits, not only for producers but for society as a whole. It provides an alternative use for food by-products. Instead of wasting huge amount in land filling these by products, they can be used biogas production.

2. Literature Review

Pakistan is situated in South Asia, having population of 176 Million, living in land of 796,095 square kilometres (estimated population as per PCO population census organization). Mainly, Pakistan has agriculture economy and has per capita income of about $1207(Zeb, 2011). Pakistan is facing severe energy crisis and energy requirements are increasing by an average of 24% annually (Hamza, 2010). The 4% of total land area is covered by forests (land cover assessment and monitoring Pakistan Volume 10). About 98% of the total wood is used as a fuel (FAO report, 1997). The annual deforestation rate of Pakistan is over 2% which is quite high. (Faisal Rahman Channa, 2011). Renewable energy sources are helpful to meet the growing energy demand and to improve the living standard of people. These resources are used for cooking and lighting purposes. Biogas is one of the most valuable energy sources, produced by biological breakdown of organic matter. Pakistan has to depend on external support to meet the energy demand. Pakistan is having huge oil resources but most of them are still unexplored. Due to lack of technical knowledge, awareness and government funding, the efforts made in utilizing renewable energy resources were not successful. The results achieved from the initial experiments were not as anticipated. There are multiple renewable energy resources in Pakistan. Among all those resources, micro-hydel, bio-energy, wind and solar energy are more feasible but still unexploited here. Pakistan must utilise these resources to meet the energy demand. Many attempts have been made in last years to use substitute energy resources but the most successful method in the field of Bio-energy is

2.1 Wastes to Energy Conversion: An Effective Way to Overcome Energy Crisis at Domestic Level:

Quantities of waste is increasing due to increase in population and changes in living standard of people, and it's a serious threat to our environment. But now due to technological development and awareness, wastes to energy plants are designed specially to produce clean environment. These plants are equipped with pollution control equipment to reduce harmful emissions.

2.2 Biomass Energy; What and How?

Biomass energy is a form of renewable energy resource which is derived from the wastes generated due to several human activities as well as animal wastes. Bio energy is stored in materials made with the help of living things e.g. wood. Biogas and Alcohol are one of the major sources of bio energy The energy derived from biogas is renewable energy because its source can be easily available again without taking a lot of time. A question may arise that fossil fuels are made by living organisms too so is it renewable or not. The answer to this question is that no doubt it takes the living organisms to convert the raw material to fossil fuels but it takes a time so long that it would take millions of years to replace it

and hence cannot be tagged as renewable energy source. Peat (a type of coal, soft brown) is another resource that is not easily replaced, taking hundreds of thousands of years to renew it. To be considered renewable, the resources must be replaceable within our lifespan. For example, the wood used in your campfire replaces itself as the forest grows. Coal on the other hand can be taken from the earth only once, and cannot be replaced. (Tiwari, G. N.; Ghosal, M. K). There are various types of waste materials available from different sources, but not all waste materials are biodegradable. Only biodegradable waste materials can produce biogas. Again, the biogas generation capacity is not the same for al1 biodegradable waste materials.

The degree of biogas generation capacity of the various types of dung is found to be in ascending order of:

Cow dung < buffalo dung < mixture of animal dung < camel dung < horse dung

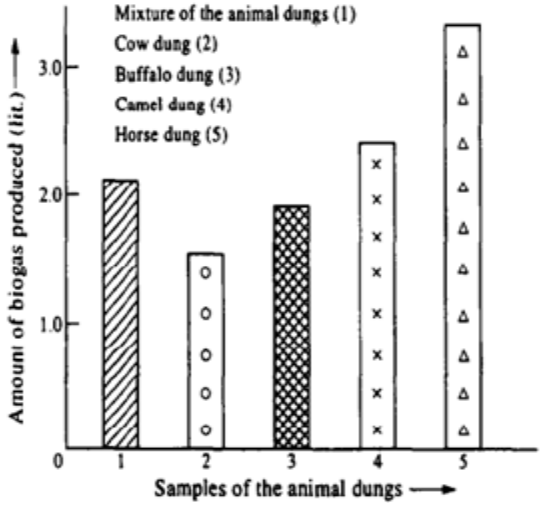


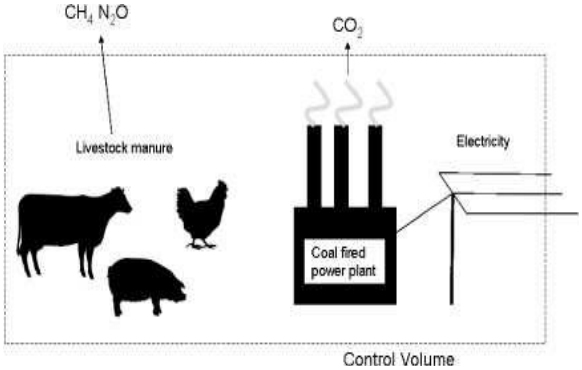
Fig. 1 Biogas Generation Capacity of the mixture of the animal dungs and its individual components

The above results were obtained at a specific temperature of 37°C. The biogas generation capacity of each waste material will be different at other temperatures (Tanusri Mandal and N.K. Mandal, 1997)

2.3 The Energy and Emissions Benefits of Converting Manure to Biogas:

To meet the energy requirements and to reduce green house gas emissions, analysis of converting animal waste into biogas could be supportive. Methane and Nitrous oxide is emitted with these disposal methods. Both of these green house effect gases have 21 and 310 times global warming potential of Carbon dioxide respectively. In 2005, emissions from this sector in US were equivalent to 7% of the total emissions in this country. Out of this, 51 to 118 Metric million tons of carbon dioxide was due to animal manure, and this amount is increasing from 1990 to 2005. Thus minimizing agricultural contributions to climate change can be helpful to mitigate the emissions from these green house gases. Anaerobic digestion can minimize GHG emissions from livestock manure. This process converts manure to methane-rich biogas. Using Biogas as alternative to fuel can replace two Green house gas emission sources i.e. manure and coal combustion, as its source is Biogas combustion, so has less carbon dioxide contribution. In US, Biogas energy potential was calculated using values from energy that can be contributed by each animal in a day and number of animals units present in the country. It was concluded that 1 quad of renewable energy can be produced from 95 million animals units, which is equivalent to 1% of total energy consumption in US. Generating electricity from biogas could contribute 88±20 billion kWh, or 2.4±0.6% of annual electricity consumption in the US. Replacing coal and manure GHG emissions with the emissions from biogas would produce a net potential GHG emissions reduction of 99±59 million metric tons or 3.9±2.3% of the annual GHG emissions from electricity generation in the US. (Amanda D Cu´ellar and Michael Ewebber, 2008).

Here is the comparison of two scenarios regarding the treatment of livestock manure.

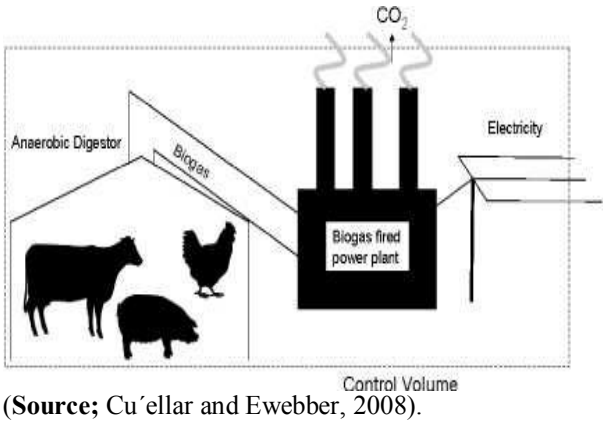


(**Source;** Cu´ellar and Ewebber, 2008).

Fig. 2 Scenario A: business as usual. Livestock manure and coal-fired power emit greenhouse gases

Scenario A shows green house gas emissions from animal waste as well as from coal combustion.

Scenario B shows treatment of waste in anaerobic digesters. The Biogas coming out of digesters is further burnt to produce electricity. In this case, carbon dioxide is only green house gas emission, going to environment.



(**Source;** Cu´ellar and Ewebber, 2008).

Fig. 2. Scenario B: biogas is produced and used for electricity generation, replacing two sources of GHGs untreated manure) with one source of GHGs (biogas combustion)

2.4 Biogas Potential in Pakistan:

In Pakistan total available biogas generation potential is round about 503 MMCFD. A lot of work is in progress all over Pakistan by PCRET (Pakistan Council of Renewable Energy Technologies) regarding biogas plants manipulation and build-up. PCRET has mounted more than 1600 plants (most of them having capacity of almost 71 ft^3/day) so far if we look into last 3 years. This development is not limited to this, almost same number of plants have also been established by various private sectors and NGO's. Before this period, operation uncertainty level for all built-up biogas plants is very high due to deficiency of available sources of maintenance and repair. For the time being, state of the art digesters are producing more appropriate gas which clearly meets the environmental circumstances with upholding internal temperature; because of this more options are available for more plants installations in the country with much better long lasting existence. In Punjab specifically in Bahawalpur region, Biogas plants are drawing high attractiveness of farmers and it's a good sign. Some more workings are also under process like a prolific project at Landhi Cattle Colony, Karachi by AEDB and its initial phase is expected to be sponsored by NZAID (New Zealand Aid). Biogas plants/generators will generate electricity & ultimately high class organic fertilizer by using waste (From around 400,000 cattle in the area). At initial stage, the plant will generate 250kW electricity and after extension it will increase up to 30MW and ultimately throughput of 1500 ton /day organic fertilizer. In Shakarganj Mill, another biogas electricity generating plant is under building phase, again with the collaboration of AEDB. Its estimated power generating capacity is 8.25MW (Munawar 2009).

3. Research Methodology

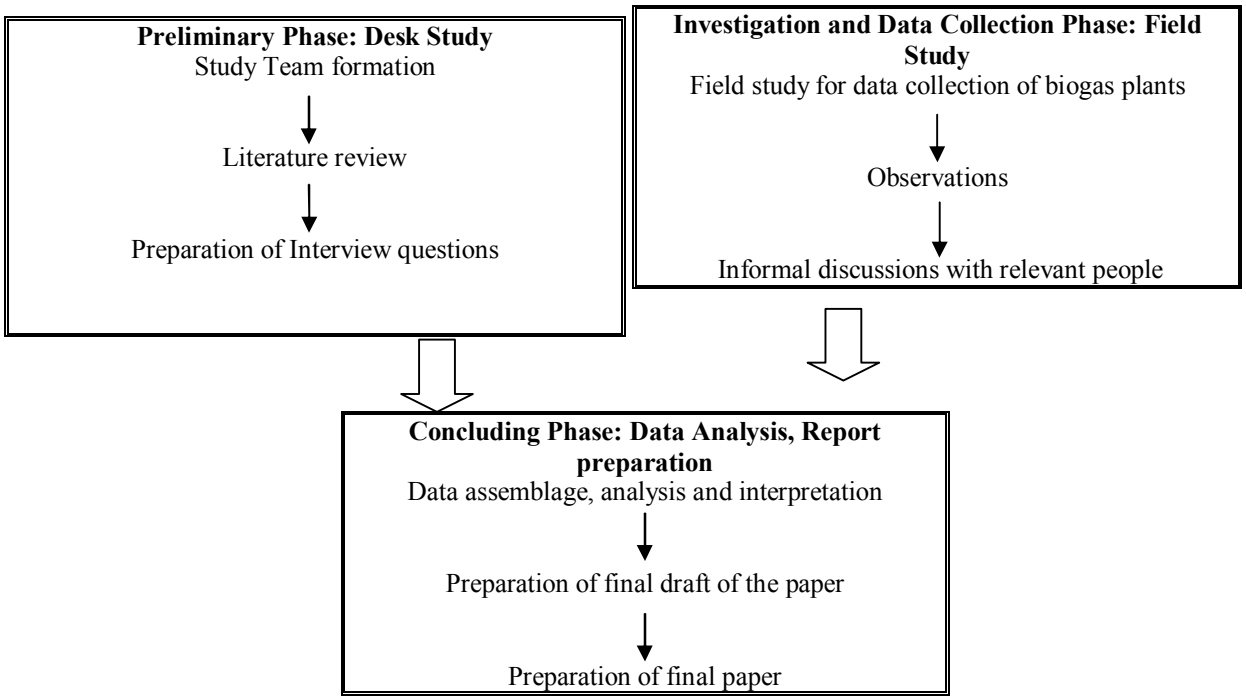


Fig. 3 Research Methodology

- The interview were conducted for one time and laddering technique was used to get deep insight of installed plants. Then the pros and cons of existing methods will be identified.

On the basis of interviews and study of existing plants, solutions will be proposed to meet the limitations.

4. Results

From the interviews conducted the following information was obtained:

4.1 Types of Biogas plants in Pakistan

Pakistan Council of Renewable Energy Technologies has installed three types of biogas plants in Pakistan. These are:

- Plants with moveable gasholder
- Plant with built-in fixed dome gasholder
- Low cost bag / balloon type plant

The installation of these biogas plants was on experimental basis to select the appropriate design based on social and cultural norms of the particular rural areas

4.1.1 Moveable Gasholder:

.

Year	No. of Biogas plants installed	Type	Results
1976	21	Chinese	Unsuccessful
1979	10	Indian	Successful
1980	100	Indian	Satisfactory
1983	350	Indian	Satisfactory
1986	4000	Indian	Failed
1993	50	-	Successful
2006	1600	-	Successful

Fig. 4 History of Biogas plants in Pakistan

4.1.2 Fixed Dome Gasholder:

Advantages	Disadvantages	Advantages	Disadvantages
• The pressure of the gas is controlled by the gasholder • Gasholder assists in stirring • Gasholder also helps in breaking down the hard crust, if formed • If the gasholder is painted black, it helps to create a greenhouse effect within the digestor which helps in keeping the temperature high in winter season • Slurry can be easily collected due to gravity flow	• Heat losses may occur due to the gasholder exposure to the atmosphere • Corrosion is the main problem for the gasholder as it dips in slurry and stays in contact with moisture • Periodic painting of the gasholder is required to avoid rusting	• Space is better utilized since it is underground • Steady temperature is easy to maintain inside the digester • Post installation maintenance is rarely needed (e.g. painting, plastering etc)	• Special construction skills are required • Stirring and froth breaking is generally difficult • Controlling the gas pressure is difficult • Leakage of gas might occur from hairline crack in the dome or from sides of manhole cover • A noticeable quantity of gas that is produced inside the slurry chamber is not fully utilized and is wasted • Slurry is to be taken out manually

Fig.5 Fixed Dome Gas holder

4.1.3 Low Cost Bag/ Balloon type plant

Low density polyethylene was used to design a bag type biogas plant. It was low cost plant and was installed for field testing. It was found from the experiments and tests that a high density polyethylene or some other composite material having better elasticity and strength is required for a sustainable bag type biogas plant. Hence this design was not very successful.

4.2 Average Capacity of Biogas plants

The annual biogas generation capacity of the biogas plants installed during last 7 years is more than 2.5 million m^3 along with production of 4 million kg/year of bio-fertilizer.

4.3 Most common Biogas Purification method

4.3.1 Water Scrubbing

Water scrubbing is used to remove H2S and CO2 from biogas. It is a well established technology and removes these gases as both of them are more soluble in water. However H2S cannot be completely removed by this process as its solubility in water is lower than that of carbon dioxide. The H2S that came in contact in water and still remained desorbed can

result in odour problems. Pre-removal of H2S is a more environmentally friendly approach.

4.4 Biogas/diesel dual fuel engine for electricity generation

A study was conducted where biogas was used in a single cylinder, compression ignition engine, which has been modified to operate under dual-fuel condition to generate electricity. The primary fuel was biogas, which is ignited by a pilot diesel liquid injection. This long term operation was carried for 2000 h. Weight of Engines components were compared by measuring it before and after this long term operation. Visual inspection was also done and rating was given for wear tear. Results shows performance between diesel and dual-fuel operations was comparable. Bio gas diesel-fuel engine proved to be successful with biogas rate above 90%. Results indicated that Biogas engine was able to generate 1.45 KW electricity. Thus dual-fuel engine proved to perform well an has potential to be used for on-farm utilization (Tippayawong et al, 2007).

4.5 Benefits of household biogas

Biogas is very beneficial for its users. It has a number of benefits which are as follows:

4.5.1 Health benefits

Biogas provides numerous health benefits. As per Integrated Environmental Impact Analysis carried out for 600 biogas users and 600 non-users, the non biogas users had 4% more respiratory diseases than biogas users. Qualitative information from various household surveys carried out by Biogas Sector Partnership (Nepal) has revealed that problems like respiratory illness, eye infection, asthma and lung problems have much decreased after installing a biogas plant.

Disease	Problems in the past (households)		Present status of households	
	Yes	No	Improved	Remained same
Eye Infection	72	18	69	3
Cases of burning	29	71	28	1
Lung problem	38	62	33	5
Respiratory problems	42	58	34	8
Asthma	11	89	9	2
Dizziness/headache	27	93	16	11
Intestinal/diarrhoea	58	42	14	44

Fig. 6 Health benefits of biogas (Source: Acharya et al, 2010)

Following are the primary organisms killed in biogas plants:

- Typhoid
- Paratyphoid
- Cholera and dysentery bacteria
- Hookworm
- Tapeworm and roundworm

The biogas can also have sound effects on dietary patterns. As this gas is cheaper and easily available, so water can be boiled more easily and regularly, thus reduces various harmful diseases.

4.5.2 Economic benefits

Biogas reduces the expenses on fuel for cooking, and over the long run. Savings of fuel expenses as a result of installing biogas plants makes it possible to recover the total plant investment cost within four to five years typically. Bio-slurry obtained from the plant has proved to be excellent organic manure. This manure is more effective and is of higher quality than traditional manure such as farm-yard manure. The use of bio slurry as manure helps in increasing farm production and the farmer's income. It also reduces the high cost of chemical fertilizers and the adverse effects arising from their use.

4.5.3 Environmental benefits

Being a smokeless fuel, biogas keeps the indoor as well outdoor environmental clean. The manure that was previously wasted or left in open (which emitted green house gases) is now properly managed. Biogas production reduces the demand of firewood and hence saves us from deforestation. It can also be used to manage wastes from poultry as well as dairy farms.

4.5.4 Other benefits

Biogas provides benefits to the rural women in their household activities by reducing their workload in collecting firewood. As it is easier to cook food using biogas and cooking time is much faster as compared to solid biomass fuels, it is very friendly. A study has showed that biogas saves approximately 2 hours per day per family due to reduction in time that was previously wasted in collecting biomass and making dung cakes. As it is a smokeless fuel, the utensils also remain clean from soot after being used for cooking on biogas. (Pandey, Bajgain 2007)

5. Analysis

Biogas technology is working tremendously all over the world, under different climatic conditions. They are very useful for rural as well as urban population. These systems well responds to industries. But still there are many countries in which this technology is not yet fully commercialized. The reason might be high capital investment. This technology is not well accepted universally because it cannot resolve every problem of a village, farm etc. Biogas plant must be able to fit the existing waste disposal and productions systems. It is unfavourable and expensive to make changes in the existing systems to best suit the production plant. This technology has many competitors. There are many others ways of producing energy like solar systems, micro-hydro-power, fuel wood and many others renewable energy sources. High quality fertilizer can be obtained from many other techniques which are closer to traditional methods. The only solution to make it most favourable and attractive method is to provide simultaneous solutions to problems.

5.1 Limitations of Biogas

The reasons why Biogas technology is in limited use are follows:

- It requires many animals for collecting daily waste to be installed in houses.
- Its initial cost is also very high.
- It requires significant economic status of the potential users.
- Its users must have animal and land resources.
- They must have mortgage ability.
- As it requires care for handling slurry and feeding plants, thus it increases workload. Continuous Water supply is required to keep the dung fresh. This production process is dependent on temperature.

6. Conclusions

In today's world there is lack of communication between Biogas stakeholders. People are unaware of each others techniques and technologies. They don't know each others ideas in this sector. Therefore there is need to share knowledge and views to establish a good communication channel between stakeholders. Big and small manufacturers should work together by sharing their ideas and technologies. Biogas is sustainable in long run. Underdeveloped countries should adopt this technology as a renewable energy source to meet their energy demands. It can be continued indefinitely in the future.

7. Recommendations

- The government should take major steps to commercialize the technology. For this purpose, the cost of the biogas plants should be reduced so that people in the lower economic brackets consider it as an economic friendly technology.
- User guides and manuals should be available to the users in the local languages. However, promotion is only effective when it is followed by technical training provided to the users.
- The users should participate in installation and successful operation of their biogas plants. They should be accountable for collecting construction supplies such as sand, stone/bricks, gravel etc., provide labours for back filling, making manure pit and regular feedings, so that the plant is properly maintained.
- Government of Pakistan should encourage the users of this technology by subsidies and loan support programs. Moreover, the government should identify the key stakeholders.
- One of the basic reasons for the failure of this technology in our country is the lack of effective promotion. It should be promoted country wide through calendars, Radio and TV advertisements, leaflets, posters etc in clear form.
- The companies constructing the biogas plants should provide after sales services, maintenance and training if required to keep the biogas functional at its best as every non functioning plant harms the reputation of the technology. (Zafar 2006).

References

1. Arif. M, Feed Resources for Livestock in Pakistan. Economics Survey 2008-09. Pp 2.
2. Jiwan Acharya .J, Bajgain.S, Subedi.S," Scaling up biogas in Nepal: what else is needed" August 2010.
3. Cu´ellar. A, Ewebber.M .Cow power: the energy and emissions benefits of converting manure to biogas. 2008 IOP Publishing Ltd. Printed in the UK, pp 1-2.
4. Food and Agriculture Organization Report 1997, Updated 23 Oct 2006
5. Global Renewable Energy Markets. August 2008. Publisher: Energy Business Reports
6. Hamza.Waqar "Country's energy demand to grow by 6pc". Pakistan Today. November 2010.
7. Intergovernmental Panel on Climate Change Third Assessment Report. January 2001.
8. Land Cover Assessment And Monitoring, Pakistan. V.(10).
9. Mandal.T, Mandal.N. "Comparative Study of Biogas Production from Different Waste Materials" Energy conversion Management 1997. Printed in Great Britain, 38. Pp 679-683
10. Munawar A. Sheikh. Energy and renewable energy scenario of Pakistan. 2009 Elsevier Ltd. Pp.360

11. National Non-Food Crops Centre. "NNFCC Renewable Fuels and Energy Fact sheet: Anaerobic Digestion". 16th February 2011. Pp 1
12. "Official Pakistani Population clock" Ministry of Economic Affairs and Statistics. Retrieved 17 January 2010.
13. Pandey. B, Bijgain. S, "Feasibility Study of Domestic Biogas in Pakistan", pp. 18
14. Population Census Organization. Government of Pakistan Updated on 19th July 2011.
15. Rehman .F. "Rampant Deforestation". The Nation. April 9th 2011.
16. Syed Zafar Ilyas "Biogas Support Program Is a Reason for its Success in Pakistan". American-Eurasian Journal of Scientific Research 2006, pp. 42-45
17. Tirmizi A, Alternative Energy Sources: Waste Productions as Fuel in Pakistan. Proceedings of 1st International Conference of IET Brunei Darussalam Network, 26-27, May 2008. pp.2
18. Tippayawong. N, Promwungkwa. A, Rerkkriangkrai. P (2007) "Long-term operation of a small biogas/diesel dual-fuel engine for on-farm electricity generation". Elsevier, pp. 26-32
19. Wong.B, 2009. "These Facts Will Help You Understand Global Warming"
20. Zeb.M, "Per capita income rises to $1,207". Daily Dawn. 6th May 2011.

2/15/2012

Molecular Study on Relatedness between Clinical and Tap Water Isolates of *Pseudomonas aeruginosa* in Two Burn Units

Mohammed S. Salama[1] Hala M. Abu Shady[1], Mohammed M. B. El-Gameal[2], Mervat G. El Anany[3] and Ghada M.Abd-El-Rehem[1]

[1.]Microbiology Laboratory, Faculty of Science, Ain Shams University, [2.] El-Maadi Military Hospital
[3.]Clinical Pathology Department, Microbiology Laboratory, Kasr Alainy Hospital, Cairo, Egypt

Abstract: Background: The role of tap water as an environmental source of disease causing *P. aeruginosa* strains was established. **Objective:** to study the relatedness between clinical and tap water *P. aeruginosa* isolates in the burn units of two different hospitals. **Materials and methods:** One hundred and fifty specimens were collected from two burn units of Cairo University Hospital and El Helmeya Military Hospital. These specimens included: 100 pus samples from infected wounds of burned patients, 25 water samples and 25 swabs were taken from hands of medical staff. The samples were cultured and *P. aeruginosa* isolates were identified as according to standards. The selected colonies were subjected to molecular identification as *P. aeruginosa* by PCR tesing using specific PAL1 andPAL2 primers, and tested for relatedness by plasmid profiling and protein electrophoresis. **Results:** All cultures from the hands of the medical personnel were negative for *P. aeruginosa.* The clinical and water samples yielded 52 *P. aeruginosa* isolates and only 19 isolates were confirmed by PCR. These isolates were from 16% of total water samples from 15% of total clinical swabs. Half of these isolates harbored plasmids. The phenotypic characteristics of isolates showed that 30% of isolates (from clinical and water specimens) were related. However, molecular studies did not prove any molecular evidence of relatedness between different clinical isolates or between clinical and environmental isolates. **Conclusions:** *P. aeruginosa* is the cause of 15% of infections in Egyptian burn units, and contaminates 16% of water samples of these units. The simple use of bacterial protein electrophoresis and plasmid profiling ruled out the relatedness between the clinical and the contaminated water samples.
[Mohammed S. Salama, Hala M. Abu Shady, Mohammed M. B. El-Gameal, Mervat G. El Anany. and Ghada M.Abd-El-Rehem. **Molecular Study on Relatedness between Clinical and Tap Water Isolates of *Pseudomonas aeruginosa* in Two Burn Units.** Life Science Journal. 2012;9(1):662-666] (ISSN:1097-8135).
http://www.lifesciencesite.com. 96

Keywords: burn, infections, water supply, plasmid profiling, *Pseudomonas aeruginosa*

1. Introduction

The burn wound represents a site susceptible to opportunistic colonization. The situation for patients with *Pseudomonas aeruginosa* infections is particularly problematic since this organism is inherently resistant to many antimicrobial classes and is able to acquire resistance to all effective antimicrobial drugs (1). Although, several authors were able to culture *P aeruginosa* from the hands of hospital personnel, however, in approximately 30% to 60% of the infection , the mode of acquisition of *P aeruginosa* remained unexplained (2).

The role of tap water as an environmental source of disease causing *P. aeruginosa* strains was established (3). The isolate occurrence in water is probably related more to ability to colonize biofilms in plumbing fixtures (i.e., faucets, shower heads, etc.).Taking the measures to reduce the contamination of water taps resulted in a significant decrease of clinical infections (4). However, the complete eradication of contaminants from these sources is nearly impossible (5).

By using phenotypic methods only, environmental *P. aeruginosa* strains, although present in many hospital locations, were not linked to strains causing clinical disease(6). This was explained by environmental factors modifying lipopolysaccharide production such as exposure to antibiotics or availability of nutrients. The advent of molecular typing techniques proved to be more suitable to study relationships between strains. Methods that have been used successfully include pulsed-field gel electrophoresis (PFGE), amplified fragment-length polymorphism (AFLP) analysis, etc.(7) The simplest of these genetic techniques has been the analysis of the plasmid profile of a given organism or group of organisms. Most bacterial species carry plasmids, which are extra-chromosomal pieces of DNA that encode a variety of genes. After isolation, plasmids are separated by electrophoresis and the pattern (number and size) of the plasmids from different organisms are compared (fingerprinting). If an organism has few or no plasmids, this technique provides little assistance.

The aim of this work was to study the relatedness between clinical and tap water *P. aeruginosa* isolates in the burn units of two different hospitals.

2. Material and Methods

Collection of samples

One hundred and fifty specimens were collected from two burn units of Cairo University Hospital and El Helmeya Military Hospital. These specimens included: 100 pus samples from infected wounds of burned patients, 25 water samples and 25 swabs were taken from hands of medical staff.

Water samples were obtained from tap water from patients' bath rooms, as well as from the central hand-washing area of the staff.

Water sampling: A 100 ml water sample was obtained from each patient-related water faucet in the patients ward, as well as from the central hand-washing area of the staff. Taps were opened, and the first flush of water was collected immediately in sterile bottle containing 50 ml of nutrient broth, and then subcultured when turbid(8).

(I) Bacterial isolation and identification

All swabs and turbid broth were cultivated on nutrient agar, blood agar and MacConkey's agar media (Oxoid Basingstoke, UK). Non-lactose fermentative colonies, on Mac Conkey's agar that showed characteristic pigmentation and hemolysis on blood agar, were tested for oxidase activity. These colonies were further studies by growth at 42˚C, oxidation test, nitrate reduction test, gelatin liquefaction test and growth on the following agar media: triple sugar iron agar, lysine iron agar, Christensen's urea, Simmon citrate (Oxoid, Basingstoke, UK) and motility- indole- ornithine. Non- fermenter, indole- negative, urease- negative, motile colonies with positive oxidase test were presumptively identified as P. aeruginosa; these colonies were picked for molecular analysis.

Antimicrobial susceptibility testing: was done according to standard methods by disk diffusion(10). Several antibiotics were tested: ampicillin, ampicillin/sulbactam, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, meropenem and imipenem (Oxoid, Basingstoke, UK).

(II) Molecular Biology Studies:

The selected colonies were subjected to molecular identification as *P. aeruginosa* by PCR tesing using specific PAL1 andPAL2 primers (11,12).The confirmed *P. aeruginosa* isolates from clinical and water samples were tested for relatedness by plasmid profiling and protein electrophoresis (13,14).

(A)Polymerase Chain Reaction(PCR)

1.Extraction of genomic DNA(11): The genomic DNA was prepared from non-transformed cells by boiling the cell suspension and by phenol-chloroform extraction. Negative control of non *P. aeruginosa* and positive control of *Pseudomonas aeruginosa* ATCC27853 were used.

2.DNA amplification(12): One type of primer was used to amplify the open reading frame of the OprL gene PAL1, 5 - ATGGAAATGCTGAAATTCGGC- 3 and PAL2, 5 - CTTCTTCAGCTCGACGCGACG- 3`. The PCR reaction was carried out using DNA thermal cycler 480(Perkin Elmer, USA) according to the following profile: 94°C for 2 minutes for1cycle, 94°C for 40 seconds, 56°C for 40 seconds for 30 cycles, 72°C for 50 seconds ,72°C for 10 minutes, finally 4°C for hold. After termination of cycles, the reaction mixture was mixed with DNA loading buffer , electrophoresed on 1.5% agarose gel and visualized using UV trans-illuminator of 312 nm wavelength.

(B)Plasmid profile(13):Plasmid DNA isolation was extracted by Quick and easy Kit for Bacterial Plasmids Isolation. Isolates with identical plasmid profiles were considered to belong to the same plasmid type.

(C) Protein electrophoresis was done according to standard methods (14).

3. Results

(I) Isolation of *P. aeruginosa*

Out of 150 samples, 52 samples yielded *P. aeruginosa* isolates, 41 (41%) from patients samples and 11(44%) from water samples taken from faucets found in patients' rooms. Other water samples were negative.

All cultures from the hands of the medical personnel were negative for *P. aeruginosa.*

The susceptibility testing of these isolates showed sensitivity as follows: ampicilin (0%), ampicillin/sulbactam (0%), cefotaxime (0%), ceftazidime (20%), ceftriaxone (0), ciprofloxacin (22%), gentamicin(22%), meropenem (100%) and imipenem (100%). The antibiogram was identical in 30% of isolates including isolates from clinical and water samples.

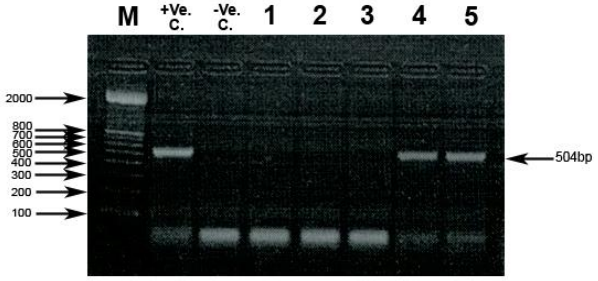


Figure (1): Agarose gel electrophoresis of specific PCR product (504 bp) resulted from amplification of genomic DNA of *Pseudomonas aeruginosa* PAL1 and PAL2 primers. Lane M represents 100 bp ladder markers. Lane 1 represent Positive control, Lane 2 represent Negative control, and Lanes from 3 to 7 represents isolates respectively.

(II) Molecular Biology Studies:

(A)-Polymerase chain reaction(PCR):

The fifty two *Pseudomonas aeruginosa* isolates were identified by PCR and only 19 (36.5% of studied isolates) *Pseudomonas aeruginosa* isolates were confirmed by PCR (Fig.1) . Only 4 isolates were from water samples (16% of total water samples), these were from water faucets of patients' room, and 15 were from clinical samples (15% of total clinical swabs).

(B)- plasmid profile:

From the19 isolates, only 9 (50%) isolates harbored plasmids. No *Pseudomonas aeruginosa* isolates had similar plasmid profiles

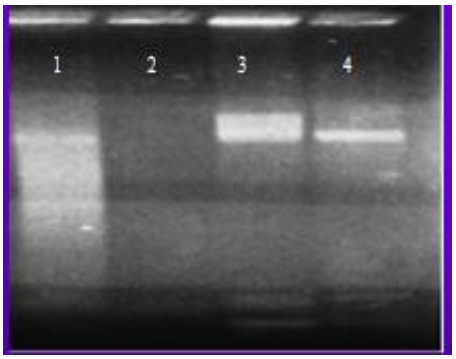


Figure (2): Plasmid profile analysis. Plasmid was detected in isolates number 3 and 4 No. 3 and 4. No plasmids in isolates 1 and 2.

(C)-protein electrophoresis:

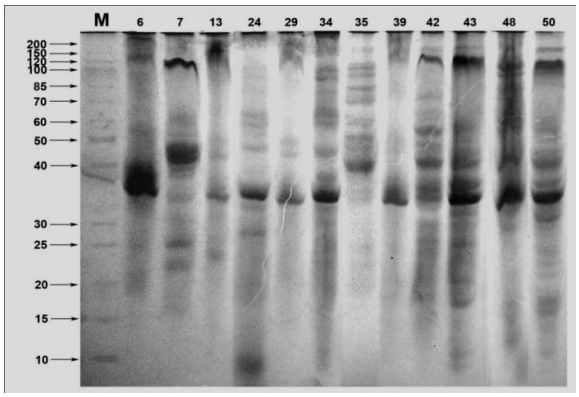
Electrophoretic separation of general protein patterns Revealed a total of 27 protein bands. Protein band of 172 KDa was present in all isolates, the common region could be related to species -specific proteins. Protein band of 45 KDa was present in 11 isolates, protein bands of 71 KDa and 31 KDa were present in 9 isolates, protein bands of 125 KDa, 81 KDa, 61 KDa, 36 KDa and 22 KDa were present in 8 isolates and protein bands of 137 KDa, 97 KDa, 75 KDa and 57 KDa were present in 7 isolates. The densitometric scanning proved diversity of the isolates.

Interpretation of results

The phenotypic characteristics of isolates (biochemical reactions and antibiogram) showed that 30% of isolates (from clinical and water specimens) were related. However, molecular studies ruled out any molecular evidence of relatedness between different clinical isolates or between clinical and environmental isolates.

4. Discussions

The ability of the laboratory to isolate and identify microorganisms is crucial to infection control studies. In this study, when *P. aeruginosa* isolates were identified by culture characteristics and conventional biochemical reactions, 41(41%) of clinical samples and 11(44%) of water samples were mis- identified as *P. aeruginosa* isolates. This falsely represented higher rates than other published studies, where *P. aeruginosa* was isolated from (4.5-26.2%) of water samples and from (21.3%) of pus samples (16,17). But using PCR- based amplification of the specific *P. aeruginosa* primers proved that *P. aeruginosa* contributed only to 4 (16%) of water samples and 15 (15%) of clinical samples, which is comparable to results of other studies.



Fig(3): Electrophoretic separation of protein patterns of *Pseudomonas aeruginosa* isolates. Isolates No. 6,29,34,35,39,42,48,50 shared in 5 bands only.

As cultures from the hands of the medical personnel were negative for *P. aeruginosa*, and *P. aeruginosa* was isolated from both patients' samples and water, we hypothesized that *P. aeruginosa* infections were related to tap water contamination. However, by using simple cheap molecular profiling methods, there was no molecular evidence of relatedness between clinical and water isolates, negating that water was the source of infection.

Other studies using advanced molecular typing techniques; RAPD and PFGE, have shown that up to 50% of nosocomial *P. aeruginosa* acquisitions may result from transmission through tap water. Carriage of *P. aeruginosa* by patients was both the source and the consequence of tap water colonization (17-19). On the other hand, some authors were unable to demonstrate a similarity by PFGE between *P. aeruginosa*, isolates recovered from faucets and the corresponding species from clinical isolates (20). Another study proved that some patients harbored strains not previously isolated from tap water. Thus; in addition to tap water, other environmental or unknown reservoirs appeared to

play a role for the epidemiology of *P. aeruginosa* infection (21).

In this study, only 50% of the isolates harbored plasmids and that have hampered making use of the results of profiling. Some authors assumed that plasmid profiling cannot be used as an epidemiological marker of *P. aeruginosa* strains in the hospital environment and that the study of the more stable and constitutive chromosomal DNA may be more predictive for clonality (22). However, in a setting with low resources, like ours, where the expensive instruments and reagents are not available, plasmid profiling can be a simple tool for epidemiologic studies.

This study has some limitations. First, the small number of water samples, as we obtained cultures from each tap water faucet once. Second, the plasmids were undetected in 50% of studied isolates, so profiling actually compared 10 isolates only.

In conclusion, the study proved that *P. aeruginosa* is the cause of 15% of infections in 2 Egyptian burn units, and contaminates 16% of water samples from the 2 burn units. These isolates were multidrug resistant. Half of the isolates harbored plasmids and the simple use of bacterial protein electrophoresis and plasmid profiling ruled out the relatedness between the clinical and the contaminated water samples. This study emphasizes the need of sterile water in burn units, and the importance of including the water surveillance in epidemiologic investigations.

Corresponding Author:
MervatG.ElAnany miroislamic@yahoo.co.uk

References

1. Livermore, D. M. (2002):Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? Clin. Infect. Dis., 34:634-640.
2. Moolenaar RL, Crutcher M, San Joaquin VH, *et al.*(2000). A prolonged outbreak of Pseudomonas aeruginosa in a neonatal intensive care unit: did staff fingernails play a role in disease transmission? Infect Control Hosp Epidemiol.,21:80-5.
3. Kolmos HJ, Thuesen B, Nielsen SV, Lohmann M, Kristoffersen K, Rosdahl VT(1993): Outbreak of infection in a burns unit due to *Pseudomonas aeruginosa* originating from contaminated tubing used for irrigation of patients. J Hosp Infect. ;24:11-21
4. Martino P, Venditti M, Papa G, Orefici G, Serra P.(1985): Water supply as a source of Pseudomonas aeruginosa in a hospital for haematological malignancies. Boll Ist Sieroter Milan; 64:109-14
5. Reuter, S.;Sigge, A.; Heidemarie, W. and Trautmann, M.(2002): Analysis of transmission pathways of *Pseudomonas aeruginosa* between patients and tap water outlets. Crit. Care Med.; 30: 2222-2228.
6. Orsi GB, Mansi A, Tomao P, Chiarini F, Visca P.(1994): Lack of association between clinical and environmental isolates of *Pseudomonas aeruginosa* in hospital wards. J Hosp Infect., 27:49-60
7. Trautmann M., Lepper P.M., Haller M. (2005): Ecology of Pseudomonas aeruginosa in the intensive care unit and the evolving role of water outlets as a reservoir of the organism AJIC, 33 (5 SUPPL. 1) S41–S49.
8. Blanc DS, Nahimana I, Petignat C, Wenger A, Bille J, Francioli P. (2004):Faucets as a reservoir of endemic *Pseudomonas aeruginosa* colonization/ infections in intensive care units. Intensive Care Med.,30:1964-8.
9. Holt, J.G. and Krieg, N.R.(1994): Bergey's Manual of determinative bacteriology,9th edition. Baltimore: Williams and Wilkins, P.179-180, 187-188.
10. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk susceptibility tests, 9th ed. 2006 Approved standard M2-A9. Clinical and Laboratory Standards Institute, Wayne, PA.
11. Elaichouni, A.; Van, E.J.;Claeys, G.;Verschraegen,G.,Verhelst, R. and Vaneechoutte, M.(1994):Study of the influence of plasmids on the arbitrary primer polymerase chain reaction fingerprint of *E. Coli* strains. FEMS. Microbiol. Lett.,115(2-3):335-339.
12. Lim, A., Jr.; De Vos, D.;Brauns, M.; Mossialos, D.; Gaballa, A.; Qing,D. and Cornelis,P.(1997): Molecular and immunological characterization of OprL,the 18 KDa outer membrane peptidoglycan-associated (PAL)lipoprotein of *Pseudomonas aeruginosa*. Microbiology, 143:1709–1716
13. Khalil, K. M. A. (2007): Quick and easy Kit for Bacterial Plasmids Isolation, Patent No: 90-2-2007 supplement in (20-2-2007).
14. Hames, B.D. (1995): One dimensional polyacrylamide gel electrophoresis. In: Gel electrophoresis of protiens, A practical approach. (ed.Hames, B.D. and Rickwood, D.), pp. 1 - 147.Oxford university press, New york
15. Adjide, C.C.; De Meyer, A.; Weyer, M.; Obin, O.; Lamory, F.; Lesueur, C.; Trouillet, L.; Biendo, M.; Eb, F.; Ganry, O. (2010): Stenotrophomonas maltophilia and

Pseudomonas aeruginosa water associated microbiologic *risk assessment* in Amiens' University Centre. Pathologie Biologie, 58(2): e1-e5

16. Cholley, P.; Thouverez,M.; Floret,N.; Bertrand,X. and Talon,D.(2008):The role of water fittings in intensive care rooms as reservoirs for the colonization of patients with *Pseudomonas aeruginosa*. Intensive Care Med.; 34(8): 1428–1433.
17. Rogues, A.M.; Boulestreau,H. ; Lashe´ras, A.; Boyer, A.D.; Gruson, D. ; Merle, C.;Castaing, Z.; Be´bear, C.M. and Gachie , J. P.(2007): Contribution of tap water to patient colonisation with *Pseudomonas aeruginosa* in a medical intensive care unit. Journal of Hospital Infection ; 67:72-78.
18. Trautmann, M.; Halder, S.; Lepper, P.M. and Exner M.(2009): Reservoirs of *Pseudomonas aeruginosa* in the intensive care unit. The role of tap water as a source of infection. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz. Mar. ; 52(3):339-44.
19. Lavenir R., Sanroma M., Gibert S. , Crouzet O. , Laurent F. , Kravtsoff J., Mazoyer M.-A. and Cournoyer B. (2008): Spatio-temporal analysis of infra-specific genetic variations among a Pseudomonas aeruginosa water network hospital population: invasion and selection of clonal complexes Journal of Applied Microbiology, 105(5):1491-1501
20. Jiun-Ling W., Mei-Ling C., Yusen E. L., Shan-Chwen C. and Yee-Chun C (2009). Association between Contaminated Faucets and Colonization or Infection by Nonfermenting Gram-Negative Bacteria in Intensive Care Units in Taiwan J. Clin. Microbiol*;* 47(10):3226.
21. Trautmann, M.; Bauer, C.; Schumann, C.; Hahn, P.; Höher, M.; Haller, M. and Lepper, P.M.(2006): Common RAPD pattern of *Pseudomonas aeruginosa* from patients and tap water in a medical intensive care unit. Int. J. Hyg. Environ. Health ; 209(4):325-31.
22. Shahcheraghi, F. ; Feizabadi, M.M.; Yamin, V.; Abiri, R. and Abedian Z. (2003): Serovar determination, drug resistance patterns and plasmid profiles of *Pseudomonas aeruginosa* isolated from burn patients at two hospitals of Tehran (IRAN). Burns; (29): 547–551

2/21/2012

Detection of Serum Kl-6 as a Tumor Marker in Hepatocellular Carcinoma

[1]Soha Z. El-Shenawy, [2]Maha M El-Sabawy, [3]Eman Abd El-Razik, [4]Maha M Allam

Departments of [1]Clinical Biochemistry, [2]Hepatology and [4]Clinical Pathology, National Liver Institute, Menofiya University, Department of [3]Oncology, Faculty of Medicine, Menofiya University, Egypt
E-mail: sohazaki69@yahoo.com

ABSTRACT: Background: Hepatocellular carcinoma (HCC) is a common malignancy affecting approximately one million of people around the world every year and represents the fifth most common cancer worldwide. Early detection of the onset of HCC would help to select more effective therapies for patients, leading to a better prognosis and longer life span. **The aim of this study** was to evaluate the efficacy of KL-6 as a diagnostic marker of HCC in Egyptian patients. **Subjects & methods:** this study was conducted on three groups. Group 1: included 57 patients (48 males and 9 females) were diagnosed as HCC by the presence of characteristic hepatic masses on abdominal MRI, CT and/or hepatic angiography. Group 2: included 46 patients (37 males and 9 females) hepatitis B virus and/or hepatitis C virus –related cirrhotic patients. Group 3: included 40 subjects (32 males and 8 females) apparently healthy as a control group with no evidence of liver disease and/or neoplasm. Serum levels of AFP and KL-6 were measured in all patients groups and control groups. **Results:** A highly statistically significant difference was found between the three groups regarding the mean serum levels of both AFP and KL-6 where the highest increase of both markers were found in the HCC group. When Spesrman's correlation coefficient was done in the patients, a significant positive correlation (*P* value <0.001) of serum levels of AFP & KL-6 was found. When analysis of the results of AFP in the patients with HCC, this study found 39 patients (68.4%) having + ve AFP level (high AFP than its normal range), while 48 HCC patients (84.2%) are + ve for KL-6 level (high KL-6 than its normal range). In patients with liver cirrhosis, 19 (41.3%) patients have + ve AFP while 24 cirrhotic patients (52.2%) are + ve for KL-6 level. Results of ROC curves analysis show that the optimal cut-off values were 437 U/ml for KL-6 (sensitivity = 91.7% & specificity = 85.7%) and 102 IU/ml for AFP with sensitivity = 84.6% and specificity = 89.4%. **Conclusion:** These results suggest that KL-6 could be a promising tumor marker for early detection of HCC in Egyptian patients. A large scale study is needed to investigate its clinical usefulness in screening cirrhotic patient for HCC.

[Soha Z. El-Shenawy, Maha M El-Sabawy, Eman Abd El-Razik, Maha M Allam. **Detection of Serum Kl-6 as a Tumor Marker in Hepatocellular Carcinoma.** Life Science Journal. 2012;9(1):667-673] (ISSN:1097-8135). http://www.lifesciencesite.com. 97

Keywords*:* Hepatocellular carcinoma (HCC), alpha-fetoprotein (AFP), tumor marker, KL-6

1. Introduction

Hepatocellular carcinoma (HCC) accounts for 80% to 90% of primary liver cancer. HCC is a major health problem worldwide. It is the fifth most common cancer in the world and the third most common cause of cancer-related death. The rates of HCC in men are 2 to 4 times higher than in women. It usually develops between 35 and 65 years of age, when people are in their most productive era in their life with the outmost family responsibilities [(1)]. Complex carcinogenesis of HCC is a multi-factorial, multi-step and complex process, which is associated with a background of chronic and persistent infection of hepatitis B virus (HBV) and hepatitis C virus (HCV) [(2)].

HCC with poor prognosis has many characteristics, such as fast infiltrating growth, metastasis in early stage, high-grade malignancy and poorly therapeutic efficacy. Early detection may offer hope for a more favorable prognosis as most of HCC patients died quickly due to the rapid tumor progression [(3)].

Current diagnosis of HCC relies on clinical information, liver imaging and measurement of serum alpha-fetoprotein (AFP) [(4)]. Serum alpha-fetoprotein (AFP) was first described as a marker for HCC by **Abelev *et al.,*** in the **1960** and used as a serum marker for HCC in humans for many years [(5)]. The first quantitative serum assays for AFP were established by **Ruoshlati and Seppala** [(6)]. It has a sensitivity of 39%–65%, a specificity of 76%–94%, and a positive predictive value of 9%–50% [(7)]. HCC patients with a high AFP concentration (≥ 400 IU/mL) tend to have greater tumor size, bilobar involvement, massive or diffuse types, portal vein thrombosis and a lower median survival rate [(8)]. Though the measurement of AFP serves as an important tool in screening of HCC, some reports have indicated that it has limited utility of differentiating HCC from benign hepatic disorders for: its high false-positive and false-negative rates,

elevated levels in patients with acute exacerbation of viral hepatitis and that tumors other than HCC may also have markedly increased AFP levels like testicular tumors [8].

AFP with its reported sensitivity and specificity are not sufficient for early diagnosis as AFP concentrations are directly correlated with tumor size. So, the development of effective marker for the diagnosis of HCC could have an impact on HCC-related cancer mortality and significant public health implications worldwide [9].

A number of serum markers have been proposed and currently used as an effective method for detecting HCC long time ago. The most urgent need was to find sensitive markers for early diagnosis and monitoring of postoperative recurrence of HCC patients, to give adequate treatment for HCC patients [10].

Mucins are large glycoproteins with high carbohydrate content and marked diversity both in the apoprotein and in the oligosaccharide moieties [11]. MUC1 mucin, one kind of mucin glycoprotein, is abundantly expressed at the surface of epithelial cells in many tissues [12]. It seems to influence various physiological or biochemical events as diminished immune response and regulate cell adhesion properties [13].

KL-6 mucin is a type of MUC1 mucin, recognized by a murine monoclonal antibody (mAb) **Kohno *et al.* (1988)** [14]. Biochemical analyses displayed that the molecular weight of kL-6 mucin was over 200 kDa because of a large amount of carbohydrate content [15].

KL-6 has been first shown to be elevated in patients with interstitial pneumonia [16]. Also, serum KL-6 is a sensitive effective marker of disease activity in fibrosing lung diseases [17]. **Hirasawa *et al.*, 1997** [18] reported in the epithelial lining fluid in small airways may cause the intra-alveolar fibrosis in fibrosing lung disease that KL-6 is one of the chemotactic factors for most fibroblasts and that the increased KL-6 in the epithelial lining fluid in small airways may cause the intra-alveolar fibrosis in fibrosing lung disease. Many investigations have shown that aberrant expression of MUC1 in gastrointestinal cancer tissue has clinicopathological and biological importance in cancer disease [19]. KL-6 mucin, was also investigated and suggested to have a significant relationship with tumor behavior especially cancer cell invasion in various gastrointestinal and primary liver cancers [20].

It was also reported to have a high positive rate in different non-hepatic malignancies and its expression was also correlated with metastatic potential of the primary tumor in some of them [21]. It has also been studied as a fibrosis marker in patients with HCV-related chronic liver disease and was found to correlate with the degree of irregular regeneration of hepatocytes. A recent study addressed its clinical significance as a tumor marker in HCV-related HCC [22]. However, all these studies investigated KL-6 in HCV-related disease only so that its actual significance as a marker for screening HCC in patients with different chronic liver disease is not yet fully understood [22]. In the light of these observations, we studied both KL-6 and AFP serum titers in a cohort of HCC patients, as well as in patients with cirrhotic liver disease, and in healthy controls. This study aims to show the efficacy of KL-6 as a more specific, sensitive and accurate biomarker than AFP to help in early diagnosis of hepatocellular carcinoma.

2.Subjects & Methods

In this study, the patients were selected from the Department of Hepatology, National Liver Institute, Minoufiya University and Department of Oncology, Faculty of Medicine, Minoufiya University. There were three groups, group (1) included 57 patients (48 males and 9 females & their mean age was 46.87±6.58 years) were diagnosed as HCC by the presence of characteristic hepatic masses on liver MRI, CT and/or hepatic angiography (i.e., enlarged tumors and/or tumors with typical arterial vascularization), group(2) [liver cirrhosis (LC)] included 46 patients (37 males and 9 females & their mean age was 42.28±9.34 years) hepatitis B virus and/or hepatitis C virus –related cirrhotic patients. A third group included 40 patients (32 males and 8 females) apparently healthy subjects as a control group with no evidence of liver disease and/or neoplasm & their mean age was 40.9±8.69 years. All the procedures used in this study were approved by the Research Ethics Committee of National Liver Institute and Faculty of Medicine, Minoufiya University, Egypt. An informed consent was obtained from all subjects in this study.

All individuals included in this study were fasting overnight. 10 ml of venous blood were withdrawn by venipuncture in the morning. It was centrifuged and the serum was frozen for subsequent analysis.

Assessment of serum AFP was performed using VIDAS instrument, BioMerieux, France using the Enzyme Linked Fluorescent Assay (ELFA). The results were expressed as IU/ml.

Serum level of KL-6 was determined, by the sandwich enzyme immunoassay method using the KL-6 antibody (Ab) as both the capture and tracer Ab, using AviBion Human KL-6 ELISA Kit, Orgenium Laboratories, Finland [23]. The results were expressed as U/ml.

Statistical analysis: Statistics were carried out using Statistical Package for Social Science program (SPSS). Data were presented as mean ± SD. Kruskal Wallis test (non parametric test) used for the values which aren't normally distributed in order to compare more than 2 groups. Determination of spearman's correlation coefficient (r) was done for correlation between serum AFP & KL-6 as a quantitative variables. Receiver Operating Characteristic (ROC) curve was produced for the measured parameters to investigate the sensitivity, specificity and the cut-off values of each AFP and KL-6. P value less than 0.05 was considered statistically significant.

3. Results

Table (1) showed the patient's background in the three studied groups regarding the presence of viral infection (either HCV, HBV, both HCV-HBV and none), Child classification and tumor size & its differentiation.

When analysis of variants were done between the three groups, a highly statistical significant difference was found between these groups regarding the mean serum levels of both AFP and KL-6 where the highest increase of both markers were found in the HCC group (Table 2). When Spesrman's correlation coefficient was done in the patients, a significant positive correlation (P value <0.001) of serum levels of AFP & KL-6 was found (Table 3).

On analysis of the results of AFP in the patients with HCC (Table 4), 39 (68.4%) patients are + ve AFP level (high AFP than its normal range), while 48 HCC patients (84.2%) are + ve for KL-6 level (high KL-6 than its normal range). In the patients with liver cirrhosis, 19 (41.3%) patients have +ve AFP while 24 patients with cirrhosis are + ve for KL-6 serum levels. As regards KL-6 serum levels in relation to Child classification in both patient groups with liver cirrhosis (103 patients), a positive correlation was detected between the patients, with a progressive increase in serum levels of KL-6 from patients with child A cirrhosis (424.5 U/ml), to patients with child B cirrhosis (1086 U/ml), up to patients with child C cirrhosis (3799 U/ml) (Table 5).

Table (6) illustrates another positive correlation which was found between the KL-6 serum levels in relation to the tumor size in the HCC group (57 patients). KL-6 was found to be (424.5 U/ml) in patients with tumor size less than 3 cm, (518.7 U/ml) in patients with tumor size 3-5 cm, and (752.3 U/ml) in patients with tumor size 5 cm.

Figure (1) represent the ROC curve of KL-6 as a test variable in comparison with AFP in the patients and figure (2) represent the ROC curve of AFP as a test variable in comparison with KL-6 in the same patient groups. Results of ROC curves analysis show that the optimal cut-off values were 437 U/ml for KL-6 (sensitivity = 91.7% & specificity = 85.7%) and 102 IU/ml for AFP with sensitivity = 84.6% and specificity = 89.4% as reported in table (7).

Table (1): Characteristics of the groups

	HCC group (N=57)		LC group (N=46)		Control group (N=40)	
	No	%	No	%	No	%
Gender: Male	48	84.2	37	80.4	32	80
Female	9	15.8	9	19.6	8	20
Viral infection:						
HCV-related	26	45.6	24	52.2	18	45
HBV-related	3	5.3	2	4.4	2	5
Both C&B-related	9	15.8	6	13	3	7.5
Non-viral	19	33.3	14	30.4	17	42.5
Child classification:						
A	12	21.1	16	34.8	00	00
B	13	22.8	11	23.9	00	00
C	32	56.1	19	41.3	00	00
Tumor size:						
< 3 cm	15	26.3	00	00	00	00
3-5 cm	25	43.9	00	00	00	00
>5 cm	17	29.8	00	00	00	00
Tumor multiplicity:						
Solitary	33	57.9	00	00	00	00
Multiple	24	42.1	00	00	00	00
Differentiation:						
Well	39	68.4	00	00	00	00
Poor	18	31.6	00	00	00	00

Table (2): AFP and KL-6 serum levels in the all studied groups

	HCC group (N=57) Mean ± SD	LC group (N=46) Mean ± SD	Control group (N=40) Mean ± SD	Test of sign. (Z-test)	*P* value
AFP (IU/ml)	4099.0 ± 1381.0	60.0 ± 53.84	2.6 ± 0.42	59.125	<0.001*
KL-6 (U/ml)	621.43 ± 257.19	319.7 ± 117.82	236.52 ± 95.68	61.9	<0.001*

P value is highly significant at <0.001*.

Table (3): Spearman's correlation coefficient between AFP and KL-6 in all patients (N=103).

	r	Sig (2-tailed)	*P* value
AFP & KL-6	0.625	0.000	<0.001*

P value is highly significant correlation at <0.001*.

Table (4): Descriptive statistics of the studied parameters (AFP & KL-6) in HCC group and LC group.

	HCC group (N=57)				LC group (N=46)			
	+ ve		-ve		+ ve		- ve	
	No	%	No	%	No	%	No	%
AFP (IU/ml)	39	68.4	18	31.6	19	41.3	27	58.7
KL-6 (U/ml)	48	84.2	9	15.8	24	52.2	22	47.8

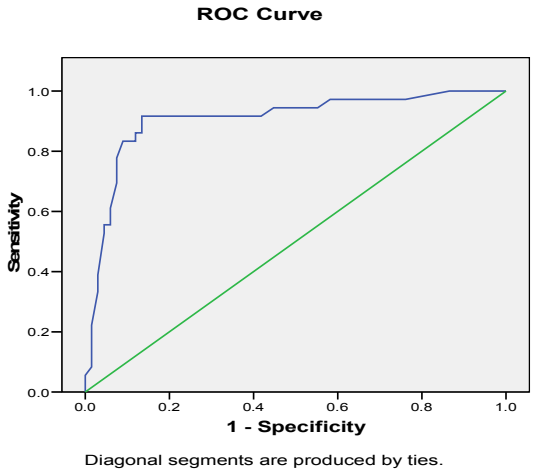
Table (5) KL-6 serum levels in relation to Child classification in all patients (No: 103 patients)

	No	%	Mean ± SD	Test of sign.	P value
Child A	28	27.2	323.5±149.15	31.3	<0.001*
Child B	24	23.3	1086.6±401.6		
Child C	51	49.5	3799.1±416.9		

P value is highly significant correlation at <0.001*.

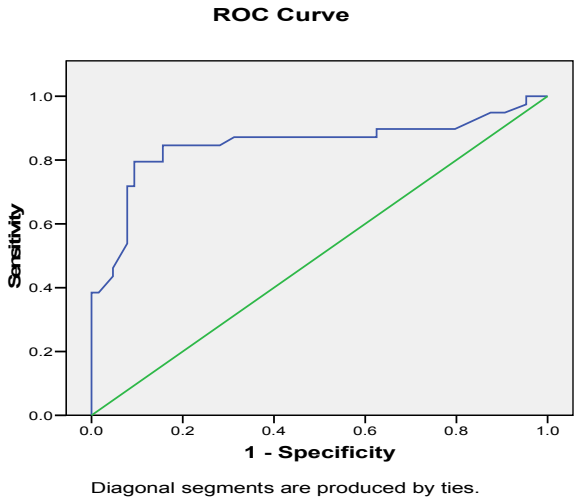
Table (6) KL-6 serum levels in relation to tumor size in HCC patients (No: 57 patients)

	No	%	Mean ± SD	Test of sign.	P value
< 3cm	15	26.3	424.5±211.9	74.3	<0.001*
3-5 cm	25	43.9	518.7±225.1		
> 5 cm	17	29.8	752.3±322.3		



Area under curve (AUC)	Standard error (SE)	95 % Confidence interval (CI)	
		Lower bound	Upper bound
0.906	0.034	0.840	0.973

Figure (1): ROC curve of KL-6 as a test variable in comparison with AFP in the patients (N= 103).



Area under curve (AUC)	Standard error (SE)	95 % Confidence interval (CI)	
		Lower bound	Upper bound
0.848	0.047	0.757	0.940

Figure (2): ROC curve of AFP as a test variable in comparison with KL-6 in the patients (N= 103).

Table (7): ROC curves analysis of the studied parameters (AFP & KL-6) in the patients (N=103).

	Sensitivity (%)	Specificity (%)	Cut-off point	PPV (%)	NPV (%)
AFP (IU/ml)	84.6	89.4	102	84.6	49.5
KL-6 (U/ml)	91.7	85.7	437	49.5	49.5

PPV: Positive predictive value NPV: Negative predictive value

4. Discussion

Hepatocellular carcinoma (HCC) is one of the commonest cancers worldwide, particularly in countries of the developing world and is increasing in incidence. The slow development and the late detection of HCC suggest that the identification of biomarkers of disease progression and early detection represents an attractive strategy for potential improvement of the outcome of HCC patients [24]. **Sherman, 2001** [25] stated that serum AFP is the most commonly used marker for HCC neoplasm, but its real clinical usefulness is unclear & furthermore, the role of AFP in HCC screening and diagnosis has lost most of the appeal that it had in the presophisticated (i.e. multislice, contrast-enhanced computed tomography, magnetic resonance imaging, etc.) imaging era. Because of the reported sensitivity (39% - 65%) and specificity (76% - 94%) of serum AFP are not sufficient for early diagnosis of HCC, additional effective markers are needed [7].

Aim of this study was to investigate the serum levels of KL-6 and AFP in HCC Egyptain patients, proving its role as a new diagnostic and prognostic marker of early detection of HCC in Egypt.

In the present study a statistically significant difference in the mean of the serum level of AFP between the three groups with highest increase in the HCC group and a slight increase in the cirrhotic group. These findings agreed with **Lau and Lai, 2008** [1] as they stated that the diagnosis of HCC is typically made by radiological liver imaging in combination with serum AFP but marginal elevations are common in patients with chronic hepatitis or cirrhosis.

Trerotoli and his colleagues, 2009 [9] stated that AFP, the only marker commonly used in clinical practice, displays poor sensitivity and a high specificity only for values higher than 400 IU/ml. Also **Farinati and his coworkers, 2006** [26] reported that because AFP concentrations are directly with tumor size, the reliability of such marker appears inadequate for early recognition of HCC.

Regarding the mean serum level of KL-6, a highly statistical significant difference was detected between HCC, LC and control groups with marked increase in the HCC group. **Gad and his coworkers, 2005** [27] agreed with these results as they found a significantly higher mean KL-6 in HCC compared

with non-HCC; either with or without LC; in addition no difference in mean KL-6 was found among HCC patients with and without LC. They stated that such findings together point to KL-6 association with HCC independent on the presence or absence of LC.

KL-6 serum levels were found to be significantly elevated with higher Child scores. This comes in accordance with **Suzuki *et al.*, in 2003** [28] found that levels of serum KL-6 of liver cirrhosis patients were significantly higher than that for the chronic hepatitis patients and when liver cirrhosis was classified according to Child's system, the level of serum KL-6 for the Child B/C patients was significantly higher than that for the Child A patients. Thus they suggested a correlation between KL-6 and liver disease, stating that this marker reflects hepatic fibrosis even better than pulmonary fibrosis.

The current study showed a highly statistical significant positive correlation between AFP and KL-6. **Gad *et al.*, 2005** [27] disagreed with these results as they stated that KL-6 serum level did not correlate with either serum AFP or PIVKA-II levels which points to its behavior independently from either of them and this may justify its clinical significance as an independent tumor marker for HCC diagnosis when considered with both AFP and PIVKA-II. This difference may be due to most patients of this study were HCV + ve as **Moriyama and his colleagues, 2003** [29] stated that there is a clinical significance of KL-6 as a tumor marker in HCV-related HCC.

The present study showed that 84.2% (48 patients) of HCC group having elevated serum level of KL-6, while 68.4% (39 patients) of the same group having elevated serum level of AFP. Regarding the cirrhotic group, 52.2% (24 patients) showed elevated serum level of KL-6 compared to 41.3% (19 patients) only showed elevated serum level of AFP in the same group. So, these data indicate that KL-6 could be a promising tumor marker for early detection for HCC.

Soresi *et al.*, 2003 [30] agreed with the above results as they stated that the false negative rate with AFP level alone may be as high as 40% for patients with early stage HCC and even in patients with advanced HCC, the AFP levels may remain normal in 15%-30% of the patients. Also, **Franca and his coworkers, 2004** [31] reported that up to 42% of patients with HCC present with serum AFP levels within normal values.

Another positive correlation in this study was found between serum levels of KL-6 and the tumor size revealing that this marker is related to the HCC tissue. this postulation does not correlate with the study done by **Gad *et al.*, 2005** [27], which revealed that no significant association with tumor size, echogenicity or multiplicity with significantly lower mean KL-6 was noticed in larger size tumors of >5 cm in comparison with tumors of less than or equal to 5 cm.

Applying the ROC curves analysis showed the best cut-off value of KL-6 to detect HCC was 437 U/ml with sensitivity 91.7% and specificity of 85.7% and the best cut-off value of AFP was 102 IU/ml with 84.6% and 89.4% in sensitivity and specificity respectively. **Gad and his coworkers, 2005** [27] used a cut-off point of 334 U/ml in his analysis of KL-6 as a tumor marker in patients with HCC. This cut-off gave the best sensitivity (60%) in their study.

Regarding the AFP cut-off level for the diagnosis of HCC**, Goma and his colleagues, 2003** [7] revealed an AFP value above 400-500 ng/ml has been considered to be diagnostic for HCC in patients with cirrhosis. Also, **Lau and Lai, 2008** [1] stated the specificity of AFP is very high when the levels are above 400 ng/ml in patients without testicular tumor. **Yao *et al.*, 2007** [32] reported that it is sometimes very difficult to make the distinction between tumors and falsely elevated AFP levels because of benign liver diseases.

In conclusion, this study suggests that KL-6 could be a good marker for detection of HCC in Egyptian patients. A large scale study is needed to investigate its clinical usefulness in screening cirrhotic patient for HCC.

Corresponding author:
Soha Zaki El-Shenawy.
Departments of [1]Clinical Biochemist, National Liver Institute, Menofiya
sohazaki69@yahoo.com.

5. References

1- Lau WY and Lai EC (2008): Hepatocellular carcinoma: current management and recent advances. Hepatobiliary & Pancreatic Dis Int.; 7: 237-257.
2- Deng-Fu Y, Zhi-Zhen D and Min Y (2007): Specific molecular markers in hepatocellular carcinoma. Hepatobiliary & Pancreat Dis Int.; 6: 241-247.
3- Jain D, Singhal S, Lee P and Xu R (2010): Molecular genetics of hepatocellular neoplasia. Am J Transl Res.; 2(1): 105-118.
4- Marrero JA (2005): Screening tests for hepatocellular carcinoma. Clin Liver Dis.; 9: 235-251.
5- Abelev GI, Perova SD, Khramkova NI, Postnikova ZA and Irlin IS (1963): Production of embryonal alpha-globulin by transplantable mouse hepatomas. Transplantation; 1: 174-180.
6- Ruoslahti E and Seppala M (1971): Studies of carcino-fetal proteins. Development of a radioimmunoassay for fetoprotein. Demonstration of fetoprotein in serum of healthy human adults. Int J Cancer; 8: 374-383.
7- Gomaa A, Khan SA, Leen E, Waked I and Taylor-Robinson SD (2003): Diagnosis of hepatocellular carcinoma. World J of Gastroenterology; 15(11): 1301-1314.

8- Daniele B, Bencivenga A, Megna AS and Tinessa V (2004): Alpha-fetoprotein and ultrasonography screening for hepatocellular carcinoma. Gastroenterology; 127: S108-S112.
9- Trerotoli P, Fransvea E, Angelotti U, Antonaci G, Lupo L, Mazzocca A, *et al.* (2009): Tissue expression of squamous cellular carcinoma antigen (SCCA) is inversely correlated to tumor size in HCC. Molecular Cancer; 8: 29.
10- Zhou L, Liu J and Luo F (2006): Serum tumor markers for detection of hepatocellular carcinoma. World J Gastroenterol.; 12: 1175–1181.
11- Hollingsworth MA and Swanson BJ (2004): Mucins in cancer: protection and control of the cell surface. Nature Rev Cancer; 4: 45-60.
12- Gendler SJ and Spieer AP (1995): Epithelial mucin genes. Annu Rev Physiol.; 57: 607-634.
13- Arase Y, Ikeda K and Tsubota A (2003): Usefulness of serum KL-6 for early diagnosis of idiopathic pulmonary fibrosis in patients with hepatitis C virus. Hepatol Res.; 27: 89-94.
14- Kohno N, Akiyama M, Kyoizumi S, Hakoda M, Kobuke K and Yamakido M (1988): Detection of soluble tumor-associated antigens in sera and effusions using novel monoclonal antibodies, KL-3 and KL-6, against lung adenocarcinoma. Jpn J Clin Oncol.; 18: 203-216.
15- Arase Y, Ikeda K and Suzuki F (2005): Usefulness of the serum KL-6 assay in patients with hepatitis C virus. Intervirology; 48:400-404.
16- Ohnishi H, Yokoyama A, Kondo K, Hamada H, Abe M, Nishimura K, *et al.* (2002): Comparative study of KL-6, surfactant protein-A, surfactant protein –D and monocyte chemoattractant protein-1 as serum markers for interstitial lung diseases. Am J Respir Crit Care Med.; 165: 378-381.
17- Kohno N (1999): Serum marker KL-6/MUC1 for the diagnosis and management of interstitial pneumonitis. J Med Invest.; 46: 151-158.
18- Hirasawa Y, Kohno N and Yokoyama A (1997): KL-6, a human MUC1 mucin, is chemotactic for human fibroblast. Am J Respir Cell Mol Biol.; 17: 501-507.
19- Inagaki Y, Xu H, Nakata M, Seyama Y, Hasegawa K, Sugawara Y, *et al.* (2009): Clinicopathology of sialomucin: MUC1, particularly KL-6 mucin, in gastrointestinal, hepatic and pancreatic cancers. Bioscience Trends; 3(6): 220-232.
20- Guo Q, Tang W, Inagaki Y, Midorikawa Y, Kokudo N, Sugawara Y, *et al.* (2006): Clinical Significance of subcellular localization of KL-6 mucin in primary colorectal adenocarcinoma and metastatic tissues. World J Gastroenterol.; 12(1): 54-59.
21- Suzuki K, Takada H, Oka S, Kanouzawa S, Limuro M, Kitazumi Y, *et al.* (2003): Clinical significance of KL-6, a marker of interstitial pneumonia, in cases of HCV-associated chronic liver disease. Intern Med.; 42: 650-654.
22- Moriyama M, Matsumura H, Watanabe A, Nakamura H, Arakawa Y, Oshiro S, *et al.* (2004): Detection of serum and intrahepatic KL-6 in anti-HCV positive patients with hepatocellular carcinoma. Hepatol Res.; 30: 24-33.
23- Hirasawa Y, Kohno N, Yokoyama A, Inoue Y, Abe M and Hiwada K (1997): KL-6, a human MUC1 mucin, is chemotactic for human fibroblasts. Am J Respir Cell Mol Biol.; 17: 501-507.
24- World Health Organization. Mortality Database. Available from: URL: http://www.who.int/whosis/en/ on 22/4/2011
25- Sherman M (2001): Alphafetoprotein: an obituary. J Hepatol.; 34: 603-605.
26- Farinati F, Marino D, DE Giorgio M, Baldan A, Cantarini M, Cursaro C, *et al.* (2006): Diagnostic and prognostic role of alpha-fetoprotein in hepatocellular carcinoma: both or neither? Am J Gastrenterol.; 524-532.
27- Gad A, Tanaka E, Matsumoto A, Abd-el Wahab M, Serwah H, Attia F, *et al.* (2005): Assessment of KL-6 as a tumor marker in patients with hepatocellular carcinoma. World J Gastroenterol.; 11(42): 6607-6612.
28- Suzuki K, Takada H, Oka S, Kanouzawa S, Imuro M, Kitazumi Y, et a. (2003): Clinical significance of KL-6, a marker of interstitial pneumonia, in cases of HCV-associated chronic liver disease. Int Medicine; 42(8): 650-654.
29- Moriyama M, Matsumura H, Mikuni M, Arkawa Y, Ohshiro S, Aoki H, *et al.* (2003): The clinical significance of serum KL-6 levels in patients with type C liver diseases. Hepatol Res.; 25: 385-395.
30- Soresi M, Magliarisi C, Campagna P, Leto G, Bonfissuto G and Rilli A (2003): Usefulness of alpha-fetoprotein in the diagnosis of hepatocellular carcinoma. Anticanver Res.; 23: 1747-1753.
31- Franca AV, Elias J, Lima BL, Martinelli AL and Carrilho FJ (2004): Diagnosis, staging and treatment of hepatocellular carcinoma. Braz J Biol Res.; 37: 1689-1705.
32- Yao DF, Dong ZZ and Yao M (2007): Specific molecular markers in hepatocellular carcinoma. Hepatobiliary Pancreat Dis Int.; 6(3): 241-247.

2/21/2012

The mechanisms by which Oxidative Stress and Free Radical Damage produces Male infertility

Magda M El-Tohamy

Department of Animal Reproduction & A.I., Veterinary Division, National Research Centre, Dokki,Cairo, Egypt
Eltohamymagda@yahoo.com

Abstract: In a healthy body, ROS (reactive oxygen species) and antioxidants remain in balance. When the balance is disrupted towards an overabundance of ROS, oxidative stress (OS) occurs. OS results from an imbalance between prooxidants (free radical species) and the body's scavenging ability (antioxidants). ROS are a double-edged sword - they serve as key signal molecules in physiological processes but also have a role in pathological processes. The production of ROS is a normal physiological event in various organs including the testis. Overproduction of ROS can be detrimental to sperm and being associated with male infertilities. The excessive generation of ROS by abnormal spermatozoa, contaminating leukocytes and by a various type of pollutants has been identified as detrimental etiologies for male infertilities Free radicals are substances with one or more unpaired electrons, which are formed as a results of many physiological and pathological cellular metabolic processes, especially in mitochondria. Enzymatic (Catalase, superoxide dismutase) and non enzymatic (vitamins A and E) natural antioxidant defense mechanisms exist; however, these mechanisms may be overcome, causing lipid peroxidation to take place. For example, breakdown in the cells results in the formation of molecules whose further metabolism in the cell leads to ROS production. Thus increased OS stimulates the activity of enzymes called cytochrome P450, which contribute to ROS production. . Oxidative stress index (OSI) was calculated as ([TOS/TAS] x 100). TOS and OSI were significantly higher and PON-1 activity and TAS were significantly lower in subfertile male with abnormal semen parameters than in male with idiopathic subfertility and fertile donors. PON-1 activity was also strongly correlated with sperm concentration, motility, and morphology in the overall group. The receiver operating characteristic curve analysis revealed a high diagnostic value for PON-1 activity with respect to male-factor sub fertility. ROS may cause infertility by two principal mechanisms, first ROS damage the sperm membrane which in turn reduces the sperm motility and ability to fuse with the oocyte secondly, and ROS directly damage sperm DNA, compromising the paternal genomic contribution to the embryo. Oxidative stress due to excessive production of ROS, impaired antioxidant defense mechanisms, or both precipitates a range of pathologies that are currently believed to negatively affect the male reproductive function. Oxidative stress-induced damage to sperm may be mediated by lipid peroxidation of the sperm plasma membrane, reduction of sperm motility, and damage to the DNA in the sperm nucleus, as the production of ROS is one of the principal mechanisms by which neutrophils destroy pathogens, it is not surprising that seminal leukocytes have the potential to cause oxidative stress. Despite the established role of OS in the pathogenesis of male infertility, there is a lack of consensus as to the clinical utility of seminal OS testing in an infertility clinic. One important reason for the inability to utilize the OS test in clinical practice is related to the lack of a standard protocol for assessment of seminal OS. Antioxidants are powerful and there are few trials investigating antioxidant supplementation in male reproduction. Several researches indicate that the diagnostic and prognostic capabilities of the seminal OS test are beyond those of conventional tests of sperm quality and function. The OS test can accurately discriminate between fertile and infertile male and identify male with a clinical diagnosis of male-factor infertility that are likely to initiate a pregnancy when followed over a period of time. We strongly believe that incorporating such a test into the routine andrology workup is an important step for the future of the male infertility practice. The resulting state of the cell, known as (OS) can lead to cell injury. ROS production and Lipid peroxidation, free radical and oxidative stress in relation to fertility are the aim of this review

[Magda M El-Tohamy. **The mechanisms by which Oxidative Stress and Free Radical Damage produces Male infertility.** Life Science Journal 2012; 9(1):674-688]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 98

Key Words: oxidative stress; free radicals; reactive oxygen species; proteins; DNA; lipids; glutathione peroxidase.

Introduction:

Reactive oxygen species (ROS), defined as including oxygen ions, free radicals and peroxides are generated by sperm and seminal leukocytes within semen and produce infertility by two key mechanisms. First, they damage the sperm membrane, decreasing sperm motility and its ability to fuse with the oocyte. Second, ROS can alter the sperm DNA, resulting in the passage of defective DNA.

The ability of sperm to produce ROS inversely correlates with their maturational state. During spermatogenesis, there is a loss of cytoplasm to allow the sperm to form its condensed, elongated form.

Immature teratozoospermic sperm are often characterized by the presence of excess cytoplasmic residues in the mid-piece. These residues are rich in the enzyme glucose-6-phosphate dehydrogenase, an enzyme which controls the rate of glucose flux and intracellular production of b-nicotinamide adenine dinucleotide phosphate (NADPH) through the hexose monophosphate shunt.

NADPH is used to fuel the generation of ROS via NADPH oxidase located within the sperm membrane**(Fisher and Aitken, 1997; Gomez *et al.*, 1998; Said *et al.*, 2005).** As a result, teratozoospermic sperm produce increased amounts of ROS compared with morphologically normal sperm.

The generation of ROS in the male reproductive tract has become a real concern because of their potential toxic effects at high levels on sperm quality and function. ROS are highly reactive oxidizing agents belonging to the class of free radicals **(Aitken, 1997)**. A free radical is defined as "any atom or molecule that possesses one or more unpaired electrons" **(Warren *et al.*, 1987)** reports have indicated that high levels of ROS are detected in semen samples of 25% to 40% of infertile male **(de Lamirande *et al.*, 1995; Padron *et al.*, 1997)**. However, strong evidence suggests that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities **(Aitken, 1999).**

Spermatozoa, like all cells living in aerobic conditions, constantly face the oxygen (O_2) paradox: O_2 is required to support life, but its metabolites such as ROS can modify cell functions, endanger cell survival, or both **(de Lamirande and Gagnon, 1995)**. Hence, ROS must be continuously inactivated to keep only a small amount necessary to maintain normal cell function. It is not surprising that a battery of different antioxidants is available to protect spermatozoa against oxidants **(Sies, 1993).**

Seminal oxidative stress (OS) develops as a result of an imbalance between ROS generating and scavenging activities **(Sharma and Agarwal, 1996; Sikka, 2001).** Spermatozoa are particularly susceptible to OS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids (**Alvarez and Storey, 1995)** and their cytoplasm contains low concentrations of scavenging enzymes **(Aitken and Fisher, 1994; de Lamirande and Gagnon, 1995; Sharma and Agarwal, 1996).** In addition, the intracellular antioxidant enzymes cannot protect the plasma membrane that surrounds the acrosome and the tail, forcing spermatozoa to supplement their limited intrinsic antioxidant defenses by depending on the protection afforded by the seminal plasma **(Zini *et al.*, 1993)**. Oxidative stress attacks not only the fluidity of the sperm plasma membrane, but also the integrity of DNA in the sperm nucleus **(Aitken, 1999).**

Even though OS has been established as a major factor in the pathogenesis of male infertility, there is a lack of consensus as to the clinical utility of seminal OS testing in an infertility clinic. One important reason for the inability to use an OS test in clinical practice may be the lack of a standard protocol to assess seminal OS. The main objective of this review in this area was to transfer this important knowledge from the research bench to clinical practice by designing studies with the following aims: 1) to understand the precise mechanism by which OS develops in semen, which we thought, could help and develop strategies to overcome the problem, 2) to establish assays for accurate and reliable assessment of seminal OS, and 3) to identify the clinical significance of testing seminal OS in the male infertility practice. In this review, we summarize the efforts of some studies to explore the role of OS in male infertility.

Reactive Oxygen Species (ROS) and Sperm Physiology

ROS were exclusively considered toxic to spermatozoa. The idea that limited amounts of ROS can intervene in a physiological manner in the regulation of some sperm functions was first evoked in a study by **Aitken *et al.* (1989).** The authors found that low levels of ROS can enhance the ability of spermatozoa to bind with zonal pellucida, an effect that was reversed by adding vitamin E. Other studies have found that incubating spermatozoa with low concentrations of hydrogen peroxide (H_2O_2) stimulates sperm capacitation, hyperactivation, and the ability of the spermatozoa to undergo the acrosome reaction and oocyte fusion **(de Lamirande and Gagnon, 1993; de Lamirande *et al.*, 1995; Kodoma *et al.*, 1996; Aitken, 1997).** ROS other than H_2O_2, such as nitric oxide and superoxide anion ($O_2^{\bullet}$), have also been shown to promote sperm capacitation and the acrosome reaction **(Zini *et al.*, 2000).**

Mechanism of Antioxidant Protection in Semen

It is interesting that seminal plasma is well endowed with an array of antioxidant defense mechanisms to protect spermatozoa against OS **(Sikka, 1996; Armstrong *et al.*, 1998)**. These mechanisms compensate for the deficiency in cytoplasmic enzymes in sperm **(Donnelly *et al.*, 1999).** Seminal plasma contains a number of enzymatic antioxidants such as superoxide dismutase (SOD) **(Alvarez *et al.*, 1987),** the glutathione peroxidase/glutathione reductase (GPX/GRD) system **(Chaudiere *et al.*, 1984)**, and catalase **(Jeulin *et al.*,**

1989). In addition, seminal plasma contains a variety of nonenzymatic antioxidants such as ascorbate **(Fraga *et al.*, 1991)**, urate **(Thiele *et al.*, 1995),** -tocopherol **(Aitken and Clarkson, 1988; Moilanen *et al.*, 1993)**, pyruvate **(de Lamirande and Gagnon, 1992),** glutathione **(Lenzi *et al.*, 1994),** taurine, and hypotaurine **(Alvarez and Storey, 1983).**

It has been reported that seminal plasma from fertile male has a higher total antioxidant capacity (TAC) than seminal plasma from infertile male **(Lewis *et al.*, 1995)**. However, pathological levels of ROS detected in semen from infertile male are more likely a result of increased ROS production rather than reduced antioxidant capacity of the seminal plasma **(Zini *et al.*, 1993).** Antioxidant defense mechanisms include three levels of protection: 1) prevention, 2) interception, and 3) repair **(Sies, 1993)**.

The term oxidative stress is applied when oxidants out-number antioxidants **(Sies, 1993)**, peroxidation products develop **(Spitteler, 1993),** and when these phenomena cause pathological effects **(Janssen *et al.*, 1993).** Oxidative stress has been implicated in numerous disease states such as cancer, connective tissue disorders, aging, infection, inflammation, acquired immune deficiency syndrome, and male infertility **(Aitken *et al.*, 1992, 1995).**

In the context of human reproduction, a balance normally exists between ROS generation and scavenging in the male reproductive tract. As a result, only a minimal amount of ROS remains, which is needed to regulate normal sperm functions such as sperm capacitation, acrosome reaction, and sperm-oocyte fusion **(Gagnon *et al.*, 1991; Griveau and Le Lannou, 1997).** Excessive ROS production that exceeds critical levels can overwhelm all antioxidant defense strategies of spermatozoa and seminal plasma causing OS **(Sharma and Agarwal, 1996; de Lamirande *et al.*, 1997; Sikka, 2001).** All cellular components including lipids, proteins, nucleic acids, and sugars are potential targets for OS. The extent of OS-induced damage depends not only on the nature and amount of ROS involved but also on the moment and duration of ROS exposure and on extracellular factors such as temperature, oxygen tension, and the composition of the surrounding environment including ions, proteins, and ROS scavengers.

Lipid Peroxidation of Sperm Plasma Membrane

Lipid peroxidation is broadly defined as "oxidative deterioration of PUFA" (ie, fatty acids that contain more than two carbon-carbon double bonds **(Halliwell, 1984)**. The LPO cascade occurs in two fundamental stages: initiation and propagation. The hydroxyl radical ($OH^{\bullet}$) is a powerful initiator of LPO **(Aitken and Fisher, 1994).** Most membrane PUFAs have unconjugated double bonds that are separated by methylene groups. The presence of a double bond adjacent to a methylene group makes the methylene C-H bonds weaker and, therefore, hydrogen is more susceptible to abstraction. Once this abstraction has occurred, the radical produced is stabilized by the rearrangement of the double bonds, which form a conjugated diene radical that can then be oxidized. This means that lipids, which contain many methylene-interrupted double bonds, are particularly susceptible to peroxidation **(Blake *et al.*, 1987).** Conjugated dienes rapidly react with O_2 to form a lipid peroxyl radical (ROO•), which abstracts hydrogen atoms from other lipid molecules to form lipid hydroperoxides (ROOH). Lipid hydroperoxides are stable under physiological conditions until they contact transition metals such as iron or copper salts. These metals or their complexes cause lipid hydroperoxides to generate alkoxyl and peroxyl radicals, which then continue the chain reaction within the membrane and propagate the damage throughout the cell **(Halliwell, 1984).** Propagation of LPO depends on the antioxidant strategies employed by spermatozoa. One of the by-products of lipid peroxide decomposition is malondialdehyde. This by-product has been used in biochemical assays to monitor the degree of peroxidative damage in spermatozoa **(Aitken and Fisher, 1994).** The results of such an assay exhibit an excellent correlation with the degree to which sperm function is impaired in terms of motility and the capacity for sperm-oocyte fusion **(Aitken *et al*., 1993; Sidhu *et al*., 1998).**

Impairment of Sperm Motility

The increased formation of ROS has been correlated with a reduction of sperm motility **(Agarwal *et al*., 1994; Armstrong *et al*., 1999).** The link between ROS and reduced motility may be due to a cascade of events that result in a decrease in axonemal protein phosphorylation and sperm immobilization, both of which are associated with a reduction in membrane fluidity that is necessary for sperm-oocyte fusion **(de Lamirande and Gagnon, 1995).** Another hypothesis is that H_2O_2 can diffuse across the membranes into the cells and inhibit the activity of some enzymes such as glucose-6-phosphate-dehydrogenase (G6PD). This enzyme controls the rate of glucose flux through the hexose monophosphate shunt, which in turn, controls the intracellular availability of nicotinamide adenine dinucleotide phosphate (NADPH). This in turn is used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH oxidase **(Aitken *et al*., 1997)**. Inhibition of G6PD leads to a decrease in the availability of NADPH and a concomitant accumulation of oxidized

glutathione and reduced glutathione. This can reduce the antioxidant defenses of the spermatozoa and increase peroxidation of membrane phospholipids **(Griveau *et al*., 1995).**

Impaired sperm function

Impaired sperm function is a general cause of male infertility and sub -fertility. A balanced and controlled generation of ROS is associated with normal sperm physiological capacitating and acrosome reaction (**de Lamirande and Gngon, 1993).** These finding stresses the importance of a balance between ROS scavenging and small, physiologic levels of ROS that are necessary for normal sperm function. An imbalance, excessive production of ROS and decreased level of antioxidant enzymes caused decreased sperm motility and viability and increased sperm defects by imitating an oxidative chain damaging protein, lipid and DNA. Seminal plasma has antioxidant system that seems to be very relevant to the protection of sperm. Seminal plasma contains small molecular mass free radicals scavenger such as vitamin C, E and tyrosine. Also, contains antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase that are active in scavenging ROS. Spermatozoa are particularly vulnerable to such stress because their plasma membranes are so enriched with unsaturated fatty acids, particularly decosohexaenoic acid.

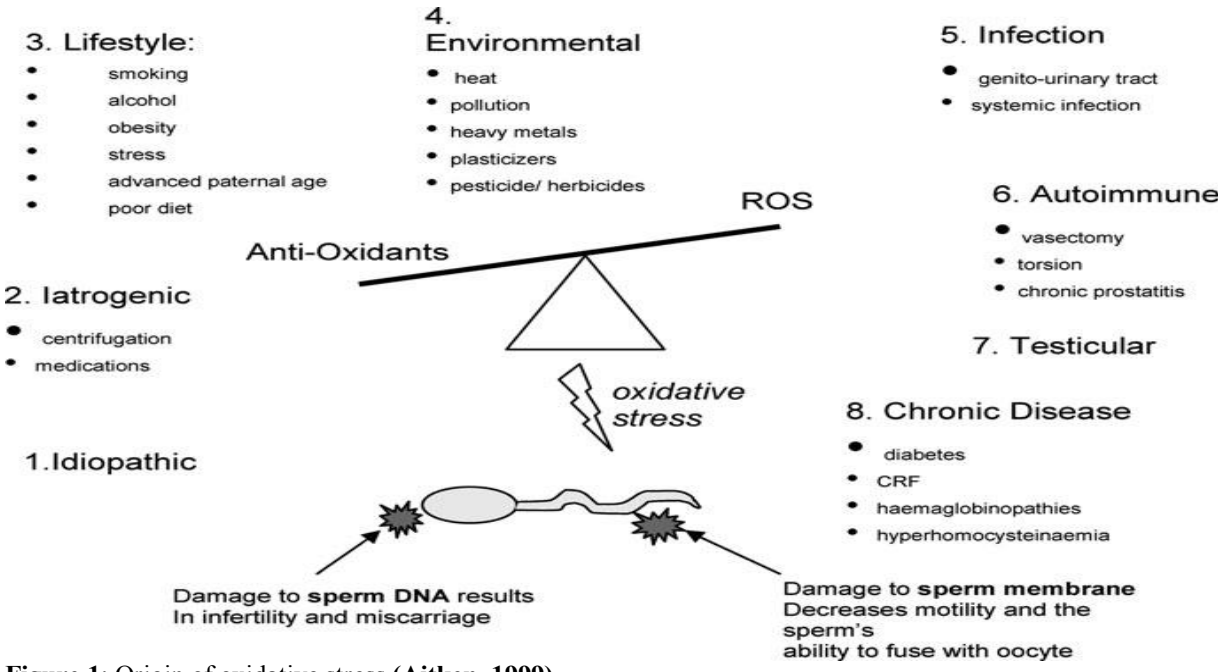


Figure 1: Origin of oxidative stress **(Aitken, 1999) .**

Origins of oxidative stress

The origins of sperm oxidative stress are summarized in **Fig. 1**. While pathologies such as genitourinary tract infection and varicocele are well established causes of oxidative stress, others such as hyper-homocysteinaemia and diabetes are only now just becoming recognized as possible causes. It is hoped that this review will stimulate further research in these less well established potential Causes of male oxidative infertility.

Oxidative stress

Oxidative stress is the presence of active oxygen species in excess of the available antioxidant buffering capacity. These products, ROS can damage proteins, lipids and DNA, altering the organism's structure and function. Not surprisingly, the organism has an efficient system to buffer these products and also not surprisingly, it will occasionally not be able to do so **(James *et al*., 2004).** When this happens, whether because of reduced antioxidant enzymes, insufficient intake of dietary antioxidants or with excessive production of ROS, oxidative stress ensues.

Oxidative stress may be defined as an imbalance between prooxidant and antioxidant forces resulting in an overall prooxidant insult. Pregnancy is a physiological state accompanied by a high energy demand of many bodily functions and an increased oxygen requirement. Because of levels of oxidative stress would be expected. Arguments for a role of oxidative stress /oxidative lipid derivatives in the pathogenesis of preeclampsia are documented in many papers other conditions such as toxic substance exposure induces oxidative stress. The oxidized lipid

products generated as a consequence of these conditions are highly reactive and cause damage to cells and cell membranes. Thus increased oxidative stress accompanied by reduced endogenous defenses may play a role in the pathogenesis of a number of diseases in the new born **(Gitto *et al*., 2002).**

Oxidative Stress-Induced DNA Damage

Two factors protect sperm DNA from oxidative insult: the characteristic tight packaging of the DNA and the antioxidants present in seminal plasma **(Twigg *et al*., 1998a).** Studies in which the sperm was exposed to artificially produced ROS resulted in a significant increase in DNA damage in the form of modification of all bases, production of base-free sites, deletions, frame shifts, DNA cross-links, and chromosomal rearrangements **(Duru *et al*., 2000).** Oxidative stress has also been correlated with high frequencies of single and double DNA strand breaks **(Twigg *et al*., 1998a; Aitken and Krausz, 2001).**

Measurement of TOS in Seminal Plasma

The TOS of semen samples was determined by using a new automated colorimetric measurement method **(Erel, 2005).** The assay is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidant species in acidic medium and the measurement of the ferric ion by xylenol orange. Within- and between-batch precision values were lower than 3%. The results were expressed as µmol hydrogen peroxide (H_2O_2) equivalent per liter **(Verit *et al*., 2006).**

Measurement of TAS in Seminal Plasma

TAS of semen samples was determined by using a novel automated measurement method developed by **Erel. (2004).** In this method, the hydroxyl radical, the most potent radical, is produced via Fenton reaction and consequently the colored dianisidinyl radical cations, which are also potent radicals, are produced in the reaction medium of the assay. Antioxidant capacity of the added sample against these colored potent free radical reactions measured the total antioxidant capacity. The assay has excellent precision values; within- and between-laboratory precision values are lower than 3%. The results were expressed as millimoles of Trolox equivalent per liter **(Verit *et al*., 2006).**

Measurement of PON-1 Activity in Seminal Plasma

PON-1 activity was determined by using paraoxon as a substrate and measured by increases in the absorbance at 412 nm because of the formation of 4-nitrophenol as already described **(Verit *et al*., 2008).** Briefly, the activity was measured at 25°C by adding 50 µL of seminal plasma to 1 mL Tris-HCl buffer (100 mM at pH 8.0) containing 2 mM $CaCl_2$ and 5.5 mM of paraoxon. The rate of generation of 4-nitrophenol was determined at 412 nm. Enzymatic activity was calculated by using the molar extinction coefficient 17 100 M–1 cm–1.

Oxidative Stress Index

The percentage ratio of TOS to TAS gave the oxidative stress index (OSI), an indicator of the degree of oxidative stress ([TOS/TAS x 100] **(Verit *et al*., 2006).** Oxidative stress (OS), as one of HS-induced response, has long believed to influence male reproductive function. Although OS was suggested as an important factor in disruption of sperm function over 50 years ago, the importance of OS has gained recently a wider understanding. Reactive oxygen species (ROS) are normal physiological event in various organs including the testis. Paradoxically, the production of ROS is both essential and detrimental to life; hence, numerous studies indicate that ROS play an important role in normal sperm function and that an imbalance in ROS production (over-production) and/or degradation (under-scavenging) by antioxidants may have serious adverse effects on sperm (**Akiyama, 1999).**

Protocol for Measurement of Total Antioxidant Capacity in Semen by Enhanced Chemiluminescence Assay— Total antioxidant capacity in the seminal plasma can be measured using an enhanced chemiluminescence assay **(Kolettis *et al*., 1999).** Frozen samples of seminal plasma are thawed at room temperature and immediately assessed for TAC. Seminal plasma is diluted 1:20 with deionized water (dH_2O) and filtered through a 0.20-µm filter (Allegiance Healthcare Corporation, McGaw Park, Ill). Signal reagent is prepared by adding 30 µL of H_2O_2 (8.8 molar/L), 10 µL of para-iodophenol stock solution (41.72 µM), and 110 µL of luminol stock solution (3.1 mM) to 10 mL of Tris buffer (0.1 M pH 8.0). Horseradish peroxidase working solution is prepared from the HRP stock solution by making a dilution of 1:1 of dH_2O. Light emission occurs when the chemiluminescent substrate luminol is oxidized by H_2O_2 in a reaction catalyzed by HRP. HRP catalyzes the reaction between a hydrogen acceptor (oxidant) and a hydrogen donor. Under normal circumstances, this reaction produces low-intensity light emission that may decay rapidly. The characteristics of the reaction can be altered substantially by the addition of para-iodophenol as an enhancer that gives a more intense, prolonged, and stable light emission. The continuous light output depends on the constant production of free radical intermediates derived from para-iodophenol (enhancer), luminol (substrate), and H_2O_2 (oxidizer)

in the presence of HRP. ROS production from spermatozoa has been measured by chemiluminescence in the two fractions of a Percoll gradient column (47 and 90%). Chemiluminescent signals were recorded in each fraction after the addition of luminol and horse-radish peroxidase (basal state), and after stimulation with formyl-methionyl-leucyl-phenylalanine and phorbol ester (PMA). Oligozoospermic samples show a higher rate of ROS production than the normozoospermic samples in both fractions of Percoll. Also, ROS were generated at a higher rate by asthenozoospermic samples in the 90% Percoll fraction than by normal samples after stimulation with PMA. Data confirm that fact of white blood cells play a major role in the production of ROS, even after purification on a Percoll gradient. Immunological cases were also found to be associated with an increased production of ROS, which may be caused by the same underlying pathological condition responsible for the production of the antibodies. Repeated centrifugation of the samples triggers a burst of ROS in excess of that produced after Percoll preparation **(Kolettis *et al*., 1999).** In addition, superoxide dismutase activity was found to be significantly increased in cases with an elevated production of ROS. It is concluded that measuring the ROS generation by semen may yield useful information on the functional capacity of spermatozoa, which may be used to improve the success of male infertility management. These findings explain the importance of a balance between ROS scavenging and small, physiologic levels of ROS that are necessary for normal sperm function.

In biological systems, a diversity of antioxidant defense systems operates to control levels of ROS. Some antioxidants synthesized within the cells themselves (endogenous) and others need to be provided in the diet (exogenous). These ROS scavengers have an important protective action on the membrane integrity and lipid stability in both seminal plasma and spermatozoa. Only a few reports have analyzed the nutritional requirement (additives) of rabbit bucks to improve both fertility and resistance to the hazards of HS.

There are many sources of oxygen radicals in cells that make them ubiquitous species. The role of oxygen radicals in physiological processes is now far from being completely understood and depends in the first place on the ability of oxygen radicals to interact with substances, drugs, proteins, lipids, enzymes, DNA and other cellular compounds. However the effect of oxygen radicals is not confirmed to these processes their action on such cell components as mitochondria microsomes plasmolemma, sarcoplasmic reticulum etc and on the cells themselves is also of great importance it is common knowledge that oxygen radicals are able to damage cells and cellular components, being possibly a primary factors of many pathologies **(Afanas 1991).**

A free radical is any molecule capable of independent usually brief existence that contains one or more unpaired electrons. Most free radicals in biology fit within the broader category of ROS which include not only oxygen centered radicals such as superoxide anion radical (O_2) Hydroxyl radical (OH), or nitric oxide (NO), but also some potentially dangerous non-radical derivatives of oxygen such as HO peroxynitrite anion (ONOO) and hypochlorous acid (HOCL) **(Halliwell, 1993).**

In healthy individual the generation of ROS appears to be counterbalanced by the antioxidant defense, although there may be some cumulative oxidative damage that contributes to the aging process and age related diseases **(Ames, 1989).** An imbalance between ROS and antioxidant defenses in favor of the former creates oxidative stress. This can happen either if the antioxidant levels are depleted and/or if the formation of ROS is high. The antioxidant defense is recruited either from endogenous systems or from the diet **(Halliwell, 1997).**

Free radicals are so reactive and short-lived that direct measurement is usually not possible. However, hundreds of biomolecules are known to be derived from the interaction of free radicals with biomolecules. Assays for some of these oxidative stress biomarkers, as well as assays for several of the body's antioxidant defense mechanisms, have been widely used. Although there are numerous tools on the market, a small number of oxidized lipids, as well as byproducts of DNA and protein oxidation, have withstood the test of time. Our major goal is to provide straightforward, reliable assays for oxidative stress biomarkers and for antioxidant capacity of biological fluids

Peng (2000) found that antioxidant supplementation decreased serum (MDA) and protein carbonyls in healthy non pregnant subject. Some of the antioxidants such as vitamin E, vitamin C, beta carotene, bilirubin and albumin trap radicals and prevent chain reactions **(Ryter, 2000).** Especially, vitamin E and beta carotene are potent antioxidant nutrients and may counteract free radical attack and thereby protect cell membranes against free radical mediated lipid and protein oxidation **(Zhang, 2000).**

Protection against ROS toxicity

Because ROS production is a naturally occurring process, a variety of enzymatic and nonenzymatic mechanisms have evolved to protect cells against ROS **(Yu, 1994).** At least some of these mechanisms are impaired after long term of consumption and may

therefore contribute to damage to organs. Enzymes involved in the elimination of ROS include superoxide dismutases (SOD), catalase and glutathione peroxidase.

Nonenzymatic mechanisms; Because of all its functions, GSH is probably the most important antioxidant present in cells. Therefore, enzymes that help generate GSH are critical to body's ability to protect itself against oxidative stress. Mitochondria cannot synthesize GSH but import it from the cytosol using a carrier protein embedded in the membrane surrounding the mitochondria **(Fernandez *et al*., 1997).**

Male germ cells may be susceptible to oxidative stress because of high concentration of polyunsaturated fatty acids and low antioxidant capacity which are associated closely with free radical generating phagocytic Sertoli cells. Decrease in sperm concentration and total sperm output may be due to direct interaction of ROS with the sperm cell membrane resulting in impairment of membrane fluidity and permeability and damage of germ cells ,spermatozoa and mature sperms **(Sarkar *et al*., 2003).** The effect of heavy metals on acrosome integrity may be attributed to the high lipid peroxidation in epididymes as a result of elevated oxidative stress, which alters the stability of plasma membrane that surrounds the acrosome through the effect on its content of polyunsaturated fatty acids and lipoproteins **(Zini *et al*., 2000).**

Decrease in swollen coiled sperm percentage may be due to direct interaction of ROS with polyunsaturated fatty acids in the cell membrane leading to a chain of chemical reactions results in the formation of various oxidatively modified products, which are toxic to cells. The spermatozoal membrane contains large amounts of polyunsaturated fatty acids, which maintain its fluidity. Peroxidation of these fatty acids leads to the loss of membrane flexibility and a reduction in the ability to swell and expand covering the tail when exposed to hypoosmotic solutions **(Amorim *et al*., 2009).**

The beneficial effects of antioxidants on sperm may be due to the decrease in the levels of TBARS present as a consequence of environmental pollution and cellular metabolism **(Castellini *et al*., 2003, 2006).** Antioxidant reduced the formation of radicals in seminal and blood plasma and different tissues. Antioxidant normalizes the activities of enzymes by protecting free radicals responsible for oxidative stress and production of ROS, prevents oxidative damage to cell membrane induced by radicals in the aqueous environment **(Arrigsoni and De Tullio, 2002).**

The decrease in MDA might be explained by enhancing the activities of antioxidant enzymes **(Reddy and Lokesh, 1994),** inhibiting the generation of ROS and mitochondrial release of cytochromes or counteracting the depletion of antioxidant enzymes **(Nagar, 2004).** The antioxidant mechanism might be attributed to scavenging or neutralizing of free radicals, interacting with oxidative cascade and preventing its outcome, quenching oxygen and making it less available for oxidative reaction, and inhibiting oxidative enzyme like cytochrome P450 and chelating and disarming oxidative properties of metal ions such as iron **(Sreejayan and Rao, 1994).**

Evidence now suggests that reactive oxygen species (ROS)-mediated damage to sperm is a significant contributing pathology in 30–80% of cases (Iwasaki and Gagnon, **(Agarwal *et al*., 2006).** ROS, defined as including oxygen ions, free radicals and peroxides, cause infertility by two principal mechanisms. First, ROS damage the sperm membrane which in turn reduces the sperm's motility and ability to fuse with the oocyte. Secondly, ROS directly damage sperm DNA, compromising the paternal genomic contribution to the embryo. Despite the common association between compromised sperm quality and oxidative damage, men are rarely screened for oxidative stress nor treated for this condition. Instead they are usually offered 'mechanical' treatments such as intracytoplasmic sperm injection (IVF-ICSI) or intrauterine insemination (IUI). This is less than Optimal as oxidative damage to sperm DNA is not directly ameliorated by either IVF-ICSI or IUI treatment. In addition, direct treatment of oxidative stress may allow for natural conception, thereby conserving scarce medical resources. This review will provide an Overview of who is at risk of oxidative stress, the mechanisms by which oxidative stress produces infertility and the methods available for its diagnosis and treatment within semen there are two principal sources of production of free radicals; leukocytes and sperm. The vast majority of semen specimens contain leukocytes, with neutrophils being the predominant leukocyte type **(Aitken and Baker, 1995).** As the production of ROS is one of the principal mechanisms by which neutrophils destroy pathogens, it is not surprising that seminal leukocytes have the potential to cause oxidative stress. However, a link between the presence of leukocytes in semen and male oxidative infertility is still under debate **(Wolff, 1995).**

Several researchers have reported a positive correlation between seminal leukocyte numbers and ROS production **(Sharma *et al*., 2001).** However, other studies have failed to find a significant difference in seminal leukocyte concentration between fertile and infertile men **(Tomlinson *et al*., 1993)** and the activation state of leukocytes must also

play an important role in determining final ROS output. This is supported by the observation of a positive correlation between seminal ROS production and pro-inflammatory seminal plasma cytokines such as interleukin IL-6 **(Rajasekaran *et al*., 1995; Martinez *et al*., 2007).**

The non-enzymatic antioxidants present within semen include ascorbic acid (Vitamin C), a-tocopherol (Vitamin E), glutathione, amino acids (taurine, hypotaurine), albumin, carnitine, carotenoids, flavenoids, urate and prostasomes. These agents principally act by directly neutralizing free radical activity chemically.

However, they also provide protection against free radical attack by two other mechanisms. Albumin can intercept free radicals by becoming oxidized itself, thereby sparing sperm from attack **(Twigg *et al*., 1998).** Alternatively, extracellular organelles (prostasomes) secreted by the prostate have been shown to fuse with leukocytes within semen and reduce their production of free radicals **(Saez *et al*., 1998).** A substantial number of researchers have reported a significant reduction in non-enzymatic antioxidant activity in seminal plasma of infertile compared with fertile men **(Song *et al*., 2006).**

Effect of Sperm Morphology on ROS Production

Gomez *et al* (1998) have indicated that levels of ROS production by pure sperm populations were negatively correlated with the quality of sperm in the original semen. The link between poor semen quality and increased ROS generation lies in the presence of excess residual cytoplasm (cytoplasmic droplet). When spermatogenesis is impaired, the cytoplasmic extrusion mechanisms are defective, and spermatozoa are released from the germinal epithelium carrying surplus residual cytoplasm. Under these circumstances, the spermatozoa that are released during spermiation are believed to be immature and functionally defective **(Huszar *et al*., 1997).** Retention of residual cytoplasm by spermatozoa is positively correlated with ROS generation via mechanisms that may be mediated by the cytosolic enzyme G6PD (Figure 2) **(Aitken, 1999).**

It is hoped that this review will stimulate further research in these less well established potential causes of male oxidative infertility.

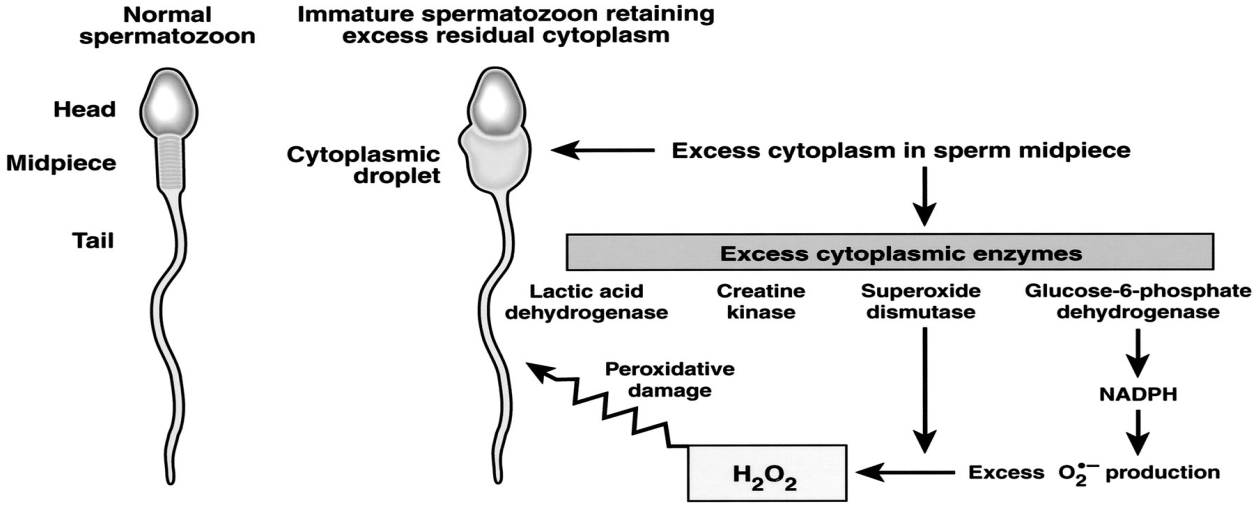


Figure 2. Mechanism of increased production of ROS by abnormal spermatozoa (spermatozoa with cytoplasmic retention).

Several environmental pollutants have been linked with testicular oxidative stress. Pesticides such as lindane **(Chitra *et al*., 2001)**, methoxychlor **(Latchoumycandane *et al*., 2002)** and the herbicide dioxin-TCDD **(Latchoumycandane *et al*., 2003)** have all been linked with testicular oxidative stress in rodent models. The commonly used preservative sulfur dioxide has also been shown to produce testicular oxidative stress in laboratory animals **(Meng and Bai, 2004).** Air pollutants such as diesel particulate matter act as potent stimuli for leukocyte ROS generation **(Alaghmand and Blough, 2007).** While no study has directly linked airborne pollutants with testicular oxidative stress, it is possible that this oxidative insult is responsible for the increase in sperm DNA damage seen following periods of airborne pollution **(Rubes *et al*., 2005).** Heavy metal exposure has been conclusively linked with sperm

oxidative damage **(Eltohamy *et al*., 2004).** Both cadmium and lead are linked with an increase in testicular oxidative stress **(Acharya *et al*., 2003)** and a resultant increase in sperm DNA oxidation **(Naha and Chowdhury, 2006).** The increase in infertility and miscarriage observed in the partners' of welders and battery/paint factory workers **(Bonde, 1993)** may be due to oxidative damage to sperm DNA initiated by the inhalation of metal fumes.

Direct methods

These assays measure damage created by excess free radicals against the sperm lipid membrane or DNA. As oxidative stress is the result of an in balance between ROS production and total antioxidant capacity (TAC), direct tests reflect the net biological effect between these two opposing forces. The most widely used method of assessing sperm membrane peroxidation is the measurement of MDA levels in sperm or seminal plasma with the thiobarbituric acid assay. MDA levels in sperm are quite low and therefore require the use of sensitive high-pressure liquid chromatography (HPLC) equipment **(Li *et al*., 2004; Shang *et al*., 2004)** or the use of iron-based promoters and spectrofluometry measurement **(Aitken *et al*., 1993).** Seminal Plasma levels of MDA are 5–10-fold higher than sperm, making measurement on standard spectrophotometers possible **(Tavilani *et al*., 2005).**

Measurement of MDA appears to be of some clinical relevance since its concentration within both seminal plasma and sperm is elevated in infertile men with excess ROS production, compared with fertile controls or normozoospermic individuals **(Tavilani *et al*., 2005; Hsieh *et al*., 2006).** Furthermore, in vitro impairment of motility, sperm DNA integrity and sperm–oocyte fusion capacity by ROS is accompanied by an increase in MDA concentration **(Aitken *et al*., 1989, 1993).** Other direct tests of sperm membrane lipid peroxidation such as measurement of the isoprostane 8-Iso-PGF2a **(Khosrowbeygi and Zarghami, 2007)** and the c11-BODIPY assay **(Aitken *et al*., 2007; Kao *et al*., 2007)** are showing promise but are not yet in common usage It is well recognized that oxidative stress is one of the major causes of sperm DNA damage **(Aitken *et al*., 1998; Oger *et al*., 2003; Saleh *et al*., 2003a, b).** However, measurement of sperm DNA damage by TUNEL or SCSA is an imperfect assessment of oxidative stress as sperm DNA can be damaged by nonoxidative mechanisms such as aberrant apoptosis and incomplete sperms protamination **(Ozmen *et al*., 2007).** The best direct assessment of sperm DNA oxidative damage is the measurement of the oxidized deoxynucleoside, 8-oxo-7, 8-dihydro 20 deoxyguanosine (8-OHdG). This can be measured in sperm or seminal plasma by HPLC **(Fraga *et al*., 1991; Loft *et al*., 2003),** enzyme-linked immunoabsorbent assay **(Nakamura *et al*., 2002)** or directly within sperm using immunoflurorescence **(Kao *et al*., 2007).** Since a large prospective study has reported that chances of natural conception is inversely correlated with sperm 8-OHdG levels **(Loft *et al*., 2003)**, measurement of this direct marker of sperm oxidative stress appears to have some clinical utility. A large number of round cells within semen may suggest the presence of oxidative stress as they may represent seminal leukocytes **(Sharma *et al*., 2001).** However, round cells may also be immature sperm rather than leukocytes, so formal identification of leukocytes requires ancillary tests such as the peroxidase test,CD45 staining or measurement of seminal elastase **(WHO manual, 1999; Zorn *et al*., 2003; Kopa *et al*., 2005).** Finally, poor sperm membrane integrity assessed by the hypo-osmolarswelling test has been linked with the presence of sperm oxidative stress **(Dandekar *et al*., 2002).**

Role of oxidative stress in male infertility

While a role for oxidative stress in male infertility is now established, many unanswered questions still remain. First, there is a clear need to develop inexpensive assays to identify sperm oxidative stress that can be easily conducted in any andrology laboratory. Secondly, large RCTs are needed to confirm the effectiveness of surgical interventions (varicocelectomy, testicular biopsy) in the management of oxidative stress.

Antioxidant defense play an important role in neutralizing various ROS. superoxide dismutase dismutase the superoxide anion (O_2^-) to H_2O_2. The enzymatic defense against H_2O_2 include catalase and family of glutathione peroxidase **(Yu, 1994)** Seminal plasma possesses antioxidant system that seems to be very relevant to the protection of spermatozoa. The sperm oxidative defense enzymes include superoxide dismutase, glutathione peroxidase and catalase **(Sikka *et al*., 2001).**

Antioxidant Effects

Antioxidants are important in maintaining the oxidant-antioxidant balance in tissues. Among the well-known biological antioxidants, superoxide dismutase, catalase, and the glutathione peroxidase/reductase system have a significant role in protecting the sperm against peroxidative damage **(De Lamirande and Gagnon, 1993; Sharma and Agarwal, 1996).** Depressed seminal antioxidant capacity has been implicated in male subfertility. TAS levels have been shown to be lower in the semen of subfertile male as compared with fertile

male **(Lewis *et al*., 1995, 1997).** More specifically, **Raijmakers *et al.* (2003)** reported significantly higher seminal plasma thiol glutathione concentrations in fertile male compared with subfertile male. In accordance with this finding, it has been reported that ascorbate levels were significantly reduced in seminal plasma of asthenozoospermic subfertile male **(Lewis *et al*., 1997).** Furthermore, studies have suggested that subfertile male empirically treated with antioxidants have demonstrated improved semen characteristics, fertilization in vitro, and higher pregnancy rates in the treatment group **(Lenzi *et al*., 1993; Geva *et al*., 1996).** In study, TAS was significantly decreased in subfertile male with abnormal semen parameters, but not in the idiopathic subfertile group.

Phenolic antioxidants from processed honey increased antioxidant activity. These effects of honey might be attributed to its antibacterial, antioxidant, anti-inflammatory, and immunomodulatory activities (**Schramm *et al.*, 2003**). At a dose of 1.2 g/kg b.wt./day for 2 weeks, honey increased antioxidant agents such as blood vitamin C concentration, β-carotene, uric acid, and glutathione reductase (GRx) (**Al-Waili, 2003**). These compounds reduce the lipid peroxidation level (**Hegazi and Abd El Hady, 2009**).

Honey increases the antioxidant parameters of the liver and kidney glutathione reduced (GSH), oxidized glutathione (GSSG) content and also decrease in (GPx) and (SOD) caused by Ochratoxin A- induced hepatotoxicity and nephrotoxicity in rats. The level of (MDA) -as lipid peroxidation marker- was also significantly decreased (**El-Khayat *et al.*, 2009).**

Honey has protective effects against oxidative stress (attenuate free radical scavenging enzymes and reduce lipid peroxidation in kidney and pancreas of streptozotocin-induced diabetic rats. The combination of two hypoglycemic drugs; glibenclamide (0.6 mg/kg b.wt) and metformin (100 mg/kg b.wt), and honey (1.0 g/kg b.wt) for 4 weeks revealed a marked increases the activities of catalase (CAT) and GRx, and the levels of total antioxidant status (TAS) and GSH in diabetic kidney (**Erejuwa *et al.*, 2011a).** The combination of glibenclamide, metformin, and honey significantly up regulated CAT activity and downregulated GPx activity while MDA levels were significantly reduced. Honey also restored SOD and CAT activities. (**Erejuwa *et al.*, 2010**).

Antioxidant activities in honey were represented by increased TAS, GSH, GSH)/GSSG ratio, GPx, and GRx in diabetic spontaneously hypertensive rats (**Erejuwa *et al.*, 2011b**) and suppressed the lipid peroxidation (**Hegazi and Abd El Hady, 2009**).

HS increased oxygen radicals, possibly by the disruption of the electron transport assemblies of the membrane. **Bruskov *et al*. (2002)** discussed that heat-induced reactive oxygen species (ROS) formation may be an additional factor that provides molecular changes in DNA, proteins, lipids and other biological molecules that may contribute to low fertility. Several studies have suggested that heat exposure could result in oxidative stress, which in turn lead to cytotoxicity **(Lord-Fontaine and Averill-Bates, 2002).**

Nichi *et al*. (2006) suggested other hypothesis to explain heat-induced ROS formation; in the absence of increased blood flow, the testicular parenchyma becomes hypoxic. Hypoxia probably increases production of ROS through the ischemia-reperfusion mechanism, thus **Filho *et al.* (2004)** had considered it a higher index of testicular oxidative stress.

There is growing evidence that oxidative stress significantly impairs sperm function, and plays a major role in the etiology of defective sperm function. This may lead to the onset of male infertility via mechanisms involving the induction of peroxidative damage to the plasma membrane; **(Griveau and Le Lannou, 1997).** Both spermatozoa and seminal plasma possess antioxidant systems capable of counteracting the harmful effects of ROS. Studies have demonstrated that infertile animals are more likely than fertile ones to have depressed total antioxidant capacity (TAC) and lower levels of individual antioxidants **(Lewis *et al*., 1995).**

Antioxidants and Fertility

Since ROS has both physiological and pathological roles, an array of antioxidants maintains a steady state 3 of ROS in the seminal plasma. Antioxidants act as free radical scavengers to protect spermatozoa against ROS. These antioxidants are SOD, catalase, and glutathione peroxidase (GPX). In addition, semen contains a variety of non-enzymatic antioxidant molecules such as vitamin C, vitamin E, pyruvate, glutathione, and carnitine **(Aitken, 2004)**. These antioxidants compensate for the loss of sperm cytoplasmic enzymes as the cytoplasm is extruded during spermiogenesis, which, in turn, diminishes endogenous repair mechanisms and enzymatic defenses. **Agarwal *et al.* (2006)** in an exhaustive review of the literature, found a total of 57 studies related to antioxidants and fertility – 10 studies were randomized controlled trials, 16 were controlled studies, and 31 were uncontrolled studies.

PON-1 is an antioxidant enzyme that is highly effective in preventing lipid peroxidation of LDL **(Mackness *et al*., 1993).** It is principally responsible for the breakdown of lipid peroxides before they accumulate on LDL **(Mackness *et al*., 1993).** PON-1 can also destroy H_2O_2; a major ROS produced under

oxidative stress during atherogenesis **(Aviram *et al*., 1998),** and increase the LDL clearance **(Shih *et al*., 2000).**

PON-1 also protects HDL against lipid peroxidation **(Mackness *et al*., 1993; Aviram *et al*., 1998; Rozenberg *et al*., 2003)**. Inhibition of HDL oxidation by PON-1 preserves the antiatherogenic effects of HDL in reverse cholesterol transport **(Aviram *et al*., 1998).** The antioxidant effect of HDL is also assumed by PON-1 **(Aviram and Rosenblat, 2004).**

The association between PON-1 activity and male infertility is unknown. PON-1 activity was significantly lower in male-factor subfertile patients compared with idiopathic subfertile male and fertile donors in the present study. There were also significant positive correlations between PON-1 activity and semen parameters such as concentration, motility, and morphology. We suggest that decreased PON-1 activity must be related to enhanced production of ROS. In addition, it has been previously shown that PON-1 activity was decreased in some diseases because of ROS pathogenesis under oxidative stress and inflammation conditions such as diabetes, coronary artery disease, and endometriosis **(Ayub *et al*., 1999; Verit *et al*., 2008).**

In conclusion the results showed that TOS was significantly higher and TAS and PON-1 activity were significantly lower in male-factor subfertility, but not in an idiopathic subfertile group. Reduced PON-1 activity may play a role in the pathogenesis of male subfertility. Therefore, both protection from oxidative stress and increases in PON-1 activity could be used as a powerful tool for the prevention of subfertility.

Future Research

Further research is also required to determine what combination and dose of antioxidant supplement provides sperm with maximal protection against oxidative stress. Finally, the development of new sperm culture media that can better protect sperm from the ravages of ROS damage is clearly required.

Although research already have gained substantial insight into the mechanisms and consequences of alcohol induced oxidative stress, additional studies are required to further clarify how alcohol produces oxidative stress in various tissues. More detailed information is needed on the mechanisms involved in some of the major proposed pathways. Finally, it still is unclear how alcohol induced oxidative stress is produced in tissues where only limited alcohol mechanism occurs. Many of these issues can study using animal models, however extrapolation of findings from animal to human will be a difficult task because ROS production and antioxidant status in human as affected by numerous .nutritional, environmental and drug influences that are difficult to reproduce in animals. To date, scattered data suggest that blood of human alcohol can contain lipids modified by radicals and other reactive molecules as well as immune molecules targeted at such modified lipids and proteins. Other questions that should be addressed in future research.

Corresponding author
Magda M El-Tohamy
Department of Animal Reproduction & A.I., Veterinary Division, National Research Centre, Dokki,Cairo, Egypt
Eltohamymagda@yahoo.com

References

Acharya UR, Acharya S, and Mishra M. (2003): Lead acetate induced cytotoxicity in magerminal cells of Swiss mice. *Ind Health*; 41:291–294.

Afanas,ev Igor (1991): Superoxide ion: chemistry and biological implications . Oxygen radicals in biology II, 135, 144-145.

Agarwal A, Ikemoto I, and Loughlin KR. (1994): Relationship of sperm parameters to levels of reactive oxygen species in semen specimens. *J Uro*; 152:107 -110.

Agarwal A, Sharma RK, Nallella KP, Thomas AJ, Jr, Alvarez JG, and Sikka SC. (2006): Reactive oxygen species as an independent marker of male factor infertility. Fertil Steril b; 86:878–885.

Aitken RJ, and Baker HW. (1995): Seminal leukocytes: passengers, terrorists or good samaritans? *Hum Reprod*;10:1736–1739.

Aitken RJ, and Clarkson JS. (1988): Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *J Andro*.;9:367 -376.

Aitken RJ, and Fisher H. (1994): Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bioassay*; 16:259 -267.

Aitken RJ, and Krausz C. (2001): Oxidative stress, DNA damage and the Y chromosome. *Reproductio*.; 122:497 -506.

Aitken RJ, Buckingham D, Brindle J, Gomez E, Baker G, and Irvine S. (1995): Analysis of sperm movement in relation to the oxidative stress created by leukocytes in washed sperm preparations and seminal plasma. *Hum Repro*; 10:2061 -2071.

Aitken RJ, Buckingham DW, and West KM. (1992): Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. *J Cell Physio*; 151:466 -477.

Aitken RJ, Clarkson JS, and Fishel S. (1989): Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Repro*.; 40:183 -197.

Aitken RJ, Fisher H, Fulton N, Gomez E, Knox W, Lewis B, and Irvine DS. (1997): Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by flavoprotein inhibitors diphenylene iodinium and quinacrine. *Mol Reprod De*.; 47:468 -482.

Aitken RJ, Harkiss D, and Buckingham D. (1993): Relationship between iron-catalyzed lipid peroxidation potential and human sperm function. *J Reprod Ferti*.; 98:257 -265.

Aitken RJ, Krausz C, and Buckingham D. (1994): Relationship between biochemical markers for residual sperm cytoplasm, reactive oxygen species generation and the presence of leukocytes and precursor germ cells in human sperm suspension. *Mol Reprod De*.; 39:268 -279.

Aitken RJ, Wingate JK, De Iuliis GN, and McLaughlin EA. (2007): Analysis of lipid peroxidation in human spermatozoa using BODIPY C11. *Mol Hum Reprod*;13:203–211.

Aitken RJ. (1995): Free radicals, lipid peroxidation, sperm function. *Reprod Fertil De*.; 7:659 -668.

Aitken RJ. (1997): Molecular mechanisms regulating human sperm function. *Mol Hum Repro*.; 3:169 -173.

Aitken RJ. (1999): The Amoroso lecture. The human spermatozoon—a cell in crisis? *J Reprod Ferti*.; 115:1 -7.

Akiyama M. (1999): *In vivo* scavenging effect of ethylcysteine on reactive oxygen species in human semen. Nippon Hinyokika Gakkai Zasshi.90: 421–428.

Alaghmand M, and Blough NV. (2007): Source-dependent variation in hydroxyl radical production by airborne particulate matter. *Environ Sci Technol*.; 41:2364–2370.

Alvarez JG, and Storey BT. (1983): Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. *Biol Repro*.; 29:548 -555.

Alvarez JG, and Storey BT. (1995): Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Mol Reprod De*.; 42:334 -346.

Alvarez JG, Touchstone JC, Blasco L, and Storey BT. (1987): Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa: superoxide dismutaseas major enzyme protectant against oxygen toxicity. *J Andro*.; 8:336 -348.

Ames BN. (1989) :Endogenous oxidative DNA damage ,aging and cancer. *Free Radical Res commun*., 7:121-128.

Amorim E, Torres C, Graham J, Amorim L, and Santos L. (2009): The hypoosmotic swelling test in fresh rabbit spermatozoa. Anim. *Reprod Sci.,* 111(2):338-343

Armstrong JS, Rajasekaran M, Chamulitrat W, Gatti P, Hellstrom WJ, and Sikka SC. (1999): Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. *Free Radic Biol Me*.;26:869 -880.

Armstrong JS, Rajasekaran M, Hellstrom WJ, and Sikka SC. (1998): Antioxidant potential of human serum albumin: role in the recovery of high quality human spermatozoa for assisted reproductive technology. *J Andro*.;19:412 -419.

Arrigoni O, and De Tullio MC. (2002): Ascorbic acid: much more than just an antioxidant. *Biochim. Biophys. Acta*: 1–9.

Aviram M, and Rosenblat M. (2004): Paraoxonases 1, 2, and 3, oxidative stress, and macrophage foam cell formation during atherosclerosis development. *Free Radical Biol Med*.; 37: 1304 – 1316.

Aviram M, Rosenblat M, Bisgaier CL, Newton RS, Primo-Parmo SL, and La Du BN. (1998): Paraoxonase inhibits high density lipoprotein (HDL) oxidation and preserves its functions: a possible peroxidative role for paraoxonase. *J Clin Invest*., 101: 1581 –1590.

Ayub A, Mackness MI, Arrol S, Mackness B, Patel J, and Durrington PN. (1999): Serum paraoxonase after myocardial infarction. Arterioscler *Thromb Vasc Biol*.;19: 330 –335.

Blake DR, Allen RE, and Lunec J. (1987): Free radical in biological systems-a review oriented to inflammatory processes. *Br Med Bul.;* 43:371 -385.

Bonde JP. (1993): The risk of male subfecundity attributable to welding of metals.Studies of semen quality, infertility, fertility, adverse pregnancy outcome and childhood malignancy. *Int J Androl* ;16 (Suppl 1): 1–29 .

Bruskov VI, Malakhova LV, Masalimov ZK, and Chernikov AV. (2002): Heat-induced formation of reactive oxygen species and 8-oxoguanine a biomarker of damage to DNA. *Nucleic Acids Research*, 30(6): 1354-1363.

Castellini C, Cardinali R, Dal Bosco A, Minelli A, and Camici O. (2006): Lipid composition of the main fractions of rabbit semen. *Theriogenology. 65: 703–712.*

Castellini C, Lattaioli P, Dal Bosco A, Minelli A, and Mugnai C. (2003): Oxidative status and semen characteristics of rabbit buck as affected by dietary vitamin E, C and n-3 fatty acids. *Reprod. Nutr.Dev., 43: 91-103.*

Chaudiere J, Wilhelmsen EC, and Tappel AL. (1984): Mechanism of selenium-glutathione peroxidase and its inhibition by mercaptocarboxylic acida and other mercaptans. *J Biol, Chem*, 259: 1043-1050.

Chitra KC, Sujatha R, Latchoumycandane C, and Mathur PP. (2001): Effect of lindane on antioxidant enzymes in epididymis and epididymal sperm of adult rats. *Asian J Androl*.;3:205–208.

Dandekar SP, Nadkarni GD, Kulkarni VS, and Punekar S. (2002): Lipid peroxidation and antioxidant enzymes in male infertility. *J Postgrad Med*.;48:186–189.

De Lamirande E, and Gagnon C. (1992): Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate (ATP) plays an important role in the inhibition of sperm motility. *J Andro*.; 13:379 -386.

De Lamirande E, and Gagnon C. (1993): Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Radic Biol Me*.;14:157-166.

De Lamirande E, and Gagnon C. (1995): Impact of reactive oxygen species on spermatozoa: a balancing act between beneficial and detrimental effects. *Hum Repro*.; 10:15 -21.

De Lamirande E, Jiang H, Zini A, Kodoma H, and Gagnon C. (1997): Reactive oxygen species (ROS) and sperm physiology. *Rev Repro*.; 2:48 -54.

De Lamirande E, Leduc BE, Iwasaki A, Hassouna M, and Gagnon C. (1995): Increased reactive oxygen species formation in semen of patients with spinal cord injury. *Fertil Steri*.; 64:637 -642

Donnelly ET, McClure N, and Lewis S. (1999): Antioxidant supplementation in vitro does not improve human sperm motility. *Fertil Steri*.;72:4

El-Khayat Z, Ezzat AR, Arbi MS, Rasheed WI, and Elias TR. (2009): Potential effects of bee honey and propolis against the toxicity of Ochratoxin A in Rats. *Macedonian J. Med. Sci*., 15: 2(4):311-318.

El-Tohamy MM, Hamam A M, and EL-Nattat W S. (2002): Cadmium toxicity consequences on testicular and prostate tissues in male rabbits fed on zinc deficient diet. *J. Egypt. Vet. Med. Ass*., 62(2): 37-50.

Erejuwa OO, Sulaiman SA, Wahab MS, Salam SK, Salleh MS, and Gurtu S. (2010): Antioxidant protective effect of glibenclamide and metformin in combination with honey in pancreas of streptozotocin-induced diabetic rats. *Int. J. Mol. Sci.*, 11(5): 2056–2066.

Erejuwa OO, Sulaiman SA, Wahab MS, Salam SK, Salleh MS, and Gurtu S. (2011a): Comparison of antioxidant effects of honey, glibenclamide, metformin, and their combinations in kidney of streptozotocin-induced diabetic rats. *Int. J. Mol. Sci*., 12(1): 829–843.

Erel O. (2004): A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem*.; 37: 277 –285.

Fernandez JC, Kaplowitz N, Colel A, and Garca RC. (1997): Oxidative stressand alcoholic liver disease . Alcohol Health and research World, 21;321-324.

Filho DW, Torres MA, Bordin AL, Crezcynski-Pasa TB, and Boveris A. (2004): Spermatic cord torsion, reactive oxygen and nitrogen species and ischemiareperfusion injury. *Mol Aspects Med*; 25:199–210.

Fisher H, and Aitken R. (1997): Comparative analysis of the ability of precursor germ cells and epididymal spermatozoa to generate reactive oxygen metabolites. *J Exp Zool*; 277:390–400.

Fraga GG, Motchnik PA, Shigenaga MK, Helbrock JH, Jacob RA, and Ames B. (1991): Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc Natl Acad Sci US*.; 88:11003 -11006.

Gagnon C, Iwasaki A, de Lamirande E, and Kovalski N. (1991): Reactive oxygen species and human spermatozoa. *Ann NY Acad Sc*.; 637:436 -444.

Gomez E, Irvine DS, and Aitken RJ. (1998): Evaluation of a spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function. *Int J Andro*.; 21:81 -94.

Griveau JF, and Le Lannou D. (1997): Reactive oxygen species and human spermatozoa. *Int J Androl*; 20:61 -69.

Griveau JF, Renard P, and Le Lannou D. (1995): Superoxide anion production by human spermatozoa as a part of the ionophore-induced acrosome reaction in vitro. *Int J Andro*.; 18:67 -74.

Halliwell B. (1984): Tell me about free radicals, doctor: a review. *J Roy Soc Me*.; 82:747 -752.

Halliwell B. (1990): How to characterize a biological antioxidant. *Free Radic Res Commu*.; 9:1 -32.

Halliwell B. (1996): Vitamin C: antioxidant or pro-oxidant in vivo? *Free Radic. Res*., 25: 439-454.

Halliwell B. (1997): Antioxidants and human disease: A General introduction. *Nutrition*, rev.55:S44-9.

Hegazi AG, and Abd El Hady FK. (2006): Egyptian propolis: 8-Antiviral and antioxidant activities in relation to flavonoid content. APIMEDICA, 1st International Forum on Apitherapy, Greece 12-15, p: 33

Hendin B, Kolettis P, Sharma RK, Thomas AJ Jr, and Agarwal A. (1999): Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. *J Uro*.;161:1831 -1834.

Hsieh YY, Chang CC, and Lin CS. (2006): Seminal malondialdehyde concentration but not glutathione peroxidase activity is negatively correlated with seminal concentration and motility. *Int J Biol Sci*.; 2:23–29.

Huszar G, Sbracia M, Vigue L, Miller DJ, and Shur BD. (1997): Sperm plasma membrane remodeling during spermiogenic maturation in men: relationship among plasma membrane beta 1,4-galactosyltransferase, cytoplasmic creatine phosphokinase and creatine phosphokinase isoform ratios. *Biol Repro*.;56:1020 -1024.

Iwasaki A, and Gagnon C. (1992): Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steri*.; 57:409 -416.

James M, Roberts Carl A, and Hubel (2004): Oxidative stress in Preeclampsia, 190;5.

Janssen YM, Van-Houton B, Borm PJ, and Mossuran BT. (1993): Cell and tissue responses to oxidative damage. *Lab Inves*.; 69:261 -274.

Jeulin C, Soufir JC, Weber P, Laval-Martin D, and Calvayrac R. (1989): Catalase activity in human spermatozoa and seminal plasma. *Gamete Re*.;24:185 -196.

Jones R, Mann T, and Sherins RJ. (1979): Peroxidative breakdown of phospholipids in human spermatozoa: spermicidal effects of fatty acids peroxidatives and protective action of seminal plasma. *Fertil Steri*.;31:531 -537.

Kao SH, Chao HT, Chen HW, Hwang TI, Liao TL, and Wei YH. (2008): Increase of oxidative stress in human sperm with lower motility. Fertil Steril.;89(5):1183-90.

Kessopoulou E, Tomlinson MJ, Banat CLR, Bolton AE, and Cooke ID. (1992): Origin of reactive oxygen species in human semen-spermatozoa or leukocytes. *J Reprod Ferti*.; 94:463 -470.

Khosrowbeygi A, and Zarghami N. (2007): Levels of oxidative stress biomarkers in seminal plasma and their relationship with seminal parameters. *BMC Clin Pathol*. ;7:6.

Kobayashi H, Gil-Guzman E, Mahran AM, Sharma RK, Nelson DR, Thomas AJ Jr, and Agarwal A. (2001): Quality control of reactive oxygen species measurement by luminol-dependent chemiluminescence assay. *J Andro*.; 22:568 -574.

Kodama H, Kuribayashi Y, and Gagnon C. (1996): Effect of sperm lipid peroxidation on fertilization. *J Andro*.; 16:151 -157.

Kodama H, Yamaguchi R, Fukuda J, Kasai H, and Tanaka T. (1997): Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steri*.; 65:519 -524.

Kolettis P, Sharma RK, Pasqualotto F, Nelson D, Thomas AJ Jr, and Agarwal A. (1999): The effects of seminal oxidative stress on fertility after vasectomy reversal. *Fertil Steri*.; 71:249 -255.

Kopa Z, Wenzel J, Papp GK, and Haidl G. (2005): Role of granulocyte elastase and interleukin-6 in the diagnosis of male genital tract inflammation. *Andrologia*; 37:188–194

Kovalski NN, de Lamirande E, and Gagnon C. (1992): Reactive oxygen species generated by human neutrophils inhibit sperm motility: protective effects of seminal plasma and scavengers. *Fertil Steri*.; 58:809 -816.

Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Veeck LL, Morshedi M, and Brugo S. (1987): New method of evaluating sperm morphology with predictive value for human in vitro fertilization. Urolog.; 30:248 -251.

Latchoumycandane C, and Mathur PP. (2002): Induction of oxidative stress in the rat testis after short-term exposure to the organochlorine pesticide methoxychlor. *Arch Toxicol*. ;76:692–698.

Latchoumycandane C, Chitra KC, and Mathur PP. (2003): Tetrachlorodibenzop-dioxin (TCDD) induces oxidative stress in the epididymis and epididymal sperm of adult rats. *Arch Toxicol*.;77:280–284.

Lenzi A, Cualosso F, Gandini L, Lombardo F, and Dondero F. (1993): Placebo-controlled, double-blind, cross-over trial of glutathione therapy, in male infertility. *Hum Reprod*.; 9:2044 -2050.

Lenzi A, Picardo M, Gandini L, Lombardo F, Terminali O, Passi S, and Dondero F. (1994): Glutathione treatment of dyspermia: effect on the lipoperoxidation process. *Hum Reprod*.; 9:2044 -2050.

Lewis SEM, Boyle PM, McKinney KA, Young IS, and Thompson W. (1995): Total antioxidant capacity of seminal plasma is different in fertile and infertile men. *Fertile Steril*.; 64:868 -870.

Li K, Shang X, and Chen Y. (2004): High-performance liquid chromatographic detection of lipid peroxidation in human seminal plasma and its application to male infertility. *Clin Chim Acta*.;346:199–203.

Loft S, Kold-Jensen T, Hjollund NH, Giwercman A, Gyllemborg J, Ernst E, Olsen J, Scheike T, Poulsen HE, and Bonde JP. (2003): Oxidative DNA damage in human sperm influences time to pregnancy. *Hum Reprod* .;18: 1265–1272.

Lopes S, Jurisicova A, Sun J, and Casper RF. (1998): Reactive oxygen species: a potential cause for DNA fragmentation in human spermatozoa. *Hum Reprod*.;13:896 -900.

Mackness MI, Arrol S, Abbott C, and Durrington PN. (1993): Protection of low-density lipoprotein against oxidative modification by high-density lipoprotein associated paraoxonase. *Atherosclerosis.*; 104: 129 –135.

Mann T, and Lutwak-Mann C. (1981): Male reproductive function andsemen. *Springer-Verlag, Berlin/Heidelberg/New York.*

Martinez P, Proverbio F, and Camejo MI. (2007): Sperm lipid peroxidation and pro-inflammatory cytokines. *Asian J Androl*.; 9:102–107.

Matson PL. (1997): Clinical value of tests for assessing male infertility. *Clin Obstet Gynecol*.; 11:641 -654.

Meng Z, and Bai W. (2004): Oxidation damage of sulfur dioxide on testicles of mice. *Environ Res*; 96:298–304.

Moilanen J, Hovatta O, and Lindroth L. (1993): Vitamin E levels in seminal plasma can be elevated by oral administration of vitamin E in infertile men. *Int J Androl*.; 16:165 –168.

Nagar A. (2004): Protective effects of Curcuma longa (turmeric) on ischemia-reperfusion induced myocardial injuries and their mechanisms. *Life Sci*., 75(14):1701-1711

Naha N, and Chowdhury AR. (2006): Inorganic lead exposure in battery and paint factory: effect on human sperm structure and functional activity. *J UOEH*;28:157–171.

Nakamura H, Kimura T, Nakajima A, Shimoya K, Takemura M, Hashimoto K,Isaka S, Azuma C, Koyama M, and Murata Y. (2002): Detection of oxidative stress in seminal plasma and fractionated sperm from subfertile male patients. *Eur J Obstet Gynecol Reprod Biol*.;105:155–160

Ochsendorf FR. (1999): Infections in the male genital tract and reactive oxygen species. *Hum Reprod*.; 5:399 -420.

Oger I, Da Cruz C, Panteix G, and Menezo Y. (2003): Evaluating human sperm DNA integrity: relationship between 8-hydroxydeoxyguanosine quantification and the sperm chromatin structure assay. *Zygote*; 11:367–371.

Ollero M, Gil-Guzman E, and Lopez MC. (2001): Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. *Hum Reprod*.;16:1912 -1921.

Ozmen B, Koutlaki N, Youssry M, Diedrich K, and Al-Hasani S. (2007):DNA damage of human spermatozoa in assisted reproduction: origins, diagnosis, impacts and safety. Reprod Biomed Online;14:384–395.

Padron OF, Brackett NL, Sharma RK, Kohn S, Lynne CM, Thomas AJ Jr, and Agarwal A. (1997): Seminal reactive oxygen species, sperm motility and morphology in men with spinal cord injury. *Fertil Steril*.; 67:1115-1120.

Park BH, Fikrig SM, and Smithwick EM. (1968): Infection and nitroblue-tetrazolium reduction by neutrophils. A diagnostic acid. *Lancet*.;2:532 -534.

Pasqualotto FF, Sharma RK, Agarwal A, Nelson DR, Thomas AJ Jr, and Potts JM. (2000a): Seminal oxidative stress in chronic prostatitis patients. *Urology*.;55:881 -885.

Pasqualotto FF, Sharma RK, Kobayashi H, Nelson DR, Thomas AJ Jr, and Agarwal A. (2001): Oxidative stress in normospermic men undergoing infertility evaluation. *J Androl*.; 73:459 -464.

Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ Jr, and Agarwal A. (2000b): Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing fertility investigation. *Fertil Steril*.;73:459 -464.

Plante M, de Lamirande E, and Gagnon C. (1994): Reactive oxygen species released by activated neutrophils, but not by deficient spermatozoa, are sufficient to affect normal sperm motility. *Fertil Steril*.;62:387 -393.

Rajasekaran M, Hellstrom WJ, Naz RK, and Sikka SC. (1995): Oxidative stress and interleukins in seminal plasma during leukocytospermia. *Fertil Steril*.;64:166 -171.

Reddy ACP, and Lokesh BR. (1994): Studies on the inhibitory effects of curcumin and eugenol on the formation of reactive oxygen species and the oxidation of ferrous iron. Mol. Cell. *Biochem*, 137 (1): 1-8.

Rozenberg O, Rosenblat M, Coleman R, Shih DM, and Aviram M. (2003): Paraoxonase (PON-1) deficiency is associated with increased macrophage oxidative stress: studies in PON1-knockout mice. *Free Radical Biol Med*.; 34: 774 –784.

Rubes J, Selevan SG, Evenson DP, Zudova D, Vozdova M, Zudova Z, Robbins WA, and Perreault SD. (2005): Episodic air pollution is associated with increased DNA fragmentation in human sperm without other changes in semen quality. *Hum Reprod*;20:2776–2783.

Saez F, Motta C, Boucher D, and Grizard G. (1998): Antioxidant capacity of prostasomes in human semen. *Mol Hum Reprod*;4:667–672.

Said TM, Agarwal A, Sharma RK, Thomas AJ Jr, and Sikka SC. (2005): Impact of sperm morphology on DNA damage caused by oxidative stress induced by beta-nicotinamide adenine dinucleotide phosphate. *Fertil Steril*;83:95–103.

Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, Nelson DR, and Thomas AJ.(2003a): Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril*.; 79(Suppl 3):1597–1605.

Saran M, Beck-Speier I, Fellerhoff B, and Bauer G. (1999): Phagocytic killing of microorganisms by radical processes: consequences of the reaction of hydroxyl radicals with chloride yielding chlorine atoms. *Free Radic Biol Med*.;26:482 -490.

Sarica M , Corduk M, Suicmez F, Cedden M, Yildirim K, and Kilinc (2007): The Effects of Dietary l-Carnitine Supplementation on Semen Traits, Reproductive Parameters, and Testicular Histology of Japanese Quail Breeders *J Appl Poult Res Summer* ,16:178-186

Sarkar M, Chaudhuri G, Chattopadhayay A, and Biswas NM. (2003): Effect of sodium arsenite on spermatogenesis, plasma gonadotrophins and testosterone in rats. *Asian J Androl.* ; 5: 27–31.

Schramm DD, Karim M, and Schrader S. (1999): Oxidative Stress, Mitochondrial Function, and Acute Glutamate Exci totoxicity in Cultured Cerebellar Granule Cells. *Journal of Neurochemistry*, 72:1394-1401.

Segal AW, and Levi AJ. (1973): The mechanism of the entry of dye into neutrophils in the nitroblue tetrazolium (NBT) test. *Clin Sci Mol Med*.;45:817 -826

Shang XJ, Li K, Ye ZQ, Chen YG, Yu X, and Huang YF. (2004): Analysis of lipid peroxidative levels in seminal plasma of infertile men by high-performance liquid chromatography. *Arch Androl*;50:411–416.

Sharma R, Pasqualotto FF, Nelson DR, Thomas AJ Jr, and Agarwal A. (2001): Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. *J Androl*.; 22:575 -583.

Sharma RK, and Agarwal A. (1996): Role of reactive oxygen species in male infertility. (Review). *Urology*.; 48:835 -850.

Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ Jr, and Agarwal A. (1999): The reactive oxygen species—total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. *Hum Reprod*.;14:2801 -2807.

Shekarriz M, Sharma RK, Thomas AJ Jr, and Agarwal A. (1995a): Positive myeloperoxidase staining (Endtz test) as an indicator of excessive reactive oxygen species formation in semen. *J Assist Reprod Genet*.;12:70 -74.

Shekarriz M, Thomas AJ Jr, and Agarwal A. (1995b): A method of human semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen species. *Eur Urol*.; 28:31 -35.

Shekarriz M, Thomas AJ Jr, and Agarwal A. (1995c): Incidence and level of seminal reactive oxygen species in normal men. *Urology*.;45:103 -107.

Shih DM, Xia YR, Wang XP, Miller E, Castellani LW, Subbana-gounder G, Cheroutre H, Faull KF, Berliner JA, Witztum JL, and Lusis AJ. (2000): Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis. *J Biol Chem*.; 275: 17527 –1753.

Sidhu RS, Sharma RK, Thomas AJ Jr, and Agarwal A. (1998): Relationship between creatine kinase activity and semen characteristics in sub-fertile men. *Int J Fertil Womens Med*.; 43:192 -197.

Sies H, Stahl W, and Sundquist RA. (1992): Antioxidant function of vitamins. Vitamins E and C, beta-carotenoids, and other carotenoids. *Ann NY Acad Sci*.;669:7 -20.

Sies H. (1993): Strategies of antioxidant defence. *Eur J Biochem*.;215:213 -219.

Sikka SC, Rajasekaran M, and Hellstrom WJG. (1995): Role of oxidative stress and antioxidants in male infertility. *J Androl*.; 16:464 -468.

Sikka SC. (1996): Oxidative stress and role of antioxidants in normal and abnormal sperm function Front *Biosci*.; 1:78 -86.

Sikka SC. (2001): Relative impact of oxidative stress on male reproductive function. *Curr Med Chem*.; 8:851 -862.

Smith R, Vantman D, Escobar J, and Lissi E. (1996): Total antioxidant capacity of human seminal plasma. *Hum Reprod*.; 11:1655 -1660.

Song GJ, Norkus EP, and Lewis V. (2006): Relationship between seminal ascorbic acid and sperm DNA integrity in infertile men. *Int J Androl.;*29:569–575.

Spitteler G. (1993): Review: on the chemistry of oxidative stress. *J Lipid Mediators*.; 7:77 -82.

Sreejayan N, and Rao MN. (1994): Curcuminoids as potent inhibitors of lipid peroxidation. *J. Pharm. Pharmacol*., 46: 1013-1016.

Stone KK, and Bermudes E. (1986): Aqueous extracts of cigarette tar containing free radicals cause DNA nicks in mammalian cells. *Environ Health Perspect*.;102:173 -177.

Sun JG, Jurisicova A, and Casper RF. (1997): Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. *Biol Reprod*.; 56:602 -600

Tavilani H, Doosti M, and Saeidi H. (2005): Malondialdehyde levels in sperm and seminal plasma of asthenozoospermic and its relationship with semen parameters. *Clin Chim Acta*;356:199–203.

Thiele JJ, Freisleben HJ, Fuchs J, and Oschendorf FR. (1995): Ascorbic acid and urate in human seminal plasma: determination and interrelationship with chemiluminescence in washed semen. *Hum Reprod*.; 10:110 -115.

Thomas J, Fishel SB, Hall JA, Green S, Newton TA, and Thornton SJ. (1997): Increased polymorphonuclear granulocytes in seminal plasma in relation to sperm morphology. *Hum Reprod;* 12:2418 -2421.

Tomlinson MJ, Barrat GLR, and Cooke ID. (1993): Prospective study of leukocytes and leukocyte subpopulations in semen suggests that they are not a cause of male infertility. *Fertil Steril.;* 60:1069 -1075.

Tomlinson MJ, White A, Barratt CL, Bolton AE, and Cooke ID. (1992): The removal of morphologically abnormal sperm forms by phagocytes: a positive role for seminal leukocytes. *Hum Reprod*.; 7:517 -522.

Twigg J, Irvine DS, and Aitken RJ. (1998a): Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Hum Reprod*.; 13:1864 -1871.

Twigg J, Irvine DS, Houston P, Fulton N, Michael L, and Aitken RJ. (1998b): Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. *Mol Hum Reprod*.;4:439 -445.

Van Voorhis BJ, and Sparks AET. (1999): Semen analysis: what tests are clinically useful? *Clin Obstet Gynecol*.; 42:957 -971

Verit FF, Erel O, and Celik N. (2008): Serum paraoxonase-1 activity in women with endometriosis and its relationship with the stage of the disease. *Hum Reprod*.; 23: 100 –104.

Verit FF, Verit A, Kocyigit A, Ciftci H, Celik H, and Koksal M. (2006): No increase in sperm DNA damage, seminal oxidative stress in patients with idiopathic infertility. *Arch Gynecol Obstet*. 274: 339 –344.

Warren JS, Johnson KJ, and Ward PA. (1987): Oxygen radicals in cell injury and cell death. *Pathol Immunopathol Res*.; 6:301 -315.

Wefers H, and Sies H. (1988): The protection by ascorbate and glutathione against microsomal lipid peroxidation is dependent on vitamin E. *Eur J Biochem*.;174:353 -357.

Wolff H, and Anderson DJ. (1988): Immunohistologic characterization and quantitation of leukocyte subpopulation in human semen. *Fertil Steril*.;49:497 -503.

Wolff H. (1995): The biologic significance of white blood cells in semen. *Fertil Steril*.; 63:1143 -1157.

Wong WY, Thomas CM, Merkus HM, Zielhuis GA, Doesburg WH, and Steegers-Theunissen RP. (2000): Cigarette smoking and the risk of male factor subfertility: minor association between continine in seminal plasma and semen morphology. *Fertil Steril*.; 74:930 -935.

World Health Organization (1999). WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. 4th ed. Cambridge, United Kingdom: Cambridge University Press .

Wu D, and Cederraum AI. (1999): Ethanol induced apoptosis to HepG2 cell lines expressing human cytochrome P450 2El. Alcoholism: Clinical and exprimantal Research. 23:67-76.

Wu D, and Cederraum AI. (2003): Alcohol, Oxidative stress and free radical damage, 27, 277-284.

Yu BP. (1994): Cellular defenses against damage from reactive oxygen species. Physiological Review, 74:139-162.

Zheng RL, and Zhang H. (1997): Effects of ferulic acid on fertile and asthenozoospermic infertile human sperm motility, viability, lipid peroxidation, and cyclic nucleotides. *Free Radic Biol Med*.;22:581–586.

Zini A, Abitbol J, Girardi SK, Schulsinger D, Goldstein M, and Schlegel PN. (1998): Germ cell apoptosis and endothelial nitric oxide synthase (eNOS) expression following ischemia-reperfusion injury to testis. *Arch. Androl*., 41: 57-65.

Zini A, de Lamirande E, and Gagnon C. (1993): Reactive oxygen species in the semen of infertile patients: levels of superoxide dismutase- and catalase-like activities in seminal plasma. *Int J Androl*.; 16:183 -188.

Zini A, De Lamirande E, and Gagnon C. (1996): Low levels of nitric oxide promote human sperm capacitation in vitro. *J Androl*.; 16:424 -431.

Zini A, Finelli A, Phang D, and Jarvi K. (2000): Influence of semen processing on human sperm DNA integrity. *Urology*.;56:1081–1084.

Zorn B, Sesek-Briski A, Osredkar J, and Meden-Vrtovec H. (2003): Semen polymorphonuclear neutrophil leukocyte elastase as a diagnostic and prognostic marker of genital tract inflammation–a review. *Clin Chem Lab MED*;41:2–12.s

2/21/2012

Design and Optimisation of Low Noise Amplifier in Superhetrodyne GPS Receiver Front End

Shafqat Hameed[1], Umar F. Khan[2]

[1]National University of Sciences and Technology(NUST), Islamabad, Pakistan
[2]University of Bradford, Bradford, UK
shafqat.hameed@ceme.nust.edu.pk; hameed.shafqat@yahoo.com

Abstract: The Global Positioning System (GPS) as it was developed for military purposes originally by the USA has become the most valuable tool for military purposes today. GPS can be used for different applications such as military, science, sports, transportation and many more. Each particular application needs a specific performance from GPS receiver. In this report the architecture of the Superhetrodyne GPS receiver front end will be discussed. The receiver includes all analogue signal paths including Low Noise Amplifier, Radio Frequency Filter, Mixer, Local Oscillator and IF Filter. Specific attention will be paid to the Low Noise Amplifier (LNA). The design issues related to LNA will be discussed as well. These issues include gain, noise figure and linearity. The discussion also includes the technological constraints for design of LNA. What particular technology is going to be used for this design? Normally LNA is designed using discrete elements, but for this particular application enhancement mode (pHEMPT) technology is used.

[Shafqat Hameed, Umar F. Khan. **Design and Optimisation of Low Noise Amplifier in Superhetrodyne GPS Receiver Front End.** Life Science Journal. 2012;9(1):689-694] (ISSN:1097-8135). http://www.lifesciencesite.com. 99

Keywords: GPS, Signal, Satellites, applications, frequency, Technology

1 Introduction

The Global Positioning System (GPS) is a constellation of 24 satellites in six orbits. They circle the earth twice each day at an inclination angle of approximately 55 degrees to the equator. The satellites continuously transmit coded positional and timing information at high frequencies (in the 1500MHz range) [1].

GPS receivers on the ground pick up the signals and use the coded information to calculate a position on an earth coordinate system. A receiver determines a position by calculating the time it takes for the radio signals transmitted from each satellite to reach the receiver. Multiplying the time by the speed of light determines how far the unit is from each of the satellites: Distance = Rate x Time. Time is determined using an ingenious code matching technique within the GPS receiver. The location of each satellite is encoded in its transmitted signal. With these data, the receiver can triangulate to calculate its location on Earth.

1.1 GPS system and its working

Space Segment: This segment is based on the constellation of satellites usually in the Medium Earth Orbit (MEO). These satellites orbit the Earth at a distance of about 20,000 km from earth's surface [2]. Satellites in this constellation use two microwave frequencies for transmission of ranging signals.

Control Segment: This segment is based on a system of ground monitors to maintain a GPS system and it is responsible for providing upload facilities and to synchronize the atomic clock on board the satellite [3].

User Segment: This segment is comprised of a user receiver. The atomic clock on board the satellite generates a unique digital code sequence and the satellite transmits that code to the ground segment. Additionally a user segment GPS antenna picks up the sequence and the GPS receiver matches the sequence with its own sequence generated inside the receiver [4]. These sequences are used to calculate the total time; the signals have taken to travel from space segment to the ground segment. The calculated time is then converted into the distance as the microwaves travel with the same speed as light (300,000 km/sec). A GPS receiver can view at least four satellites at the same time and knows the exact location of these satellites. The receiver clock is synchronized with the GPS time standard.

2 GPS receiver Introduction

We no longer have to do what people used to do few years before to find a location. If you have to go somewhere you do not need to get map references. Now things have become simpler and faster. If you want to trace a particular place just buy £100 tom-tom and it will take you exactly where you want. When people discuss GPS they actually mean the GPS receiver. The GPS receiver actually uses the process called trilateration to calculate a particular position or place. How does it work? Let us take this example.

Let us say that you are at a location and you do not know where you are. You ask someone and they tell you that you are about 100 KM away from A. You can be within a circle the radius of which is 100 km. Now if you enquire from somebody else the

location he tells you that you are about 130 km from B so it means you are now in a circle the radius of which is about 130 km. Similarly a third person tells you that you are at 95 km from C. Here you are in a circle whose radius is 95 km. Now you will know the exact location which is shown as D below, because the third circle will intersect at only one point. So this is how GPS receiver determines the location by using four satellites. The satellites functionality matches the analogy discussed in the above example.

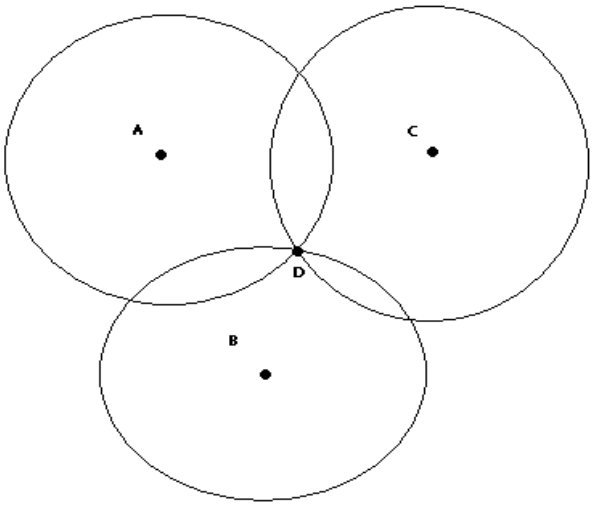


Figure 2.1: Working of GPS to find the distance

2.1 GPS Receiver Architecture

RF signals have very small wavelength and they are shorter then ordinary signals like audio signals. Even a moderate RF signal has a very short wavelength. As the value of frequency changes the values of reactive components like capacitors and inductors also change because they are frequency dependant. The practical circuits do not contain only capacitors or only inductors but they contain both of them. At microwave frequencies their effects are very sensitive. So when we are designing an RF circuit it is very different from the ordinary circuit.

As discussed earlier the amount of gain we shall have at various blocks of the GPS receiver is always a trade off. High gain at early stages of the receiver will help in getting better performance from the receiver [5]. If we get high gain at active antenna and LNA then of course it will help a lot to reduce the noise figure by minimizing the contribution of noise to the mixer. On the other hand if the gain is low then it will improve the linearity and power consumption, as these blocks will consume power as well. For a GPS receiver front end non linearity is not a big problem. As the signal is coming from far away, so its strength is very low. Therefore for our design we would rather get better gain then get better linearity.

In the receiver technology section we shall discuss the GPS receiver components overview.

In this section we shall focus on the following GPS receiver components:

- Antenna
- Low noise amplifiers
- RF Filters
- Mixers
- Oscillators
- IF Filter
- IF Amplifier

As the ADC part of the receiver is beyond the scope of our study, because our main focus will be on the RF part of the receiver, we shall not discuss the digital part of the receiver.

Before we look in depth at the GPS receiver we must first look at its basic architecture. The idea here is to understand the basic architecture of how different components of the receiver are attached to get the desired functionality.

Below is the basic architecture of the GPS receiver.

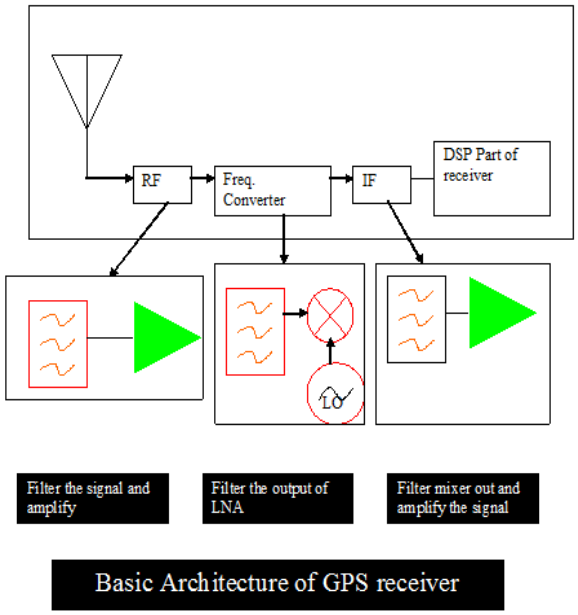


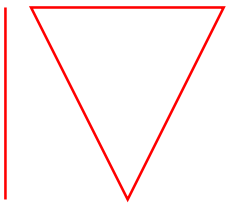
Figure 2.2: Basic block diagram of GPS Receiver [7]

The next section will discuss the receiver components one by one.

2.2 GPS Receiver Components

2.2.1 Antenna

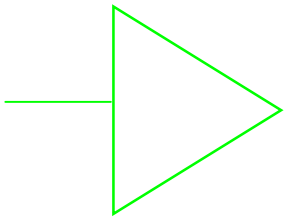
The symbol for the antenna is



The antenna is the first part of the receiver. It receives the signal coming from the satellite. The design is working at 1.57GHZ, so it detects the signal coming at this frequency from the satellite. It is actually a narrow band device of the receiver. Its sensitivity is very important, meaning that it should be highly sensitive because the signal coming from the satellite is at about 20,000 KM, as the satellite is at MEO distance so there will be a huge path loss as the signal that arrives at the receiver will be very weak.[7]

2.2.2 Low Noise Amplifier (LNA)

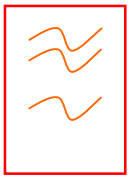
The symbol for LNA is.



The low noise amplifier is the component of the receiver which is placed earliest in the receiver. The reason for it being put at the earliest stage is that it amplifies the signal. As the signal is coming from far away it is very weak as it reaches at the antenna of the receiver. So if the signal is weak at the initial stage of the receiver then it will affect the whole performance of the receiver. Thus the LNA amplifies the signal and then it goes to the other components of the receiver. There is another problem with these components which is that they have their own internal noise as well, so if the signal strength is low at the initial stage of the receiver, then noise may overcome the actual signal which can be a problem for us as well so for that reason we shall have to put the LNA at the earlier stages of the receiver [6]. As the focus will be on the LNA of the receiver so the detailed study of LNA will be discussed later on.

2.2.3 Filters

The symbol for the filter is.



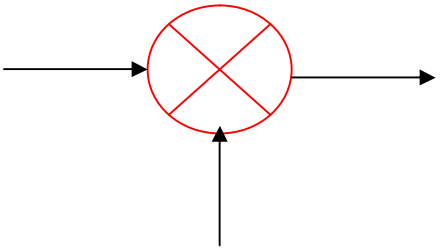
The word filters is used instead of filter because we use this component more then once and it can have different types. We use the one based on requirement.

Filters can have the following three types:

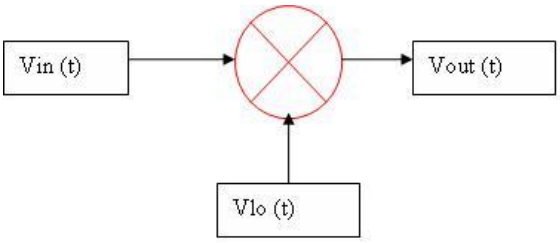
i) Low pass filter
ii) Band pass filter.
iii) High pass filter.

Which particular type of filter we are going to use depends upon the requirement, e.g. a low pass filter will only pass low frequencies and will filter out all other frequencies. A high pass filter will pass high frequencies and will reject all others. Whereas a band pass filter will pass a band of frequencies and will filter out the frequencies below and above that [7].

2.2.4 Mixers



The mixer is another important receiver component. The mixer is used for frequency conversion of the information and can be used at different places in the receiver chain. Its function is actually multiplication. The Mixer multiplies the input signal and the local oscillator signal. Multiplication in the time domain becomes a convolution in the frequency domain. Information in the input signal can be up converted which is called sum or it can be down converted which is called difference. The ratio of output signal power (Intermediate frequency) to the input signal power gives us the conversion gain [6][7].



Vout (t) = Vin (t).Vlo (t) [7].

2.2.5 Oscillator

Oscillators are sine wave or square wave generators. An oscillator provides a signal at a particular frequency. It consists of an amplifier and a feed back circuit. The frequency of the Oscillator can be tuned by varying the feed back circuit.

An ideal Oscillator provides a single sine wave**.** It may consist of thermal noise and phase noise etc.

These are some of the important components of the Superhetrodyne receiver. There are of course other components which include the DSP part of the

receiver as well but we are not going to discuss them here.

2.2.6 IF Filter

We use an IF Filter at this stage (IF stage) to filter the incoming signal and to remove those unwanted harmonics which are generated by the mixer. It is difficult to remove the unwanted harmonics because they lie very close to the wanted signal. Thus their performance is critical.

.

2.3 GPS Receiver Link Budget

Before you design any communication system you need to know the receiver requirements in terms of its sensitivity, selectivity, link margin, gain, path losses and noise figure. So the calculation of these values is called the link budget. Below are the specifications for the link budget for this particular application:

The total received power is given by the following equation:

Prx = Ptx + Gtx +G rx +FSL +Ap

Where,

Prx = is received power.

Ptx = Transmitted power at the satellite, (assume Ptx=14dBw)

Gtx = Gain of the antenna of the satellite, (assume Gtx=13 dBw)

FSL is a free space loss= $[\lambda/4\pi R]^2$= -184 dBw.

λ is the wave length= 19cm.

R is satellite altitude=25000 km

Ap is a propagation losses because of rain, atmosphere. (Assume Ap = -2 dBw).

G rx is a GPS receiver antenna gain =0Dbw

So,

Prx =14 + 13 + 0 - 184 - 2

= - 159dBw

= - 127 dBm

Now we need to calculate the sensitivity of a receiver which is also known as minimum received power as following:

Pmin = Pn + NF+ C/N min

Where,

Pmin is a minimum received power

Pn is Noise power = 10 log K Ta B= - 133.97 dBw

K is Boltezman constant =1.38x 10^{-23} J/K

Ta is Antenna noise temperature= 290 k (assumption).

B is IF signal bandwidth=10 MGZ.

NF is the noise figure of the receiver =9 dBw (assumption).

C/N min is the minimum carrier to noise ratio at the detector input =10 dBw. (Assumption).

Pmin = -133.97+9+ 10

= - 114.97 dBw

= -84.97 dBm

Finally the dynamic range for the receiver should be measured. Typically it is between 40 dB and 80 dB [5].

Dynamic range = P1dB - Pmin

Where,

P1dB is the input 1dB compression point.

Pmin is the sensitivity of the receiver.

P1dB= 1dB output compression point –Gain +1

Where,

Gain = (GLNA +G RF filter) = 15-3=12dBw

P1dB=0 dB-(12) +1= -11 dBw

So,

Dynamic range = -11-(-84.97) = 73.97 dBw.

3 LNA Design and Introduction

Before going into the details of the LNA look at the basic block diagram again.

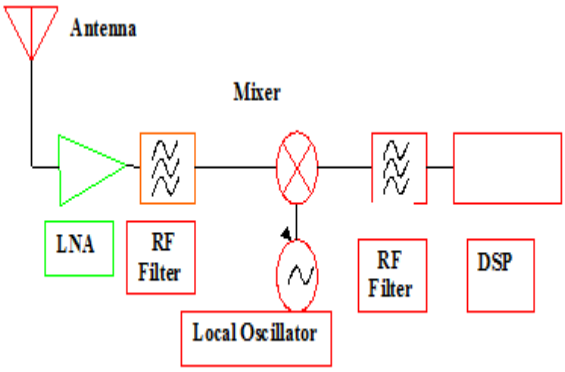


Figure 3.1: Receiver Block Diagram

Usually for a radio receiver front end the first stage element is a low noise amplifier (LNA). The LNA is very important for a high performance Radio frequency receiver. It plays a very important role to match the impedance as well. How to design a high performance LNA, depends upon the application, but there are different factors such as, Gain, stability, linearity, noise etc which determines its optimization. The design of the LNA has involved much effort during recent years to get the noise figure as low as possible and to get linearity to its highest possible limit. Our GPS receiver design is based on cascaded components. The noise performance of the given circuit is determined by the first few stages.

According to the formula for the calculation of the noise figure as follows:

$$F(Total) = F1 + \frac{F2-1}{G1} + \frac{Fn-1}{G1G(n-1)}$$

From the given equation it can be seen that noise factor of the first element clearly dominates the later stage elements provided that G >>1 which is of course

in that case, so we design the first stage as low noise amplifier.

In this project LNA is at 1.57 MHz Typically GPS signal at receiver antenna are in the order of -150 dBm, while signal to noise ratio is around -20dB. This is very weak and it makes noise performance very poor as well. The bandwidth is between 2 to 20 MHz so the design will focus on noise performance of LNA, impedance matching linearity [3.1].

3.1 LNA Block Diagram

Below is the block diagram for LNA. It is a simple block diagram which gives the idea of how the circuit for LNA will look.

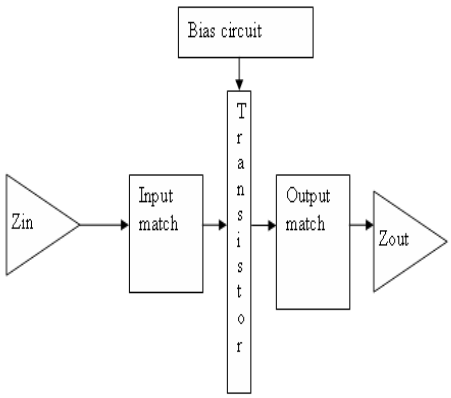


Figure 3.2: LNA Block Diagram

3.2 LNA Parameters

LNA has the following parameters.

i) Gain
ii) Noise Figure
iii) Unconditional stability
iv) Linearity
v) Input voltage

3.3.1 Gain

It is the ratio of output to input. For the LNA the value of gain is 15dB. It is not necessarily a fact that an LNA with maximum gain is very good. LNA is a nonlinear device and to make it linear there is a trade-off between gain and linearity. Because of the non linearity intermodulation products arise in LNA and as well in the receiver. So non linearity increases with gain, for that we choose an appropriate amount of gain depending upon the application to get the functionality appropriate.

3.3.2 Noise Figure

The GPS receiver is a combination of cascaded components. The LNA is the one of the components. Noise figure is the ratio which determines the LNA noise power to total noise for the receiver. The weakest signal a receiver can detect indicates the sensitivity of the receiver. If the LNA has more noise, then the noise floor will be will be higher and will make the receiver less sensitive. In this specification the noise figure of LNA should be <= 1.6 dB.

3.3.3 Unconditional stability

Whether the circuit is unconditionally stable or not depends upon the value of K. K is the factor whose value is determined by the following formula

$K = (1 + |Ds|^2 - |S11|^2 - |S22|^2) / 2 * |S21| * |S12|$

If K > 1 then circuit is unconditionally stable.

3.3.4 Linearity

Linearity is very important parameter of LNA. LNA is a non linear device. The transfer function for linear device can be written as.

$V_{out.} = C * V_{in.}$

If $V_{in.}$ is a signal having only one frequency component, then there will be no intermediation product. But active circuits are never completely linear. We need to understand non linearity in the way that it can cause intermodulation products to arise which can distort the output. Too large an input signal can overdrive the device which will distort the output. The transfer function for non linearity can be written as

$V_{out.} = C * V_{in.} + c_2 * Vin^2$

It shows clearly that there is a frequency component which if not present in the input signal which will distort the output.

3.3.5 Input Voltage

The LNA will operate at around 3Volts. The input voltage coming from GPS receiver will be 12Volts. LNA has a voltage regulator that converts 12Volts to 3Volts.

The GPS LNA can be designed by different methods depending upon the application process. The design can be of using discrete elements or lumped elements. By using a discrete transistor we get low noise figures in amplifiers. These days the requirements are small circuits and should be available very quickly in the market so as to fulfil these requirements pHEMPT technology is used. Still discrete elements have the lowest noise figure but pHEMPT has a noise figure

almost same discrete components but they have other advantages as well which are:

1) Circuit is unconditionally stable at which frequency range.
2) Internal feedback which makes impedance matching easier
3) Their linearity is consistently high and they have low noise as well.

3.4 Selective Active LNA Device

Selection of LNA device is the first and most important step in LNA design as known to us these are:

Gain = 15dB
f = 1.57 MHz
N.F <=1.0 dB
Current assumption = 15 mA
Input voltage = +3V

The rule for any electronic design is that if you want to make a circuit faster then you have to keep in mind two things

1) The smaller the circuit the faster it is.
2) The less components there are the faster it is, and also less power consumption.

This means that the size of the circuit should be kept small and if the number of components is less than it will count towards faster speed as well.

To minimize the NF of amplifier the input matching should be tuned in such a way that we get the optimized value of ' Γ '(reflection coefficient). As simulations are not to be done at this stage but we need to note the values of Input Output reflection coefficient and $\Gamma_{opt.}$

3.5 How to extract Maximum Gain

In the design where NF is not the important parameter, we can attempt to conjugate a match to get the maximum possible gain from the transistor. At the same time we can provide good values for S_{11} and S_{22}. There are certain circuit topologies which can be considered, these will be described in the future work not at this stage.

3.6 Final approaches for LNA Design

The design of a LNA involves many issues addressed throughout the discussion. This is the problem with any RF circuit design. The technology chosen for the LNA device for this application is pHEMPT technology. The discrete elements have the lowest noise figure of all technologies, but they have other disadvantages as well, such as linearization and gain etc. by using pHEMPT we can improve gain and linearity over a frequency range. The design will be made using advance system design.

4. Conclusion and Future Work

The GPS receiver is now in increasing demand for civil purposes. Its cost is falling and it has become more user friendly. An initial study has been done for a Superhetrodyne GPS receiver front end. The market demand is increasing, so the objective is to introduce a low power, efficient user friendly and low cost receiver. We have discussed the architecture of the Superhetrodyne receiver and its functionality. The main focus of the project was on the Low noise amplifier of the receiver. We have discussed the LNA architecture, its design and the issues involving in its design. The LNA gain is 15dB and its noise figure should be below 1.6dB. Input voltage is kept small to LNA to avoid overdriving because LNA is a non linear device and also the most important part of the receiver. So we need to avoid any intermodulation produced by non linear devices including mixer. An enhancement mode pHEMPT technology will be used to design the LNA.

The design will be completed by using the software called advance system design. This is part of the future work. On the basis of the study done at this stage an LNA for a Superhetrodyne receiver will be designed. The results of the simulation should meet the LNA specifications mentioned above and in the end the whole simulation will be converted to a hardware circuit and will be ready to use.

References

1. Ahmed, R(2006), "Introduction to GPS: The Global Positioning System", Artech House, pp 56, 2006.
2. Getting,I.A.(1993), Perspective/navigation-The Global Positioning System", IEEE Spectrum, pp 36 38, Dec 1993.
3. Mohammad, S and Xiangdong L. (2001) "Method and system of determining user terminal position using multiple satellites", US patent 34235705, Hughes Electronics Corporation, Nov 13, 2001.
4. NAVSYS Corporation (2002), "High Gain Advanced GPS Receiver", White Paper, Sep. 2002.
5. Xiao, F., Craig, J and Schaik, A (2002) "A Low Noise Amplifier Optimized for a GPS Receiver RF Front End", School of Electrical and Information Engineering, The University of Sydney, Feb 2002.
6. Ragnar V. Reynson " Receiver Front End 1:Signals and Imperfections" Radio frequency Integrated Systems and Circuits Division, http://kom.aau.dk/~dpl/courses/mm07_slides.pdf. NAVCOM
7. Umar F. K., Hameed, S., and Badii, A. (2008), "Design and implementation of Broadband Low Noise Amplifier (LNA) for radiometer", Proceedings of the 8th European and Mediterranean Conference on Information Systems (EMCIS), Dubai, UAE, May 25-26, 2008
8. Siraj, M & Bakar, A, K (2011), "Link establishment and performance evaluation in IEEE802.16 wireless mesh networks", International Journal of the Physical Sciences Vol. 6(13), pp.3189–3197, ISSN 1992-1950 ©2011 Academic Journals

Reactions of 5-Bromo-4,6-Dimethyl-2-Thioxo-1,2- Dihydropyridine -3- Carbonitrile with Organophosphorus Reagents

Hoda A. Abdel – Malek[1*], Marwa. S. Salem[2], and Leila S. Boulos[1]

[1]Department of Organometallic and Organometalloid Chemistry, National Research Centre, El – Behoos St., P.O. 12622, Dokki, Cairo, Egypt
[2]Synthetic Organic Chemistry Laboratory, Chemistry Department, Faculty of Science, Ain Shams University, Abbasiya , Cairo, Egypt. *hodanwar@yahoo.com

Abstract: The reaction of 5-bromo-4,6-dimethyl-2-thioxo-1,2-dihydropyridine-3-carbonitrile (**1**) with phosphorus ylides **2a,b** afforded the new phosphonium ylides **7a,b**. Wittig – Honer reagent **3** reacts with **1** to give the olefinic product **8**. On the other hand, the dimeric compound **9** and the alkylated products **10**, **11** were isolated from the reaction of **1** with dialkyl phosphite **4**, trialkyl phosphite **5** and tris(dimethylamino) phosphine **6**. Possible reaction mechanisms are considered, and the structural assignments are based on analytical and spectroscopic evidence.
[Hoda A. Abdel – Malek, Marwa. S. Salem, and Leila S. Boulos **Reactions of 5-Bromo-4,6-Dimethyl-2-Thioxo-1,2- Dihydropyridine -3- Carbonitrile with Organophosphorus Reagents.** Life Science Journal 2012; 9(1):695-700]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 100

Keywords: Phosphorus ylides, Wittig-Horner reagent, phosphites, dihydropyridines, nicotinonitriles.

Introduction:

It has been reported that pyridine carbonitrile derivatives exhibit a wide spectrum of biological and pharmacological activities [1-3]. This together with our interest in organosphosphorus chemistry [4-11] has encouraged the synthesis of new organophosphorus compounds incorporating such important unclei that may possibly lead to further biological activity. The present study deals with the reaction of 5-bromo-4,6-dimethyl 2-thioxo-1,2-dihydropyridine-3-carbonitrile (**1**) with phosphorus ylides **2a,b** Wittig – Horner reagent **3** , dialkyl phosphite **4**, trialkyl phosphite **5** and tris(dimethylamino) phosphine **6** (Scheme 1).

1

$Ph_3P{=}CHCOR$

2a, R= OCH_3
b, R=OC_2H_5

$(C_2H_5O)_2P(O)CH_2CN$

3

$H{-}\overset{\overset{O}{\|}}{P}(OCH(CH_3)_2)_2$

4

$P(OCH_3)_3$

5

$P[N(CH_3)_2]_3$

6

Scheme 1

2. Experimental

Melting points were determined in open glass capillaries using an Electrothermal IA 9000 series digital melting point apparatus (Electrothermal, Essex. UK) and were uncorrected. The IR spectra were measured in KBr pellets with a Perkin-Elmer Infracord Spectrophotometer model 157(Grating). The ^{1}H and ^{13}C-NMR spectra were recorded in $CDCl_3$ as solvent on a Joel-500 MHz spectrometer, and the chemical shifts were recorded in δ values relative to TMS. The ^{31}P-NMR (125 MHz) spectra were taken with a Varian CFT-20 (vs. external 85% H_3PO_4 standard). The mass spectra were performed at 70 eV on a Shimadzu GCS-OP 1000 Ex spectrometer provided with a data system. Elemental analyses

were performed using an Elmenter Varu EL Germany Instrument.

Reaction of 5-bromo-4,6-dimethyl-2-thioxo-1,2-dihydropyridine -3- carbonitrile (1)[12] with carbmethoxymethylenetriphenylphosphorane (2a).

A mixture of **1** (0.24g , 1 m mol) and the phosphonium ylide **2a** (0.32g , 1 m mol) was refluxed in boiling DMF (20 mL) for 6h. The reaction mixture was evaporated under reduced pressure. The residue was washed several times with petroleum ether (b.r. 60-80 °C) to give product **7a** [methyl (5-bromo-3-cyano-4,6-dimethyl-2- sulfanyl -1,2- dihydropyridin-2-yl) (triphenyl -λ^5- phosphanylidene) acetate (**7a**, $C_{29}H_{26}BrN_2O_2PS$). Crystallized from ethyl acetate **7a** was separated as colorless crystals, yield 78%, and m. p. 240 – 241 °C. IR [ν, cm^{-1}, KBr] : 3420 (NH), 2219 (CN), 1700, (C=O, ester), 1630, 1515 (C=P), and at 1435, 990 (P-C-phenyl). ^{1}H-NMR (500 MHz, δ ppm, $CDCl_3$) : 2.46, 2.54 (2s, 6H, CH_3), 2.22 (s, H, NH), 2.32 (s, 1H, SH), 3.20 (s, 3H, $COOCH_3$), 7.68 – 7.74 (m, 15H, Ar) ^{13}C-NMR (125 MHz, δ ppm, $CDCl_3$): 12.3 , 13.5 ($2CH_3$), 45.6 (d, $^2J_{CP}$ = 16.4 Hz, HS-C-C=P), 53.4 (d, $^3J_{CP}$ = 7.5 Hz, OCH_3), 91.9 (C-Br), 100.10 (C-CN), 118.91 (CN) 126.3 ((d, $^1J_{C-P}$ =89.2 Hz, P=C), 127.5 – 133.8 (Ar), 145.8 (C=C-Br), 152.1 (CH_3-C=C), 168.3 (d, $^2J_{CP}$ =14.5 HZ, C=O, ester), ^{31}P-NMR (δ ppm, $CDCl_3$): 22.52. MS m/z (%) 574 [M-3]$^+$ (50). Anal. Calcd. for $C_{29}H_{26}BrN_2O_2PS$(576.06) : C, 60.32 ; Br, 13.84 ; N, 4.85 ; P, 5.36 ; S, 5.55. Found: C, 60.62 ; Br, 13.54 ; N, 4.45 ; P, 5.58 ; S, 5.84.

Reaction of 1 with carbethoxymethylene triphenyl phosphorane (2b).

A mixture of 1 (0.24g, 1 m mol) and the phosphonium ylide 2b (0.34g , 1 m mol) was refluxed in boiling DMF (20 mL) for 6 h. The reaction mixture was evaporated under reduced pressure. The residue was washed several times with petroleum ether (b. r. 60 – 80 °C) to give product **7b** [ethyl (5-bromo-3-cyano-4,6-dimethyl-2-sylfanyl-1,2- dihydro pyridin -2-yl) (triphenyl - λ^5- phosphanylidene) acetate) (**7b**, $C_{30}H_{28}BrN_2O_2PS$).

Crystallized from ethylacetate, 7b was separated as colorless crystals, yield 66%, m. p 293 – 294 °C. IR [ν, cm^{-1}, KBr] : 3420 (NH), 2219 (CN), 1700 , (C=O), 1630, 1515 (C=P), 1434, 990 (P-C-phenyl). ^{1}H-NMR (500 MHz, δ ppm, $CDCl_3$) : 2.40, 2.50 (2s, 6H, $2CH_3$), 2.21 (s, H, NH), 2.3 (s, 1H, SH), 1.29 (t, 3H, $COOCH_2CH_3$), 4.21 (q, 2H, $COOCH_2CH_3$), 7.68 – 7.74 (m, 15H, Ar). ^{13}C-NMR (125 MHz, δ ppm, $CDCl_3$): 12.3 , 13.5 (2CH3), 45.6 (d, $^2J_{CP}$ = 16.4 Hz, HS-C-C=P), 14.1 (OCH_2CH_3), 61.6 (OCH_2CH_3), 100.1 (C-CN), 118.9 (CN) 126.3 (d, $^1J_{C-P}$ =90.24 Hz, P=C). 127.4 – 133.8 (Ar), 168.42 (d, $^2J_{CP}$ =14.5 HZ, C=O, ester), ^{31}P-NMR (δ ppm, $CDCl_3$): 22.55. MS m/z (%) 517 [M-$COOC_2H_5$]$^+$ (55). Anal. Calcd. for $C_{30}H_{28}BrN_2PO_2S$(590.08) : C, 60.92 ; H, 4.77 ; Br, 13.51 ; N, 4.74 ; P, 5.24 ; S, 5.42. Found : C, 60.58 ; H, 4.53 ; Br, 13.76 ; N, 4.95 ; P, 5.31 ; S, 5.45.

Reaction of 1 with diethyl (cyano methylene) phosphonate (3).

Diethyl (cyanomethylene) phosphonate (**3**) (0.17, 1m mol) was dissolved in very dry xylene (25mL) and the sodium hydride (0.024g, 1m mol) was added carefully with stirring. The carbonitrile **1** (0.24g , 1m mol) was added to the mixture and refluxed for 10h. after evaporation of the volatile materials under reduced pressure, the residue was washed several times with petroleum ether (b.r. 60-80 °C) to give product **8** [5-bromo-3-cyano-4,6-dimethyl -1,2-dihydropyridine-2-yl) maleonitrile] (**8**, $C_{12}H_9BrN_4$). Crystallized from ethylacetate, **8** was separated as colorless crystals, yield 75% and m.p 253 – 254 °C. IR [ν, cm^{-1}, KBr] : 3422 (NH), 2220 (CN), 1644 , (C=C). ^{1}H-NMR (500 MHz, δ ppm, $CDCl_3$) : 2.46, 2.54 (2s, 6H, $2CH_3$), 2.22 (s, H, NH), 3.99 (s, 1H, CH), 5.97 (s, 1H, CH=C). ^{13}C-NMR (125 MHz, δ ppm, $CDCl_3$): 11.6 , 15.3 ($2CH_3$), 47.0 (CH), 91.9 (C-Br), 102.8 (C-CN), 108.3(CH-CN), 117.3 (CN), 145.8 (C=C-Br), 152.1 (CH_3-C=C). MS m/z (%) 288 [M$^+$] (45). Anal. Calcd. for $C_{12}H_9BrN_4$ (288.00) : C, 49.85 ; H, 3.14 ; Br, 27.64 ; N, 19.38. Found : C, 49.49 ; H, 3.25 ; Br, 27.55 ; N, 19.72.

Reaction of 1 with diisopropyl phosphite (4)

Excess of diisopropyl phosphite (**4**) (≈ 3 mL). was added to 1 (0.24g , 1 m mol) without solvent and reaction mixture was refluxed for 3h. After evaporation the volatile material under reduced pressure, the residue washed several times with petroleum ether (b.r. 60-80 °C) to give product **9** [2,2'-thiobis (5-bromo-4,6-dimethylnicotinonitrile] **(9**, $C_{16}H_{12}Br_2N_4S$).

Crystallized from ethyl acetate, **9** was separated as colorless crystals, yield 70% and m. p 190 - 191°C. IR [ν, cm^{-1}, KBr] : 2219. ^{1}H-NMR (500 MHz, δ ppm, $CDCl_3$) : 2.46, 2.54 (2s, 6H, $2CH_3$), ^{13}C-NMR (125 MHz, δ ppm, $CDCl_3$): 14.1 , 23.5 ($2CH_3$), 103.9 (C-CN), 117.0 (CN), 119.8 (C-Br), 155.0 (CH_3-C=C). 165.4 (C=C-Br), 182.6 (N=C-S). MS m/z (%) 449 [M$^+$] (40). Anal. Calcd. for $C_{12}H_9BrN_4$ (449.91) : C, 42.50 ; H, 2.67 ; Br, 35.34 ; N, 12.39 ; S, 7.09 Found : C, 42.73 ; H, 2.29 ; B, 35.76 ; N, 12.43 ; S, 7.18.

Similarly, the reaction of carbonitrile 1 with excess of trimethyl phosphite (≈ 3 mL) was refluxed for 3h to give product **10** [5-bromo-4,6-dimethyl-2-(methylthio) nicotinonitrile] (**10**,$C_9H_9BrN_2S$).

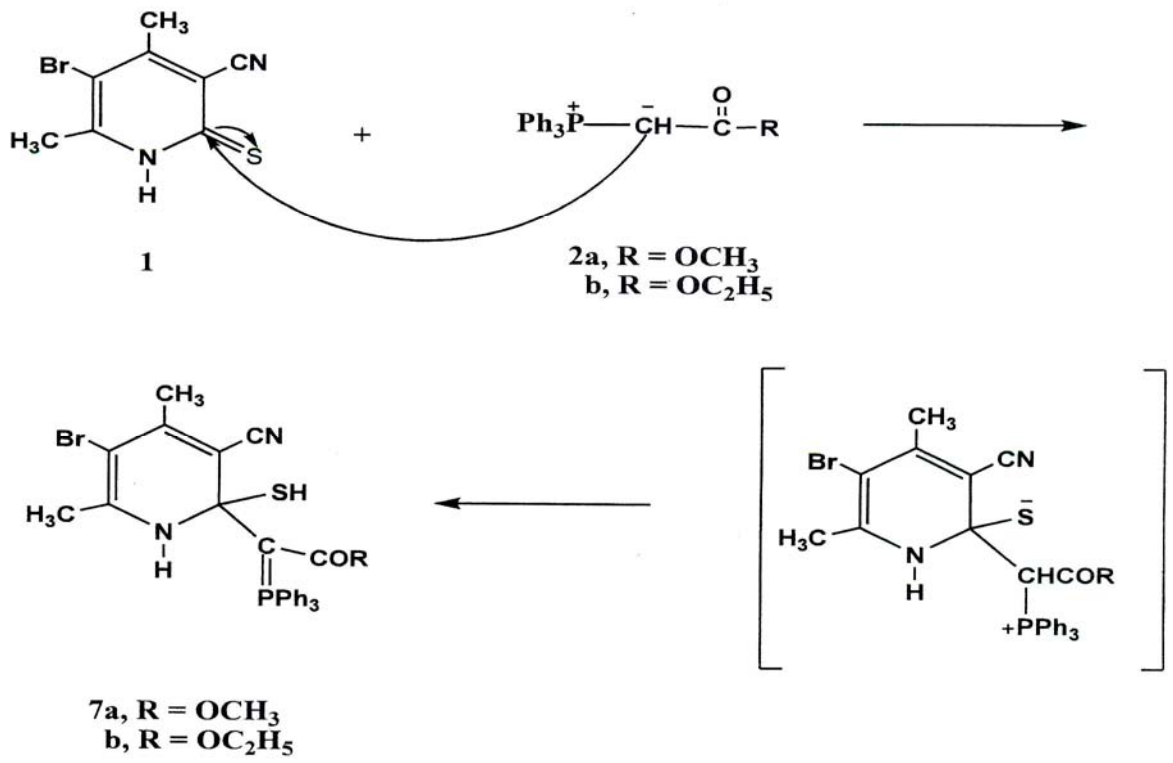
Crystallized from ethyl acetate, 10 was separated as colorless crystals yield 73% and m.p 268 – 269 °C. IR [ν, cm^{-1}, KBr] : 2220 (CN) ^{1}H-NMR (500 MHz, δ ppm, $CDCl_3$) : 2.36 , 2.53 ($2CH_3$) , 2.51 (S-CH_3) ^{13}C-NMR (125 MHz, δ ppm, $CDCl_3$): 14.1 , 23.5 ($2CH_3$), 13.1 (S-CH_3), 106.7 (C-CN), 117.0 (CN), 117.4 (C-Br), 154.0 (C=C-CN). 162.7 (C=S-CH_3), 164.9 (C=C-Br), MS m/z (%) 255 [M^+] (23), [M-15]$^+$ (70). Anal. Calcd. for $C_9H_9BrN_2S$ (255.97) : C, 42.04 ; H, 3.53 ; Br, 31.07 ; N, 10.89 ; S, 12.47 Found : C, 42.33 ; H, 3.59 ; Br, 31.47 ; N, 11.03 ; S, 12.53.

Similarly, carbonitrile 1 was reacted with excess tris (dimethylamino) phosphine (**6**) to give product **11** [5-bromo-2- (dimethylaminothio)-4,6-dimethyl-nicotinonitrile] (**11**,$C_{10}H_{12}BrN_3S$).

Crystallized from ethyl acetate, **11** was separated as colorless crystals yield 75% and m.p 95 - 96 °C. IR [ν, cm^{-1}, KBr] : 2219 (CN). ^{1}H-NMR (500 MHz, δ ppm, $CDCl_3$) : 2.36 , 2.53 (2s, 6H, CH_3) , 2.47 (s, 6H, $N(CH_3)_2$) ^{13}C-NMR (125 MHz, δ ppm, $CDCl_3$): 14.1 , 25.5 ($2CH_3$), 43.1 ($N(CH_3)_2$) , 103.9 (C-CN), 117.0 (CN), 119.8 (C-Br), 155.0 (CH_3-C=C), 165.4 (C=C-Br) , 182.6 (C-S). MS m/z (%) 284 [M^+] (75). Anal. Calcd. for $C_{10}H_{12}BrN_3S$ (284.99) : C, 41.97 ; H, 4.23 ; Br, 27.92 ; N, 14.68 ; S, 11.20. Found : C, 41.88 ; H, 4.29 ; Br, 27.53 ; N, 14.93.

3- Results and Discussion

We have found that carbmethoxymethylene-triphenylphosphorane (**2a**) reacts with 5-bromo-4,6-dimethyl-2-thioxo-1,2 -dihydropyridine -3-carbonitrile (**1**), in boiling dimethylformamide, to give the new phosphorane product **7a** as the sole reaction product. Triphenylphosphine and / or triphenylphosphine oxide are neither isolated nor detected in the reaction medium (Scheme 2). Compound **7a** consists of pure crystals and has a sharp melting point. Structure elucidation of the new phosphorus ylide 7a is based on the following evidence: elemental analyses and molecular weight determination (MS) of **7a** support the molecular formula $C_{29}H_{26}BrN_2O_2PS$ (576.06) ; accordingly, MS : m/z = 574 [M-3]$^+$, 50%.

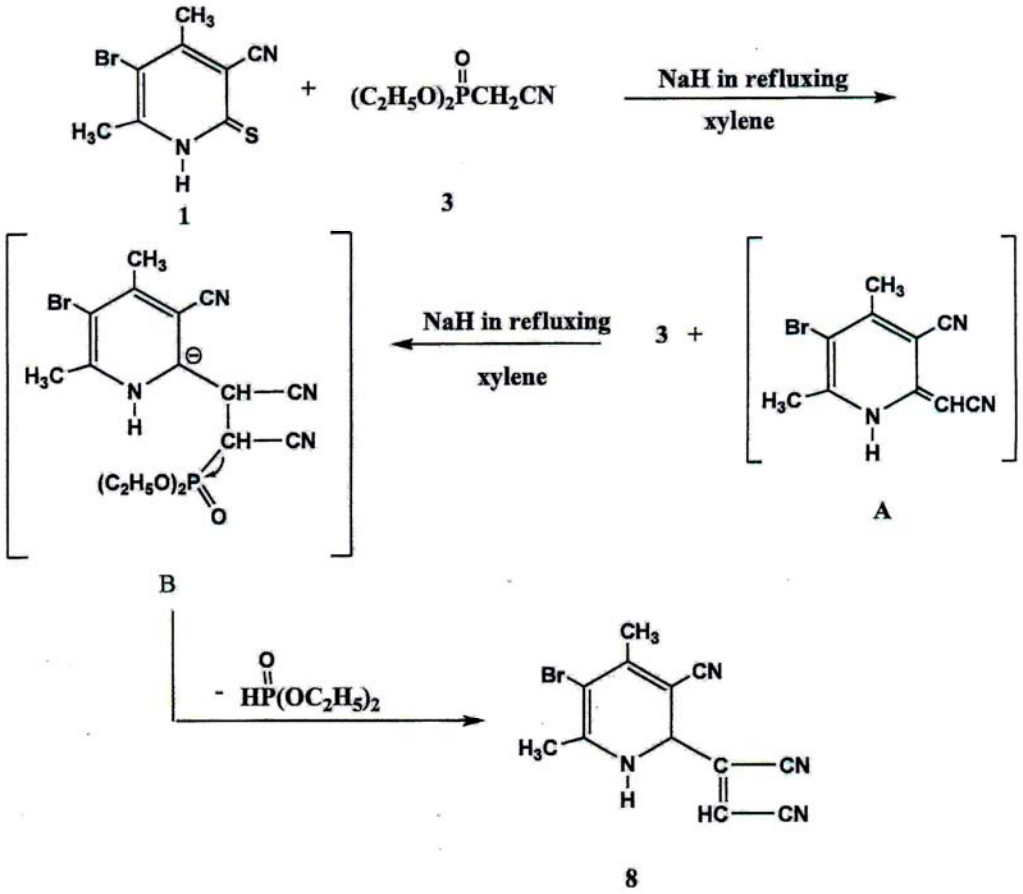


Scheme 2

Its IR spectrum, in KBr, exhibits strong absorption band at 1700 cm^{-1} (C=O, ester), 1630, 1515 cm^{-1} (C=P), and at 1435, 990 cm^{-1} (P-C-phenyl) [13] ^{31}P-NMR δ = 22.52, a value that falls in the range frequently recorded for this class of compounds [13 , 14]. The ^{1}H-NMR spectrum of **7a** in $CDCl_3$ discloses the presence of signals at δ = 3.20 ppm (s, 3H, $COOCH_3$), 2.46 , 2.54 ppm (2s, 6H, $2CH_3$), 2.20 (s, H, NH) , 2.30 (s, 1H , SH) , 7.68 – 7.74 (m, 15H, aromatic). Actually, the structure assigned for compound **7a** is based on ^{13}C-NMR spectroscopy, which indicate the presence of signals at 168.3 (d, $^{2}J_{CP}$ = 14.5 Hz) allocated to the C=O of the ester group, at 53.4 ppm (d, $^{3}J_{CP}$ = 7.5 Hz for the OCH_3 group), at 126.3 (d, $^{1}J_{CP}$ = 89.22 Hz, P=C) [15], at 45.6 ppm (d, $^{2}J_{CP}$ = 16.4 Hz (HS-C-C=PPh_3) at 118.9 (CN) 100.1 ppm (C-CN), 12.3, 13.5 ppm ($2CH_3$).

Similarly, the reaction product of **1** with carbethoxymethylene triphenylphosphorane **2b** was assigned analogous structure **7a** on the basis of comparable spectroscopic arguments (cf. Scheme 2, Experimental section). Products **7a,b** are presumably formed via addition of the ylide species **2a,b** to the active methine carbon in compound **1** to afford the new phosphonium ylieds **7a,b** (Scheme 2).

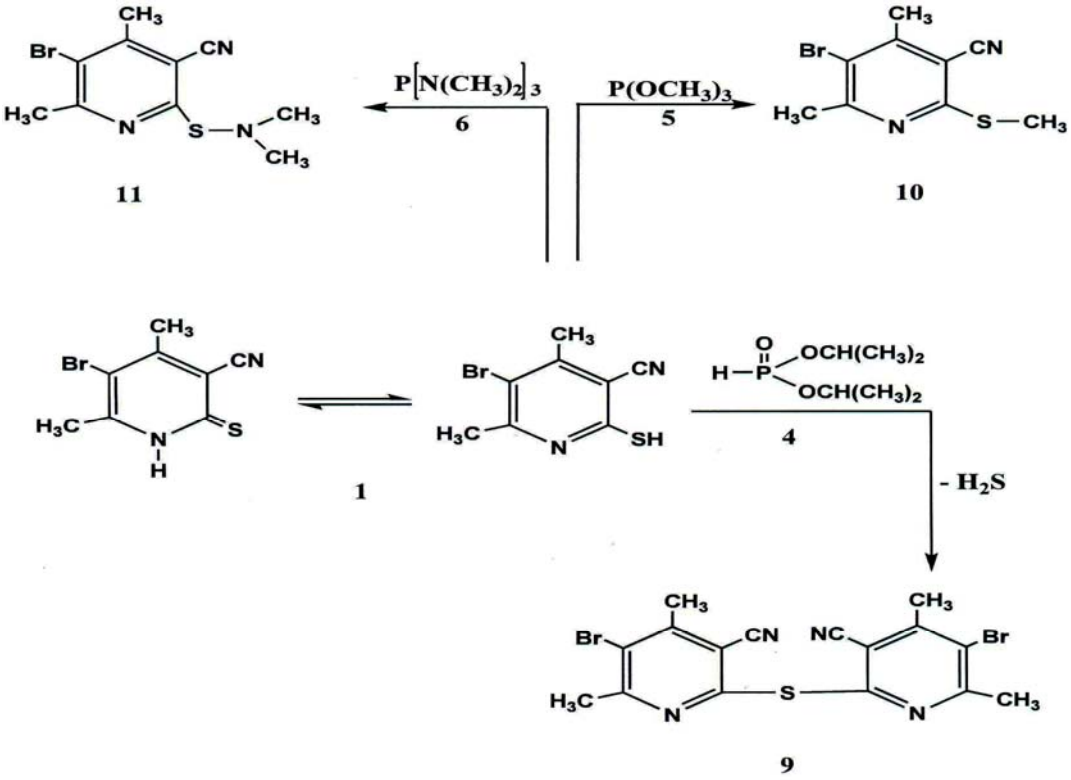
Next, when **1** was allowed to react with one mol equivalent of diethyl (cyanomethyl) phosphonate (**3**), in the presence of sodium hydride in xylene, at reflux temperature for 10h, adduct **8** was isolated in 75% yield (scheme 3). The structure of 2-(5-bromo -3-cyano-4,6-dimethyl -l,2- dihydropyridin-2-yl) maleonitrile is derived from its spectral data (cf. Experimental Section).



Scheme 3

A possible explanation for the reaction course of **1** with Wittig – Horner reagent **3** in the presence of sodium hydride as base is shown in Scheme 3. Thio-olefinatin of compound **1** with Wittig – Horner reagent **3** gave the intermediate (**A**) which reacted with another molecule of **3** to give intermediate (**B**). Under the influence of the base present in the reaction medium, elimination of dialkyl phosphite after a suitable proton transfer gives the final product **8** (scheme 3).

The reaction of 5-bromo-4,6-dimethyl-2-thioxo-1,2-dihydropyridine-3-carbonitrile (**1**) with diisopropyl phosphite (**4**), trimethyl phosphite (**5**), and tris (dimethylamino) phosphine (**6**) was also investigated. We have found that the reaction of diisopropyl phosphite (**4**) with **1** proceeds without solvent at reflux temperature to give the dimeric product **9** in 70% yield (Scheme 4). Structure elucidation for compound **9** was attested by the following evidence (a) elemental analyses and molecular weight determination (MS) for compound **9** correspond to $C_{16}H_{12}Br_2N_4S$. (b) The IR spectrum of **9**, in KBr, discloses the absence of both NH and C=S bands appeared at 3419 cm^{-1} and 1165cm^{-1} in the starting material.



Scheme 4

Moreover, the ^{1}H and ^{13}C-NMR of 2,2' – thiobis (5-bromo-4,6-dimethyl nicotinonitrile) furnish strong evidence in support of the dimeric structure **9** (cf. Experimental Section). Trimethyl phosphite (**5**) and tris(dimethylamino) phosphine (**6**) on the other hand, reacted with **1** without solvent at reflux temperature to give the alkylated [16] products **10**, **11**, respectively (Scheme 4). The structures of the new compounds **10**, **11** are assigned on the basis of the full set of their spectral data (cf. Experimental Section).

4- Conclusion

From the results of the present investigation, it could be concluded that the Wittig reagent **2a,b** preferentially attacked the thiocarbonyl carbon in **1** to give the phosphonate products **7a** and **7b**. Meanwhile, it has been found that the reaction of **1** with Wittig-Horner reagent **3** proceeds according to the Wittig reaction to give the olefinic product **8**, where as, in the reaction of **1** with dialkyl phosphite, trialkyl phosphite, and tris(dialkylamino) phosphine the dimeric compound **9** and the alkylated products **10**, **11** are the sole reaction products.

Corresponding author:
Hoda Anwar Abdel - Malek
Department of Organometallic and Organometalliod Chemistry
National Research Centre,
El – Behoos St., P.O. 12622, Dokki, Cairo , Egypt.
E-mail: hodanwar@yahoo.com

References

1. Dawoud , N. T. A. (2011) : Synthesis , reaction and antimicrobial activity of some substituted 4.6 – diphenyl pyridine -2- thione derivatives. Nature and Science. 9(7): 202-210.

2. Johns , B. A., Gudmundsson , K. S., Turner, E. M., Allen, S.H., Jung, D. K, Sexton, C. J, Bayd, Jr. F. L., and Peel, M.R. (2003) : Pyrazalo [1,5-a] pyridines : synthetic approach to a novel class of antiherpetics . Tetrahedron. 59 : 9001 – 9011.
3. Magedov, I. V., Manapadi , Ogasawara , M. A., Dhwan, A. S. and Rogadi, S. (2008) : Structural implication of bioactive natural products with multicomponent synthesis. Antiproliferative and antitubolin activities of pyran [3,2-c] quinolines. J. Med . Chem. 51: 2561 – 2570.
4. Boulos, L. S., Ewies, F. E. and Fahmy, A. F. M. (2011): Synthesis of new bisphosphonate and bisphosphonic acid derivatives and Heterocyclic and dialkyl carbamoyl oxazolones derivatives with anticancer and antischistosmal activity. Z. Naturforsch, 666: 1056-1068.
5. Boulos, L. S., Abdel-Malek, H. A., El-Sayed, N.F., Moharm, M. E. (2012): Reactions of 1,1'-(Azodicarbonyl) dipiperidine with organophosphorus reagents. Phosphorus, Sulfur, Silicon Relat. Elem., 187 (2) : 225-237.
6. Boulos, L. S., Abdel-Malek, H. A., El-Sayed, N.F., and Moharm, M. E. (2011) : Scope and limitation of the reactions of 3-imino derivatives of pentane -2,4-diones with organophosphorus reagents. Phosphorus, Sulfur, Silicon, Relat. Elem. (in press).
7. Abdel-Malek, H. A. (2012) : Reaction of 2-thioxo-4-thinazolidinones toward Lawesson's reagent, phosphorus pentasulfide, dialkyl amino phosphines and phosphorus ylides. Phosphorus, Sulfur, Silicon Relat. Elam., 187 (4) : 506 – 514.
8. Abdel-Malek, H. A. (2011) : The behavior of 1,7,7-trimethyl bicyclo [2.2.1] heptane-2,3-dione and 3-(2-phenylhydrazone)-1,7,7- trimethyl bicyclo [2.2.1] heptane -2-one toward organophosphorus reagents. Journal of American Science. 7(12): 864-869.
9. Abdel-Malek, H. A. (2005) : Comparative Studies of phosphonium and phosphonate carbanion reagents in reactions with fluorenone. Egypt. J. Chem., 48(1): 129-134.
10. Boulos, L. S., Abdel-Malek, H. A., (2005): The behavior of trisdialkylamino phosphines and alkylphosphites towards 5,6-diphenyl-4- cyano pyridazin -3-thione. Indian J. Heterocycl. Chem., 14: 245-48.
11. Boulos, L. S., Abdel-Malek, H. A., (2004): The behavior of 3,4-diphenyl-5-cyanopyridazine-6-thione toward phosphorus ylides. New synthesis of furopyridazine derivatives, phosphorus, Sulfur and Silicon, 179: 97-105.
12. Modkour, H. M. F., Afify, A. A. E., Abdalha, A. A., Elsayed, G.A. and Salem, M. S. (2009): Synthetic utility of enaminonitrile moiety in heterocyclic synthesis : Synthesis of some new thienopyrimidines. Phosphorus, Sulfur, Silicon Relat. Elem., 184 (3) : 719-732.
13. Mark, V., Dungan, C. H., Crutchfield, M. M., Van Wazer, J. R., Grayson, M., Griffith, E. J. (1976): Eds., Topics in phosphorus, Chemistry. Interscience publishers : New York, vol. 5, pp. 227-447.
14. Ramirez, F., Madan, O. P., Smith, C. P. (1965): Trialkyl – and triaryl – alkylidene phosphoranes from the reaction of tertiary phosphines with trans – dibenzoylethylene. Tetrahedron Lett,. V. 6(3) : 201.
15. Kalinowski. H. O., Berger, S., Brauy, S., (1984) : ^{13}C-NMR Spectroscopic, Geroge thiene Verlage, Stuttgart, New York.
16. Sidky, M. M., Mahran, M. R., Zayed, M. F., Abdou, W. M., Hafez, T. S. (1983): Alkyl phosphites and phosphonates as alkylating agents for 1,3,4-thiazolidine-2,3-dithiones, Organic preparations and procedures. Int. 14(4): 225-232.

2/18/2012

Forecasting gamma radiation levels using digital image processing

Abou-Bakr M.Ramadan [1], Ahmed M. El-Garhy [2], Fathy Z.Amer [2], and Mazhar M. Hefnawi [1 *]

[1] Department of National Network for Monitoring Radioactivity, Atomic Energy Authority of Egypt, Cairo, Egypt
[2] Department of Electronics, Communications and Computers, Faculty of Engineering, Helwan University, Cairo, Egypt.
mmaazz_2222@yahoo.co.uk

Abstract: This work introduces a new way for data visualization. Its name is " Digital 'application name' Image". Normal digital image is created by digital camera or digital scanner but digital application name image is created by measurements of monitoring data. This work uses the data which is measured by some radiation monitoring stations and classifies it using fuzzy logic rules to create some digital radiation images. The main unique advantage of digital radiation image is that it expresses thousands of measurements in a very clear form through only one picture while the maximum number of measurements does not exceed 100 for other conventional visualization methods. This feature gives a facility to view one year of all recorded measurements in only one photo. This picture helps the user to observe the behavior of thousands of measurements in few minutes instead of spending few hours in reviewing hundreds of charts for the same measurements. This work also introduces a new way for forecasting Gamma radiation levels. This way uses image restoration technique to predict the gamma levels. Of course, this technique is used after creating digital radiation image. The quality for the output result from this model is at least accepted for forecasting and covering lost data. The main feature from this model is that it needs only one kind of data while other prediction models need at least three kinds of data. Therefore this model covers the common limitation in famous prediction models and saves money, time and effort.
[Abou-Bakr M. Ramadan, Ahmed M. El-Garhy, Fathy Z.Amer, and Mazhar M. Hefnawi. **Forecasting gamma radiation levels using digital image processing.** Life Science Journal 2012;9(1):701-710]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 101

Keywords: Data Visualization; Digital Image Processing; Digital Radiation Image; Environmental Forecast.

1. Introduction:

This work describes a novel approach for encoding a set of continuous numerical observations in a form of a color image, where the coordinates of each pixel encode time of a specific observation and color represents magnitude of an observation. This work also uses this color image to generate a new approach for predicting future or missing observations from collected ones and compares this approach to artificial neural network and various deterministic classification algorithms. It is preferred to start with reviewing the basic definition of an image because it is a necessary part for explaining the definition of the digital measurements image.

An image may be defined [1] as a two-dimensional function, f(x,y) , where x and y are spatial (plane) coordinates and the amplitude of f at any pair of coordinates (x,y) is called intensity of the image at that point. The term gray level is used often to refer to the intensity of the monochrome images. Color images are formed by a combination of individual 2-D images such as RGB color system, a color image consists of three (red, green, and blue) individual component images. So converting such an image to digital form requires that the coordinates, as well as the amplitude, be digitized. Digitizing the coordinate values is called sampling; digitizing the amplitude values is called quantization. Thus when x,y and the amplitude values of f are all finite, discrete quantities. We call the image a digital image as shown in Fig. 1a for digital representation for monochrome image [1] and Fig. 1b for digital representation of RGB color system image [1].

2. Experimental

Digital measurements image like digital image has two-dimensional function, f(x,y) , but x and y are spatial (plane) coordinates indicate the date and time for each measurement. The amplitude of f at any pair of coordinates (x,y) is called intensity of the image at that point which is the value of the measurement as shown in Fig. 2a.

Construction of digital radiation image

Digital radiation image is a digital RGB color measurements image for radiation levels in ambient air. These measurements are measured by a radiation monitoring station. This station is in constant place and operating for 24 hours daily. It measures a radiation level in ambient air every 15 minutes [2]. As shown in Fig. 2b the digital RGB color measurements image may be viewed as a "stack" of three gray-scale images that, when fed into red, green and blue inputs of a color monitor, produce a color image on the

screen. By convention, the three images forming an RGB color image are referred to as the red, green, and blue component images. The x and y coordinates for this image represent the date and time for each measurement. The color for each pixel in this image represents the measurement value.

Representing digital radiation image using different colors is better than representing it using 16 bit grayscale because it is easier and faster for human to watch and analyze the behavior of Gamma radiation levels in form of different colors than grayscale. The color image is a good choice for measurements of radiation levels because the nature behavior in time series for these measurements varies in non-smoothly form. Therefore if a 16 bit grayscale image is used for these measurements then the low values which their time is very close to the time of the high values will not clearly appear in grayscale image. The reason for this phenomenon is due to the basic colors used in grayscale image which are white, black and other colors in which their intensity degree are between them. So, the pixels which have high darkness intensity reflect their darkness to their neighborhood pixels which have very low darkness intensity. Maybe grayscale image is a good choice for other kinds of measurements which their nature behavior in time series varies in smooth form such as measurements of ambient temperature.

Digital Radiation Image Creation

The processes for creating the digital radiation image are as follows:

a. Collecting all radiation measurements in one year from radiation monitoring station.
b. Putting these measurements in a 365 × 288 array of radiation measurements. The number of rows is equal the number of days in one year and the number of columns equals to the number of measurements in one day which equals to twelve measurements per hour multiplied by 24 hours daily.
c. Creating another three arrays. The size of each array is the same as the pervious array. The elements of the first array represent the red components, the elements of the second array represent the green components, and the elements of the third array represent the blue components.
d. Converting each radiation measurement to fuzzy number or linguistic status [3] according to allowed limit that is set up by environmental law number four in Egypt [4]. This is necessary to establish a meaningful system for creating a digital radiation image. The value for this allowed limit does not exceed 2.3×10^{-7} Sv / hr. Table 1 shows all fuzzy numbers and their linguistic expressions used in this study.
e. Determining the color for each radiation measurement by using rule based structure of fuzzy logic [5]. The series of fuzzy rules for all measurements were recorded in one year defines the digital radiation image. Defining that Radiation Measurement as RM. These rules are as follows:-

R_1: IF RM IS UL_ST1 THEN RM_color IS WHITE.
R_2: IF RM IS UL_ST2 THEN RM_color IS LIGHT BLUE SKY.
R_3: IF RM IS UL_ST3 THEN RM_color IS BLUE SKY.
R_4: IF RM IS UL_ST4 THEN RM_color IS LIGHT BLUE.
R_5: IF RM IS UL_ST5 THEN RM_color IS BLUE.
R_6: IF RM IS UL_ST6 THEN RM_color IS DARK BLUE.
R_7: IF RM IS NL_ST1 THEN RM_color IS LIGHT GREEN.
R_8: IF RM IS NL_ST2 THEN RM_color IS GREEN.
R_9: IF RM IS NL_ST3 THEN RM_color IS DARK GREEN.
R_{10}: IF RM IS AL_ST1 THEN RM_color IS VERY DARK GREEN.
R_{11}: IF RM IS AL_ST2 THEN RM_color IS LIGHT YELLOW.
R_{12}: IF RM IS AL_ST3 THEN RM_color IS YELLOW.
R_{13}: IF RM IS AbL_ST1 THEN RM_color IS LIGHT ORANGE.
R_{14}: IF RM IS AbL_ST2 THEN RM_color IS ORANGE.
R_{15}: IF RM IS AbL_ST3 THEN RM_color IS BROWN.
R_{16}: IF RM IS OL_ST1 THEN RM_color IS LIGHT PINK
R_{17}: IF RM IS OL_ST2 THEN RM_color IS PINK
R_{18}: IF RM IS OL_ST3 THEN RM_color IS DARK PINK.
R_{19}: IF RM IS OL_ST4 THEN RM_color IS LIGHT RED.
R_{20}: IF RM IS VOL THEN RM_color IS RED.
R_{21}: IF RM IS NO_DATA THEN RM_color IS BLACK.

f. Putting the value for red component in the first array, green component in the second array and blue Component in the third array according to radiation measurement color produced from step number five. Hence the three images are ready for forming the RGB image which is the digital radiation image.
g. To make the final image more clear increase its width by repeating each pixel in every

row four times. So, the resulted image dimension is 365 × 1512.

Fig. 3a shows how to implement the pervious steps for Gamma radiation station located in Cairo city in Egypt. The starting date at one January 2007 from 12:00 am to 1:30 am and the ending date is at three January 2007 from 12:00 am to 1:30 am.

The final image resolution in Fig. 3a is sex columns and three rows. Gamma levels measurements with negative values means missed data or unregistered measurement.

Fig. 3b shows the final output result which is Digital Radiation image for Gamma radiation station located in Cairo city in Egypt at 2007. This digital radiation image expresses the all registered and unregistered Gamma radiation measurements at year 2007.

Using image restoration technique for prediction of Gamma radiation levels

Previous section led us to use an image processing technology to integrate the digital radiation image using image restoration technique. The main job from this technique is considering the black points in the digital radiation image as noisy points then covering those points. Our technique for restoration this points is accomplished by dividing the Gamma radiation image into two groups of micro images [6, 7]. All micro images in both groups have the same size. The first group contains all micro images in which these images do not include any missed point. The second group contains small sub-groups of micro images in which any image of them include at least one missed point. Each sub-group contains all micro images which are taken around every one missed point. Fig.4 shows how these processes are performed [8, 9]. The color suggested for the missed point is the color of point of the same location in the same micro image from the first group. The next step is getting the color for every missed point by the color, which has the highest membership function value. The initial membership function of each color is determined by determining the number of all matching points n between each micro image from the sub-group which is from second group to each complete micro image from first group. Then the initial membership function is calculated from the equation (1) [10].

$$\text{Initial Membership} = 10^{n} \quad \ldots\ldots\ldots (1)$$

Many of initial membership functions for each color are created by repeating the previous step for the reminder of micro images of the sub-group which is from second group. The final membership function for any color is the sum of all initial membership functions that occurred with this color as shown from equation (2)

$$\text{Final Membership} = \sum_{i=1}^{i=k} \text{Initial Membership}_i \quad (2)$$

K is the number of occurrences for one color.

The color for the missed point is the color which has the highest final member ship value [11, 12]. This algorithm is summarized in the following steps:-

a. Suppose we have a digital radiation image X where image X has some missed points.
b. Divide image X to a group of micro images Yi. Where i = 0, 1, 2, 3, Number of micro images.
c. The dimension of any Y_i is (3 col × 3 row).
d. All Yi images do not include any missed point.
e. For every missed point $N_{(J,K)}$ take a micro images Z_j surrounding it.
f. Dimension of Z is equal to dimension to Y. For all i , for all j , Compare each point in Z_j with each point in Y_i [8].
g. If missed point coordinates in Z_j is (u,v) then the color suggested for this point is the color of point coordinate (u,v) in Yi [9].
h. IF number of matching points for any Z_J and Y_I = n then color initial member ship function =10^n [10]. Accumulate all initial member ship values for each color to get final member ship value for this color [11].
i. The color of the missed point in Z_j is the color, which has the highest member ship function [12, 13].
j. Repeat those steps for all reminder-missed points.

Fig. 4 shows how to determine micro images for both of missed points and unmissed points.

When there are some 3×3 neighborhoods for desired observation are unavailable this prediction algorithm will start to process all the missed points which their 3×3 neighborhoods for desired observation are available. The output result from this step is decreasing the number of missed points. This means that some of 3×3 neighborhoods for desired observation that were unavailable become available. So all unavailable 3×3 neighborhoods for desired observation can become available by repeating the prediction algorithm several times until the number of unavailable 3×3 neighborhoods is zero.

3. Results and Discussion

This model is implemented for forecasting the Gamma radiation levels measurements in ambient air. Fig. 5a shows a complete digital radiation image; Fig. 5b shows this image after making about 9105 missed pixels which represent about 8.5% of the all pixels in the image via evenly distributed random function and Fig. 5c shows the image resulted after processing the image in Fig. 5b using the restoration technique in this model. The total number of accepted

points with error less than 10 % is 90 % from total missed point. Table 2 classifies the predicted missed pixels in Fig. 5c to three groups. This classification is performed according to prediction quality for each pixel. The first group represents the percentage of the predicted pixels in which their prediction quality is 100% or their error is 0%. The second group represents the percentage of the predicted pixels in which their prediction quality is 90% or their error is 10%. The third group represents the percentage of the predicted pixels in which their prediction quality is less than 90% or their error is more than 10%. The values of the prediction quality in table 2 are computed by getting the percentage error between each predicted value in Fig. 5c and its actual value from Fig. 5a.

This model has a powerful feature in which this model can accomplish its task by only one type of measurements while other prediction models need at least three types of measurements. This means that this model produces accepted results by only one type of measurements and this is verified with measurements of gamma levels in this work. In the other side famous prediction models cannot be operated with one type of measurements. They need at least three types of measurements therefore the output result from this model is the best because other famous models will not produce an accepted output with the same data that is used by our prediction model. This means also that this model does not contradict the theory of modeling by suggesting reaching same accuracy that is resulted from famous models with less information.

This feature makes this model less expensive than other models such as deterministic models and neural network models [14- 22]. The deterministic models are mathematical models, physical models and chemical models. Both of deterministic model and neural network models require a large amount of data from different types of measurements. These types of measurements may be not available for the user. In this case, the user must buy the unavailable data to operate his or her model. On the other hand, the user can use only one type of data to operate Long term predication model or Short term predication model.

This feature also saves a lot of money, effort and time because any other model requires money to buy the unavailable data and effort for insuring the quality for this data. These two steps will consume a lot of time. In the other hand this prediction model works with only one kind of data. This means that a lot of money, lot of effort and lot of time are saved by using this predication models.

The major advantage from this model is that this model carries a great useful feature for some continuous monitoring systems. This continuous monitoring systems watch a unique elements such as Gamma radiation levels (which is covered in this study), earth vibration levels, sea levels. These elements are unique because when using other models such as regression model or neural network model many kinds of data are required to initialize their operation and if these kinds of data are few or not exist as in case of continuous monitoring system for earth vibration levels then this prediction model becomes more suitable model for predicting the unique elements than regression model or neural network model. Table 3 summarizes the pervious comparison between this model and other famous models.

The operations of this model are like the operations of the Artificial Neural Network ANN. The micro images in the first group are considered as a training dataset but this model needs only one training cycle in case of all 3×3 neighborhoods for desired observation are available while ANN needs a lot of training cycles. The micro images in the first group which is described in "Using image restoration technique for prediction of Gamma radiation levels" section contains the necessary training dataset for neighborhoods for desired observation as a predictor and training dataset for non- neighborhoods for desired observation as other predictor.

Since both of this model and ANN model are constructed from training dataset this model has the same problem of ANN model. The performance of this model is affected by the amount of the training data. If the amount of the training dataset is low or the number of missing pixels is high the quality of the performance for this model will be reduced and if the amount of the training dataset is low or the number of missing pixels is low the quality of the performance for this model will be increased. This fact is verified by applying experimentation to the prediction of this model with different ratios of missing pixels. When the ratio of missing pixels is less than or equal 15% the performance of the prediction quality is varying linearly from 95% to 90%. When the ratio of missing pixels is greater than 15% and less than or equal 30% the performance of the prediction is varying linearly from 90% to 83%. When the ratio of missing pixels is greater than 30% and less than or equal 40% the performance of the prediction quality is varying linearly from 83% to 70%. When the ratio of missing pixels is greater than 40% the performance of the prediction quality is not accepted. In other wards the limitation of this model is done when the ratio of missing pixels is greater than 40%.

There is a special problem with this model or another limitation. This limitation is seldom

happened when the frequency distribution for the magnitude of one value in the training dataset is very high. In this case the output from the prediction model also has high frequency distribution for the same value. The good illustration of this limitation is a period between October and November on Fig. 6b. The output data of this period seems to be non-meaningful but the prediction performance is not affected from this limitation because this output reflects the nature behavior of the training dataset which is also the nature behavior of the actual output.

Important information can be obtained in just few minutes by watching the digital radiation image in Fig. 5a such as the value of maximum and minimum measurements in the year and date of them, the most repeated measurement value in the year and the number of times that the radiation station did not work with their date and time. As shown in Fig. 5c the following information can be estimated in few minutes about Gamma radiation measurements in Cairo city in 2007.

a. Maximum measurements value is recorded in the period from 7th March to 15th November from 8:00 am to 10:00 am and their values are AL_ST1 and AL_ST2 in 2007.
b. Minimum measurements value is UL_ST2 at various times in the year.
c. The most repeated value in year 2007 is UL_ST6.
d. The number of days that the station did not work in year 2007 is about seven days, two days in the first week of June, two days in the third week of July and three days in the fourth week of July.

Fig. 6a shows the digital radiation image for Cairo city at 2010. This digital radiation image shows that there are some days do not have any measurements. Fig. 6b shows the same digital radiation image after applying our model. Fig. 5d shows the keys for colors in each image with their fuzzy values.

Table 1: Fuzzy values for gamma radiation levels.

Fuzzy Number	Linguistic meaning	Fuzzy value
UL_ST1	Under allowed limit stage one	0<= UL_ST1 <= (0.044* Allowed limit)
UL_ST2	Under allowed limit stage two	UL_ST1< UL_ST2 <= (0.087* Allowed limit)
UL_ST3	Under allowed limit stage three	UL_ST2< UL_ST3 <= (0.13* Allowed limit)
UL_ST4	Under allowed limit stage four	UL_ST3< UL_ST4 <= (0.174* Allowed limit)
UL_ST5	Under allowed limit stage five	UL_ST4< UL_ST5 <= (0.217* Allowed limit)
UL_ST6	Under allowed limit stage six	UL_ST5< UL_ST6 <= (0.261* Allowed limit)
NL_ST1	Near from allowed limit stage one	UL_ST6< NL_ST1 <= (0.304* Allowed limit)
NL_ST2	Near from allowed limit stage two	NL_ST1< NL_ST2 <= (0.348* Allowed limit)
NL_ST3	Near from allowed limit stage three	NL_ST2< NL_ST3 <= (0.393* Allowed limit)
AL_ST1	At allowed limit stage one	NL_ST3< AL_ST1<= (0.435* Allowed limit)
AL_ST2	At allowed limit stage two	AL_ST1< AL_ST2<= (1.304* Allowed limit)
AL_ST3	At allowed limit stage three	AL_ST2< AL_ST3<= (2.174* Allowed limit)
AbL_ST1	Above allowed limit stage one	AL_ST3< AbL_ST1<= (3.04* Allowed limit)
AbL_ST2	Above allowed limit stage two	AbL_ST1< AbL_ST2<= (3.91* Allowed limit)
AbL_ST3	Above allowed limit stage three	AbL_ST2< AbL_ST3<= (4.34* Allowed limit)
OL_ST1	Over allowed limit stage one	AbL_ST3< OL_ST1<= (13.04* Allowed limit)
OL_ST2	Over allowed limit stage two	OL_ST1< OL_ST2<= (21.73* Allowed limit)
OL_ST3	Over allowed limit stage three	OL_ST2< OL_ST3<= (30.43* Allowed limit)
OL_ST4	Over allowed limit stage four	OL_ST3< OL_ST4<= (34.18* Allowed limit)
VOL	Very over allowed limit	VOL > OL_ST4
NO_DATA	No data recorded at this date	Ø

Table 2: Classifying the predicted pixels according to their predication quality

Groups from predicted pixels	% of The amount of each group to amount of all predicted pixels.
First Group: Predicted pixels that their prediction error is 0%	53%
Second Group : Predicted pixels that their prediction error is less than 10%	37%
Third Group: : Predicted pixels that their prediction error is more than 10%	10%
Average Error	9.5%
Total accepted output	90 %

Table 3: Forecasting using digital image processing and other famous models

	Forecasting gamma radiation levels using Digital image processing	Deterministic models and Neural network models
Input	One kind of measurements	At least three kinds of measurements
Performance	Maximum average error = 9.5% Prediction quality = 90%	With the same dataset that is used with our forecasting using digital image processing both of deterministic models and ANN models will not operate because this dataset is insufficient input to initialize their operations [14- 22].
Cost	Only one kind of measurements	Total price of all unavailable kinds of necessary measurements.
Time	Few minutes for operation time	Time spent for getting and insuring the quality of the unavailable kinds of necessary measurements + Few minutes for operation time = few days
Applications	Continues monitoring systems, market forecast, environmental decision-making, and Continues monitoring systems for unique elements	Continues monitoring systems, market forecast, environmental decision-making

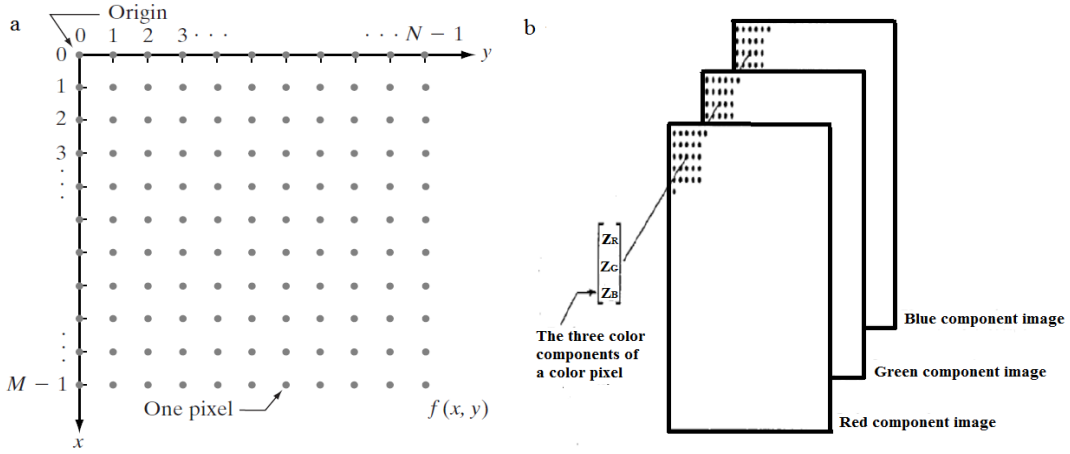


Figure 1 Presentation of digital image by (a) digital monochrome image, and (b) digital (RGB) color image

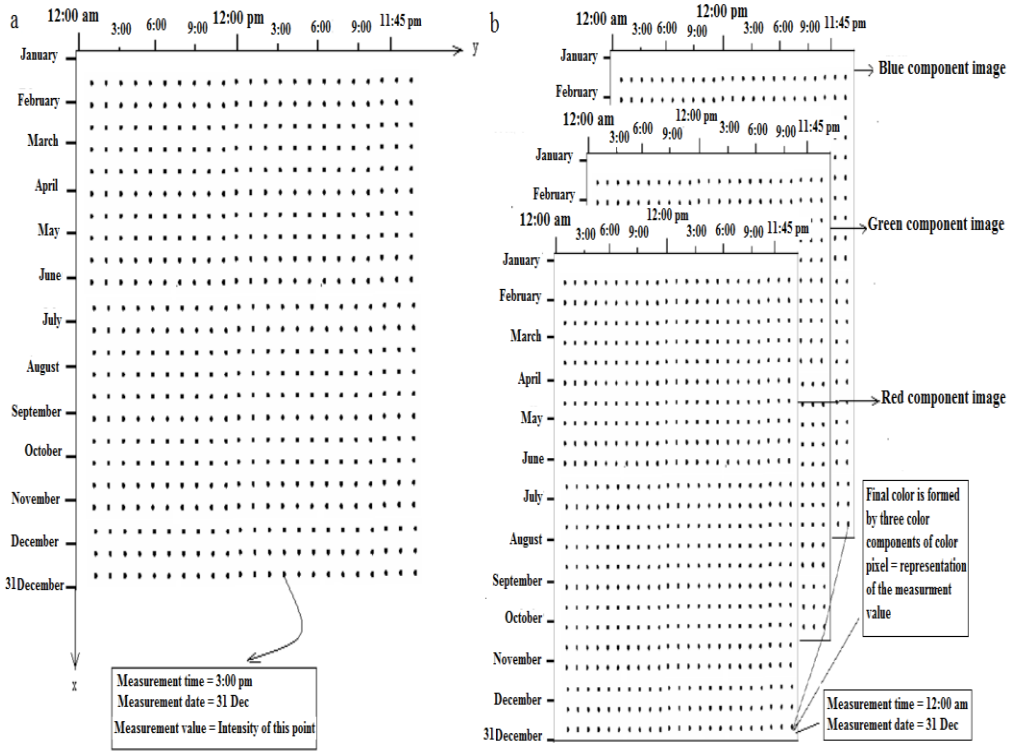


Figure 2 Presentation of digital measurements image by (a) digital monochrome measurements image and, (b) digital (RGB) measurements color image

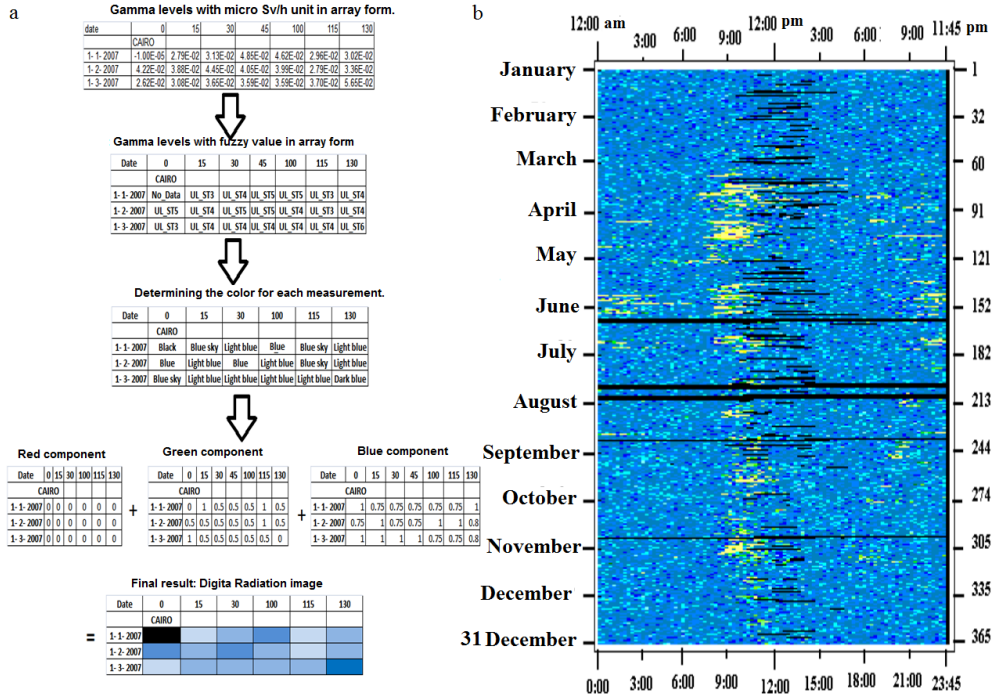


Figure 3 Steps for creating small digital radiation image as shown in (a) and Digital Radiation image for Cairo city at year 2007 as shown in (b)

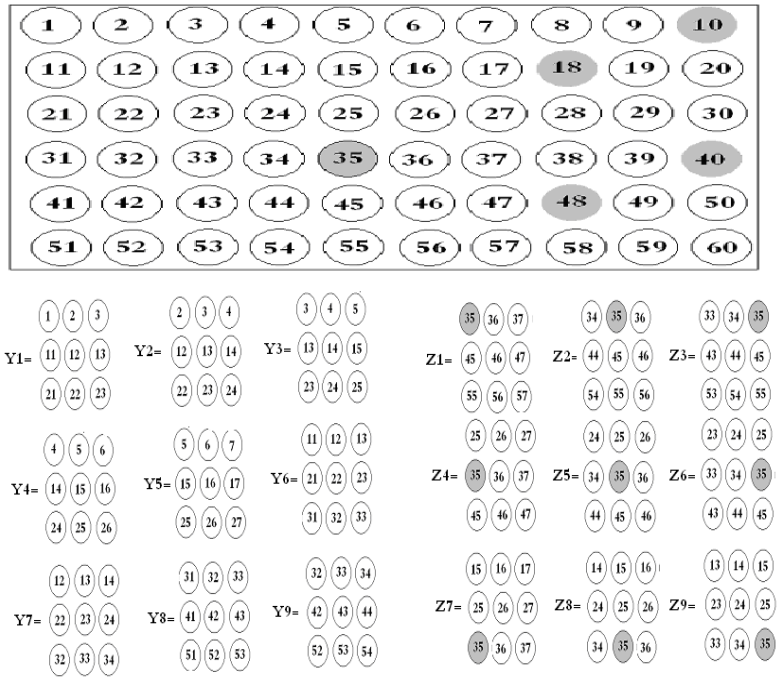


Figure 4 Digital radiation images X which is presented by a rectangle, gray circles are missed points, Y1 to Y9 is micro images and Z1to Z9 is micro images that are surrounding the point number 35.

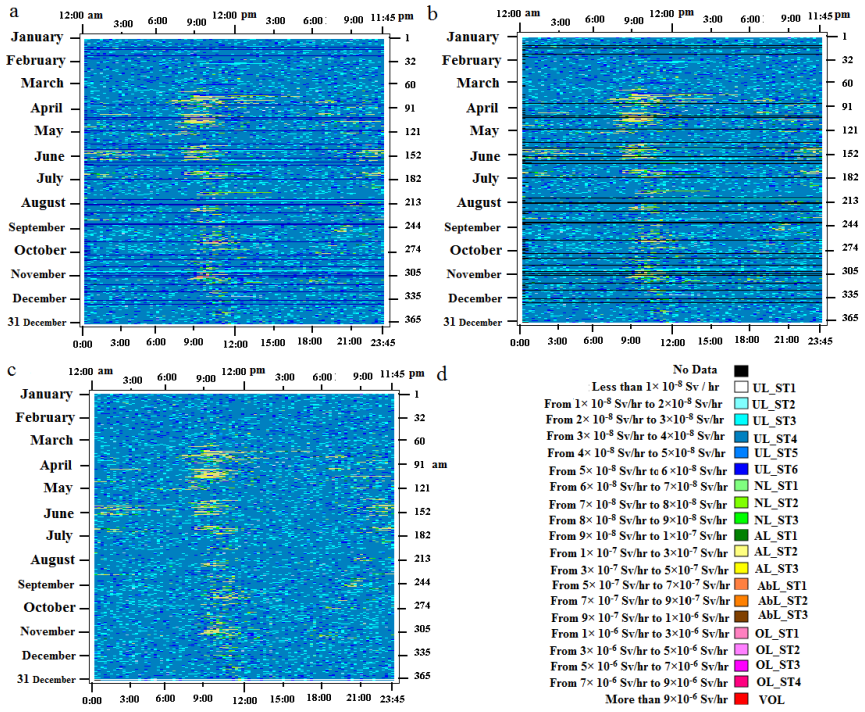


Figure 5 Digital radiation image for Cairo at 2007 without missed points (a), digital radiation image for Cairo at 2007 after making some missed day (b), Image resulted after processing the image in (b) using the restoration technique in this research (c) and color keys for each point in digital radiation image with their fuzzy values (d).

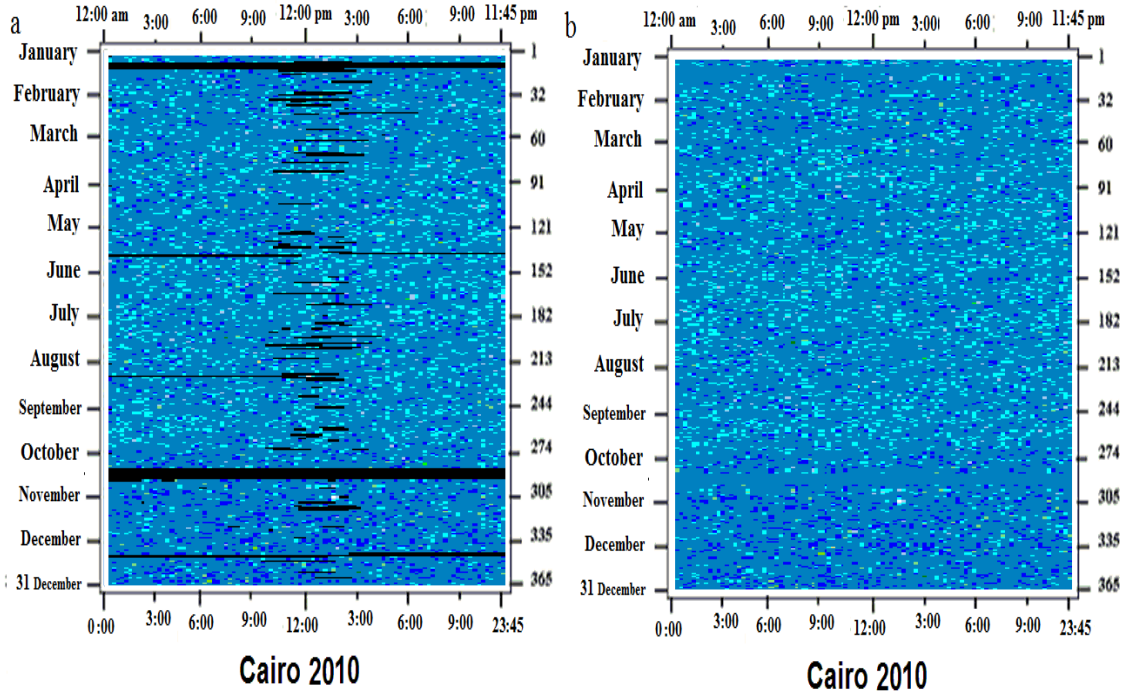


Figure 6 Actual digital radiation image for Cairo at 2010 (a) and digital radiation image for Cairo at 2010 after processing all missed points (b), Image resulted after processing all missed points in image (a) using the restoration technique in this research.

Conclusions

The results from this model are good enough to depend on them for forecasting, recognizing the artificial or strange phenomena, covering lost or missing data and making a temporally monitoring system. This model is better than other famous models when the prediction is only required and only one type of measurements is available because this model is designed to overcome the common limitation with famous prediction models. This common limitation is that the famous prediction models need at least three types of measurements to start their operations.

This model can be used in many applications such as continuous monitoring systems, market forecast and environmental decision-making and this model becomes more suitable model for predicting the unique elements than regression model or neural network model.

Digital Radiation image is more useful tool than other conventional data visualization tools for watching a huge number of measurements which is included in a database of any digital monitoring system. It is a good tool for making a quick analysis for radiation measurements because it has the ability to include thousands of measurements in a very clear form through only one picture. In the other side, the maximum number of measurements does not exceed 100 for other conventional data visualization methods. This picture helps the user to study and analyze the behavior of these measurements in few minutes instead of spending few hours in reviewing hundreds of charts for the same measurements. Therefore, a huge amount of effort and time is saved by using the digital radiation image.

The novel components in this work which are measurement image and prediction model which is derived from measurement image can be used for other purposes. The measurement image can be used as a visual database for measurements. The main advantage from this database is that the user can perform a quick query without using a computer. Our prediction model can be used to integrate or assist the preparation processes to operate the famous prediction models when there are some missing data in one or more than one type of measurements. The two novel components which are introduced in this work also presented a new field for modeling, data analysis and data mining.

Corresponding author
Mazhar M. Hefnawi
Department of National Network for Monitoring Radioactivity, Atomic Energy Authority of Egypt, Cairo, Egypt
mmaazz_2222@yahoo.co.uk

References

[1] Gonzalez R, Woods R. Digital Image Processing 2nd ed. Prentice Hall, 2002;1: 10–10.
[2] Eberline Environmental Monitoring System Software Operation Manual. USA, Eeberline, 1990, pp 1-100.
[3] William S, Buckley J. Fuzzy Expert system and Fuzzy reasoning. Willey Interscience, 2005; 2: 100-20.
[4] Law number Four -- Environmental law in Arab Republic of Egypt. Egypt government 1995.
[5] Weinschenk J, Combs W, Marks R. Proc IEEE Int'l Conference on Fuzzy Systems. Missouri, 2003; 5: 43–5.
[6] Julien M,Michael E, Guillermo S. Sparse Representation for Color Image Restoration. Institute for Mathematics and its Applications (IMA). 2006; 7:120-31.
[7] Elad M , Aharon M. Image denoising via learned dictionaries and sparse representation. IEEE Computer Vision and Pattern Recognition (CVPR), 2006; 6:200-7.
[8]Russo L, Gonzalo N, Arlindo L, Pedro M. Approximate String Matching with Compresed Indexes, Algorithms, 2009; 2: 1105-150
[9] Dmitriy Z, Daniela R, Jacob F, Samuel G. Armato. Predicting Radiological Panel Opinions Using a Panel of Machine Learning Classifiers. Algorithms, 2009; 2:1473-40.
[10] Marcel T, Tomàs P, Mercè T, Jordi P. Using the Optical Mouse Sensor as a Two-Euro Counterfeit Coin Detector. Sensors, 2009; 9: 7083-13.
[11] Zhen E, Michelle X. Visualization and Graphics Technical Committee. IEEE, 2008; 3: 55-8.
[12] Portilla J, Strela V, Wainwright M, Simoncelli E. Image denoising using scale mixtures of gaussians in the wavelet domain. IEEE Transactions on Image Processing, 2004;13(4): 496–12.
[13] Chiappini L, Cossu R, Lorenzo M. Computer Graphics International. IEEE,2004; 10(7): 505-7.
[14] Azzi M, Johnson G, Hyde R, Young R. Prediction of NO_2 and O_2 and O_3 concentrations for NOx plumes photochemically reacting in urban air. Mathematical and Computational Modelling, 1995; 21(9):39–50.
[15] Abdul-Wahab S, Bouhamra W, Ettouney H, Sowerby B, Crittenden H.Development of statistical model for prediction of ozone levels in Shuaiba Industrial Area in Kuwait. Environmental Science and Pollution Research (ESPR), 1996; 3(4):195–10.
[16] Hsu K, Time series analysis of the interdependence among air pollutants. Atmospheric Environment, 1992; 26(4): 491–20.
[17] Comrie A .Comparing neural networks and regression models for ozone forecasting. Journal of the Air and Waste Management (J. Air & Waste Manage.), 1997; 47: 653–8.
[18] Boznar M, Lesjak M, Mlakar A. A neural network-based method for short-term predictions of ambient SO2 concentrations in highly polluted industrial areas of complex terrain. Atmospheric Environment, 1993; 27: 221–9.
[19] Ruiz-Sua J, Mayora I, Torres J, Ruiz M. Short-term ozone forecasting by artificial neural networks. Advances in Engineering Software, 1995; 23:143–6.
[20] Elkamel A. Measurement and prediction of ozone levels around a heavily industrialized area: a neural network approach. Advances in Environmental Research, 2001; 5: 47 -12.
[21] Walle B, Turoff M. Fuzzy relations for the analysis of traders preferences in an information market game. European Journal of Operational Research (EUR J OPER RES), 2009; 195: 905–8.
[22] Ido E, Eyal E, Alvin E. A Choice Prediction Competition for Market Entry Games: An Introduction.Games, 2010;(1): 117-20.

2/1/2012

Microwave-Assisted Synthesis of Novel Pyrazole, Pyrimidine and Pyrazolo[1,5-a]pyrimidines Containing Aryl Sulfone Moiety

Ahmed A. El-Kateb[1], Naglaa M. Abd El-Rahman[1,2,*], Tamer S. Saleh[1,3], Ibrahim F. Zeid[4], Mohamed F. Mady[1]

[1]Green Chemistry Department, National Research Centre, Dokki, Cairo, 12622, Egypt
[2]Chemistry department, Faculty of Science, Jazan University, Jazan, Saudi Arabia
[3]Chemistry Department, Faculty of Science, King AbdulAziz University, Jeddah, Saudi Arabia
[4]Chemistry Department, Faculty of Science, El-Menoufia University, Shebin El-Koam, El-Menoufia, Egypt
naglaa_r@yahoo.com

Abstract: A facile, rapid and efficient microwave-assisted procedure for the synthesis of novel pyrazole, pyrimidine, and pyrazolo[1,5-a]pyrimidines linked to sulfonyl dibenzene moiety *via* the reaction of *E*-3-(*N*,*N*-dimethylamino)-1-(4-(phenylsulfonyl)phenyl)prop-2-en-1-one **3** with the appropriate amines described here. In general, microwave procedure as an eco-friendly energy source offers advantages such as a shorter reaction time, simple workup, reaction selectivity and improves an overall yield compared with conventional methods. The structures of all the compounds were confirmed by analytical and spectral data.
[Ahmed A. El-Kateb, Naglaa M. Abd El-Rahman, Tamer S. Saleh, Ibrahim F. Zeid, Mohamed F. Mady. **Microwave-Assisted Synthesis of Novel Pyrazole, Pyrimidine and Pyrazolo[1,5-a]pyrimidines Containing Aryl Sulfone Moiety.** Life Science Journal 2012; 9(1):711-718]. (ISSN: 1097-8135). http://www.lifesciencesite.com.102

Keywords: Green chemistry; Enaminone; Pyrazolo[1,5-a]pyrimidine; Aryl Sulfone; Microwave irradiation

1. Introduction:

Pyrazolopyrimidines are found to possess a wide important pharmacophore and adjective structure in medicinal chemistry due to their biological importance. Functionalized pyrazolopyrimidines are known to exhibit several pharmacological activities such as CNS depressant **[1]**, antihypertensive **[2],** adenosine receptors **[3],** tuberculostatic **[4],** antibacterial and antifungal **[5].** Some of the pyrazolopyrimidine derivatives are known to inhibit enzymes such as xanthine oxidase **[6,7].**

The pyrazolo[1,5-a]pyrimidines frame work, are attractive compounds for drug discovery since many of them have been shown to exhibit excellent biological activities **[8].** In addition, the pyrimidines and pyrazoles have received much attention over their years because of their interesting pharmacological properties **[9,10]**.

Aryl sulfone structure is featured in a variety of pharmacological and biological active compounds. These compounds include antifungal, antibacterial, or antitumor agents **[11,12],** and inhibitors for several enzymes **[13,14**]. In addition, the Sulfone moiety is usually incorporated as an active part in many analgesic anti-inflammatory molecules available as drugs in market such as celecoxib **[15,16],** valdecoxib [**17],** rofecoxib **[18],** parecoxib **[19],** etoricoxib **[20**], tenoxicam **[21],** piroxicam **[22],** meloxicam **[23],** lornoxicam **[24],** ampiroxicam **[25]** and nimesulide **[26]**.

The development of simple and eco-friendly synthetic procedures constitutes an important goal in organic synthesis. Microwave assisted organic synthesis (MAOS) is a fast growing area of research, due to the generally short reaction times, high purities and yields of the resulting products when compared to conventional methods **[27].** In addition, Solvent-free reactions under microwave irradiation are the subject of constant development because of its ease of set-up, mild conditions, and increased yields of products, cost efficiency and environment friendliness compared to their solution counterparts **[28].**

In continuation of our recent work aiming at the synthesis of a variety of heterocyclic systems using green chemistry tools **[27,29-31**], we focus in this article on a practical, rapid, and efficient microwave (MW) promoted synthesis of novel pyrazole, pyrimidine and pyrazolo[1,5-a]pyrimidines containing the aryl sulfone moiety compared with conventional methods.

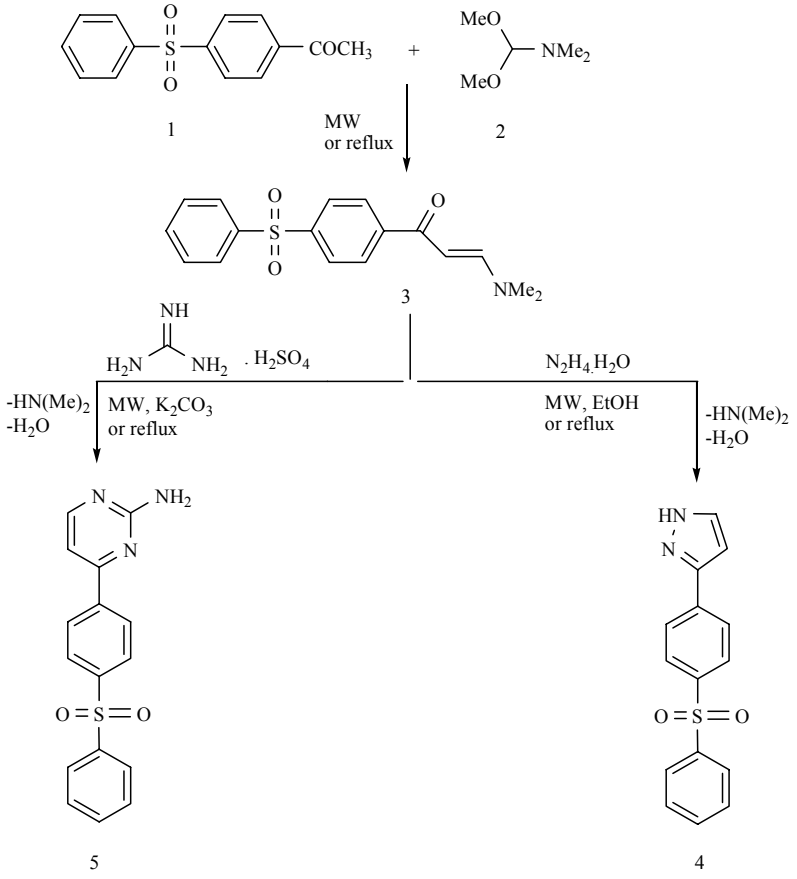
2. Results and Discussion

Firstly, a simple, efficient and solvent-free reaction for condensation of 1-acetyl-4-(phenylsulfonyl)benzene **1** with dimethylformamide-dimethylacetal (DMFDMA) **2** under microwave irradiation for 20 min. providing the corresponding *E*-3-(dimethylamino)-1-(4-(phenylsulfonyl)phenyl) prop-2-en-1-one **3** in 90 % yield compared with conventional heating reaction in dry toluene for 12hr in 70% yield (Scheme 1). The structure of the enaminone **3** was established on the basis of their elemental analysis and spectral data. The ^{1}H NMR spectrum of **3** displayed a singlet signals at δ 2.91

and 3.14 due to *N*,*N*-dimethyl protons, two doublets at δ 5.78 and 7.74 (*J*=12.3 Hz) due to olefinic protons, in addition to an aromatic multiplets in the region δ 7.63-8.06, the value of the coupling constant (*J* = 12.3 Hz) for the ethylenic protons indicates that the enaminone **3** exists exclusively in the *E*-configuration **[32].**

Enaminones are valuable intermediates in synthetic organic chemistry **[33,34]**. Thus, the enaminone **3** underwent cyclocondensation on treatment with hydrazine hydrate under microwave irradiation in presence of ethanol or refluxing in ethanol to afford, in each case, 3-(4-(phenylsulfonyl)phenyl)-1H-pyrazole **4** (Scheme 1). In case of conventional method the product was obtained in good yield up to 71% within 5h. While under microwave irradiation the obtained yield was excellent up to 89% in 30 min. The structure of 3-(4-(phenylsulfonyl)phenyl)-1H-pyrazole **4** was established on the basis of its elemental analysis and spectral data. For example, its IR spectrum showed NH absorption band at 3059 cm^{-1}. the ^1H NMR spectrum of the same compound revealed two doublet signals at δ 6.83 and 7.60 with *J* values = 1.5 Hz due to pyrazole protons and D_2O-exchangeable signal at δ 13.11 due to NH proton, in addition to an aromatic multiplet at δ 7.59-8.05.

Additionally, treatment of enaminone **3** with guanidine sulfate in presence of potassium carbonate as an efficient catalyst under microwave irradiation, afforded 2-amino-4-(4-(phenylsulfonyl)phenyl) pyrimidine **5** in 95% yield within 1 h. Similarly, repeating the same reaction under the conventional heating conditions by refluxing in ethanol in presence of potassium carbonate, the yield into 85% and increase the time up to 7 h (scheme 1).

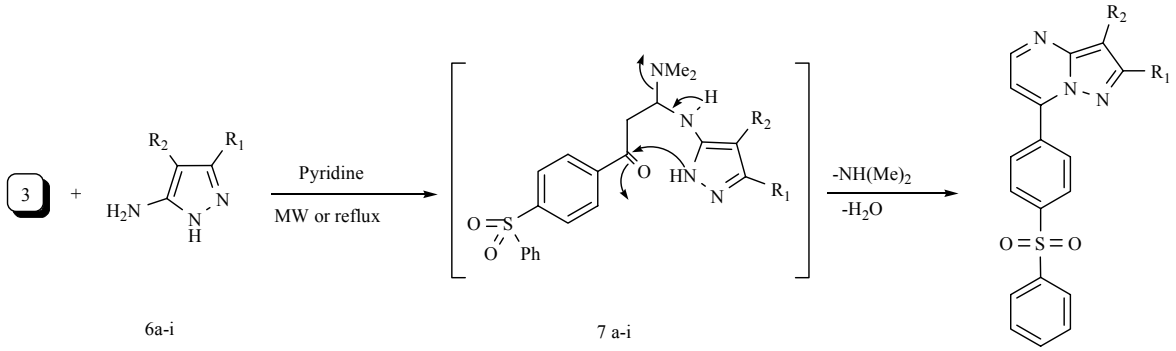


Scheme 1. Synthesis of 3-(4-(phenylsulfonyl)phenyl)-1H-pyrazole **4** and 2-amino-4-(4 (phenylsulfonyl)phenyl)pyrimidine **5** under microwave irradiation or conventional conditions.

On the other hand, the behavior of enaminone towards some aminopyrazole derivatives as potential precursors for interesting biologically active pyrazolo[1,5-a]pyrimidine derivatives was investigated **[35]**. Thus, when enaminone **3** was treated with aminopyrazoles **6a-i** under microwave irradiation in presence of a catalytic amount of pyridine or conventional heating under reflux in

pyridine, afforded, in each case, pyrazolo[1,5-a]pyrimidine **8a-i** in high yield as shown in table 1 (Scheme 2).

A plausible mechanism for the formation of compounds 8a-i is therefore assumed to take place *via* an initial Michael addition of the exocyclic amino group in the aminopyrazoles **6a-i** to the α,β-unsaturated moiety in the enaminone **3** to give the acyclic non-isolable intermediates **7a-i** followed by cyclization and aromatization of the latter intermediates, under the reaction condition *via* the loss of a water and dimethyamine molecules to afford the pyrazolo[1,5-a]pyrimidines **8a-i** (Scheme 2). The products **8a-i** were obtained in good yields up to 70% in case of conventional method. While under microwave irradiation the yields obtained were excellent up to 93% as illustrated in table 1. The structures of compounds **8a-i** were established on the basis of their elemental analyses and spectral data. For example the IR spectrum of compound **8f** shows two bands at 3423, 2928 cm^{-1} due to amino group. The ^1H NMR spectrum of the same compound exhibited singlet signal at δ 2.36 (CH_3), also, one singlet signal (D_2O-exchangeable) assigned to NH_2 protons at δ= 7.12, and two doublet signals at δ= 7.24, 8.62 (J = 4.5 Hz) due to pyrimidine protons (CH-6 and CH-5 respectively), in addition, aromatic protons as a multiplet at δ 7.23-8.25 ppm. The mass spectrum of compound **8f** reveals a molecular ion peak at m/z 468 (*cf*. experimental part).



6-8	R_1	R_2
a	C_6H_5	H
b	C_6H_4Cl-*p*	H
c	$C_6H_4OCH_3$-*p*	H
d	Me	Ph
e	NH_2	-N=N-C_6H_5
f	NH_2	-N=N-$C_6H_4CH_3$-*p*
g	NH_2	-N=N-$C_6H_4OCH_3$-*p*
h	NH_2	-N=N-C_6H_4F-*p*
i	NH_2	-N=N-C_6H_4Br-*p*

Scheme 2. Synthesis of pyrazolo[1,5-a]pyrimidine derivatives **8a-i** under microwave irradiation and conventional conditions.

Table 1. Synthesis of pyrazolo[1,5-a]pyrimidine derivatives **8a-i** under microwave irradiation and conventional conditions

Product	**R_1**	**R_2**	**Microwave**		**Conventional**	
			Time (min)	Yield (%)	Time (h)	Yield (%)
8a	C_6H_5	H	30	89	5	70
8b	C_6H_4Cl-*p*	H	15	92	5	80
8c	$C_6H_4OCH_3$-*p*	H	20	90	5	75
8d	Me	C_6H_6	30	90	5	72
8e	NH_2	-N=N-C_6H_5	45	91	5	77
8f	NH_2	-N=N-$C_6H_4CH_3$-*p*	60	89	5	70
8g	NH_2	-N=N-$C_6H_4OCH_3$-*p*	45	90	5	75
8h	NH_2	-N=N-C_6H_4F-*p*	30	93	5	83
8i	NH_2	-N=N-C_6H_4Br-*p*	45	92	5	80

Table 1 indicated that there was a remarkable microwave effect on the reaction of enaminone **3** with some aminopyrazole derivatives, the target products **8a-i** were afforded in excellent yields greater than 92% within dramatically shortened time (15-60 min). Evidently, the Microwave effect might be the significant factor to the highly efficient synthesis of pyrazolo[1,5-a]pyrimidine derivatives.

3. Experimental Section

The chemicals used in this work were obtained from Fluka and Merck and were used without purification. Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. Microwave experiments were carried out using a CEM Discover Labmate Microwave apparatus (300 W with Chem. Driver software). Reactions were monitored by thin layer chromatography using Fluka GF254 silica gel plates with a fluorescent indicator, and detection by means of UV light at 254 and 360 nm. IR spectra were recorded on KBr disks on a Perkin Elmer 2000 FTIR spectrometer. The NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer at 300.13 and 75.47MHz. ^{1}H NMR and ^{13}C NMR spectra were recorded in deuterated chloroform ($CDCl_3$) or dimethylsulphoxide (DMSO-d6) using TMS as the internal standard. ^{13}C chemical shifts were related to that of the solvent. Mass spectra were recorded on a Shimadzu GCMS-QP 1000 EX mass spectrometer at 70 eV. Elemental analyses were carried out at the Micro analytical Center of Cairo University, Giza, Egypt.

1-acetyl-4-(phenylsulfonyl)benzene**1[36],** 5-amino-3-methyl-1H-pyrazole **6a[37**], 5-amino-3-methyl-4-phenyl-1H-pyrazole **6d[38],**and 4-(arylhydrazono)-3,5-diamino-1H-pyrazole **6e–i[37**], were prepared according to literature procedures.

Synthesis of *E*-3-(dimethylamino)-1-(4-(phenylsulfonyl)phenyl)prop-2-en-1-one 3

Method A: under microwave irradiation

A mixture of 1-acetyl-4-(phenylsulfonyl)benzene **1** (26.0 g, 100 mmole) and DMFDMA **2** (13.4 g, 100 mmol) was irradiated by focused microwaves (at 180 °C, 300 W) for 20 min. After the irradiation, the reaction was cooled until the temperature had fallen below 50 °C. The solid product, so formed, was collected by filtration and crystallized from ethanol to give compound **3** in 90% yields.

Method B: under conventional conditions

To a solution of 1-acetyl-4-(phenylsulfonyl)benzene **1** (26.0 g, 100 mmole) in dry toluene (150 ml) was added dimethylformamide-dimethylacetal (DMF-DMA) **2** (13.4 g, 100 mmol) and the mixture was refluxed for 12 h. The solvent was distilled off at reduced pressure and the residual reddish brown viscous liquid was taken in petroleum ether (bp. 60-80 °C) (20 ml) then the resulting reddish yellow crystal was collected by filtration, washed thoroughly with ether, dried and finally crystallized from ethanol to afford *E*-3-(dimethylamino)-1-(4-(phenylsulfonyl)phenyl) prop-2-en-1-one **3** in 70% yield.

E-3-(dimethylamino)-1-(4-(phenylsulfonyl)phenyl) prop-2-en-1-one **(3).**

m.p.; 125-127 °C; IR (KBr) νmax/cm^{-1}:1643 (C=O), 1300, 1155 (SO_2); ^{1}H NMR (DMSO-d6): δ 2.91 (s, 3H, NCH_3), 3.14 (s, 3H, NCH_3), 5.78 (d, 1H, J = 12.3 Hz, -CO-CH=), 7.62-8.06 (m, 9H, Ar-H), 7.74 (d, 1H, J = 12.3 Hz, =CH-N); ^{13}C NMR (DMSO-d6): δ 44.63, 91.06, 120.55, 127.51, 128.23, 129.34, 133.80, 140.83, 142.37, 144.68, 155.01, 184.01; MS (*m/z*): 315 (M^+). (Found: C, 64.74; H, 5.43; N, 4.44; S, 10.17 $C_{17}H_{17}NO_3S$ requires C, 64.72; H, 5.45; N, 4.46; S, 10.16).

Synthesis of 3-(4-(phenylsulfonyl)phenyl)-1H-pyrazole 4

Method A: under microwave irradiation

To an equimolar amounts of *E*-3-(dimethylamino)-1-(4-(phenylsulfonyl)phenyl)prop-2-en-1-one **3**, hydrazine hydrate (2 mmol, 100%), ethanol (10 ml). The mixture was then subjected to microwave irradiation (at 80 °C, 300 W) for 30 min. until consumption of reactants as determined by TLC. After cooling to room temperature, the precipitated product was recrystallized from ethanol, to give the corresponding 3-(4-(phenylsulfonyl)phenyl)-1H-pyrazole **4** in 89% yields.

Method B: under conventional Conditions

To a mixture of *E*-3-(dimethylamino)-1-(4-(phenylsulfonyl)phenyl)prop-2-en-1-one **3** (0.315 g, 1mmol) and hydrazine hydrate (2 mmol, 100%), in ethanol (25 ml), the reaction mixture was refluxed for 5h. The solid product was filtered off, washed with ethanol and recrystallized from ethanol to afford the pure product **4** in 71% yields. The physical and spectral data of the synthesized compound **4** is listed below.

3-(4-(phenylsulfonyl)phenyl)-1H-pyrazole **(4).**

m.p.; 238-240°C; IR (KBr) υmax /cm^{-1}: 3059 (NH), 1312, 1154 (SO_2); ^{1}H NMR (DMSO-d6): 6.83 (d, 1H, J = 1.5 Hz pyrazole -4-CH), 7.59-8.05 (m, 9H, ArH's), 7.60 (d, 1H, J = 1.5 Hz pyrazole -5-CH), 13.11 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d6): δ 101.25, 125.31, 127.08, 127.30, 129.60, 129.64, 133.51, 138.44, 139.06, 141.43,

144.62; MS (*m/z*): 284 (M^+). (Found: C, 63.36; H, 4.25; N, 9.85; S, 11.28 $C_{15}H_{12}N_2O_2S$ requires C, 63.30; H, 4.27; N, 9.87; S, 11.29).

Synthesis of 2-amino-4-(4-(phenylsulfonyl)phenyl) Pyrimidine 5

Method A: under microwave irradiation

To an equimolar amount of enaminone **3** (0.315 g, 1mmol) and guanidine sulfate (1 mmol), 145 mg of K_2CO_3. The mixture was subjected to microwave irradiation (at 180 °C, 300 W) for 1h until completion of the reaction (monitored by TLC). The mixture was dissolved in hot CH_2Cl_2. the catalyst was removed by filtration and washed with hot CH_2Cl_2 and the solvent was evaporated under reduced pressure. The residue was purified by crystallization from ethanol to afford the pure product **5** in 95% yields.

Method B: under conventional Conditions

A solution of guanidine sulfate (1 mmol) in absolute ethanol (15 ml) was added to a stirred solution of the enaminone **3** (1 mmol) in boiling absolute ethanol (10 ml), stirring was continued for 20 min. This mixture was added to the appropriate K_2CO_3 (145 mg) in absolute ethanol (10 ml) and the reaction mixture was refluxed for 7 h. The solution was allowed to cool at room temperature and the precipitate was removed by filtration followed by concentration of the filtrate under reduced pressure. The solid products that formed was collected by filtration, washed with water and dried. Recrystallization from ethanol afforded **5** in 85% yields.

2-amino-4-(4-(phenylsulfonyl)phenyl)pyrimidine **(5).**
m.p.; 252-254°C; IR (KBr) υmax /cm^{-1}: 3453, 3158 (NH_2), 1563 (C=N), 1298, 1151 (SO_2); ^{1}H NMR (DMSO-d6): δ 6.82 (s, 2H, NH_2, D_2O exchangable), 7.15 (d, 1H, *J* = 5.1 Hz pyrimidine-6-CH), 7.61-8.35 (m, 9H, ArH's), 8.36 (d, 1H, *J* = 5.1 Hz pyrimidine-5-CH); ^{13}C NMR (DMSO-d6): δ 106.43, 127.35, 127.78, 127.87, 129.76, 133.80, 140.76, 141.77, 142.25, 159.58, 161.65, 163.75; MS (*m/z*): 311 (M^+). (Found: C, 61.72; H, 4.21; N, 13.50; S, 10.30 $C_{16}H_{13}N_3O_2S$ requires C, 61.70; H, 4.20; N, 13.51; S, 10.31).

General procedure for the reaction of enaminone 3 with pyrazoles 6a-i

Method A: under microwave irradiation

An equimolar amount of *E*-3-(dimethylamino)-1-(4-(phenylsulfonyl)phenyl)prop-2-en-1-one **3** (0.315 g, 1mmol) and pyrazoles **6a-i** (1 mmol), in presence of catalytic amount of pyridine (0.25 ml), then the reaction mixture was subjected to microwave irradiation (at 180 °C, 300 W) for the appropriate time as listed in table 1 until consumption of reactants as determined by TLC. After cooling to room temperature, the residual solid was recrystallized from ethanol/DMF (1:1) to give pyrazolo[1,5-a]pyrimidine derivatives **8a-i**.

Method B: under conventional Conditions

To a mixture of enaminone **3** (0.315 g, 1mmol) and pyrazoles **6a-i** (1 mmol) in pyridine (25 ml). The reaction mixture was refluxed for 5h. The solid product was filtered off, washed with cold ethanol and recrystallized from ethanol/DMF to afford the pure products **8a-i**. The physical and spectral data of the synthesized compounds are listed below.

2-phenyl-7-(4-(phenylsulfonyl)phenyl)pyrazolo[1,5-a]pyrimidine **(8a).**
m.p.; 239-241°C; IR (KBr) υmax /cm^{-1}: 1599 (C=N), 1307, 1155 (SO_2); ^{1}H NMR (DMSO-d6): δ 7.31 (d, 1H, *J* = 4.2 Hz pyrimidine-6-CH), 7.38 (s, 1H, pyrazole-3-CH), 7.46-8.46 (m, 14H, ArH's), 8.63 (d, 1H, *J* = 4.5 Hz pyrimidine-5-CH); ^{13}C NMR (DMSO-d6): δ 93.68, 108.64, 126.24, 127.39, 128.77, 129.08, 129.87, 130.76, 132.14, 134.04, 135.30, 140.45, 142.84, 143.29, 149.68, 150.46, 154.78; MS (*m/z*): 411 (M^+). Found: C, 70.05; H, 4.16; N, 10.21; S, 7.79 $C_{24}H_{17}N_3O_2S$ requires C, 70.02; H, 4.18; N, 10.21; S, 7.80).

2-(4-chlorophenyl)-7-(4-(phenylsulfonyl)phenyl) pyrazolo[1,5-a]pyrimidine **(8b).**
m.p.; 214-216°C; IR (KBr) υmax /cm–1: 1596 (C=N), 1317, 1158 (SO_2); ^{1}H NMR (DMSO-d6): δ 7.31 (d, 1H, *J* = 4.5 Hz pyrimidine-6-CH), 7.40 (s, 1H, pyrazole-3-CH), 7.51-8.44 (m, 13H, ArH's), 8.64 (d, 1H, *J* = 4.5 Hz pyrimidine-5-CH); ^{13}C NMR (DMSO-d6): δ 93.92, 108.86, 127.42, 127.61, 127.92, 128.83, 129.88, 130.77, 131.06, 133.69, 134.05, 135.24, 143.36, 149.85, 153.56; MS (*m/z*): 445 (M^+). Found: C, 64.64; H, 3.62; N, 9.42; S, 7.19 $C_{24}H_{16}ClN_3O_2S$ requires C, 64.66; H, 3.60; N, 9.40; S, 7.21).

7-(4-(phenylsulfonyl)phenyl)-2-(4-methoxyphenyl) pyrazolo[1,5-a]pyrimidine **(8c).**
m.p.; 245-247°C ; IR (KBr) υmax /cm^{-1}: 1598 (C=N), 1306, 1156 (SO_2); ^{1}H NMR (DMSO-d6): δ 3.77 (s, 1H, OCH_3), 6.99 (d, 1H, *J* = 8.7 Hz pyrimidine-6-CH), 7.23 (s, 1H, pyrazole-3-CH), 7.61-8.56 (m, 13H, ArH's), 8.41 (d, 1H, *J* = 4.5 Hz pyrimidine-5-CH); ^{13}C NMR (DMSO-d6): δ 55.45, 94.15, 110.87, 114.35, 127.84, 127.34, 128.12, 128.54, 129.17, 130.22, 132.15, 133.79, 134.50, 136.15, 138.24, 144.54, 146.04, 149.12, 155.04; MS (*m/z*): 441 (M^+). (Found: C, 68.01; H, 4.34; N, 9.52; S, 7.26

$C_{25}H_{19}N_3O_3S$ requires C, 68.03; H, 4.30; N, 9.50; S, 7.30).

2-methyl-3-phenyl-7-(4-(phenylsulfonyl)phenyl) pyrazolo[1,5-a]pyrimidine **(8d).**
m.p.; 260-262°C; IR (KBr) υmax /cm^{-1}: 1599 (C=N), 1310, 1153 (SO_2); ^{1}H NMR (DMSO-d6): δ 2.52 (s, 1H, CH_3), 7.24 (d, 1H, *J* = 4.2 Hz pyrimidine-6-CH), 7.31-8.31 (m, 14H, ArH's), 8.61 (d, 1H, *J* = 4.2 Hz pyrimidine-5-CH); ^{13}C NMR (DMSO-d6): δ 14.09, 108.47, 120.49, 127.39, 127.57, 128.32, 128.58, 129.86, 130.72, 134.02, 135.44, 140.60, 142.80, 143.28, 146.54, 149.59, 149.60, 151.32,168.48; MS (*m/z*): 425 (M^+). (Found: C, 65.31; H, 4.33; N, 12.03; S, 9.18 $C_{25}H_{19}N_3O_2S$ requires C, 65.29; H, 4.35; N, 12.00; S, 9.21).

2-Amino-3-phenylazo-7-(4-(phenylsulfonyl)phenyl) pyrazolo[1,5-a]pyrimidine **(8e).**
m.p.; 223-225°C; IR (KBr) υmax /cm^{-1}: 3425, 2922 (NH_2), 1549 (C=N), 1310, 1157 (SO_2); ^{1}H NMR (DMSO-d6): 7.15 (s, 2H, NH_2, D_2O exchangeable), 7.27 (d, 1H, *J* = 4.5 Hz pyrimidine-6-CH), 7.45-8.34 (m, 14H, ArH's), 8.69 (d, 1H, *J* = 4.5 Hz pyrimidine-5-CH); ^{13}C NMR (DMSO-d6): δ 109.31, 115.59, 120.45, 121.85, 126.34, 127.78, 129.57, 130.28, 133.15, 136.74, 139.24, 141.46, 142.96, 144.64, 148.68, 150.51, 151.54, 152.90 ; MS (*m/z*): 454 (M^+). (Found: C, 63.42; H, 3.99; N, 18.49; S, 7.05 $C_{24}H_{18}N_6O_2S$ requires C, 63.44; H, 4.01; N, 18.50; S, 7.01).

2-Amino-3-(4-methylphenylazo)-7-(4-(phenyl sulfonyl)phenyl)Pyrazolo[1,5-a]pyrimidine **(8f).**
m.p.; 260-262°C; IR (KBr) υmax /cm^{-1}: 3423, 2928 (NH_2), 1547 (C=N), 1305, 1154 (SO_2); ^{1}H NMR (DMSO-d6): δ 2.36 (s, 3H, CH_3), 7.12 (s, 2H, NH_2, D_2O exchangeable), 7.24 (d, 1H, *J* = 4.5 Hz pyrimidine-6-CH), 7.23-8.25 (m, 13H, ArH's), 8.62 (d, 1H, *J* = 4.5 Hz pyrimidine-5-CH); ^{13}C NMR (DMSO-d6): δ 20.81, 109.38, 114.36, 120.60, 121.05, 127.28, 127.55, 129.55, 130.87, 134.02, 135.20, 138.33, 140.55, 142.83, 143.254, 147.34, 150.54, 150.90, 151.82 ; MS (m/z): 468 (M^+). (Found: C, 64.09; H, 4.30; N, 17.94; S, 6.84 $C_{25}H_{20}N_6O_2S$ requires C, 64.04; H, 4.32; N, 17.96; S, 6.85).

2-Amino-3-(4-methoxyphenylazo)-7-(4-(phenyl sulfonyl)phenyl)Pyrazolo[1,5-a]pyrimidine **(8g).**
m.p.; 230-232°C; IR (KBr) υmax /cm^{-1}: 3403, 3261 (NH_2), 1552 (C=N), 1302, 1153 (SO_2); ^{1}H NMR (DMSO-d6): δ 2.82 (s, 3H, OCH_3), 7.13 (s, 2H, NH_2, D_2O exchangeable), 7.04 (d, 1H, *J* = 4.5 Hz pyrimidine-6-CH), 7.21-8.58 (m, 13H, ArH's), 8.04 (d, 1H, *J* = 4.5 Hz pyrimidine-5-CH); ^{13}C NMR (DMSO-d6): δ 55.38, 109.07, 114.08, 120.57, 123.82, 127.27, 129.87,134.02, 135.25, 136.04, 140.54, 142.80, 143.15, 147.09, 149.52, 150.35, 151.81, 153.03, 159.85; MS (*m/z*): 484 (M^+). (Found: C, 61.97; H, 4.16; N, 17.34; S, 6.62 $C_{25}H_{20}N_6O_2S$ requires C, 61.93; H, 4.17; N, 17.30; S, 6.63).

2-Amino-3-(4-fluorophenylazo)-7-(4-(phenyl sulfonyl)phenyl)pyrazolo[1,5-a]pyrimidine **(8h).**
m.p.; 250-252°C; IR (KBr) υmax /cm^{-1}: 3421, 3262 (NH_2), 1547 (C=N), 1315, 1149 (SO_2); 1H NMR (DMSO-d6): δ 7.20 (s, 2H, NH_2, D_2O exchangeable), 7.64 (d, 1H, *J* = 6.0 Hz pyrimidine-6-CH), 7.18-8.58 (m, 13H, ArH's), 8.14 (d, 1H, *J* = 6.0 Hz pyrimidine-5-CH); ^{13}C NMR (DMSO-d6): δ 109.53, 114.53, 115.63, 122.97, 127.25, 129.54, 130.86, 135.12, 140.55, 142.84, 143.26, 147.44, 149.61, 150.58, 133.99, 151.79, 160.37, 163.63; MS (*m/z*): 472 (M^+). (Found: C, 61.01; H, 3.63; N, 17.79; S, 6.79 $C_{24}H_{17}FN_6O_2S$ requires C, 61.03; H, 3.65; N, 17.74; S, 6.80).

2-Amino-3-(4-bromophenylazo)-7-(4-(phenyl sulfonyl)phenyl)pyrazolo[1,5-a]pyrimidine **(8i).**
m.p.; 241-243°C; IR (KBr) υmax /cm^{-1}: 3418, 3287 (NH_2), 1545 (C=N), 1299, 1148 (SO_2); ^{1}H NMR (DMSO-d6): 7.20 (s, 2H, NH_2, D_2O exchangeable), 7.75 (d, 1H, *J* = 6.9 Hz pyrimidine-6-CH), 7.26-8.63 (m, 13H, ArH's), 8.96 (d, 1H, *J* = 6.9 Hz pyrimidine-5-CH); ^{13}C NMR (DMSO-d6): δ 109.89, 114.97, 120.58, 122.96, 127.94, 128.38, 129.78, 133.84, 134.03, 136.05, 140.90, 143.40, 146.24, 150.79, 131.96, 151.925, 152.87, 150.59; MS (*m/z*): 532 (M^+). (Found: C, 54.04; H, 3.21; N, 15.76; S, 6.01 $C_{24}H_{17}BrN_6O_2S$ requires C, 54.05; H, 3.20; N, 15.77; S, 6.00).4.

4. Conclusions

In conclusion, we have synthesized novel pyrazole, pyrimidine and fused pyrazolo[1,5-a]pyrimidines incorporating the aryl sulfone moiety under influence of microwave irradiation and conventional conditions. The clean protocol minimizes the organic solvent and energy demands, as well as, the reaction time could be reduced to a few minutes using MW irradiation and also this technique was found to be very useful to improve an overall yield and reaction selectivity.

Corresponding author:
Naglaa M. Abd El-Rahman
[1]Green Chemistry Department, National Research Centre, Dokki, Cairo, 12622, Egypt
[2]Chemistry department, Faculty of Science, Jazan University, Jazan, Saudi Arabia
naglaa_r@yahoo.com

References

1. (a) Julino, M.; Stevens, F. G. M. Antitumour polycyclic acridines. Part 5.1 Synthesis of 7H-pyrido[4,3,2-kl]acridines with exploitable functionality in the pyridine ring. *J. Chem. Soc. Perkin Trans. 1* **1998**, 1677-1684; (b) Abdou, I. M.; Saleh, A. M.; Zohdi, H. F. Synthesis and antitumor activity of 5-trifluoromethyl-2,4-dihydropyrazol-3-one nucleosides. *Molecules* **2004,** *9*, 109-116.
2. El-Feky, S. A.; Abd el-Samii, Z. K. Synthesis and antihypertensive activity of novel 1-(4-benzyl-1-phthalazinyl)-pyrazolo[3,4-d]. pyrimidines. *Pharmazie* **1996**, *51*, 540-543.
3. (a) Davies, L. P.; Brown, D. J.; Chow, S. C.; Johnston, G. A. Pyrazolo[3,4-d] pyrimidines, a new class of adenosine antagonists. *Neurosci. Lett.* **1983**, *41*, 189-193; (b) Davies, L. P.; Chow, S. C.; Skerritt, J. H.; Brown, D. J.; Johnston, G. A., Pyrazolo[3,4-d]pyrimidines as adenosine antagonists. *Life Sci.* **1984**, **34**, 2117-2128.
4. Ghorab, M. M.; Ismail, Z. H.; Abdel-Gawad, S. M.; Abdel Aziem, A. Antimicrobial activity of amino acid, imidazole, and sulfonamide derivatives of pyrazolo[3,4-d]pyrimidine. *Heteroatom Chem.* **2004**, *15*, 57-62.
5. Holla, B. S.; Mahalinga, M.; Karthikeyan, M. S.; Akberali, P. M.; Shetty, N. S. Synthesis of some novel pyrazolo[3,4-d]pyrimidine derivatives as potential antimicrobial agents. *Bioorg. Med. Chem.* **2006,***14*, 2040-2047.
6. Gupta, S.; Rodrigues, L. M.; Esteves, A. P.; Oliveira-Campos, A. M.; Nascimento, M. S.; Nazareth, N.; Cidade, H.; Neves, M. P.; Fernandes, E.; Pinto, M.; Cerqueira, N. M.; Bras, N. Synthesis of N-aryl-5-amino-4-cyanopyrazole derivatives as potent xanthine oxidase inhibitors. *Eur. J. Med. Chem.* **2008,** *43*, 771-780.
7. Tamta, H.; Thilagavathi, R.; Chakraborti, A. K.; Mukhopahyay, A. K. 6-(N-benzoylamino)purine as a novel and potent inhibitor of xanthine oxidase: inhibition mechanism and molecular modeling studies. *J. Enzyme Inhib. Med. Chem.* **2005,** *20*, 317-324.
8. Bouabdallah, I.; M'Barek, L. A.; Zyad, A.; Ramdani, A.; Zidane, I.; Melhaoui, A. Anticancer effect of three pyrazole derivatives. *Nat. Prod. Res.* **2006,** *20*, 1024-1030.
9. Coimbra, C.; Boris-Moller, F.; Drake, M.; Wieloch, T. Diminished neuronal damage in the rat brain by late treatment with the antipyretic drug dipyrone or cooling following cerebral ischemia. *Acta Neuropathol.* **1996,** *92*, 447-453.
10. (a) Shaaban, M. R.; Saleh, T. S.; Mayhoub, A. S.; Mansour, A.; Farag, A. M. Synthesis and analgesic/anti-inflammatory evaluation of fused heterocyclic ring systems incorporating phenylsulfonyl moiety. *Bioorg. Med. Chem.* **2008,** *16*, 6344-6352; (b) Shaaban, M. R.; Saleh, T. S.; Mayhoub, A. S.; Farag, A. M. Single step synthesis of new fused pyrimidine derivatives and their evaluation as potent Aurora-A kinase inhibitors. *Eur. J. Med. Chem.* **2011,** *46*, 3690-3695.
11. Otzen, T.; Wempe, E. G.; Kunz, B.; Bartels, R.; Lehwark-Yvetot, G.; Hansel, W.; Schaper, K. J.; Seydel, J. K. Folate-synthesizing enzyme system as target for development of inhibitors and inhibitor combinations against Candida albicans-synthesis and biological activity of new 2,4-diaminopyrimidines and 4'-substituted-4-aminodiphenyl sulfones. *J. Med. Chem.* **2004,** *47*, 240-253.
12. Sun, Z. Y.; Botros, E.; Su, A. D.; Kim, Y.; Wang, E.; Baturay, N. Z.; Kwon, C. H. Sulfoxide-containing aromatic nitrogen mustards as hypoxia-directed bioreductivecytotoxins. *J. Med. Chem.* **2000,** *43*, 4160-4168.
13. Neamati, N.; Mazumder, A.; Zhao, H.; Sunder, S.; Burke, T. R., Jr.; Schultz, R. J.; Pommier, Y. Diarylsulfones, a novel class of human immunodeficiency virus type 1 integrase inhibitors. *Antimicrob. Agents Chemother.* **1997,** *41*, 385-93.
14. Doherty, G. A.; Kamenecka, T.; McCauley, E.; Van Riper, G.; Mumford, R. A.; Tong, S.; Hagmann, W. K. N-aryl 2,6-dimethoxybiphenylalanine analogues as VLA-4 antagonists. *Bioorg. Med. Chem. Lett.* **2002,** *12*, 729-731.
15. Clemett, D.; Goa, K. L. Celecoxib: a review of its use in osteoarthritis, rheumatoid arthritis and acute pain. *Drugs* **2000,** *59*, 957-980.
16. Bing, R. J.; Lomnicka, M. Why do cyclo-oxygenase-2 inhibitors cause cardiovascular events? *J. Am. Coll. Cardiol.* **2002,** *39*, 521-522.
17. Sikes, D. H.; Agrawal, N. M.; Zhao, W. W.; Kent, J. D.; Recker, D. P.; Verburg, K. M. Incidence of gastroduodenal ulcers associated with valdecoxib compared with that of ibuprofen and diclofenac in patients with osteoarthritis. *Eur. J. Gastroenterol. Hepatol.* **2002,** *14*, 1101-1111.
18. Langman, M. J.; Jensen, D. M.; Watson, D. J.; Harper, S. E.; Zhao, P. L.; Quan, H.; Bolognese, J. A.; Simon, T. J. Adverse upper gastrointestinal effects of rofecoxib compared with NSAIDs. *JAMA* **1999,** *282*, 1929-1933.
19. Cochrane, D. J.; Jarvis, B.; Keating, G. M. Etoricoxib. *Drugs* **2002,** *62*, 2637-2651; discussion 2652-2653.

20. Dallob, A.; Hawkey, C. J.; Greenberg, H.; Wight, N.; De Schepper, P.; Waldman, S.; Wong, P.; DeTora, L.; Gertz, B.; Agrawal, N.; Wagner, J.; Gottesdiener, K. Characterization of etoricoxib, a novel, selective COX-2 inhibitor. *J. Clin. Pharmacol.* **2003,** *43*, 573-585.
21. Todd, P. A.; Clissold, S. P. Tenoxicam. An update of its pharmacology and therapeutic efficacy in rheumatic diseases. *Drugs* **1991,** *41*, 625-646.
22. Lee, C. R.; Balfour, J. A. Piroxicam-beta-cyclodextrin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in rheumatic diseases and pain states. *Drugs* **1994,** *48*, 907-929.
23. Fleischmann, R.; Iqbal, I.; Slobodin, G. Meloxicam. *Expert Opin. Pharmacother.* **2002,** *3*, 1501-1512.
24. Zhao, H.; Ye, T. H.; Gong, Z. Y.; Xue, Y.; Xue, Z. G.; Huang, W. Q. Application of lornoxicam to patient-controlled analgesia in patients undergoing abdominal surgeries. *Chin. Med. Sci. J.* **2005,** *20*, 59-62.
25. Kurumaji, Y., Ampiroxicam-induced photosensitivity. *Contact Dermatitis* **1996,** *34*, 298-299.
26. Bernareggi, A. Clinical pharmacokinetics of nimesulide. *Clin. Pharmacokinet.* **1998,** *35*, 247-274.
27. Abd El-Rahman, N. M.; El-Kateb, A. A.; Mady, M. F. Simplified approach to the uncatalyzedknoevenagel condensation and michael addition reactions in water using microwave irradiation. *Synth. Commun.* **2007**, *37*, 3961-3970.
28. Campo, C. J.; Tato, V. P. M.; Seijas, A. J. Microwave-Promoted, One-Pot, Solvent-Free Synthesis of 4-Arylcoumarins from 2-Hydroxybenzophenones. *Eur. J. Org. Chem.* **2010**, 4130–4135.
29. Abd El-Rahman, N. M.; Saleh, T. S.; Mady, M. F. Ultrasound assisted synthesis of some new 1,3,4-thiadiazole and bi(1,3,4-thiadiazole) derivatives incorporating pyrazolone moiety. *Ultrason. Sonochem.* **2009,** *16*, 70-74.
30. Saleh, T. S.; Abd El-Rahman, N. M. Ultrasound promoted synthesis of substituted pyrazoles and isoxazoles containing sulphone moiety. *Ultrason. Sonochem.* **2009,** *16*, 237-242.
31. Saleh, T. S.; Eldebss, T. M.; Albishri, H. M., Ultrasound assisted one-pot, three-components synthesis of pyrimido[1,2-a]benzimidazoles and pyrazolo[3,4-b]pyridines: A new access via phenylsulfonesynthon. *Ultrason. Sonochem.* **2012,** *19*, 49-55.
32. Fischer, P.; Schweizer, E.; Langner, J.; Schmidt, U. ^{13}C, ^{1}H long-range coupling constants in configuration assignment of some trisubstituted alkenes. *Magn. Reson. Chem.* **1994**, *32*, 567–568.
33. Al-Mousawi, S. M.; El-Apasery, M. A.; Elnagdi, M. H. Enaminones in heterocyclic synthesis: a novel route to tetrahydropyrimidines, dihydropyridines, triacylbenzenes and naphthofurans under microwave irradiation. *Molecules* **2010,** *15*, 58-67.
34. Stanovnik, B.; Svete, J. Synthesis of heterocycles from alkyl 3-(dimethylamino)propenoates and related enaminones. *Chem. Rev.* **2004,** *104*, 2433-2480.
35. Novinson, T.; Hanson, R.; Dimmitt, M. K.; Simon, L. N.; Robins, R. K.; O'Brien, D. E. 3-Substituted- 5,7-dimethylpyrazolo(1,5-a) pyrimidines, 3',5'-cyclic-AMP phosphodiesterase inhibitors. 1. *J. Med. Chem.* **1974,** *17*, 645-648.
36. Zou, J.; Li, F.; Tao, F. G. Microwave-assisted synthesis of diaryl or aryl-alkyl sulfones without catalyst. *Chin. Chem. Lett.* **2009**, *20,* 17-20.
37. Hori, I.; Igarashi, M. The Paal-Knorr Condensation of Acetonylacetone with 5-Aminopyrazoles. *Bull. Chem. Soc. Jpn.* **1971**, *44*, 2856-2858.
38. Elfahham, H. A.; Sadek, K. U.; Elgemeie, G. E. H.; Elnagdi, M. H. Novel synthesis of pyrazolo[5,1-c]-1,2,4-triazoles, imidazo[1,2-b]pyrazoles, and [1,2,4]-triazolo[4,3-a]benzimidazoles. Reaction of nitrite imines with amino- and oxo-substituted diazoles. *J. Chem. Soc. Perkin Trans. 1* **1982**, 2663-2666.

2/1/2012

Lipid Profile in Tuberculous Patients: A Preliminary Report

Mohamed M. Metwally[1] and Hesham Abdel Raheem[2]

[1]Chest Diseases Department and [2]Clinical Pathology Department, Assiut University Hospital, Assiut, Egypt

Abstract: Background: Serum cholesterol in pulmonary TB patients is lower than healthy controls, but this is not clear if it is a risk factor or a consequence of the disease itself. **Study Objectives:** To detect any differences in lipid profile between Egyptian TB patients and controls and to test whether this difference changes after treatment or not aiming to prove if the difference is a risk factor for tuberculosis or a consequence of the disease itself. **Patients and Methods:** For new TB patients, we did a fasting serum lipid profile of serum cholesterol (SC), triglycerides (TG), low density lipoproteins (LDL) and high density lipoproteins (HDL). All patients received the same four antiTB drugs for 8 weeks then a follow up lipid profile was done. Samples for lipid profile from healthy controls were also taken. **Results:** We recruited 30 new TB patients, 14 women and 16 men with a mean age (±SD) of 33.4 ±13.25 years. There were 16 pulmonary TB and 14 other forms as pleural (5), TB lymphadenitis (5), TB peritonitis (4) plus 15 controls. Regarding the whole studied group, only serum triglyceride was significantly lower before treatment than control group ($P<0.01$) while both serum cholesterol and HDL showed a significant increase after treatment than before it ($P<0.01$ for both). Regarding pulmonary tuberculosis patients, both serum cholesterol and triglycerides were significantly lower on diagnosis than healthy controls ($P<0.05$ for both) and only serum cholesterol increased significantly after treatment than before it ($P<0.01$). **Conclusions and recommendations:** Hypocholesterolemia in Egyptian patients with pulmonary TB is present at the time of diagnosis. However, it proved to be a consequence of the disease rather than a risk factor as serum cholesterol significantly increased in both pulmonary TB and in the whole group after treatment.

[Mohamed M. Metwally and Hesham Abdel Raheem. **Lipid Profile in Tuberculous Patients: A Preliminary Report.** Life Science Journal 2012; 9(1):719-722]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 103

Keywords: Serum; cholesterol; pulmonary; patient; healthy; treatment

1. Introduction

Pulmonary tuberculosis is one of the oldest diseases, afflicting the human race since ancient times. A milestone in therapy was the discovery of drugs with antimycobacterial activity, beginning in 1944 with Streptomycin. With the currently available drugs, about 90% of pulmonary tuberculosis cases can be cured[1]. However, the success of the treatment depends on the use of appropriate antituberculous drugs, the adherence of the patient to treatment, the sensitivity of mycobacteria to drugs, and the control of associated diseases[2]. An additional factor that could negatively affect the efficacy of the antitubercular treatment is a deficiency in cellular immunity, which in turn can be influenced by nutritional status[3].

Guzman *et al.*[4] and ***Pérez-Guzmán***[5], found that most patients with pulmonary tuberculosis had low total serum cholesterol levels, and that values of about 90 mg/dL were strongly associated with mortality in those patients with miliary disease. Although very scantily investigated, these associations have been already mentioned by others. For example, ***Taylor*** and ***Bamgboye***[6] ***and Padmapriyadarsini***[7], found low cholesterol levels in Nigerian tuberculous patients.

Cholesterol constitutes up to 30% of the total lipid content in the cell membrane, and participates in the fluidity of this structure[8].***Thomas***[9] consequently, cholesterol is involved in the activity of membrane-bound enzymes and membrane functions such as phagocytosis and cell growth. In this context, ***Drabowsky*** *et al.* [10] demonstrated that cholesterol content in the cell membrane of human lymphocytes is important for their cytotoxic function. Moreover, in a work published by ***Gatfield*** and ***Pieters***[11] a clear derangement of the ability of the macrophage to phagocytose mycobacteria was observed when they were depleted of cholesterol. All of these findings are important in patients with pulmonary tuberculosis, inasmuch as activated lymphocyte subsets, such as CD4, CD8, and T cells, recruit macrophages and release molecules, such as interferon and tumor necrosis factor that render them more efficient in killing mycobacteria. In addition, cytotoxic lymphocytes (either CD4 or CD8) undergo phagocytosis of macrophages that have already internalized mycobacteria[12].

However, taking into account the above-mentioned clinical observations and in vitro studies, it was evident for us that in the case of pulmonary tuberculosis, a low-cholesterol level might have a detrimental effect. Unfortunately, this kind of studies cannot unveil whether hypocholesterolemia in tuberculosis is a consequence of the disease or a contributory factor. In a recent randomized clinical

trial in pulmonary tuberculous patients (new cases) hospitalized during the intensive phase of the four-drug antitubercular treatment, ***Guzman*** *et al.,* 2005 demonstrated that a cholesterol-rich diet notably accelerated the bacteriological sterilization of sputum[13].

Aim of the work:

To detect any differences in lipid profile between Egyptian TB patients and controls and to test whether this difference changes after treatment or not aiming to prove if the difference is a risk factor for tuberculosis or a consequence of the disease itself.

2. Patients and Methods

This study was conducted in Chest diseases department, Assiut University from May 2006 to November 2006. We recruited patients with newly diagnosed Tuberculosis of any type (never treated, newly diagnosed patients with bacteriologic confirmation of the disease) and excluded those with a history of diabetes mellitus.

For every patient who accepted entry to the study, we did a 12- hours fasting blood sample from a peripheral vein for serum cholesterol, triglycerides, low density lipoproteins, high density lipoproteins and fasting blood sugar (Hitachi 912 autoanalyzer, Roche Boehringer, Mannheim, Germany).

Diabetic patients were excluded from the study. All patients received the same four drugs antituberculous treatment during the intensive phase of 8 weeks on outpatient bases. They received a short-course regimen with four antituberculous drugs, which were administered daily at standard doses (Isoniazid, Rifampicin, Pyrazinamide, and Ethambutol, according to body weight), under the directly observed therapy strategy, as proposed by the World Health Organization.[14] Drugs were administered in the morning, approximately 30 to 60 min before breakfast. After 8 weeks, another follow up blood sample was taken under the same conditions. Blood samples from healthy controls were also taken.

Statistical Analysis:

Differences between the control group and the experimental groups were assessed by using the independent sample t-test while differences before and after treatment in the same patient was done by using paired sample t-test. Statistical analysis was performed using a statistical software package (SPSS version 10 for windows). Statistical significance was set at $p<0.05$ (two-tailed test). Data in the text and figures are expressed as frequencies or as the mean ± SD.

3. Results

We recruited 30 new TB patients, 14 women and 16 men with a mean age ± SD of 33.4 ± 13.25 years. There were 16 pulmonary TB and 14 other forms as pleural (5), TB lymphadenitis (5), TB peritonitis (4) (**Figure 1**) plus 15 controls.

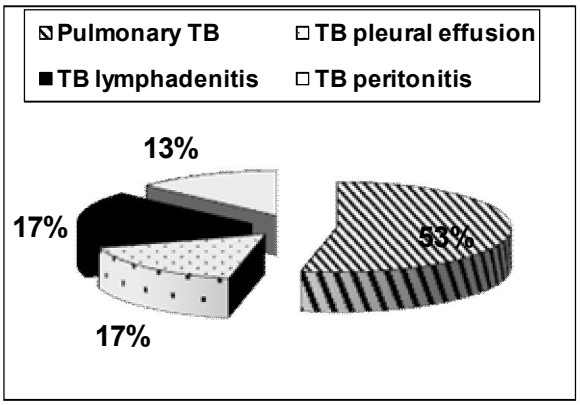


Figure 1. Percentage distribution of different recruited tuberculous cases

The mean ± SD in mg/dl of different serum lipid parameters for control group as well as the test group (before and after anti TB treatment) are shown in ***table 1***.

Table 1. Serum lipid profile in control and the whole test group before and after anti tuberculous treatment

Group Item	Control (I)	Whole Test Group		Significance	
		Before II	After III	I vs II	II vs III
Cholesterol…	144±34.6	135.8±30.8	155.7±28.4	NS	**S**
Triglycerides…	113.6±39.8	80.1 ± 23.5	76 ± 20	**S**	NS
HDL…	39.2±3.9	42.6 ± 15.1	58.3 ± 16.6	NS	**S**
LDL…	82±30.7	77.16±24.9	82 ± 23.6	NS	NS

S= Significant NS= Non-significant

Regarding the whole studied group, only serum triglyceride was significantly lower before treatment than control group ($P<0.01$) while both serum cholesterol and HDL showed a significant increase

after treatment than before it (P<0.01 for both). On evaluating the subgroup of pulmonary tuberculosis patients alone, we found that both serum cholesterol and triglycerides were significantly lower on diagnosis than healthy controls (P<0.05 for both) and only serum cholesterol increased significantly after treatment than before it (P<0.01) as shown in ***table 2***.

Table 2. Serum lipid profile in control and the pulmonary TB group before and after anti tuberculous treatment

Group Item	Control I	Pulmonary TB Group		Significance	
		Before II	After III	I vs II	II vs III
Cholesterol	144±34.6	120±26	148.6±30	**S**	**S**
Triglycerides	113.6±39.8	78 ±20.8	80.8±23	**S**	NS
HDL	39.2±3.9	37.7±16	53±16.5	NS	NS
LDL	82±30.7	66.9±18.8	79±28.6	NS	NS

S= Significant NS= Non-significant

4. Discussion

It is estimated that about one third of the world's population is infected with Mycobacterium tuberculosis[15], yet only a small proportion of these individuals (~10% of those not receiving preventive therapy) will develop active tuberculosis. Therefore, it is evident that some specific conditions predispose these individuals to develop the disease. In this sense, several studies have confirmed that patients with pulmonary tuberculosis often have low cholesterol levels[4,6]. This comes in agreement with the results of our study that showed a significantly lower level of serum cholesterol and triglycerides in pulmonary TB patients than controls. However, on evaluating the whole group with different TB presentations, there were only lower serum triglycerides than controls. To our knowledge, this is the first report that discusses lipid profile in tuberculosis in general so, there were no available results of previous researches to compare with. On trying to address the question of hypocholesterolemia if being a risk factor as suggested by ***Guzman*** *et al.*[16] or a consequence of the disease itself, we did a follow up serum cholesterol after stabilization of the disease. We hypothesized that if hypocholesterolemia is corrected after disease stability, then the patient would have normal serum cholesterol before being diseased and consequently, hypocholesterolemia would be considered as consequence of the disease rather that a risk factor as risk factors should be present before the disease and continue to be present after treating the disease. In this context, we found that serum cholesterol increased significantly after treatment in pulmonary TB as well as whole TB groups to a comparable level to that of healthy controls with no statistic significance (data not shown in tables). In this same line of thoughts, we may find that hypocholesterolemia is a common feature shared by several conditions traditionally considered as risk factors for developing tuberculosis, such as malnutrition[17], aging, gastrectomy,[18] intravenous drug use, leukemias and other cancers and chronic renal failure[19]. On the contrary, patients with diabetes mellitus, a disease considered as an important risk factor for tuberculosis, there is usually associated hypercholesterolemia.

In conclusion, according to our results, we found that patients with pulmonary tuberculosis have hypocholesterolemia that proved to be a consequence of the disease itself rather than a risk factor. This hypocholesterolemia proved to be correctable to normal levels with regular intake of anti TB treatment and normal diet. Further research is needed with larger number of patients and longer follow up periods in order to provide additional support to this assertion.

Corresponding author
Hesham Abdel Raheem[2]
Clinical Pathology Department, Assiut University Hospital, Assiut, Egypt

5. References

1- Rieder HL. (2002): Interventions for tuberculosis control and elimination. Paris, France: International Union Against Tuberculosis and Lung Diseases; 15–93.
2- Friedman LN, Selwyn PA. (1994): Pulmonary tuberculosis: primary, reactivation, HIV related, and non-HIV related. In: Friedman LN, ed. Tuberculosis: current concepts and treatment. Boca Raton, FL: CRC Press; 93–112.
3- Martin TR. (1987): The relationship between malnutrition and lung infections. Clin Chest Med.; 8:359–372.
4- Perez-Guzman C, Vargas MH, Torres-Cruz A, *et al.* (2002): La hipocolesterolemia: un hallazgo frecuente que se asocia a la tuberculosis. Rev Inst Nac Enf Respir.; 15:7–11 (Caught from Perez-Guzman and Mario H. Vargas: Cholesterol-Rich Diet Accelerates Bacteriologic Sterilization in Pulmonary Tuberculosis. CHEST 2005; 127:643–651).

5- Pérez-Guzmán C, Vargas MH, Salas-Mártir C, Trejo-Santacruz T, Gallegos-Discua C, Flores-López F (2008): [Lipid profile in household contacts of patients with pulmonarytuberculosis]. Rev Med Inst Mex Seguro Soc. May-Jun;46(3):247-52.
6- Taylor GO, Bamgboye AE. (1979): Serum cholesterol and diseases in Nigerians. Am J Clin Nutr.; 32:2540–2545.
7- Padmapriyadarsini C, Ramesh Kumar S, Terrin N, Narendran G, Menon PA, Ramachandran G, Subramanyan S, Venkatesan P, Wanke C, Swaminathan S. (2011): Dyslipidemia among HIV-infected Patients with tuberculosis taking once-daily nonnucleoside reverse-transcriptase inhibitor-based antiretroviral therapy in India. Clin Infect Dis., Feb 15;52(4):540-6. Epub 2011 Jan 20.
8- Devlin TM. (1992): Biological membranes: structure and membrane transport. In: Devlin TM, ed. Textbook of biochemistry with clinical correlations. New York, NY: John Wiley & Sons; 226–236.
9- Thomas ST, VanderVen BC, Sherman DR, Russell DG, Sampson NS (2011): Pathway profiling in *Mycobacterium tuberculosis*: elucidation of cholesterol-derived catabolite and enzymes that catalyze its metabolism. J Biol Chem., 2011 Dec 23;286(51):43668-78. Epub Nov 1.
10- .Drabowsky MP, Peel WE, Thomson AER. (1980): Plasma membrane cholesterol regulates human lymphocyte cytotoxic function. Eur J Immunol.; 10:821–827.
11- Gatfield J, Pieters J. (2000): Essential role for cholesterol in entry of mycobacteria into macrophages. Science; 288:1647–1650.
12- Schluger NW, Rom WN. (1998): The host immune response to tuberculosis. Am J Respir Crit Care Med.; 157:679–69.
13- Perez-Guzman and Mario H. Vargas (2005): Cholesterol-Rich Diet Accelerates Bacteriologic Sterilization in Pulmonary Tuberculosis. CHEST; 127:643–651.
14- World Health Organization (1997): Treatment of tuberculosis: guidelines for national programmes; WHO/TB/97.220. Geneve, Switzerland: World Health Organization.
15- Sudre P, Dam GT, Kochi A. (1992): Tuberculosis: a global overview of the situation today. Bull World Health Org.; 70: 149-159.
16- Carlos Pérez-Guzmán, Mario H Vargas, César Salas-Mártir, et.al,: Lipid Profile In Household Contacts Oftuberculous Patients. Journal of Medical Hypothesis. Article in press.
17- Bonnefoy M, Abidi H, Jauffret M, Garcia I, Surrace JP, Drai J. (2002): Hypocholesterolemia in hospitalized elderly: relations with inflammatory and nutritional status. Rev Med Interne; 23: 991-998.
18- Yagi T, Yamagishi F, Sasaki Y, *et al.* (2004): A study on cases developed pulmonary tuberculosis after receiving gastrectomy. Kekkaku; 79: 355-359.
19- Iseki K, Yamazato M, Tozawa M, Takishita S. (2002): Hypocholesterolemia is a significant predictor of death in a cohort of chronic hemodialysis patients. Kidney Int.; 61: 1887-1893.

2/25/2012

Dynamic Characteristics of a Hollow Femur

B.W. Huang[1,2], C.H. Chang[3], F.-S. Wang[4], A.D. Lin[1], Y.C. Tsai[1], M.Y. Huang[1], J.-G. Tseng[2]*

[1]Graduate Institute of Mechatronics Engineering, Cheng Shiu University, Taiwan, ROC
[2]Medical Mechatornics Engineering Program, Cheng Shiu University, Taiwan, ROC
[3]Neurosurgery department, Mackay Memorial Hospital Taitung Branch, Taiwan, ROC
[4]Kaohsiung Chang Gung Memorial Hospital, Center for Laboratory Animals, Taiwan, ROC
*E-mail: james.tseng@csu.edu.tw. (NSC 99-2632-E-230-001-MY3)

Abstract: The femur is the largest, longest, and strongest bone of the human skeleton and has the ability to support up to 30 times the weight of an adult. This paper examines the fundamental dynamic characteristic of both the solid and hollow femur, experimentally and numerically. Reverse engineering is applied to obtain the outer geometry of synthesis femur. Noble's Canal Flare Index (CFI) is applied to excavated marrow material of the femur canal and to build a more realistic human hollow femoral model. Both solid and hollow femur are imported to the finite element package, ANSYS, to perform the analysis. The mechanical properties of real human femur are substituted into the FE model to achieve the final goal of finding dynamic response of human solid and hollow femur. The comparison of natural frequencies, stress, strain, and displacement of both solid and hollow femur are matched to the physical rules.

[B. W. Huang, C.H. Chang, F.-S. Wang, A.D. Lin, Y.C. Tsai, M.Y. Huang, J.-G. Tseng. **Dynamic Characteristics of a Hollow Femur.** Life Science Journal 2012; 9(1):723-726]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 104

Keywords: Human Hollow Femur, Geometry, Femoral Canal, Reverse Engineering

1. Introduction

The femur, or thigh bone, extending from the hip to the knee, is the most proximal bone of the leg in vertebrates capable of walking or jumping. The femur is the largest, longest, and strongest bone of the human skeletons. Its rounded, smooth head fits into a socket in the pelvis called the acetabulum to form the hip joint. The head of the femur is joined to the bone shaft by a narrow piece of bone known as the neck of the femur. The neck of the femur is a point of structural weakness and a common fracture site. The lower end of the femur hinges with the tibia (shinbone) to form the knee joint.

Many studies have focused on geometry, biomechanical properties, fractural type, etc. of a human femur [1~6]. A typical femur structure includes compact bone, sponge bone, medullary cavity, yellow marrow, periosteum, articular cartilage, etc, as shown in Fig. 1 [7]. The average adult male femur is 48 centimeters in length and 2.84 cm in diameter at the mid-shaft, and has the ability to support up to 30 times the weight of an adult. Yan et al. measure the parameters of femur from 52 normal adults' X-ray photograph. The total length of femur medullary cavity of male and female are 33.51±0.63 cm and 33.13±0.64 cm, respectively. The length of the narrow point are 5.61±0.49 cm (male) and 5.17±0.46 cm (female). The narrowest parts are located on the proximal end of medullary cavity and the distance of them are 3.55±0.15 cm (male) and 3.52±0.27 cm (female). The sagittal diameter is longer than the coronal diameter of the narrow point of the medullary cavity [8].

Ho Bo Tho models bone and joints with individualized geometric and mechanical properties derived from medical images [9]. Brekelmans et al. [10], proposed the finite element method (FEM) to analyze the mechanical behavior of skeletal structure, and built a 2D FEM models of the femur (226 triangular elements and 146 nodes) in 1972 which showed roughly the development and transmission of stress and marked the beginning of FEM applied to the orthopedic biomechanics. Gross et al [11], build a hollow femoral stem by the hexahedral elements of ANSYS software and discuss the stress shielding effect. Yerry and Shephard [12] propose the Octree method to create three-dimensional finite element model which more able to show the true dynamic behavior of femur. Taylor et al. [13] estimated the orthogonal bone material properties through modal experiments and ultrasound bone density measurements, and then verify the results with Finite Element Analysis (FEA). Zhang et al [14], construct a 3D FE model which solid elements are used to model bone, soft tissue and the lining. On the other hand, contact elements are employed to simulate the interface of bone and internal lining to get more realistic dynamical behavior at the interface. Kleemann et al [15], discuss Total Hip Arthroplasty (THA) loading arising from increased femoral anteversion and offset may lead to critical cement stresses. Noble [16, 17] measures 200 femurs and

defines a method called canal flare index (CFI) to scan the shape of the proximal femoral canal. CFI is the ratio of the canal width, 20 mm above lesser trochanter, to the canal width in the middle of diaphysis. Noble classifies the canal into three types: stove pipe (CFI<3), normal (3<CFI<4.7), champagne-fluted (CFI>4.7). Stephen and Eckrich [18] propose the relationship between femoral dimensions and its rotation under X ray.

In reverse engineering field, Song and Kim [19] propose an autonomous digitization of free-form surface on a CNC coordinate measuring machine. Kruth and Kerstens [20] establish a free surface from limited boundary conditions of point database. These geometric boundary conditions are then merged into a CAD model according to NURBS theory and to form a free surface model [21]. Peng and Loftus [22] propose an integrated photometric stereo photography illumination algorithm to reconstruct the three-dimensional surface.

This research employs reverse engineering, Noble CFI method and FEM to obtain the outer geometry, to excavate the cancellous bone according to inner canal shape, and to mesh the model of the hollow femur. The results are compared with Liang's outcome which is focused on solid femur [23].

2. Methodology

The flow chart of this research is shown Figure 2. First, it employs reverse engineering by using 3D white light scanner to obtain the outer profile of a teaching synthesis femur (made by plastic material).

The repair software, Studio, is used to patch the holes on the scanned 3D femur diagram which is then imported into finite element analysis software ANSYS [24]. The simulation results are verified with the experimental data to make sure the accuracy of the FEM model.

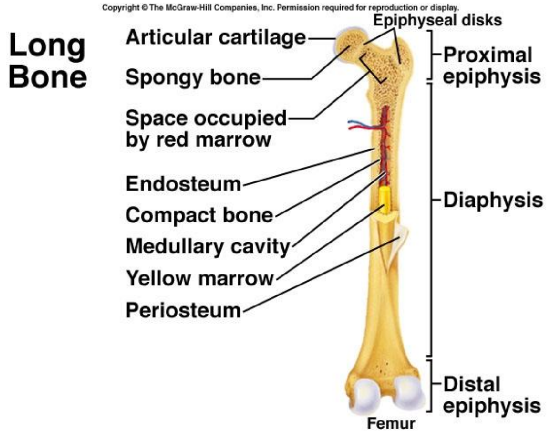


Figure 1. Schematic diagram of the femur structure (source: http://dc260.4shared.com/doc/lQlSf0m5/preview.html) [7]

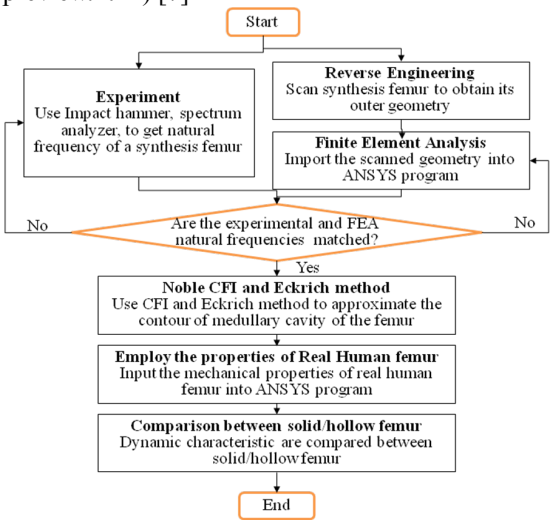


Figure 2. Flow chart of this research

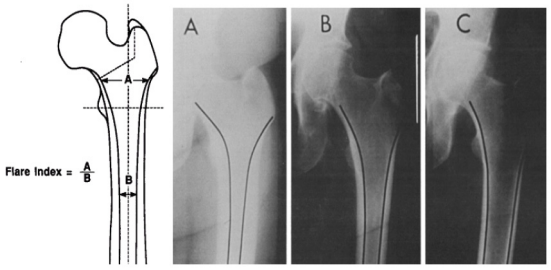


Figure 3. Proximal femur bone marrow cavity shape scanning method [25]

Proximal medullary cavity shape of the femur is sketched by Noble's CFI method. The femur is classified into three types (as shown in Figure 3): (A) Chimney-type (stovepipe) (CFI is less than 3), (B) normal type (CFI is between 3 to 4.7), and (C) inverted champagne-type (champagne-flute) (CFI is greater than 4.7). The cancellous bone and marrow are then cut off by drawing technique according to the above obtained the profile of femoral canal.

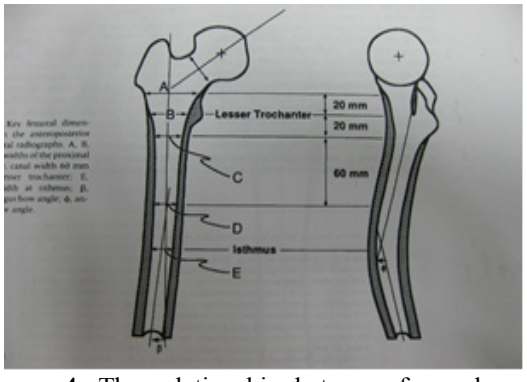


Figure 4. The relationship between femoral canal dimension and rotation [18]

Since Young's modulus of cortical bone is from dozens to hundred times higher than cancellous bone [9] and even much higher than the marrows, this hollow femur model contains only compact bone, the marrow are cut off. It is then analyzed in ANSYS program with the mechanical properties of real human femur. Hollow femoral model is very important to much accurately simulate real human femur. The dynamic characteristic of real human hollow femur is obtained and is compared with the solid femoral model.

3. Femoral canal contour drawing process

According to radiographic appearance of the femoral canal proposed by Eckrich et al., A is the canal width 20 mm above lesser trochanter, B is the canal width at lesser trochanter, C is the canal width 20 mm below lesser trochanter, D is the canal width 60 mm below lesser trochanter, E is the canal width at isthmus of the femur, βis the angle between vertical line passing through D and center line parallel to femoral canal below D, φis the angle between upper and lower line parallel to the upper and lower femoral canal, respectively, cross at isthmus and measured in the side view of the femur, as shown in Figure 4. The calculated CFI value is 3.07 of the proposed femoral model which is normal type femur. Assume canal width A at front view is 40 mm and side view is 30 mm, then use the angle β and φ, arithmetical progression, and CFI value to calculate each point from A to E and the corresponding canal width of the distal (lower part) femur, as shown in Table 1.

Table 1. Dimensional of femoral canal

Item	Front view		Side view	
	Parameters	Value	Parameters	Value
Angle	β	0.1±0.3°	φ	7.9±0.6°
Canal width	A	40 mm	A	30 mm
	B	30.31 mm	B	24.07 mm
	C	20.1 mm	C	20.1 mm
	D	15.66 mm	D	17.8 mm
	E	13 mm	E	17.34 mm

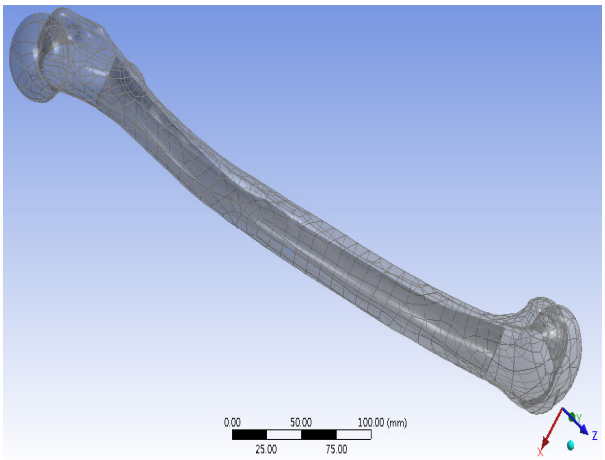


Figure 5. Hollow femoral model

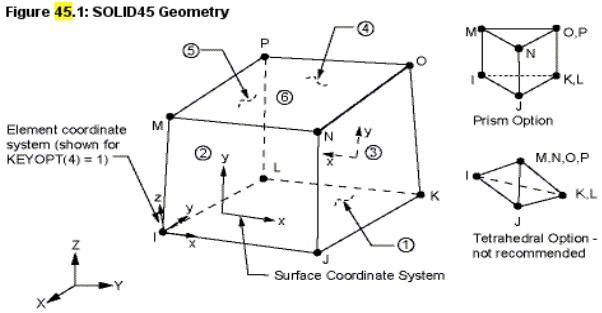


Figure 6. ANSYS Solid45 element

According to the above Table 1., the hollow femur model is built as shown in Figure 5.

4. Finite Element Analysis

ANSYS provides different type of elements, such as point element, line elements, surface elements and body elements, etc. All the elements are selected according to the construction of the graph and the solution needed for the analysis [27, 28]. ANSYS higher order 3-D, 8-node solid element SOLID45 (Figure 6), suitable for irregular grid, is chosen for this hollow femur model.

There are free mesh and mapped mesh in ANSYS. In free mesh, solid models requires not much, just to specify mesh size, density and type, then use the Mesh Generator to produce grid. However, the mapped mesh is more stringent. To meet a certain requirements in a complex geometric will spend a lot of time for detail cutting to complete the entire grid.

Nevertheless, the mapped mesh will turn out more rigorous and accurate results. Finer grid means more elements to be analyzed and will need longer process time. This study uses mapped mesh to form the finite element model, as shown in Figure 7.

The mechanical properties of the homogenous, isotropic synthesis femur (Polyethylene) are: Young's modulus: 1.3 GPa, density, 950 kg/m3, and poisson's ratio: 0.42. The mechanical properties of real human femur (assume homogenous and isotropic material) are used according to Hsu's result [26]: Young's modulus: 17 GPa, density, 2132.6 kg/m3, and poisson's ratio: 0.3.

5. Results and Discussions

Convergence test is performed before doing further investigation. The fundamental natural frequency is converged when the hollow femoral model is divided into 18,000 elements. Therefore, more than 30000 elements are used to simulate the hollow femoral model.

After the several lower natural frequencies of

FEM model are matched with experimental results, the material properties of real human femur are substitute into the FEM model. Both solid femoral model and hollow femoral model are conducted for the analysis. The vibration natural frequencies of these two models are compared and shown in Table 2.

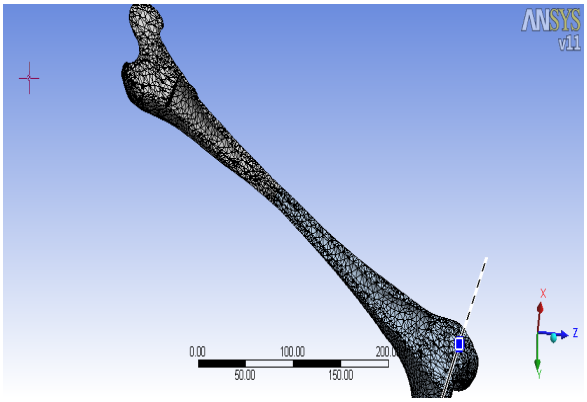


Figure 7. ANSYS meshed hollow femur model

Table 2. Natural frequency comparison between solid and hollow femoral model

Mode	Frequency (Hz)		Difference Value(%)
	Solid femur	Hollow femur	
1	265.53	271.85	-2.38
2	286.91	291.11	-1.46
3	609.21	564.08	7.41
4	881.44	901.55	-2.28
5	926.71	957.18	-3.29

Table 3. Stress, strain, displacement comparison between solid and hollow femoral model when apply a force of 1000N

Item	Solid femur	Hollow femur	Difference Value
Stress	40.613 Mpa	42.741 Mpa	2.128 Mpa
Strain	0.00239	0.00251	0.00012
Displacement	5.15 mm	8.66 mm	3.51 mm

The natural frequencies of hollow femur are basically higher than solid femur, since the moment of inertia of hollow femur is higher than that of solid femur and the mass of hollow femur is lower than that of solid femur. When apply 1000N force on the distal part of both solid and hollow femur and the response stress, strain, and displacement at the same place is shown in Table 3. The stress, strain and displacement of a solid femur are lower than that of the hollow femur. Because stress (σ), moment of inertia (I), moment of cross section (M), and distance with neutral axis (y) in bending motion have the relationship: σ=(M*y)/I. Therefore,σis inverse proportional to I, andσis proportional toε(strain). In other words, I is the ability of an object to resist deformation, also is the stiffness of the object. The greater value of the moment of inertial (I), the harder of the material to be deformed. The first mode shapes of both solid and hollow femur are shown in Figure 8 and 9, respectively.

7. Conclusion

The numerical simulation of synthesis femur, solid human femur, hollow human femur, and experiments of the synthesis femur are investigated in this study. The major conclusions drawn from the this study are summarized as follows:

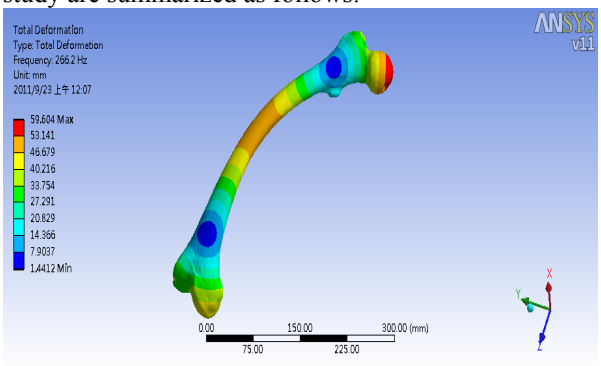


Figure 8. First mode shape of a solid femur (266Hz)

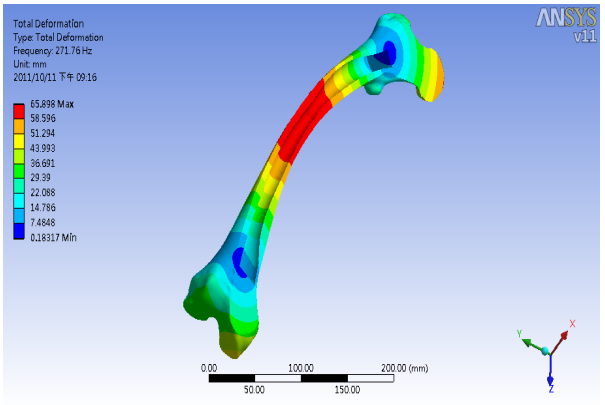


Figure 9. First mode shape of a hollow femur (272Hz)

(1) Both FEA and experimental results are quite match for a synthesis femur. Therefore, the outer geometry femur model is confirmed and can be used to calculate the dynamic properties of the real human femur.

(2) The drawing process of excavation the femur canal is built up to much approximate real human hollow femur situation.

(3) The natural frequencies, stress, strain, and displacement when apply a 1000N force are compared between the solid and hollow femur. All of them are matched with the physical rules.

(4) The advantage of this method is to avoid analyze the fresh or dry human bones and still can get quite promising results.

Acknowledgement

The financial support by the National Science Council, Republic of China, through Grant NSC 99-2632-E-230-001-MY3 of the Cheng Shiu University

Corresponding Author:
Jung-Ge Tseng, Ph.D.
E-mail : james.tseng@csu.edu.tw

References

1. N. Hraiech, C. Boichon, M. Rochette, T. Marchal, M. Horner, "Statistical Shape Modeling of Femurs Using Morphing and Principal Component Analysis," ASME J. Med. Devices, Vol. 4 (2), 027534, 2010.
2. Y. Jun and K. Choi, "Design of patient-specific hip implants based on the 3D geometry of the human femur," Advances in Engineering Software, Vol. 41, pp. 537–547, 2010.
3. M. Peacock, K.A. Buckwalter, S. Persohn, T.N. Hangartner, M.J. Econs, S. Hui, "Race and sex differences in bone mineral density and geometry at the femur," Bone, Vol. 45, pp. 218–225, 2009.
4. J. Panula, M. Sävelä, P.T. Jaatinen, P. Aarnio, S.-L. Kivelä, "The impact of proximal femur geometry on fracture Type – a comparison between cervical and trochanteric fractures with two parameters," Scandinavian Journal of Surgery, Vol. 97, pp. 266–271, 2008.
5. Z. Chen, T.J. Beck, J.A. Cauley, C.E. Lewis, A. LaCroix, T. Bassford, G. Wu, D. Sherrill and S. Going, "Hormone therapy improves femur geometry among ethnically diverse postmenopausal participants in the Women's Health Initiative hormone intervention trials," J. Bone Miner Res. 23(12), pp.1935-45, 2008.
6. C.S. Duncan, C.J.R. Blimkie, A. Kemp, W. Higgs, C.T. Cowell, H. Woodhead, J.N. Briody, and R. Howman-Giles, "Mid-femur geometry and biomechanical properties in 15- to 18-yr-old female athletes," Official Journal of the American College of Sports Medicine, Medicine & Science in Sports & Exercise, pp. 673-681, 2002.
7. Schematic diagram of the femur structure, source: http://dc260.4shared.com/doc/lQlSf0m5/preview.html
8. H. Yan, J. Zhang, K. Liu, K. Wang, "Morphological study of the femur medullary cavity and its significance," Journal of Xi'an Jiaotung University (Medical Sciences), Vol. 27, No. 3, pp. 236-239, 2006.
9. MC. Ho Bo Tho, "Bone and joints modelling with individualized geometric and mechanical properties derived from medical images," CMES, Tech Science Press, Vol. 4, no.3&4, pp.489-496, 2003.
10. W. Brekelmans, H. Poort, T. Slooff, "A new method to analysis the mechanical behavior of skeletal parts," Acta Orthop Scand, 301-317, 1972.
11. S, Gross, E.W. Abel, "A finite element analysis of hollow stemmed hipprostheses as a means of reducing stress shielding of the femur," Journal of Biomechanics, Vol. 34, pp. 995-1003, 2001.
12. M.A. Yerry and M.S. Shephard, "Automatic three-dimensional mesh generation by the modified-octree technique", International Journal for Numerical Methods in Engineering, Vol. 20, pp.1965-1990, 1983.
13. W.R. Taylor, E. Roland, H. Ploeg, D. Hertig, R. Klabunde, M.D. Warner, M.C. Hobatho, L. Rakotomanana, S.E. Clift, "Determination of orthotropic bone elastic constants using FEA and modal analysis", Journal of Biomechanics, Vol. 35, pp. 767-773, 2002.
14. M. Zhang, "Development of a non-linear finite element modelling of the below-knee prosthetic socket interface," Medical Engineering & Physics, Vol. 17, pp. 559-566, 1995.
15. R.U. Kleemann, M.O. Heller, U. Stoeckle, W.R. Taylor, and G.N. Duda, "THA loading arising from increased femoral anteversion and offset may lead to critical cement stresses", J. Orthop. Res., Vol. 21, pp. 767-774, 2003.
16. PC Noble, JW Alexander, U. Lindahl, "The anatomic basis of femoral component design," Clin. Orthop. Vol. 235, pp. 148-65, 1988.
17. PC Noble, E Kamaric, N Sugano, Matsubara Ma, Harada Y, Ohzono K, Paravic V, "Three-Dimensional Shape of the Dysplastic Femur: Implications for THR," Clin Orthop Vol. 417, pp. 27-40, 2003.
18. S. G.J. Eckrich, PC. Noble, H.S. Tullos, "Effect of rotation on the radiographic appearance of the femoral canal, Original Research Article," The Journal of Arthroplasty, Vol. 9 (4), pp. 419-426, 1994.
19. C. K. Song and S. W. Kim, "Reverse Engineering : Autonomous Digitization of Free-form Surface on A CNC Coordinate Measuring Machine," Int. J. Math. Tools Manufact, Vol. 37, pp. 1041-1051, December, 1997.
20. J. P. Kruth, A. Kerstens, "Reverse engineering modeling of free-form surfaces from point clouds subject to conditions", Journal Materials Processing Technology Vol.76, pp. 120-127, 1998.
21. Jeng-Nan Lee and Kuan-Yu Chang, "An Integrated Investigation of CAD/CAM for the Development of Custom-made Femoral Stem," Life Science Journal, Vol 7, No 1, 56 – 61, 2009.
22. Q. J. Peng, M. Loftus, "A new approach to reverse engineering based on vision information," International Journal of Machine Tools & Manufacture Vol.38, pp. 881-899, 1998.
23. S.H. Liang, An Investigation on Dynamic Characteristics of a Human Leg Bone, Master Thesis, Institute of Mechatronics Engineering, Cheng Shiu University, 2011.
24. B.W. Huang, H.K. Kung, K.Y. Chang, P.K. Hsu,

J.-G. Tseng, "Human Cranium Dynamic Analysis," Life Science Journal, 6(4): 15– 22, 2009.
25. Y.E. Lee, "The Proximal Femoral Canal Geometry of Osteoporosis, Osteoarthritis, and Osteonecrosis," Master Thesis, Institute of Biomedical Engineering , National Yang Ming University, 2005.
26. J.-T. Hsu, C.-H. Chang, S.-I Chen, "Investigate the influence of material properties under various loading mode: finite element simulations," Engineering Science and Chinese-Western medical Application Conference, 2002.
27. Y.K. Tu, Y.Y. Hong, Y.C. Chen, "Finite element modeling of kirschner pin and bone thermal contact during drilling," Life Science Journal, 6(4): 23-27, 2009
28. Chang Kuan Yu, Chang Hong Chang, Yu Pu Ping, Yen Ke Tien, Huang Bo Wun, "Natural Properties in a Micro Drill Cutting into Bones," Life Science Journal, 6(4): 28–33, 2009.

2/1/2012

Assessment of Ischemia in Asymptomatic Type II Diabetics versus Asymptomatic patients with other Risk Factors of Coronary Artery Disease. Preliminary Data from GSPECT Tc^{99m} sestaMIBI Myocardial Perfusion Imaging Study in Egyptian Patients.

Akram Abd Elbary,[1]; Mohamed Khaled,[1]Wael Sami[1]; Mohga Said[2], Hala Zakaria[2], Lamiaa Hamed[3], Shaban Mohamed[4]; and Alia Abd El-Fattah[4]

[1] Critical Care Medicine Department, Faculty of Medicine, Cairo University, Cairo, Egypt
[2] Lab Department Critical Care Medicine Department, Faculty of Medicine Cairo University, Cairo, Egypt
[3] Statistics Critical Care Medicine Department, Faculty of Medicine, Cairo University, Cairo, Egypt
[4] Medical physicians Critical Care Medicine Department, Faculty of Medicine, Cairo University, Cairo, Egypt
akram_bary@yahoo.com

Abstract: Background and Aim: Diabetes mellitus is a recognized risk factor for coronary artery disease (CAD). However ischemia in diabetics may express their ischemia as atypical symptoms. We aimed at comparing incidence and extent of diabetic vs. non diabetic with other risk factors for CAD. **Patients:** The study included 46 pts with 1 or 2 risk factors for CAD mean age 55±6 years, 18 males. **Methods:** Patients were subjected to laboratory assessment including lipid profile, HbA1C, microalbuminuria. Patients were subjected to myocardial perfusion imaging [(MPI) study using 2 day (stress-rest) protocol patients were injected 25 mCi Tc^{99m} sestaMIBI intravenously at peak of stress. Rest study was acquired in a separate day. Gated SPECT was acquired 30-60 minute post-stress for estimation of LVEDV, LVESV and LVEF. Processing and analysis were done to get the classic short axis, vertical long axis and horizontal long axis slices with application of 20 segment scoring system for semiquantitative analysis of defect size to get summed stress score (SSS), summed rest score(SRS),and summed difference score (SDS). The study was interpreted as negative when SSS = 0-3, mild (SSS>3& ≤8), moderate (SSS=8 &≤12), and severe >12)]. **Results:** Patients were subdivided into two groups; Group1 (Diabetic): 24 pts and Group 2 (non Diabetic): 22 pts. Laboratory data showed comparable lipid profile in G1 vs G2; Serum cholesterol (196±58 vs. 200±58 mg/dl, p>0.05), triglycerides 163±66 vs. 187±90, p>0.05), HDL (45±14vs. 50+14, p>0.05) & LDL (127±48vs 135+44, p>0.05). HbA1c level was 9+3% in G1 vs 7+2 in G2, p=0.01 and was abnormal in 19 pts of G1 vs. 10 pts in G2, p=0.03. Microalbuminuria was detected in 13/24 pts of G1 vs 6/22 pts in G2. Mean exercise duration was 7±3 min in G1 vs 7±2 minutes in G2 P>0.05, mean Mets achieved was 10.6±3 vs 9.4±2 in G2, P > 0.05. Myocardial perfusion imaging was interpreted as positive in 41% of all pts (19 pts) (46% in G1 vs. 36% of G2), Six pts had mild ischemia (4 diabetics), Nine had moderate ischemia (4 diabetics) and 4pts had severe ischemia (3 diabetics [75%]), Mean SSS, SRS,& SDS were comparable (4.7±4vs 3.6±4, (0.3±1.6 vs 0.2±0.9),& (4.4±4 vs 3.4±3.7%) in G1vs G2 respectively. left ventricular ejection fraction, LVEDV and LVESV were comparable in G1vs G2 (68.1±10.8% vs 70.6±8.2%, P = 0.3), (103±24 vs 97±17, P = 0.3) & (32±16 vs 28±12, P = 0.4). Neither microalbuminuria nor HbA1c abnormality correlated with severity or abnormality of MPI. **Conclusion:** Preliminary data from our ongoing study suggest high incidence of silent ischemia in patient with type II diabetes mellitus and non diabetic patients with other risk factors for CAD in our developing country, with tendency of more severe ischemia and higher post-stress LV volumes in diabetic patients.

[Akram Abd Elbary; Mohamed Khaled; Mohga Said, Hala Zakaria, Lamiaa Hamed, Shaban Mohamed and Alia Abd El-Fattah. **Assessment of Ischemia in Asymptomatic Type II Diabetics versus Asymptomatic patients with other Risk Factors of Coronary Artery Disease. Preliminary Data from GSPECT Tc^{99m} sestaMIBI Myocardial Perfusion Imaging Study in Egyptian Patients.** Life Science Journal 2012;9(1):727-732]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 105

Key Words: Diabetics, Coronary Artery Disease, sestaMIBI, Myocardial Perfusion Imaging

1. Introduction

Diabetes mellitus is a major source of cardiovascular morbidity and mortality in developed and developing countries.

Currently, the worldwide prevalence of diabetes is estimated to be around 194 million. This figure is expected to rise to almost 333 million by the year 2025[1]. Type 2 diabetes constitutes 85–95% of all patients with diabetes. Cardiovascular disease is the cause of death in 65–70% of persons with diabetes.

In general, diabetic patients have more extensive atherosclerosis with a higher prevalence of multi-vessel coronary artery disease (CAD), frequent silent myocardial ischaemia, and infarction with a higher cardiac event rate when compared with non-diabetic patients[2–4]. Some studies have even suggested that diabetic patients without CAD have

the same risk for future cardiac death as non-diabetic patients with established CAD[5]. Even once CAD becomes manifest clinically, diabetic patients continue to have a worse prognosis compared with non-diabetic patients both acutely after the event and during long-term follow-up [4,6]. Proposed strategies that may favorably affect CAD risk and outcomes in this patient population include identifying diabetic patients with subclinical disease at high risk of future cardiac events. Such subjects are likely to be good candidates for aggressive risk factor management.

Stress echocardiography and MPI are well-established functional imaging techniques for assessing patients with suspected CAD and for evaluating prognosis in patients with known CAD[7,8].

Reversible left ventricular regional wall motion abnormalities, either stress-induced or spontaneous, in the absence of angina, provide evidence for silent myocardial ischemia. Reversible myocardial thallium perfusion defects at rest or during dipyridamole or exercise stress occur rather frequently without associated angina. On the basis of wall motion abnormalities, metabolic dysfunction, reversible scintigraphic perfusion defects and ischemic changes on the stress electrocardiogram (ECG), the incidence rate of silent myocardial ischemia has been estimated to be approximately 34% in patients with coronary artery disease[9].

Aim of the Work:

Comparing incidence and extent of silent ischemia in diabetics versus non diabetics assessed by myocardial perfusion imaging (MPI).

2. Patients and Methods

Our study was conducted in Critical Care Department, Cairo University on 46 patients, 24 had diabetes 22 patients had one or two risk factor, CAD.

All pts will subject to:

1. Full medical history.
2. Lipid profile including serum cholesterol, triglycerides levels (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL).
3. HbA1c: Sample collection two to three milliliters of whole blood in EDTA tube were collected from the patient and transferred to the laboratory in ice box. Samples are known to be stable for one week in refrigerator in 2-8^{o}C. By quantitative colorimetric determination of glycohemoglobin in samples using Teco-Glycohemoglobin kits procedures No. 0350. the apparatus is dimension RXL band produced by Siemens health care diagnostics.
4. Detection of microalbumin in urine:
5. Myocardial perfusion imaging (MPI): study using 2 day (stress-rest) protocol patients were injected 25 mCi Tc^{99m} sestaMIBI intravenously at peak of stress. Rest study was acquired in a separate day. Gated Single Photon Emission Computed Tomography (gSPECT) was acquired 30-60 minute post-stress for estimation of left ventricular end diastolic volume (LVEDV), left ventricular end systolic volume (LVESV) and left ventricular ejection fraction (LVEF).

Processing and analysis were done to get the classic short axis, vertical long axis and horizontal long axis slices with application of 20 segment scoring system for semiquantitative analysis of defect size to get: 1) Summed stress score (SSS) representing the total defect size, 2) Summed rest score (SRS) representing infarct size, and 3) Summed difference score (SDS) representing extent of ischemia.

The study was interpreted as negative when SSS = 0-3, mild (SSS > 3 & $\leq$8), moderate (SSS = 8 & $\leq$12), and severe > 12)].

Statistical methods:

Data were statistically described in terms of mean ± standard deviation (± SD), frequencies (number of cases) and percentages when appropriate. Comparison of numerical variables between the study groups was done using Student *t* test for independent samples. For comparing categorical data, Chi square (χ^2) test was performed. Correlation between various variables was done using Spearman rank correlation equation for non-normal variables. P values less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel 2007 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows.

3. Results:

Our study was conducted on 46 patients with mean age 55$\pm$64 yrs 18 males and 28 females and divided into two groups.

Group I (G1): 24 diabetic patients with diabetes duration more than 5 years.

Group 2 (G2): 22 non diabetic patients having one or two risk factors of CAD.

Risk factors for coronary artery disease in group II (non diabetic patients).

- Hypertension.
- Dyslipidemia.
- Positive family history for CAD.

Lipid profile was comparable in both groups.

HbA1c: Showed statistically significant higher values in Group 1 patients compared to G2 patients (9$\pm$3% vs 7$\pm$2%, P = 0.01)

Table (1): Lipids profile in both groups.

	G1	G2	P value
Cholesterol mg/dl	196±58	200±58	P > 0.05
Triglycerides mg/dl	163±66	187±90	P > 0.05
HDL mg/dl	45±14	50±14	P > 0.05
LDL mg/dl	127±48	135±44	P > 0.05

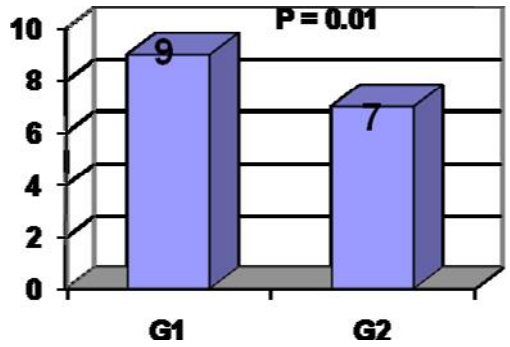


Figure (1): HBA1c in both groups

Microalbuminuria was detected in 13 Patients out of 24 in group I versus 6 out of 22 patients in group II with statistical significant comparison, P = 0.02

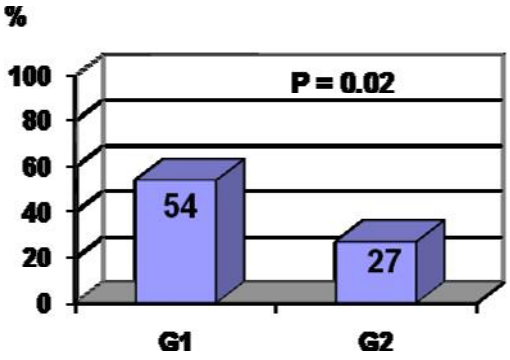


Figure (2): Microalbuminuria in both groups

Exercise data:

Exercise duration:

There was no statistical significant difference between the two groups as regards exercise duration in G1 7+3 min and in G2 7+2 min.

Mean Mets:

There were no statistical significant difference between G1 patient and G2 patients as regards mean mets achieved during excuses (10.6+3 m vs 9.4+2 m, P = 0.85) (Fig. 2).

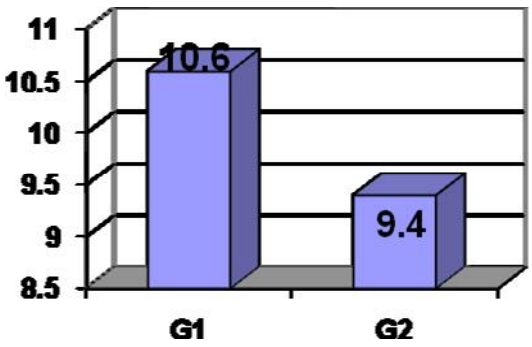


Figure (3): Metabolic equivalents in both groups.

MPI was considered positive in 41% of all patients (19 pts), 46% of G1 vs 36% of G2 pts (P > 0.05).

Severity of ischaemia:

A total of 6 pts showed mild ischemia (4 diabetics), moderate ischaemia was detected in 9 patients (4 diabetics) while severe ischaemic was detected in 4 patients (3 diabetics) (Table 2).

Table (2): Severity of ischemia by MPI

	G1	G2	P value
Mild Ischemia	4	2	
Moderate Ischemia	4	5	
Severe Ischemia	3	1	

Quantification of ischemia:

G1 pts showed insignificantly higher mean SSS & SDS and SSS (Table 3).

Gated SPECT data:

Both LVEDV and LVESV in both groups were comparable with higher LVEDV and LVESV in G1 without statistical significance.

As regards LVEF there were no statistical significant between the two groups (68.1±10.8% vs 70.6±8.2, P = 0.3) G1 vs G2.

In our study we didn't found significant correlation – between level of HbA1c and presence of microalbuminuria and severity of myocardial ischemia by MPI.

Table (3): Quantification of ischemia in both groups

	G1	G2	P value
SSS	4.7±4%	3.6±4%	0.7
SRS	0.3±1.6%	0.2±0.9%	0.9
SDS	4.4±4%	3.4±3.7%	0.8

Table (4): LVEDV & LVESV in both groups

	G1	G2	P value
LVEDV	103±24	97±17	0.3
LVESV	32±19	28±12	0.4

4. Discussion

This study is a part of multicenter trial to evaluate asymptomatic diabetic patients, we found high incidence of positive scans in both diabetic and non diabetic patients with one risk factor. However there is a tendency towards more severe perfusion defects in diabetic patients with higher LV volumes.

There is solid evidence that number and size of defects in stress MPI carries a prognostic value for hard cardiac events with higher incidence in diabetic patients but the value of treating asymptomatic patients with low and medium risk scans is not evident.

We believe that this study when completed with follow up can guide us to a management strategy for these patients.

Myocardial perfusion imaging is recognized that perfusion abnormalities precede abnormalities in systolic function in the ischemic cascade[10]. In pooled studies including both diabetic and non-diabetic patients and symptomatic as well as asymptomatic patients, an unequivocally normal stress MPI has been associated with a cardiac event rate of ,1% per year[11]. With abnormal stress MPI studies, the extent and severity of myocardial ischemia strongly predicts short and long-term risks of coronary events[8]. **Felsher *et al.*[12]** were the first to confirm that this same pattern is found in diabetics and that an abnormal stress MPI predicts a poor cardiac prognosis. At least eight subsequent studies have confirmed that event rates vary with the size of perfusion defect[13–20]. In a single-centre retrospective study using dual-isotope MPI (rest thallium-201/stress technetium-99 m sestamibi), with exercise or adenosine pharmacological testing. **Kang *et al.*[39]** showed that hard cardiac event rates in diabetics with mild, moderate, and severe perfusion defects were 1–2, 3–4, and .7% per year, respectively. In general, diabetic patients had an approximately two-fold higher hard event rate when compared with non-diabetic patients (4.3 vs. 2.3%; P = 0.001). **Giri *et al.*[15]** also showed that despite the higher rates of revascularization, diabetic patients had an almost two-fold increase in the hard cardiac event rate (8.6%) when compared with non-diabetics (4.5%). However, similar to the stress echocardiography trials, the earlier mentioned studies also demonstrate that a normal MPI is less reassuring in diabetics than in non-diabetics, providing a limited 'warranty period' of 2 years at most.[21]

Three studies have examined the relationship between myocardial perfusion abnormalities and prognosis in asymptomatic diabetic patients. **De Lorenzo *et al.*[17],** showed that an abnormal MPI significantly increased the annual incidence of hard cardiovascular events (9%) when compared with a normal MPI (2%). Furthermore, in this study, established risk factors were related neither to the extent of abnormalities on MPI nor to the cardiovascular events. In a subsequent larger study comprising 1737 patients, **Zellweger *et al.*[18]** showed that frequency of abnormal MPI (39%) and annual critical event rate in asymptomatic diabetics was comparable to that of diabetic patients with angina (44%). Similarly, **Miller *et al.*[22]** studied 27165 patients, of whom 4736 were diabetic, and found that the prevalence of an abnormal MPS was the same in asymptomatic and symptomatic diabetic patients (58.6 vs. 59.5%); this was significantly higher than in asymptomatic non-diabetic (46.2%) and symptomatic non-diabetic (44.4%) patients. A subsequent follow-up study confirmed the increased prevalence of severe angiographic CAD and mortality in those diabetic patients with severe asymptomatic ischaemia.[19]

The large prospectively designed study of asymptomatic ischaemia in unselected type 2 diabetics was Detection of Ischaemia in Asymptomatic Diabetics (DIAD) study by **Wackers *et al.*[23]** This multi-centre study more than 1000 patients, there was a 22% prevalence of an abnormal MPI study, with marked perfusion abnormalities occurring in 6% of patients. Similar to our study the DIAD also demonstrated that traditional cardiovascular risk factors and novel biomarkers (hs-C-reactive protein, homocysteine, lipid subfractions,

and plasminogen activator inhibitor-1) were not predictive of abnormal myocardial perfusion.

In 2007 the follow up revealed resolution of ischemia in 79% of patients with medical treatment alone[24].

Conclusion

Preliminary data from our ongoing study suggest high incidence of silent ischemia in patient with type II diabetes mellitus and non diabetic patients with other risk factors for CAD in our developing country, with tendency of more severe ischemia and higher post-stress LV volumes in diabetic patients.

Corresponding author
Akram Abd Elbary
Critical Care Medicine Department, Faculty of Medicine, Cairo University

References

1. Diabetes e-Atlas. International Diabetes Federation. http://www.idf.org/e-atlas/home/ (30 October 2004).
2. Goraya TY, Leibson CL, Palumbo PJ, Weston SA, Killian JM, Pfeifer EA, Jacobsen SJ, Frye RL, Roger VL. Coronary atherosclerosis in diabetes mellitus: a population-based autopsy study. J Am Coll Cardiol., 2002; 40:946–953.
3. Nesto RW, Philips RT, Kett KG, Hill T, Perper E, Young E, Leland OS Jr. Angina and exertional myocardial ischemia in diabetic and non-diabetic patients: asssessment by exercise thallium scintigraphy. Ann Intern Med., 1988; 108:170–175.
4. Stamler J, Vaccaro O, Neaton JD, Wentworth D. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial. Diabetes Care, 1993;16:434–444.
5. Haffner SM, Lehto S, Ronnemaa T, Pyorala K, Laakso M. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. N Engl J Med., 1998; 339:229–234.
6. McGuire DK, Emanuelsson H, Granger CB, Magnus Ohman E, Moliterno DJ, White HD, Ardissino D, Box JW, Califf RM, Topol EJ. Influence of diabetes mellitus on clinical outcomes across the spectrum of acute coronary syndromes. Findings from the GUSTO-IIb study. Eur Heart J., 2000;21:1750–1758.
7. Poldermans D, Fioretti PM, Boersma E, Bax JJ, Thomson IR, Roelandt JR, Simoons ML. Longterm prognostic value of dobutamine–atropine stress echocardiography in 1737 patients with known or suspected coronary artery disease: a single centre experience. Circulation, 1999;99:757–762.
8. Hachamovitch R, Berman DS, Shaw LJ, Kiat H, Cohen I, Cabico AJ, Friedman J, Diamond GA. Incremental prognostic value of myocardial perfusion single photon emission computed tomography for the prediction of cardiac death: differential stratification for risk of cardiac death and myocardial infarction. Circulation, 1998;97:535–543.
9. Gu K, Cowie CC, Harris MI. Mortality in adults with and without diabetes in a national cohort of the US population, 1971–1993. Diabetes Care, 1998; 21: 1138– 1145.
10. Nesto RW, Kowalchuk GJ. The ischaemic cascade: temporal sequence of hemodynamic, electrocardiographic and symptomatic expressions of ischaemia. Am J Cardiol., 1987; 59:23C–30C.
11. Iskander S, Iskandrian AE. Risk assessment using single-photon emission computed tomographic technetium-99 m sestamibi imaging. J Am Coll Cardiol., 1998; 32: 57– 62.
12. Felsher J, Meissner MD, Hakki AH, Heo J, Kane-Marsch S, Iskandrian AS. Exercise thallium imaging in patients with diabetes mellitus. Prognostic implications. Arch Intern Med., 1987;147:313–317.
13. Vanzetto G, Halimi S, Hammoud T, Fagret D, Benhamou PY, Cordonnier D, Denis B, Machecourt J. Prediction of cardiovascular events in clinically selected high-risk NIDDM patients: prognostic value of exercise stress test and thallium-201 single-photon emission computed tomography. Diabetes Care, 1999; 22:19–26.
14. Kang X, Berman DS, Lewin HC, Cohen I, Friedman JD, Germano G, Hachamovitch R, Shaw LJ. Incremental prognostic value of myocardial perfusion single photon emission computed tomography in patients with diabetes mellitus. Am Heart J., 1999;138:1025–1032.
15. Giri S, Shaw LJ, Murthy DR, Travin MI, Miller DD, Hachamovitch R, Borges-Neto S, Berman DS, Waters DD, Heller GV. Impact of diabetes on the risk stratification using stress single-photon emission computed tomography myocardial perfusion imaging in patients with symptoms suggestive of coronary artery disease. Circulation, 2002; 105:32–40.
16. Schinkel AFL, Elhendy A, Van Domburg RT, Bax JJ, Vourvouri EC, Sozzi FB, Valkema R, Roelandt JRTC, Poldermans D. Prognostic value of dobutamine–atropine stress myocardial

perfusion imaging in patients with diabetes. Diabetes Care, 2002; 25:1637–1643.
17. De Lorenzo A, Lima RSL, Siqueira-Filho AG, Pantoja MR. Prevalence and prognostic value of perfusion defects detected by stress technetium-99 m sestamibi myocardial perfusion single-photon emission computed tomography in asymptomatic patients with diabetes mellitus and no known coronary heart disease. Am J Cardiol., 2002;90:827–832.
18. Zellweger MJ, Hachamovitch R, Kang X, Hayes SW, Friedman JD, Germano G, Pfisterer ME, Berman DS. Prognostic relevance of symptoms versus evidence of coronary artery disease in diabetic patients. Eur Heart J., 2004; 25:543–550.
19. Frans J.TH. Wackers, Lawrence H. Young, Mdsilvio E. Inzucchi, *et al.:* Detection Of Silent Myocardial Ischemia In Asymptomatic Diabetic Subjects The DIAD Study. Diabetes Care, 2004; Volume 27, Number 8, August. 1954-61
20. Cosson E, Guimfack M, Paries J, Paycha F, Attali JR, Valensi P. Prognosis for coronary stenoses in patients with diabetes and silent myocardial ischemia. Diabetes Care, 2003; 26:1313.
21. Hachamovitch R, Hayes S, Friedman JD, Cohen I, Shaw LJ, Germano G, Berman DS. Determinants of risk and its temporal variation in patients with normal stress myocardial perfusion scans. What is the warranty period of a normal scan? J Am Coll Cardiol., 2003; 41:1329–1340.
22. Miller TD, Rajagopalan N, Hodge DO, Frye RL, Gibbons RJ. Yield of stress single photon emission computed tomography in asymptomatic patients with diabetes. Am Heart J., 2004;147:890–896.
23. Wackers FJ, Young LH, Inzucchi SE, Chyun DA, Davey JA, Barrett EJ, Taillefer R et al. for the Detection of Ischemia in Asymptomatic Diabetics (DIAD) Investigators. Detection of silent myocardial ischemia in asymptomatic diabetic subjects: the DIAD study. Diabetes Care, 2004; 27:1954–1961.
24. Frans J. Th. Wackers, Deborah A. Chyun, Phd Lawrence H. Young, *et al.*: Resolution of Asymptomatic Myocardial Ischemia in Patients With Type 2 Diabetes in the Detection of Ischemia in Asymptomatic Diabetics (DIAD) Study. Diabetes Care, Volume 30, Number 11, November 2007. online issue pages 1-18.

2/1/2012

Transplantation of bone marrowderived stromal cells to chronic cerebral ischemia rats on the influence ofCognitive function and proteins Nogo-A and NgR expression in the hippocampus.

Yanlin Wang, Songbo Yang, Huili Zhang, Guangming Gong, Qin Jie, Yuming Xu

Department of Neurology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan province, 450052, P. R. China. xuyuming@zzu.edu.cn

Abstract: TO study the transplantation of bone marrowderived stromal cells (mesenchimal stem cells,MSC) to chronic cerebral ischemia rats on the influence of proteins Nogo-A and NgR expression in the hippocampus and explore the mechanism of its function in improving the Cognitive function. BMSCs were harvested and purified by flCOLLC density gradient centrifugation. The chronic cerebral ischemia models were produced by permanent occlusion and snip of bilateral common carotid arteries. Thirty male rats were randomly divided into three groups: model group ,BMSCs transplantation group (Brdu marker)and sham operate group.All of the rats were trained in Morris Water Maze to find the changes of spatial learning and memory ability. The expression of Nogo-A and NgR in the hippocampus were measured by immunohistochemical technique. The model group exhibited serious spatial learning and memory deficits in both navigation test and spatial probe test. In the former test，the mean escape latency of BMSCs transplantation group was significantly shorter than that of model groups and the frequency of crossing the former platform was significantly higher. The expressions of Nogo-A and NgR in BMSCs transplantation group were significantly lower than those of model groups and significantly higher than sham operated group, which was accordant with the change of spatial learning and memory ability. BMSCs transplantation effectively promote spatial learning and memory ability for the rats of chronic cerebral ischemia, which maybe induced by the decrease of proteins Nogo-A and NgR.

[Yanlin Wang, Songbo Yang, Huili Zhang, Guangming Gong, Qin Jie, Yuming Xu. **Transplantation of bone marrowderived stromal cells to chronic cerebral ischemia rats on the influence ofCognitive function and proteins Nogo-A and NgR expression in the hippocampus.** Life Science Journal. 2012;9(1):733-739] (ISSN:1097-8135). http://www.lifesciencesite.com. 106

Keywords: BMSC; Chronic cerebral ischemia; Nogo-A ; NgR ; Cognitive function

Introduction

Chronic cerebral ischemia is cognitive impairment caused by the vasospasm, stenosis, occlusion of carotid artery system or vertebral - basilar artery system caused by various factors and long-term cerebral blood flow hypoperfusion, causing tissue ischemia and hypoxia, and further leading to impaired nerve function [1]. A series of pathophysiological changes caused by injury of nerve will lead to the release of a large number of inhibitory proteins against axon regeneration, which is not conducive to the functional rehabilitation of nerve [2]. Nogo-A is currently the strongest known inhibitory proteins against axon regeneration, with strong axonal growth inhibitory function after myelin sheath injury [3]. NgR is its receptor, expressing the inhibitory action of nerve growth, and is an important pathway affecting nerve repair [4]. Research has shown that Nogo-A and NgR protein expression both increase after rat ischemia and hypoxia, strongly inhibiting the regeneration and connection of nerve fibers and resulting in subsidence of nerve ability [5].

BMSCs is a kind of stem cells with multidifferentiation potential, a variety of sources, easy collection, preparation and preservation. It can avoid immunological rejection and at the same time is not related to social, ethical and legal issues, etc. It has extensive foreground of clinical application. Research has shown that BMSCs transplantation can bring down nerve injury through reducing Nogo-A and NgR expression of rats of spinal cord injury model and cerebral infarction model [6] [7]. In addition, studies have shown that BMSCs can improve rat's cognition dysfunction from chronic cerebral ischemia model, but it is not yet known that whether the improvement of cognitive status is related to the decreased NgR and Nogo-A expression.

The chronic cerebral ischemia models were produced by permanent occlusion and snip of bilateral common carotid arteries ,the changes of spatial learning and memory ability were detected by BMSCs transplantation ,the influence of proteins Nogo-A and NgR expression in the hippocampus were measured by immunohistochemical technique in our research to explore the mechanism of its function in improving the Cognitive function .

Materials and methods

1.1 Animal Grouping and Model Preparation

30male SD rats of clean grade at the age of 12-14 months, purchased from Experimental Animal Center of Zhengzhou University, were randomly divided into sham operation group, model group and BMSCs

transplantation group, 10 rats for each group. Sham operation group: just separate the bilateral common carotid artery without ligation after anesthetizing rats. Model group: after fasting and water deprivation and anesthesia, take the median incision in cervical part of rats, the proximal end and distal end of the bilateral common carotid artery were separated and double ligated and then cut, keeping the rectal temperature at 37℃. BMSCs transplantation group: after seven days of model preparation, rats were injected 1ml BMSCs single cell suspension, which contains 1×10^7 cells, by caudal vein.

1.2 The Separation and Culture of the BMSCs

Separate the thighbones and shankbones of the rats on aseptic condition after they were anesthetized. The cell suspension was obtained by repeatedly flushing the bone marrow cavity with DMEM-F12 (Gibco 11330). Mononuclear cells were obtained by adopting flCOLLC density gradient centrifugation method and were incubated in the incubator (37℃, 5%CO2 saturated humidity). After one night, the suspended growth hematopoietic stem cells were removed. The liquid was changed every three days. When the cell density reached 80%～90%, cells were transferred of culture according to 1:2 to 1:3. Tested the BMSCs surface antigen CD34(-), CD44(+), CD45(-) and CD105(+) of rats with flow cytometry. Dissolved 10mg Brdu(Solarbio b8010) into 15ml distilled water. Took 50ul Brdu solution into 5ml cell culture solution at 48 hours before transplantation and cultured it in dark for 48 hours. Counted cells before transplantation and the number of the transplanted cells was about 1×10^7.

1.3 Ethology Test

After one month of modeling, all rats were performed by the Morris (8) water maze test . place navigation test: before training, made rats swim in the pool for two days, two minutes for each day to adapt them to the pool environment. Rats of each group had five days. There were nine periods for swimming training and four trainings for each period. Everyday, threw rats into the pool from different quadrants and recorded the time they found the platform, which was recorded as the escape latency. The longest time limit was 120 seconds. If rats couldn't find the platform in 120 seconds, they would be guided to the platform by the experimental personnel and the escape latency would be 120 seconds. On the fifth afternoon, rats were thrown into the pool from the same quadrant with the one of the first day, to test and record the escape latency. The spatial probe trial: removed the platform after recording the escape latency, and recorded the times that they crossed the platform in 120 seconds.

1.4 Specimens Collection and Tissue Section Preparation:

One month later after the behavioral test, take ten rats from each group, expose the heart upon excessive anesthesia, intubate the left ventricular and incise the right auricle and perfuse 200ml of 37℃ sterile saline through the left ventricular within 10 - 20min, then perfuse 250ml of PBS stationary liquid of 4℃, 40g/L paraformaldehyde and finish this perfusion within 1 - 2h, then decollate and take the brain. Take brain tissue in hippocampal region at the point 3mm after the optic chiasma, embed it with paraffin, then do continuous coronal sections with the thickness of 3 μm each, and do HE stain and Nogo-A and NgR immunohistochemical stain respectively.

1.5 Test Brdu with Immunofluorescence Stain and Test Nogo-A and NgR Protein Expression with Immunohistochemical Stain

(1) Take the paraffin sections for immunostaining, conventional dewaxing to water progressively. Enclose peroxidase in 3％ hydrogen and conduct microwave antigen retrieval for 15min, and enclose peroxidase in goat's blood serum for 20min. Add rabbit anti-Brdu FITC antibody (Beijingboaoshen bs-0489R) (1:100) respectively, and keep them at 4℃ overnight. Drop FITC-marked Brdu fluorescence secondary antibody upon PBS cleaning and drying and incubate for 3h at 37°C. After cleaning with PBS for three times, observe under 200 magnified visual field of fluorescence microscope that the ones with green fluorescence are Brdu-marked bone marrow derived mesenchymal stem cells.

(2) Take the paraffin sections for immunostaining, conventional dewaxing to water progressively. Enclose peroxidase in 3％ hydrogen and conduct microwave antigen retrieval for 15min, and enclose peroxidase in goat's blood serum for 20min. Rabbit anti-Nogo-A antibody (Beijingboaoshen bs-0134R) and rabbit anti-NgR antibody (Beijingboaoshen bs-0129R, 1:100), kept at 4℃ overnight. Drop goat anti-rabbit secondary antibody and horseradish peroxidase-marked strepto-avidin respectively upon PBS incubate for 3h at 37°C, dehydrate it after 3min DAB coloration and mount upon lucidity. Replace primary antibody with PBS for negative control. Observe with optical microscope (Germany, LEICA) that brown granules are immunoreactive cells. Choose 5 visual fields for each section under 400 times magnified visual field and do optical density analysis on them by image analysis system.

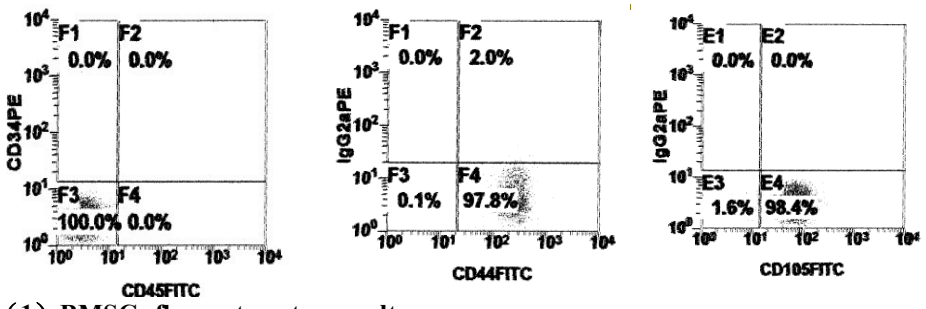
1.6 Data Analysis and Processing

Data is shown with $\overline{x} \pm s$. Firstly, conduct normality test and test of homogeneity variance for the data, do statistical analysis with SPSS17.0 software, T test and single factor analysis of variance are conducted for pairwise comparison between groups, take $P<0.05$ as difference and of statistical meaning.

2. Results:

2.1 BMSCs flow cytometry result

Tested the BMSCs with flow cytometry, the CD34(-) was 100%, CD44(+) was 97.8%, CD45(-) was 100% and CD105(+) was 98.4%.



Figure（1）**BMSCs flow cytometry result**

2.2 The Morris water maze result

The model group exhibited serious spatial learning and memory deficits in both navigation test and spatial probe test. In the former test,the mean escape latency of BMSCs transplantation group was significantly shorter（P<0.01）than model groups and the frequency of crossing the former platform was significantly higher（P<0.05）. In navigation test ,the mean escape latency of model groups was significantly longer（P<0.01）than sham-operated group and the frequency of crossing the former platform was significantly lower（P<0.01）.

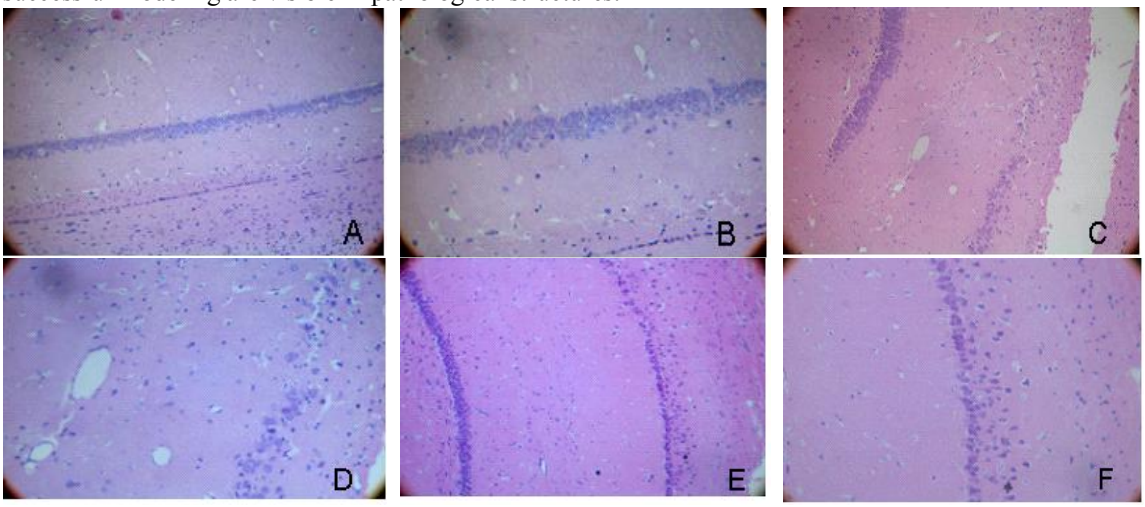
Table 1. The Morris water maze result

GROUPS	escape latency（S）	cross times
Model	53.10±1.85*	2.90±1.29★
BMSCs	32.20±1.32△	4.70±1.49▲
Sham	15.20±2.15□	7.20±1.93■

*compare with△*P*<0．01；*compare with□*P*<0．01；△compare with□*P*<0．01；★compare with ▲*P*<0．05；★compare with■*P*<0．01；▲compare with■*P*<0．01；

2.3 HE Stain Result

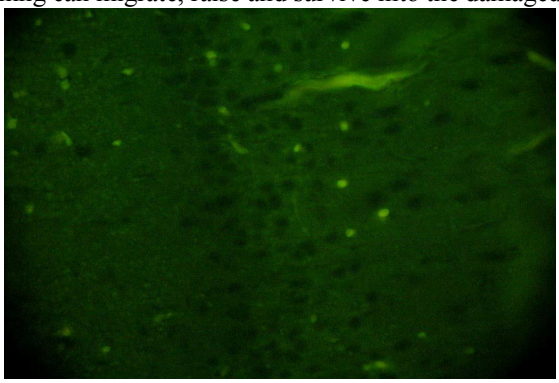
HE stain result in hippocampus tissue of model group shows that cone cells are arranged disorderly, and even shows a linear fracture, the original structure is damaged, a large number of cell necrosis and apoptosis and vacuolization, cone cells of hippocampus in transplantation group are more chaotic arranged, but we can also see a large number of nuclear hyperchromatism and vacuolization, cells in sham-operated group are arranged normally, the successful modeling are visible in pathological structures.



Figure（2）**HE Stain Result :**hippocampus tissue of sham operation group in A(×100) and **B**(×200); hippocampus tissue of model group in **C**(×100) and **D**(×200); hippocampus tissue of BMSCs transplantation group in **E**(×100) and **F**(×200);

2.4 Brdu Immunofluorescence Stain Result

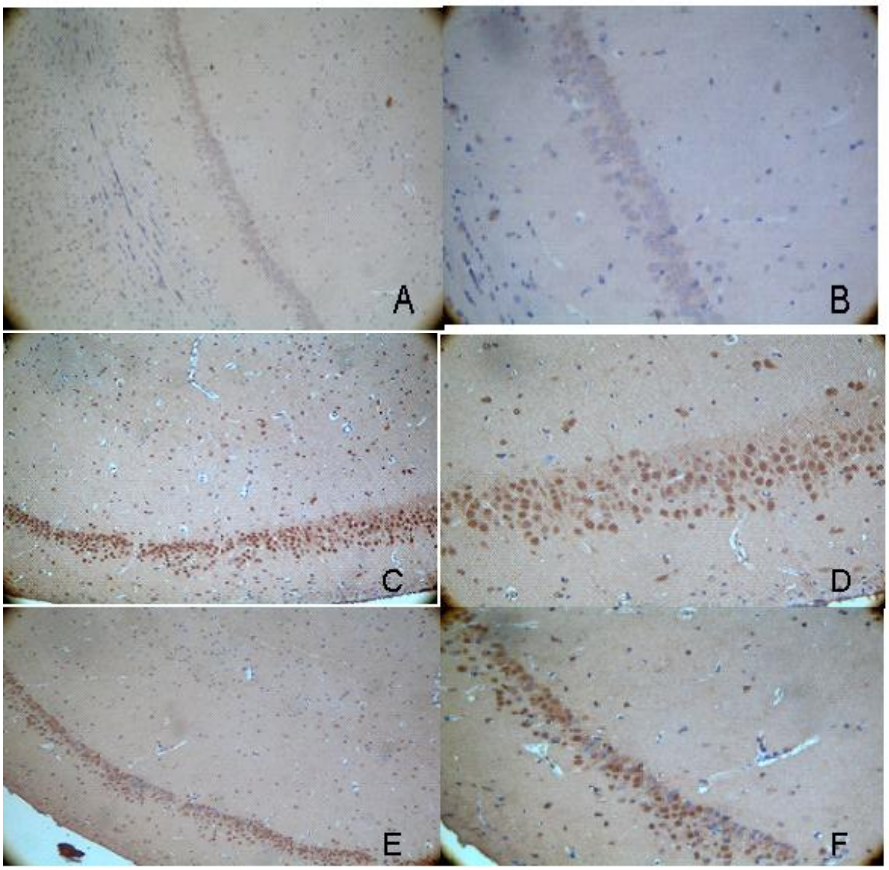
As shown in Figure 3 in the hippocampal tissue of BMSCs transplantation group, it can be observed that positive BMSCs of Brdu fluorescent staining can migrate, raise and survive into the damaged area of cerebral ischemia.



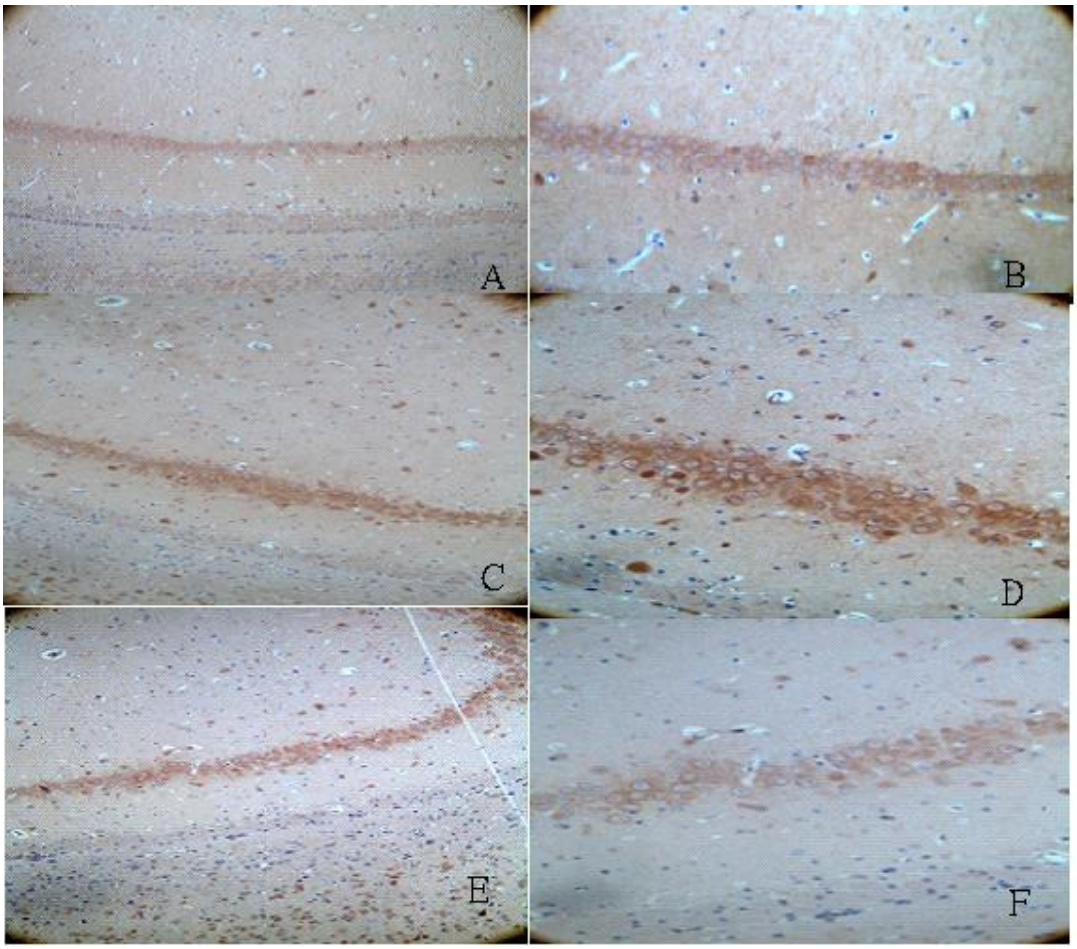
Figure（**3**）Brdu fluorescent staining positive cells are BMSCs;

2.5 Nogo-A and NgR Immunohistochemical Stain Result in Various Hippocampal CA1 Regions

Brown granules can be seen in neurons cell membrane of hippocampal CA1 region, indicating that there are both Nogo-A and NgR expression; The expressions of Nogo-A and NgR in BMSCs transplantation group and model group were significantly higher（$P<0.05$） than those of sham operated group;tThe expressions of Nogo-A and NgR in BMSCs transplantation group were significantly lower（$P<0.05$）than those of model group ;



Figure（**4**）Nogo-A Immunohistochemical Stain Result：A(DAB×100) and **B**(DAB×200) : the expression of Nogo-A in sham operation group ; **C**(DAB×100) and **D**(DAB×200): the expression of Nogo-A in model group ; **E**(DAB×100) and **F**(DAB×200) :the expression of Nogo-A in BMSCs transplantation group ;



Figure（**5**）NgR Immunohistochemical Stain Result：A(DAB×100) and **B**(DAB×200) : the expression of NgR in sham operation group ; **C**(DAB×100) and **D**(DAB×200): the expression of NgR in model group ; **E**(DAB×100) and **F**(DAB×200) :the expression ofNgR in BMSCs transplantation group;

Table 2. Optical density resuit

GROUPS	Nogo-A	NgR
Model	170.15±4.41*	178.56±7.56★
BMSCs	145.76±3.94△	136.29±3.56▲
Sham	125.50±3.02□	115.62±4.45■

*compare with△P<0. 01；*compare with□P<0. 01；△compare with□P<0. 01；★compare with▲P<0. 05；★compare with■P<0. 01；▲compare with■P<0. 01；

3. **Discussions**

In this study, cone cells of hippocampus in transplantation group are more chaotic arranged than model group in HE stain result. The mean escape latency of BMSCs transplantation group was significantly shorter（P<0.01）and the frequency of crossing the former platform was significantly higher （P<0.05） than those of model groups in the Morris water maze ,the model group and BMSCs transplantation group both exhibited cognitive function deficits than sham operated group, but BMSCs transplantation group was better than model group. Brdu fluorescent staining positive cells were observed in hippocampal tissue of BMSCs transplantation group, indicates that the transplanted BMSCs can migrate, raise, and survive into the damaged area of cerebral ischemia. Indicates that BMSCs transplantation effectively promote cognitive function for the rats of chronic cerebral ischemia.

The damaged central nervous system is difficult to form effective regeneration, which may be related to inadequate secretion of nerve growth factor and over-expression of nerve growth inhibitory factor, the vast majority of stem cell transplantation researches

currently are focused on promoting nerve growth factor expression, while there are few studies on nerve growth inhibitory factor. Nogo-A has been confirmed to be the strongest axonal growth inhibitory factor, through p75NTR and NgR receptor, it plays inhibitory effect [8]. After integrating Nogo-A and NgR-p75 complex, acting on Rho-kinase-RhoA pathway increases the level of RhoA, leading to retraction of growth cone cell body, reducing simultaneously the levels of the other two members of the Rho family, namely, Rac1 and Cdc42 level, can retract the filopodium and pseudopodia on growth cones, NgR also can make the Rac1 and Cdc42 change to the RhoA, and thus leading to growth cone collapse [9]. Studies found that the usage of neutralizing Nogo-A antibody or NgR antagonist effectively reduce the Nogo-A and NgR expression after nerve injury, and induce long-distance axonal regeneration, increasing structural plasticity and promoting functional recovery [10,11]. The above study suggests that reducing Nogo-A and N gR expression can promote synapse regeneration and reduce nerve injury, with the neuroprotective effect.

In the paper, it can be seen that expression of Nogo-A and NgR protein in hippocampal nerve cells of model group increased. This may be involved in the rat's cognitive dysfunction, which is coincident with the few documentary reports [12] [13]. In this study, Nogo-A and NgR protein expression in hippocampal of transplantation group was less than those of model group, indicating that BMSCs transplantation may promote the rebirth of axon of damaged nerve cells and then improve the cognitive functions by lowering down Nogo-A and NgR protein. Possible mechanism of the lower down of Nogo-A and NgR protein expression after BMSCs transplantation may lie on those: 1: BMSCs may inhibit relating inhibitive factor of myelin sheath by adjusting the expression of MMP-1, for example, the generation of Nogo-A [14]. 2: BMSCs may also be able to improve the expression of synaptophysin, which will make the feedback to inhibition to the generation of Nogo-A [7]. In the spinal cord injury model and cerebral infarction model, BMSCs transplantation may protect nerve function, and it also can be seen that it reduced the expression of Nogo-A and NgR [6] [7] at the same time, which is similar to the result of this study.

Suppression and regeneration mechanism of central nervous system is complicated. There are lots of factors which may promote the regeneration or inhibit the growth of nerve cells in the central system, and specific mechanism affecting the learning and memory activities is still not completely clear. And the promoting effect of bone marrow derived mesenchymal stem cells to cognitive function may be a result of co-working of various approaches, Nogo-A and NgR is just one of them, and there are still many factors affected by bone marrow derived mesenchymal stem cells in the upstream and downstream. As for how bone marrow derived mesenchymal stem cells acts on Nogo-A and NgR and whether it brings into play via other factors, further study is still in need.

Conclusion:

We have for the first time to expole whether improvement of cognitive function by BMSCs transplantation is related to decreased NgR and Nogo-A expression.We find that BMSCs transplantation effectively promote spatial learning and memory ability for the rats of chronic cerebral ischemia,which maybe induced by the decrease of proteins Nogo-A and NgR.

Corresponding author:
Dr. Yuming Xu, xuyuming@zzu.edu.cn.
Mailing address:1 East Jianshe Road，Zhengzhou, Henan province, 450052, P. R. China

5.**References**

[1] Huesa, G., Baltrons MA,Gomez-Ramos P, et al. Altered distribution of RhoA in Alzheimer's disease and AbetaPP overexpressing mice[J]. J Alzheimers Dis, 2010. 19(1): p. 37-56.
[2] Cafferty, W.B.J., et al., MAG and OMgp synergize with Nogo-A to restrict axonal growth and neurological recovery after spinal cord trauma. J. Neurosci. 2010.30 (20),p. 6825–683
[3] ChenM S, Huber AB, Schwab M E *et al*. Nogo-A is a myelin Associated neurite outgross inhibitor and an antigen for monoclonal antibody IN 21. Nature, 2000; 403 (6768) :p. 434- 439
[4] Budel, S., et al., Genetic variants of Nogo-66 receptor with possible association to Schizo phrenia block myelin inhibition of axon growth. J. Neurosci. 2008 28 (49), p.13161 –13172.
[5]WangH,YaoY,JiangX,et al.Expression of NogoA and NgR in the developing rat brain after hypoxiaischemia. BrainRes, 2006; 1114 (1) :p. 212-220□.
[6]Chihiro Tohda , Tomoharu Kuboyamae,et al.Current and future therapeutic strategies for functional repair of spinal cord injury.Pharmacology & Therapeutics;2011 (132) p.57–71
[7] Shen LH, Li Y, Chen J, et al. One-year follow-up after bone marrow stromal cell treatment in middle-aged female rats with stroke[J]. Stroke;2007,38(7):p. 2150—2156.
[8] Kilic E, ElAli A, Kilic U, et al. Role of Nogo-A in neuronal survival in the reperfused ischemic brain. J Cereb Blood Flow Metab 2010;30:p. 969–984.
[9] Feras Akbik, William B.J. Cafferty,et al., Myelin

associated inhibitors: A link between injury-induced and experience-dependent plasticity,Exp. Neurol; 2011, 10(1016).
[10]Tianzhu Wang, Jing Wang, Cheng Yin,et al. Down-regulation of Nogo receptor promotes functional recovery by enhancing axonal connectivity after experimental stroke in rats. BrainRes ;2010 (1360);p. 147-158
[11]Freund, P, et al;Anti-Nogo-A antibody treatment promotes recovery of manual dexterity after unilateral cervical lesion in adult primates re-examination and extension of behavioral data. Neurosci;2009. 29 (5), p. 983–996.
[12] Heather D. VanGuilder , Julie A, et al;Hippocampal dysregulation of synaptic plasticity-associated proteins with age-related cognitive decline；Neurobiology of Disease；2011(43) p.201–212.
[13] Rebecca L. Gillani, Shih-Yen Tsaic, et al; Cognitive recovery in the aged rat after stroke and anti-Nogo-A immunotherapy BrainRes 2010 (208)p. 415–424.
[14] Wright KT, El Masri W, Osman A, et al.;Bone marrow stromal cells stimula te neurite outgrowth over neural proteoglycans(CSPG), myelin associated glycoprotein and Nogo-A [J]. Biochemical and Biophysical Research Communications，2007,354:p. 559—566.

2/22/2012

Nurses' Performance, isolation policy and HCV Sero-conversion among Hemodialysis Patients in Egyptian Hospitals

Sahar Yassin[1], Manal El Dib[2] and Dalia Roshd[2]

Departments of [1]Public Health and [2]General Medicine, Faculty of Medicine, Cairo University, Egypt
Yassins1966@yahoo.com

Abstract: HCV infection remains highly prevalent both in developed and less-developed countries. In spite of considerable decline in the incidence and prevalence of HCV infection among HD patients in many countries, this infection still remains a major problem among patients on maintenance HD especially in Egypt. Clearly, strict adherence to standard precautions including isolation precautions is the key stone in the prevention of nosocomial transmission of HCV in hemodialysis units. **Study design and aim of the work**: This study was conducted in the HD units at four Egyptian hospitals, two public and two private hospitals. First phase; cross section – exploratory phase to document nurses' knowledge, practice and application of isolation policy in the units. Second phase; prospective phase to the HCV sero-negative cases to detect sero-conversion rate. **Statistical analysis:** the public units (Group A) was compared with private units(group B) using suitable significant tests with significant level P value<0.05, calculation of HCV period prevalence, incidence and incidence density. **Results:** group B units follow isolation policy for HCV positive patients while group A units don't, nurses training sessions, knowledge and practices are significantly better in group B units than in group A, seroconversion rate is higher significantly in group A units than in group B. **Conclusion and recommendations:** HDUs that conform to policies and regulation related to infection control and isolation of HCV sero-positive cases had significant low incidence rate for HCV sero-conversion.

[Sahar Yassin, Manal El Dib and Dalia Roshd. **Nurses' Performance, isolation policy and HCV Sero-conversion among Hemodialysis Patients in Egyptian Hospitals.** Life Science Journal. 2012;9(1):740-749] (ISSN:1097-8135). http://www.lifesciencesite.com.107

Key words: HCV seroconversion, isolation policy, hemodialysis units, infection control standard

1. Introduction:

Hepatitis C virus (HCV) infection is especially problematic in patients with end-stage renal disease (ESRD) who are undergoing hemodialysis (HD) (1). The prevalence of HCV infection is higher among HD patients than in the general population, and several routes of transmission are thought to originate from HD units (1, 2). There is wide variation in the prevalence of HCV infection among different HD units and countries as shown by Dialysis Outcomes and Practice Patterns Study (DOPPS). The mean prevalence of HCV in different HD facilities is 13.5% and varied among countries from 2.6% to 22.9% (3). On the other hand **Kellerman and Alter,** mentioned that; the worldwide data suggest that the prevalence of hepatitis viruses among chronic renal failure (CRF) cases could be as high as 80% (4).

Zakaria reported that Egypt has the highest countrywide prevalence of hepatitis C virus in the world. This prevalence exceeds other countries in the region and elsewhere with comparable socioeconomic living conditions (5). At the community level, Egypt Demographic and **Health Survey** (2008) showed that the percentage of HCV antibody positive cases was 14.7% and the percentage positive on HCV RNA test was 9.8% (6). In Egypt the prevalence ranges from 13-48% among HD units patients; MOH report (Egyptian Ministry of Health), unpublished data 2006. The main reasons for high incidence of infections is the high prevalence of HCV infection in the general population, lack of standard of infection prevention precautions and effective vaccination, inadequate disinfection procedures of dialysis machines and other medical equipment, as well as spread of infection from patient to patient, especially in dialysis centers with a high percentage of infected patients (7,8). **A.Mele et al said that** "A high prevalence of infected patients with HCV in the dialysis setting increases the risk for HCV nosocomial transmission" (9)

HCV infection is associated with greater mortality (10-12). As a cause of death, hepatocellular carcinoma and liver cirrhosis are significantly more frequent among anti-HCV-positive than anti-HCV-negative dialysis patients (12). Risk factors for HCV infection in dialysis patients include number of blood transfusions, duration of HD, and mode of dialysis, prevalence of HCV infection in the dialysis unit, previous organ transplantation, intravenous drug use, male gender, older age, and nosocomial transmission of HCV in HD units (13-16). Clearly, strict adherence to standard precautions "universal precautions" and careful attention to hygiene are the key stone in the prevention of nosocomial transmission of HCV in

hemodialysis units (17) . However, those hemodialysis units with a high HCV prevalence or in which there is no fulltime infection control personnel dedicated to the infected patients during the hemodialysis sessions, could have a greater risk of sero-conversion. Therefore, isolation in special units or dialyzing patients in specific sessions must be considered (18). The role of service providers especially nurses in to assure following the standard precautions is crucial in reducing the prevalence of HCV infection in the HD units in hospitals. Periodic assessment of the prevalence and incidence of HCV infection among HD patients presents important indicators for compliance of the service providers towards the standards of infection prevention in hospitals.

The study objectives:

1- Identify the period prevalence of HCV infection in the HD units,
2- Assess the nurses' knowledge about the standard of infection control precautions in Hemodialysis Units,
3- Explore the nurses' practices regarding the infection prevention precautions in Hemodialysis Units,
4- Identify incidence of sero-conversion of viral hepatitis C infections among hemodialysis patients,
5- Examine the association between following the standards of infection prevention precautions by HDUs with the frequency of occurrence of HCV infection.

Study hypothesis:

HDUs that fulfill standards of infection control and having nurses with adequate knowledge and skills and follow the standard precautions to prevent infection in HD units especially isolation of HCV positive patients, could contribute in reducing the incidence of HCV infections among HD patients indicated by sero- conversion rate.

2. Methodology:

Study setting:

This study was conducted in the Hemodialysis Units at four Egyptian hospitals .Two of the selected hospitals were public general hospital that provide fee-free services. The other two hospitals are private hospitals. The study was conducted in the HD units of the selected hospitals. The investigators considered that private hospitals have good surveillance system and ensure compliance of service providers to standard infection control precautions in the HD units.

Study population:

All renal failure patients admitted to the hemodialysis units of the four hospitals from January to December 2008 and investigated for viral markers at least three times (one at the time of admission and the others each 3 months) had been included in the study. All nurses working in the HD units in the four hospitals had been included in the study.

Study design:

The study was composed of two phases:

1- **Cross section – exploratory (situation analysis)phase:**

The objectives of this phase were to assess the prevalence of HCV infection among HD admissions, and to assess the level of nurses' knowledge and the current practices regarding commitments to the standards of infection prevention protocols.

Sample Size and Techniques

-HD patients: All admissions to the HD units in the four hospitals throughout a period of January to December 2008 had been included in the study.
-Nurses: All nurses working in the HD units in the four hospitals had been included in the study

Types and Sources of data: quantitative data had been collected through:

-Direct observation of the HD unit work environment, equipment and operations related infection control (including isolation policies)
-Structured interview with patients,
-Reviewing patients' hospital records, especially lab investigations related to HCV infection (anti-HCV antibodies detected by ELISA)
-Structured interview with nurses to identify levels of knowledge regarding infection control
-Direct observation of operations using a checklist to assess the nurses' performance.

Data collection Instruments:

- Checklist for HD unit that included items related to availability and proper operation of equipment and isolation policies.
- A questionnaire form had been used to identify the demographic characteristics, Exposure to risks of hepatitis C infection among hemodialysis patients. The form included items to be completed from the patients' records regarding lab investigation findings from time of admission and at a frequency of every three months for HCV infection.
- A questionnaire to assess nurses' knowledge concerning route of transmission, methods of effective prevention of hepatitis infection and previous training in infection control.
- Observational check list to record performance of nurses regarding application of isolation policy, methods of disinfection and the

conforming to standard precautions recommended by CDC (19).

Methods of Data Collection:

- Observation and recording information about HD unit facilities for infection control
- Structured interview with patients was done using the patient questionnaire form. The form included also information derived from the patients' hospital records regarding anti-HCV detected by ELISA
- Structured interview with nurses to assess their knowledge about standards infection control in HD units
- Direct observation of operations by observing and recoding nurses' performance according to standard checklist

Data analysis plan:

The analysis of data derived from the first phase was aiming at allocating patients into two groups and hospitals into two groups:

Patients' groups:

1. Seropositive cases: they are the patients who showed positive serological test to HCV at time of admission to the hospital.
2. Sero-negative cases: they are the patients who showed negative results by ELISA at time of admission to the hospital.

Hospital Groups:

Group A: The two hospitals that showed less satisfactory fulfillment of standard requirements for HDU-infection control facilities and didn't apply isolation policy for positive HCV patients.

Group B: The two hospitals that showed satisfactory fulfillment of standard requirements for HDU-infection control facilities and applied isolation policy for positive HCV patients.

2- Prospective/follow up phase to the HCV sero-negative cases

The follow up phase was 12 months in duration. It started from January to the end of December 2009. During this phase, all the records of the sero-negative patients determined at the first phase were reviewed every 3 months to check for sero-conversion. (It is to be noted that the serological analysis is done routinely every 3 months in the four units for all patients.)

The objectives of this phase were to detect the frequency/time of sero-conversion of sero-negative cases detected in the first phase, study the characteristics of patients with sero- conversion and association between the frequency of sero-conversion, and performance level of the HDUs in Infection control especially adoption of HCV isolation policy.

Data management and statistical analysis:

The data was coded and entered using the statistical package SPSS version 16. The data were summarized using descriptive statistics: mean, standard deviation, minimal and maximum values for quantitative variables and percentage for qualitative values. Statistical differences between groups were tested using T- test for quantitative normally distributed variables, Nonparametric Mann Whitney test for abnormally distributed quantitative variables and Chi Square test for qualitative variables. P-value less than 0.05 were considered statistically significant.

Period prevalence and sero-conversion rate of HCV were calculated.

Cumulative incidence and incidence density were calculated to describe the rate at which new infections are occurring. The duration of exposure to HD/days had been calculated for each patient throughout the study period to calculate the incidence density.

Incidence density= Number of new cases (sero-conversion) during a given period * 10000
Total person-days of exposure to dialysis sessions

Comparison between groups considered the independent variables (patients' characteristics, Type of HDUs regarding commitments to infection control standards and the independent variables (HCV sero-conversion).

Ethical consideration:

All the included subjects were treated according to the Helsinki Declaration of biomedical ethics (20) Written consent was obtained after proper orientation of the subjects regarding the objectives of the study. Presentation of the results for all dialysis units, hospitals and for all patients was anonymous, to be sure of data confidentiality.

3. Results:

Information derived from the study had been presented in three main themes:

1- HD Units' Facilities for CDC Standard for Infection Control:

Findings from study the HD units in the four hospitals came to categorize the two public hospital dialysis units as "Group A HDU" (57 dialysis machines) they have dedicated machines (two at each unit) for HBV positive patients but they doesn't follow isolation policy for HCV positive patients. The two private dialysis units Group B units" (49

dialysis machines) apply isolation policy for both HBV (two at each unit) and HCV positive patients (five machines at each unit). Group A units use chemical disinfection as rinsing of the machine at the end of the day, while in group B units they rinse the machine using heat disinfection after each patient.

The total number of nurses in group A HDUs was 38 nurses while at group B units they were 36 nurses. The ratio of nurses to HDU machines was 10:15 in the Group A HDUs, and 10:14 in the Group B HDUs

2-**Nurses' Performance in infection control in HDUs:**

Table (1) shows that the proportion of nurses in group B HDUs who received training courses on infection control was significantly higher than that for nurses of group A HDUs. The table shows also that significant proportions of Group B HDUs compared to Group A HDUs nurses had demonstrated satisfactory performance related to application of the standard precautions and CDC guidelines

3- **Nurses' knowledge about Infection control in HDUs:**

Table (2) illustrates the nurses' knowledge score about the mode of transmission of HCV among dialysis patients. They were asked to rank the mode of transmission to "one" as least likely and "10" as the most likely. In group B units nurses ranked "nurses practice" then "blood transfusion" as highly responsible for HCV transmission while group A nurses ranked "dialysis in other center" as the highest cause responsible for HCV transmission, nurses in group B showed significant better scores in several points.

4- Prevalence of HCV infection among HDUs' patients at time of hospital admission:

During the first phase of the study, 957 patients were undergoing hemodialysis but the study including only 914 (484 from general hospitals [group A] and 430 from the private hospitals [group B] and 43 patients were excluded either due to their admission less than 3 months or their fate were unknown. Accordingly the average number of patients per machine per year (turnover) was 8.5 patients /machine for group A and 8.88.5 patients /machine for group B. Figure (1) demonstrates that out of the total HDUs patients (n=914), 531 patients (58%) were HCV antibodies positive. There was no statistically significant differences between Group A (59%) and Group B (57%) regarding the prevalence of HCV infection at the time of HDUs admission. chi-square=0.42, P value <0.52. This considers the period prevalence of HCV antibodies among patients attending dialysis units within a period of one year (From January to December 2008).

5- HCV Sero-conversion among HDUs' patients:

The sero-negative patients (group A=198, group B=185) identified during the first phase of the study had been followed up for 12 months and all patients were tested at least twice for HCV antibodies by ELISA. Group A (with no isolation of HCV infected patients) had 46 seroconverted patients (23%) i.e. was negative at the start of this period then become positive for HCV antibodies while group B (with isolation of HCV infected patients) had 24 seroconverted patients (13%) with chi-square=6.74, P value = 0.009 with odds ratio (95% CI)= 2.03(1.18-3.49) (Figure 1).

Information pertaining to the age and sex structure of HCV sero-negative cases for the studied two groups of HDUs patients is illustrated in Table (3). The table shows that more than half of sero-negative cases were in the age group 50 - <70 years old. The proportion of males is nearly equal to females regarding the prevalence of HCV infection. There were no statistically significant difference between the Group A and Group B HDU regarding the percent distribution of patient with HBV negative ELISA results at time of admission in the study.

The characteristics of HDUs' patients whose reports showed HCV sero-conversion had been illustrated in Table (4). It is obvious from the table that the incidence of HCV sero-conversion across the age groups indicates that the age group 30-<50 had reported the highest incidence compared for other age groups and for both group A patients (32.4%) and group B patients. Nevertheless, there were no statistically significant differences regarding the incidence of sero-convergence at different age groups for the studied group A and B HDUs' patients. However, Group A had reported a significantly high incidence of sero- conversion among males (25.7%) than males in group B (10.4%) (p=0.005).

Table (5) shows that, different percentages of both group A and group B seroconverted patients gave a history of exposure to risk factors for HCV infections especially blood transfusion (more than 60%). However, there was no statistically significant difference between the two groups of patients regarding the proportion of seroconverted patients who had been exposed to the three types of risks (i.e. blood transfusion, surgical procedure and /or dental care).

Estimation of the incidence density rate was done by calculating number of seroconverted patients in relation to the sum of duration of exposure to HD services (from starting day of dialysis of each patient to the end of study period). Table (6) The incidence density of sero-conversion was significantly higher among Group A (18.9/10000) than group B (5.01/10000).

Figure (2) Added another dimension to HCV nosocomial infection. The figure illustrate the cumulative percentage of positive anti-HCV antibody cases of all studied patients according to duration of dialysis (from January 2008 to December 2009) it shows that patients on dialysis for one year had 16.7% positive cases while patients with five years dialysis had 51.1% positive cases.

4. Discussion

HCV infection remains highly prevalent both in developed (21) and less-developed countries (22). In spite of considerable decline in the incidence and prevalence of HCV infection among HD patients in many countries, this infection still remains a major problem among patients on maintenance HD (23, 24) especially in our country. Patients undergoing hemodialysis are at the highest risk of becoming infected with HCV, HBV and HIV. While infection with HCV was identified more frequently than HBV and HIV (25). Also varying prevalence rates have been reported from different Middle Eastern countries and the figures have varied from as low as 26.5% from Oman to as high as 80% from Egypt (26).

Table (1): Percent of Nurses in the two studied groups according to history of training and observed performance in infection control in the HDUs

	Group A Nurses (No=38)		Group B Nurses(No=36)		X^2	*P* value
Previous infection control training courses	21	55.2%	29	80.6%	5.4	0.02
Clean surface of machine after each patient	21	55.2%	34	94.4%	14.9	<0.001
Clean tables after each patient	18	47.4%	29	80.6%	8.79	0.003
Change gloves after each practice	5	13.2%	25	69.4%	24.3	<0.001
Sharing supplies between patients	38	100%	30	83.3%	0.01*	<0.001
Place supplies on the top of dialysis machine	32	84.2%	12	33.3%	19.85	<0.001

*Fisher exact test was used as there is one cell with less than 5 observations

Table (2): Achieved mean score of knowledge about items of infection transmission in the HDUs for the two studied groups of nurses in the four hospitals.*

Items of nurses' opinion	Mean ± SD of Nurses opinion		t-test	P value
	Group A	Group B		
Nurses think that blood transfusion is responsible for the infection	6±4	9±2	3.43	0.001
Nurses think that HCV is transmitted by contaminated hemodialysis machine	6±2.9	7±3.5	1.34	0.18
Nurses think that HCV is transmitted by contaminated hemodialysis chairs	3.6±3	3.9±3	0.43	0.67
Nurses think that HCV is transmitted by contaminated dialysis tables	4±3	4±3	0.00	1.0
Nurses think that nurse's practice which transmit the HCV from patient to patient	5.8±3.6	9.6±3.4	4.66	<0.001
Nurses think that HCV is transmitted by sharing supplies and instruments	2.8±3	6±4	3.91	<0.001
Nurses think that HCV is transmitted by dialysis in other centers	7.8±2.7	6.3±3.2	2.17	0.033
Nurses think that HCV is transmitted by contaminated food	2.9±2.8	3.6±3.2	1.0	0.319

*They were asked to rank their opinion on scale of 10, as 1 is the least likely and 10 as the most likely.

Table (3): Percent distribution of HCV sero-negative HDUs patients in Groups A and B by age and sex

Age group (year)	Group A (–ve) HCV patients (198)		Group B (–ve) HCV patients(185)		X^2	P value
< 30	17	8.7%	18	9.7 %	0.17	0.98
30- <50	37	18.8%	35	18.1 %		
50- < 70	104	52.4%	95	51.3 %		
> = 70	40	20.1%	37	19.9 %		

Table (4) Percent of HCV sero-converted patients by age, sex and exposure to risk factors for the two studied A and B HDUs groups

Items	Group A Seroconverted (N0=46)		Group B Seroconverted (No=24)		X^2	P value
Age group (years)						
< 30	2/17	11.8 %	1/18	5.5 %	0.6	0.5
30- <50	12/37	32.4%	6/35	17.1%	2.24	0.13
50- < 70	25/104	24.0%	15/95	12.6%	2.10	0.14
> = 70	7/40	17.5 %	2/35	14.2%	0.16*	0.11
Sex						
Males	**26/101**	**25.7%**	**10/96**	**10.4%**	**7.74**	**0.005**
Females	**20/97**	**20.6%**	**14/89**	**15.7%**	**0.74**	**0.38**

*Fisher exact test was used as there is one cell with less than 5 observations

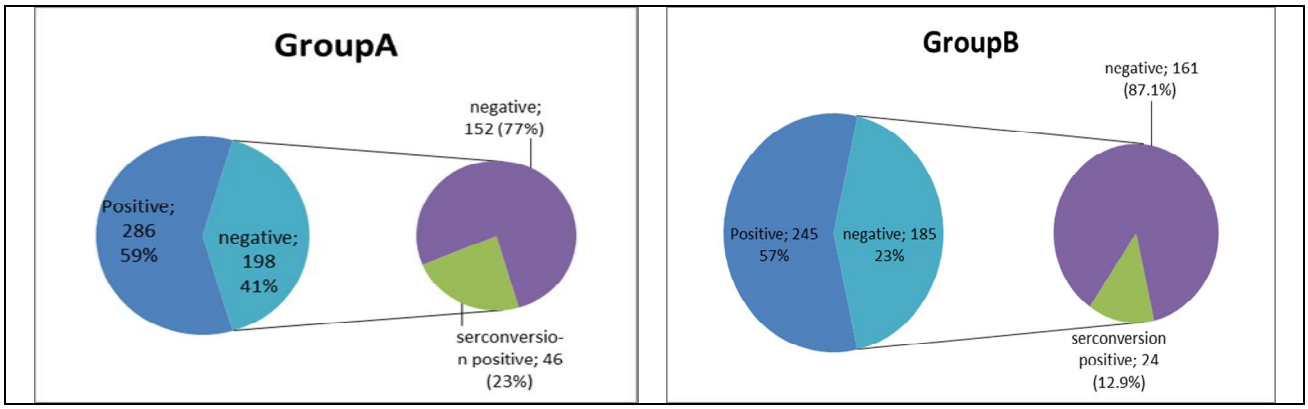


Figure (1) Period prevalence and Incidence of +ve sero-conversion among HD patients in the study groups (group A=484 group B=430)

Table (5) Percent of HCV sero-converted patients by age, sex and exposure to risk factors for the two studied A and B HDUs groups

Exposure to HCV risk of Infection	Group A Seroconverted (N0=46)		Group B Seroconverted (No=24)		X^2	P value
Blood transfusion	30 / 46	(65%)	16 / 24	(66.7%)	0.01	0.9
Surgical procedures	2 / 46	(4.3%)	1/ 24	(4.1%)	1.0*	0.97
Dental care	7 / 46	(15.2%)	4 / 24	(16.7%)	0.03	0.87

*Fisher exact test was used as there is one cell with less than 5 observations

Table (6) Incidence density of HCV sero-conversion for the studied group A and B of HDUs' patients

Group A (198 sero-negative patients)			Group B(185 sero-negative patients)			X^2	P value
HCV Seroconverted patients	Total HD days	Incidence density	HCV Seroconverted patients	Total HD days	Incidence density	32.12	<0.001
46	24314	18.9 /10000	24	47885	5.01 / 10000		

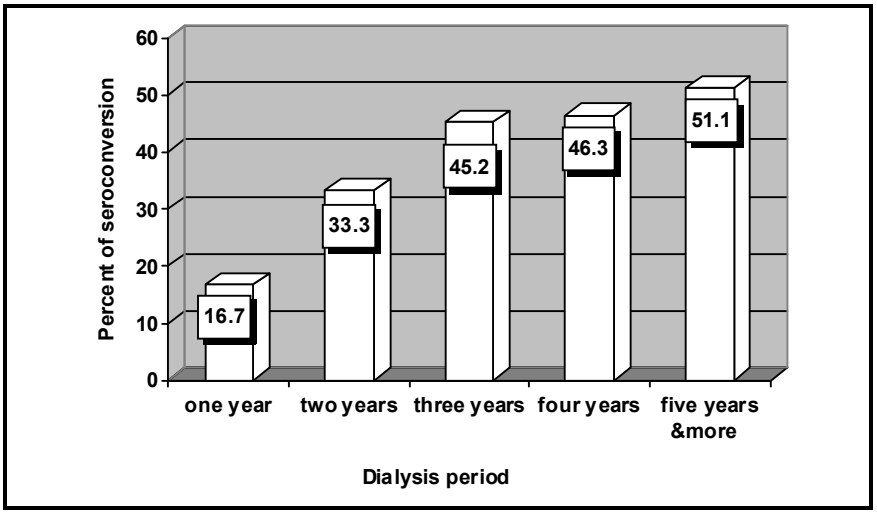


Figure (2) Cumulative percentage of HCV seroconverted patients according to the duration of exposure to dialysis sessions

In the current study, the prevalence of HCV infection among HD patients (59% in group A units while 57% in group B units) using ELISA technique, was higher than that seen in other representative study conducted at Egyptian HD units using random sample of MOH dialysis units composed of 48 HD units on the average the prevalence was 52% (27).. The high prevalence of hepatitis C in Egyptian studies is in concordance with high prevalence of the HCV infection in Egypt. **Frank *et al.***. reported that Egypt has the highest countrywide prevalence of hepatitis C virus in the world (28). A study conducted in Egypt showed that the prevalence of anti-HCV was 24.5% (5). **Another study** reported that 78% of

patients with ultrasound-detected cirrhosis of Egyptian cross sectional survey were anti HCV positive in the Nile Delta [29]. On the other hand in **Kosova a** study was conducted on 583 ESRD patients on maintenance HD from six HD centers, they reported an anti-HCV antibody prevalence rate of 43%(30`). In a descriptive study conducted in Iran, the prevalence of positive HCV antibodies decreased from 14.4% in 1999 to 4.5% in 2006 (31). In Izmir, Turkey, **the** sero-prevalence of HCV infection was 19% among 437 HD patients (32). While in Salvador, Northeastern Brazil , the anti-HCV seroprevalence among HD patients was 10.5% (33). At Northern Part of Iran, 18.4% of 163 studied HD patients were found positive for anti-HCV antibody by ELISA (34). In a descriptive study conducted at northern Iran, they mentioned that; the prevalence of HCV infection in HD patients has decreased significantly during the past decade in most HD units in the province of Mazandaran,; in December 2001, the prevalence of antibody against HCV was 18%, whereas by December 2006, it was 12% (35). In Saudi Arabia, **study was conducted on** 180 patients at HD unit, 34 (18.9%) patients were positive for anti-HCV antibody (36). While another study, in Casablanca reported higher rate, a retrospective study performed on 186 chronic HD patients, reported a high prevalence of HCV infection (76%) (37). In the study performed by **Silva.** HCV-RNA was detected in 92 (73.6%) of 125 anti-HCV-positive patients (31). **Dattolo** showed that HCV-RNA was positive in 18 (75%) of 24 anti-HCV-positive subjects (38). As confirmed in many studies that the spread of HCV infection in HD units is mainly due to nosocomial transmission from patient to patient (39-47). The importance of this route of transmission is evidenced by the high HCV prevalence in some HD units.(48) In our study after prospective follow up of the antibody HCV negative patients group A units 46 patients out of 198 (23%) suffered from seroconversion (Fig.1) with incidence density 18.9/10000 patient's days; while in group B units out of 185 patients 24 (12.9%) were HCV seroconverted with incidence density 5.01/10000 patient's days (*P* value <0.001) Table 6 By checking the isolation policy at each group, it was noticed that group A units doesn't follow isolation policy for HCV positive patients while group B units adopts HCV isolation policy. However, there is no consensus on the necessity of infection control isolation of HCV positive patients as they conclude that strict adherence to the universal infection control precautions is enough to fully prevent HCV nosocomial transmission (49-54), but recently, some reports have recommended the adoption of infection control isolation measures in centers with high HCV prevalence (55-56) which is applicable on our country situation and as recommended in January 2007 by Egyptian **Ministry of Health (MOH)**, Infection Control Program in Egypt (**ICP**) (57). A study conducted on a representative sample of 48 Egyptian HD units recommended adoption of isolation policy; as they found significant higher incidence rate of HCV seroconversion among units which do not adopt isolation policy (206/4154) comparing with (61 / 3989) at units adopt isolation policy; with p value < 0.01.(28) So, the usage of dedicated machines for HCV infected patients in a defined area of the unit is important, provide that they are attended by devoted personnel to avoid nosocomial transmission of HCV to uninfected patients (58,59). Although hemodialysis machines can act as vertical HCV transmission vectors (53,60), their disinfection can be adequately performed (61 - 64), as it was noticed in our study that all nurses in group A units use chemical disinfection to rinse the machine at the end of the day. While group B units' policy, is using heat disinfection to rinse the machine after each patients. As was mentioned in APIC guideline 2010; there are two methods of disinfecting the dialysate pathways (internal) of the HD machine: heat and chemical. The standard as recommended by HD machine manufacturers is to perform disinfection of the dialysate pathways at the end of each treatment day using heat disinfection. (65).However, nowadays, vertical HCV transmission by monitors is exceptional, being the horizontal patient-to-patient transmission the most important; CDC (66) has recommended a training and educational program for HD personnel before they begin working in the units. From this study, it was interest to find only 55.2% of group A units' nurses received infection control sessions while group B nurses 80.6% had previous training on infection control with p value =0.02. This could explain practices noticed in group B nurses were significantly better than group A nurses' practice (Table 1) as cleaning surfaces of the machine and tables after each patient, 69,4% of nurses in group B changes gloves after each patients while 13.2 % from group A did, 33.3% of group B places supplies on the top of the dialysis machine which is forbidden by CDC recommendations but they are significantly lower than group A percentage (84.2%). In spite of the significant difference between the two groups in sharing supplies between patients both had very high percentage in a contraindicated practice (Group A 100%, Group B 83.3%) this poor practice share in increasing of the probability of HCV transmission and positive serocoversion of HD patients so as mentioned in other study we conclude that inadequate application of standard precautions primarily responsible for spread of the infection in HD units (66-67). Thus, lack of proper and targeted

education could explain nurses' perception about how HCV is transmitted. The knowledge of group B nurses is significantly better in considering nurses practices are involved in transmission of HCV from patient to patient, also in using shared instruments and supplies and finally in considering blood transfusion one of the important method of HCV transmission while in contrary nurses in group A significantly consider HCV is more transmitted by dialysis in other center while there is no significant difference between two groups concerning other modes of transmission. In our study we tried to find other significant different risks between Group A units and group B units associated with seroconversion; (Table 3) Showed no significant difference between age groups in both study groups concerning HCV negative patients (patients have been followed in the prospective part of the study). Tab. 4 showed that no significant difference in HCV seroconversion rate between each age group in the studied units after one year follow up, similar finding was reported by Bassem etal in Egypt (28). While in the current study an increasing cumulative seroconversion was found ranging from 16.7% among patients who were on dialysis for one year to 51.1% among those on HD for five or more years (figure 2). This was similar to the findings of a study conducted in Taiwan; the annual incidence of HCV infection was 14.6% reaching a cumulative prevalence of 60% after six years [68]. In another one in Saudi Arabia in which a significantly increasing annual prevalence of HCV infection among dialysis patients was found. It ranged from 16.4% among patients who were on dialysis for one year to 94.5% among those on dialysis for three or more years. They added that the annual HCV seroconversion rate in the Kingdom of Saudi Arabia was 7 to 9%[69]. In Kuwait, it was estimated that there is an annual increment in the incidence of positive anti-HCV seroconversion that is equal to 11.5% of patients on dialysis [70]. On the other hand, the current study showed that; there is no statistically significant difference between studied groups of units, concerning sex distribution among HCV negative patients (Table 3). While concerning seroconversion after one year, we found significant difference between A & B groups of units in males only while no difference found between females (Table 4). Concerning other risk factors for HCV transmission we didn't find significant difference between the study groups A&B; as blood transfusion, surgical procedures and dental care (Table 5).

The strengths of the study were related to the selection of the topic of HCV as a nosocomial infection among the vulnerable patients with high frequency of exposure to the risk of HD. The study design considered estimation of the incidence of HCV sero-conversion as indicator of nosocomial infection in HDUs. Despite testing the knowledge and practice of nurses in the studied HDUs, the study considered nosocomial infection as multifactorial where service providers' performance as well as infection control policies and regulations are pivotal in determining the incidence of seroconversion. The study raised an important issue related to disparity regarding access to quality health services and acquiring HCV infection. The Group A-HDUs in public hospitals with fee-free service, being not conforming to quality infection control standards, especially isolation of HCV positive patients, thus exposes poor patients to the risk of HCV infection.

Limitations of the study is related to depending on the hospital regulations regarding frequency and type of lab tests. Serological test for anti-HCV antibody were performed routinely each three months on the recruited HD patients using third generation enzyme-linked immunosorbent assay (ELISA) kit. With no consecutive confirmation of positive cases by polymerase chain reaction (PCR) or recombinant immunoblot assay (RIBA), as recommended by CDC (70)

Conclusion and recommendations:

The study concluded that HDUs that conform to policies and regulation related to infection control and isolation of HCV sero-positive cases had significant low incidence rate for HCV sero-conversion.

The study recommended that, surveillance system should include both ensuring conforming to CDC policies and regulations related to infection control in HDUs, as well as periodic estimation of the incidence of HCV seroconversion, for timely decision making to improve the quality of services.

Corresponding author
Sahar Yassin
Departments of [1]Public Health and [2]General Medicine, Faculty of Medicine, Cairo University, Egypt. Yassins1966@yahoo.com

References:

1. Fabrizi F, Poordad FF, Martin P (2002). Hepatitis C infection and the patient with end-stage renal disease. Hepatology, 36(1):3-10
2. Fabrizi F, Lunghi G, Ganeshan SV, Martin P, Messa P (2007). Hepatitis C virus infection and the dialysis patient. Semin Dial., 20(5):416-22.
3. Fissell RB, Bragg-Gresham JL, Woods JD (2004). Patterns of hepatitis C prevalence and seroconversion in hemodialysis units from three continents: the DOPPS. Kidney Int.,65(6):2335-42

4-Kellerman S and Alter MJ (1999): Preventing hepatitis B and hepatitis C virus infections in end-stage renal disease patients: Back to basics. Hepatol.; 29:291-293.
5-Zakaria S., Esmat G., Al-Boraey Y. (2005): A Community-based study of viral hepatitis infection in Giza Governorate, Egypt: Seroprevalence, risk factors and associated morbidity. Med. J. Cairo Univ., 73 (4): 899-912.
6-El-Zanaty F and Way A (2009): Egypt Demographic and Health Survey 2008. Ministry of Health, Unicef, El-Zanaty & Associates and USAID,
7- Sulowicz W, Radziszewski A, Chowaniec E (2007): Hepatitis C virus infection in dialysis patients. Hemodial Int., 11(3):286-95.
8-Lu SN, Tung HD, Chen TM, *et al*. (2004): Is it possible to diagnose acute hepatitis C virus (HCV) infection by a rising anti-HCV titre rather than by seroconversion? J Viral Hepat.,;11(6):563-70.
9- A. Mele, G. Ippolito, A. Craxì, R.C. Coppola, N. Petrosillo, M. Piazza, V. Puro, M. Rizzetto, L. Sagliocca, G. Taliani, A et al.(2001) Risk management of HBsAg or anti-HCV positive healthcare workers in hospital Digestive and Liver Disease, Volume 33, Issue 9, Pages 795-802.
10- Fabrizi F, Takkouche B, Lunghi G, Dixit V, Messa P, Martin P (2007): The impact of hepatitis C virus infection on survival in dialysis patients: meta-analysis of observational studies. J Viral Hepat., 14(10):697-703.
11- Fabrizi F, Martin P, Dixit V, Bunnapradist S, Dulai G(2004): Meta-analysis: Effect of hepatitis C virus infection on mortality in dialysis. Aliment Pharmacol Ther., 20(11-12):1271-7.
12- Henderson WA, Shankar R, Gill JM, (2010): Hepatitis C progressing to hepatocellular carcinoma: the HCV dialysis patient in dilemma. J Viral Hepat.;17(1):59-64.
13- Nicolardi E, Grieco A, Rapaccini GL, Pompili M (2010): [Natural history, diagnosis and treatment of chronic hepatitis B and C in hemodialysis patients]. G Ital Nefrol.,;27(3):262-73.
14- Tu AW, Buxton JA, Whitlock M. (2009): Prevalence and incidence of hepatitis C virus in hemodialysis patients in British Columbia: Follow-up after a possible breach in hemodialysis machines. Can J Infect Dis Med Microbiol., 20(2):e19-23.
15- Sun J, Yu R, Zhu B, Wu J, Larsen S, Zhao W (2009): Hepatitis C infection and related factors in hemodialysis patients in China: systematic review and meta-analysis. Ren Fail.,;31(7):610-20.
16- Natov SN, Pereira BJ (2005): Hepatitis C virus in chronic dialysis patients. Minerva Urol Nefrol., 57(3):175-97.
17-Pereira B.G.,Levey A.S. (1999): HCV infection in dialysis and renal transplantation. Kidney Int., 51:98-.
18-Yang,C.S.,Chang H.H.,Chou C.C.,Peng S.J. (2003): Isolation effectively prevents the transmission of HCV in the hemodialysis units. J. Formos. Med. Assoc., 102:79-85.
19-CDC (2001): Recommendations for preventing transmission of infections among chronic hemodialysis patients. MMWR, 50 (No. RR-5); 1-43 .
20- World Medical Association Declaration of Helsinki (2000): Ethical Principles for Medical Research Involving Human Subjects. Revised by the 52nd WMA General Assembly, Edinburgh, Scotland, October 2000. http://www.wma.net/e/policy/b3.htm
21. Petrosillo N, Gilli P, Serraino D, (2001): Prevalence of infected patients and understaffing have a role in hepatitis C virus transmission in dialysis. Am J Kidney Dis., 37(5):1004-10.
22. Broumand B, Shamshirsaz AA, Kamgar M, *et al*. (2002): Prevalence of hepatitis C infection and its risk factors in hemodialysis patients in tehran: preliminary report from "the effect of dialysis unit isolation on the incidence of hepatitis C in dialysis patients" project. Saudi J Kidney Dis Transpl., 13(4):467-72.
23. Nemati E, Alavian SM, Taheri S, Moradi M, Pourfarziani V, Einollahi B(2009): Hepatitis C virus infection among patients on hemodialysis: a report from a single center in Iran. Saudi J Kidney Dis Transpl., 20(1):147-53.
24. Espinosa M, Martin-Malo A, Alvarez de Lara MA, Gonzalez R, Rodriguez M, Aljama P(2002): Natural history of acute HCV infection in hemodialysis patients. Clin Nephrol., 58(2):143-50.
25-Ballester JM, Rivero RA, Villaescusa R, (2005): Hepatitis C virus antibodies and other markers of blood-transfusion-transmitted infection in multi-transfused Cuban patients. J. Clin. Viro.; 34(2): 39-46.
26-Huraib S. O. (2003): Hepatitis C in dialysis patients. Saudi J. Kidney Disease and Transplantation,14 (4): 442-450.
27-Basem A.E., Mohssen G., Sahar S.(2009):Incidence of anti HCV positive seroconversion in isolated versus non isolated hemodialysis patients in Egypt. MD thesis in epidemiology, Faculty of medicine, Ain Shams University, Cairo, Egypt.
28- Frank C., Mohamed M., Strickland G., (2000): The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. Lancet.; 355: 887 – 91.
29- Darwish M., Faris R., Darwish N. (2001): Hepatitis C and cirrhosis liver disease in the Nile Delta of Egypt: a community – based study. Am. J. Trop. Med. Hyg.; 64 : 147 – 53.
30- Telaku S, Fejza H, Elezi Y, Bicaj T(2009): Hepatitis B and C in dialysis units in Kosova. Virol J. 6:72.
31- Alavian SM, Bagheri-Lankarani K, Mahdavi-Mazdeh M, Nourozi S. (2008): Hepatitis B and C in dialysis units in Iran: changing the epidemiology. Hemodial Int., 12(3):378-82.
32- Olut AI, Ozsakarya F, Dilek M(2005): Seroprevalence of hepatitis C virus infection and evaluation of serum aminotransferase levels among haemodialysis patients in Izmir, Turkey. J Int Med Res., 33(6):641-6.
33- Silva LK, Silva MB, Rodart IF, *et al.(*2006): Prevalence of hepatitis C virus (HCV) infection and HCV genotypes of hemodialysis patients in Salvador, Northeastern Brazil. Braz J Med Biol Res., 39(5):595-602.
34- Mansour-Ghanaei F, Sadeghi A, Mashhour M, (2009): Prevalence of Hepatitis B and C Infection in Hemodialysis Patients of Rasht (Center of Guilan Province, Northern Part of Iran). Hepat Mon., 9(1):45-9.
35- Taziki O, Espahbodi F. (2008): Prevalence of hepatitis C virus infection in hemodialysis patients. Saudi J Kidney Dis Transpl., 19(3):475-8
36- Hussein MM, Mooij JM, Hegazy MS, Bamaga MS(2007): The impact of polymerase chain reaction assays for the detection of hepatitis C virus infection in a hemodialysis unit. Saudi J Kidney Dis Transpl., 18(1):107-13.
37- Boulaajaj K, Elomari Y, Elmaliki B, Madkouri B, Zaid D, Benchemsi N(2005): [Prevalence of hepatitis C, hepatitis B and HIV infection among haemodialysis patients in Ibn-Rochd university hospital, Casablanca]. Nephrol Ther., 1(5):274-84.
38- Dattolo P, Lombardi M, Ferro G, Michelassi S, Cerrai T, Pizzarelli F(2006): [Natural history of HCV infection and

risk of death in a cohort of patients on long-term hemodialysis]. G Ital Nefrol., 23(6):585-90.
39-CDC(1998): Recommendation for prevention and control of HCV infection and HCV – related chronic diseases. MMWR, 47(NO.RR-19):1-39
40-Abacioglu, Y.H., Bacaksiz, F., Bahar, I.H., Simmonds, P.(2000): Molecular evidence of nosocomial transmission of hepatitis C virus in haemodialysis units Eur. J. clin. Microbiol. Infect. Dis., 19:182-186.
41-Irish, D.N., Blake, C., Christophers, J.(1999): Identification of hepatitis C virus seroconversion resulting from nosocomial transmission on a hemodialysis unit :implications for infection control and laboratory screening J. Med. Virol., 59:135-140
42-Izopet, J., Pasquier, C., Sandres, K., Puel, J., Rostaing, L.(1999): Molecular evidence for nosocomial transmission of hepatitis C virus in French hemodialysis unit J. Med. Virol., 58: 139-144.
43-Kaldor, J.M., Dore, G.J., correll, P.K(2000): Public health challenges in hepatitis C virus infection , J. Gastroenterol. Hepatol., 15: E83-E90 .
44-Olmer,M., Bouchouareb, D., Zandotti, C., de Micco, P., de Lamballerie, X.,1997. Transmission of the Hepatitis C virus in an hemodialysis unit: evidence for nosocomial infection. Clin. Nephrol., 47:263-270.
45-Taskapan,H., Osymak, O., Dogukan,A., Utas, C., 2001.Patient to patient transmission of hepatitis C virus in hemodialysis units. Clin. Nephrol., 55:477-481.
46-Me´ndez-Sa´nchez N., Motola-Kuba D., Chavez-Tapia N.C. et al.,(2004): Prevalence of hepatitis C virus Infection among hemodialysis patients at a tertiary-care hospital in Mexico City, Mexico. J. Clinical Microbiology, 42(9): 4321–4322.
47-Sanchez-Tapias, J.M., (1999). Nosocomial transmission of HCV. J. Hepatol., 31 (Suppl. 1). 107-112.
48-Flett, A., Teo, M., Mah, Y.I., (1998). Low seroconversions for HCV antibody achieved by universal precautions alone. EDTNA ERCA J. , 24: 40-42.
49-Gilli, P., Soffritti, S., De Paoli Vitali, E., Bedani, P.L., (1995). Prevention of HCV in dialysis units. Nephron 70, 301-306.
50-Jadoul, M., 1996. Transmission routes of HCV infection in dialysis. Nephrol. Dial Transplant. 11 (suppl. 4), 36-38.
51-Jadoul, M., Cornu, C., van Ypersele de Strihou, C., 1998. Universal precautions prevent hepatitis C virus transmission: a 54 month follow- up of the Belgian Multicenter Study. Kidney Int., 53: 1022-1025.
52-Natov, S.N., Pereira, B.J., 1996. HCV in dialysis patients. Adv. Ren. Replace. Ther., 3: 275-283.
53-Valtuille, R., Fernandez, J.L., 1998. Evidence of hepatitis C virus passage across dialysis membrane. Nephron, 80:194-196.
54-Valtuille, R., Moretto, H., Lef, L., Rendo, P., Fernandez, J.L., 2002. Decline of high hepatitis C prevalence in a hemodialysis unit with no isolation measures during a 6 year follow- up. Clin. Nephrol., 57: 371-375.
55-Wrong. J.B., McQuillan, G.M., Mc Hutchison, J.G., Poynard, T., 2000. Estimating future HCV morbidity, mortality, and cost in the United States. Am. J. Public Health, 90:1562-1569.
56-Yang, C.S., Chang. H.H., Chou, C.C., Peng, S.J., 2003. Isolation effectively prevents the transmission of HCV in the hemodialysis unit. J. Frormos. Med. Assoc., 102:79-85.
57- Egyptian National guidelines for Infection control second version, Ministry of Health & Population MOHP Egypt, 2007.
58-Alfurayh, O., Sabeel, A., Al Ahdal, M.N., 2000. Hand contamination with HCV in staff looking after hepatitis C positive hemodialysis patients. Am. J. Nephrol., 20:103-106.
59-Saxena, A.K., Panhotra, B.R., Sundaram, D.S., 2003. Impact of dedicated space, dialysis, equipment, and nursing staff on the transmission of hepatitis C virus in a hemodialysis unit of the middle east. Am. J. Infect. Control, 31: 26-33.
60-Agarwal, S.K., Irshad, M., Dashh, S.C., 1998. HCV infection during replacement machines? Nephron, 79:478-480.
61-Aucella, F., Vigilante, M., Vlente, G.L., Stallone, C., 2000. Systematic monitor disinfection is effective in limiting HCV spread in hemodialysis. Blood Purif., 18: 110-114.
62-Barril, G., 2000. Hepatitis C virus –induced liver disease in dialysis patients. Nephrol. Dial. Transplant., 15 (Suppl. 8):42-45.
63-Caramelo, C., Navas, S., Alberola, M.L., Bermejillo, T., Reyero, A., Carreno, V., 1994. Evidence against transmission of hepatitis C virus through hemodialysis ultrafiltrate and peritoneal fluid. Nephron, 66:470-473.
64-Lombardi, M., Cerrai, T., Dattolo, P., 1995. Is the dialysis membrane a safe barrier against HCV infection ? Nephrol. Dial. Transplant., 10: 578-579.
65-APIC Guide 2010 "Guide to the Elimination of Infections in Hemodialysis"
66-Recommendation for preventing transmission of infection among chronic hemodialysis patients. MMWR Recom. Rep., 2001; 50(RR05):1-43.
67-McLaughlin KJ, Cameron SO, Good T. Nosocomial transmission of hepatitis C virus within a British dialysis center. Nephrol Dial Transplant, 1997;12:304
68- Lin D.Y., Lin H.H., Huang C.C., Liaw Y.F.(1993): High incidence of hepatitis C virus infection in hemodialysis patients in Taiwan. Am J Kidney Dis.; 21(3): 288-91.
69- Al-Jiffri A. M. Y., Fadag R. B., Ghabrah T. M., Ibrahim A. (2003): Hepatitis C virus infection among patients on hemodialysis in Jeddah: A single center experience. Saudi J. Kidney Disease and Transplantation,14 (1): 84-89.
70- El-Reshaid K., Kapoor M., Sugathan T..(1995): Hepatitis C virus infection in patients on maintenance dialysis in Kuwait: epidemiological profile and efficacy of prophylaxis. Saudi J Kidney Dis Transplant., 6 (2): 144-150.
71- CDC Guidelines for laboratory testing and result reporting of antibody to hepatitis C virus. MMWR, 2003;52(No.RR-3)

Study on the molecular mechanisms of the inflammation induced by β-amyloid in vivo

Jun Wu[1, 2], Zhe Min[2], Yongjie Xiong[2], Qiuyue Yan[2], Yuming Xu[1,*], Suming Zhang[2,*]

[1] Department of Neurology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, China;
[2] Department of Neurology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China;
*Co corresponding authors:Yuming Xu. Email: xuyuming@zzu.edu.cn; Suming Zhang. Email: suming_zhang@hotmail.com.

Abstract: *Background*. The study is to explore the molecular mechanisms of the inflammation induced by β-amyloid (Aβ) in the tissue of cortex and hippocampus of mice. *Methods*. The tissue of cortex and hippocampus of mice, were exposed to $A\beta_{1-42}$ with or without Pyrrolidinedithiocarbamate ammonium (PDTC), then Enzyme linked immunosorbent assay (ELISA) for interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα); Quantitative real time polymerase chain reaction (RT-PCR) for IL-1β and TNFα mRNA, western blot analyses of IκBα and NF-κB p65. *Results*. PDTC inhibited the protein expression of IL-1β, TNFα, IL-1β and TNFα mRNA, inhibited the decreasion of IκBα in the cytoplasm, and also inhibited elevation of NF-κB p65 in the nucleus in Aβ-stimulated tissue of cortex and hippocampus of mice. *Conclusion***.** Our findings suggest that the IL-1β, TNFα protein and mRNA were induced by Aβ via the NF-κB signal pathway in the tissue of cortex and hippocampus of mice.
[Jun Wu, Zhe Min, Yongjie Xiong, Qiuyue Yan, Yuming Xu, Suming Zhang. **Study on the molecular mechanisms of the inflammation induced by β-amyloid in vivo.** Life Science Journal. 2012;9(1):750-755] (ISSN:1097-8135). http://www.lifesciencesite.com. 108

Keywords: Aβ; PDTC; IL-1β; TNFα; NF-κB

1. Introduction

AD is a progressive neurodegenerative disease characterized by cognitive impairment. Even after centuries of research and exploration, the exact etiology of AD remains to be determined, so, there is no effective treatment until now. The risk of AD increases with age, and it reduced the quality of life of people. With the rapid expansion of aged population, AD will be one of the most common cause of death. Therefore, it is imperative to study the pathogenesis in depth. In recent years, studies increasingly indicate that inflammation contributes to the progression of AD (Hoozemans JJ, 2006; Eikelenboom P, 2006), and in the pathogenesis of AD, Aβ plays an important role. Aβ activates microglia, and the activated microglia promotes neuronal injury through the release of proinflammatory factors (Graeber MB, 2010; Moore AH, 2002; Wang MJ, 2002). In this study, the proinflammatory effect of Aβ on the cortex and hippocampus of mice of and the possible signal transduction pathways were discussed.

2. Subjects and Methods

2.1 Reagents

NF-κB p65 antibodies were from Cell Signaling Technology Inc.(Beverly, MA, USA), IκBα antibodies were from Abcam Inc.(Cambridge, MA, UK), PDTC was from Sigma-Aldrich Co. LLC.(Ronkonkoma, America), goat anti-rabbit IgG-PE was from Santa Cruz Biotechnology (Santa Cruz, CA), goat anti rabbit IgG (H+L) HRP was from Thermo Fisher Scientific Inc. (Rockford, IL, USA), IL-1β, TNFα ELISA assay kits were purchased from Wuhan Boster Bioligical Technology, LTD (Wuhan, Hubei, China).

2.2 Preparation of $A\beta_{1-42}$

$A\beta_{1-42}$, obtained from AnaSpec, Inc (Fremont, CA, USA), was dissolved in sterile double-distilled water to a concentration of 0.1 mM, incubated for 5 days at 37°C to allow fibril formation, and then further diluted in PBS to 5 μM.

2.3 Animals and Intracerebroventricular injection (ICV)

The experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Tongji Medical College, Huazhong University of Science and Technology, which abides by the Institute of Laboratory Animal Resources (ILAR) guide. The intracerebroventricular (ICV) infusion method was established by Laursen and Belknap (Laursen SE, 1986). Five groups of 3-4 month male C57BL/6 mice weighing 20~25g were anesthetized and administrated with ICV infusion of $A\beta_{1-42}$ 50 μg with and without the presence of PDTC (pretreated with PDTC at 5μg, 50μg for 30 min) into mice. Each group of the animals consisted of 5 mice. The control group were injected with physiological saline, and administrated with ICV infusion for seven consecutive days. The mice were sacrificed at the 8th day after the ICV infusion. Sections of cortex and hippocampus of the mice were collected.

2.4 Enzyme linked immunosorbent assay (ELISA) for IL-1β and TNFα

IL-1β and TNFα, which existed in the tissue homogenate, were measured by ELISA kit according to the manufacturer's instructions. The optical density was measured at 450 nm using a microplate reader, and the values were obtained from standard curves using recombinant IL-1β and TNFα in the ELISA kits.

2.5 Quantitative real time polymerase chain reaction (RT-PCR) for IL-1β and TNFα mRNA

Total RNA was extracted from tissues using TRIzol Reagen (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the instructions. Each reverse transcription reaction was made to a final volume of 25 μl with the following reagents: 1 μg of total RNA and 1μl of M-MLV reverse transcriptase (Invitrogen), 4 μl of M-MLV RT 5 × buffer, 20 U of RNasin, 1 μl of Random primer (10 pmol/μl),1 μl of dNTP mix (10 mM) and DEPC water. They were mixed and incubated at 37°C for 50 min and at 70°C for 15 min. The cDNA thus obtained was then stored at -20°C. The real time RT–PCR was carried out using LightCycler DNA master plus SYBR Green I kit (TOYOBO Co., LTD, Osaka, Japan) according to the protocols. After an initial denaturation step (95°C for 1 min), the PCR was performed as follows: 40 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and elongation at 72°C for 45 s. Primer sequences (Invitrogen) were as follows (orientation 5′ to 3′): β-actin (forward): CCGTGAAAAGATGACCCAG, β-actin (reverse): TAGCCACGCTCGGTCAGG; IL-1β (forward): TTTGAAGTTGACGGACCCC, IL-1β (reverse): GTGCTGCTGCGAGATTTGA; TNFα (forward): CGGGCAGGTCTACTTTGGAG, TNFα (reverse): CAGGTCACTGTCCCAGCATC. For the relative comparison of each gene, the data of real time PCR were analyzed with ΔΔCt method.

2.6 Western blot analyses of IκBα and NF-κB p65

After the tissues were resuspended in the lysis buffer, and the nuclear protein and the cytoplasmic protein were extracted. The protein samples were electrophoresed by SDS-PAGE gel, and transferred to a nitrocellulose membrane, and assayed firstly using rabbit polyclonal antibodies against IκBα or NF-κB p65, then with anti-rabbit horseradish peroxidase-coupled secondary antibody, and at last with an enhanced chemiluminescence detection reagent.

2.3 Statistical analysis

Data were subjected to statistical analysis using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). Data are shown as means±S.D. for at least three independent experiments. Statistical significance was analyzed by one-way ANOVA, followed by the Scheffe's test for comparison of multiple comparison. Difference with p value < 0.05 was considered statistically significant.

3. Results

3.1 PDTC inhibited IL-1β and TNFα production stimulated by Aβ in the tissue of cortex and hippocampus of mice

In this study, we investigated whether PDTC inhibits the production of IL-1β and TNFα stimulated by Aβ in the cortex and hippocampus of mice. The effects of PDTC on the production of IL-1β and TNFα are depicted in (Fig 1). The expression of IL-1β and TNFα markedly increased in the cortex and hippocampus of mice exposed to Aβ. In contrast, when pretreated with PDTC before being exposed to Aβ, their production was found to greatly decrease. PDTC alone did not affect the production of IL-1β and TNFα. Analysis showed that IL-1β and TNFα production stimulated by Aβ in the cortex and hippocampus of mice could be inhibited by PDTC.

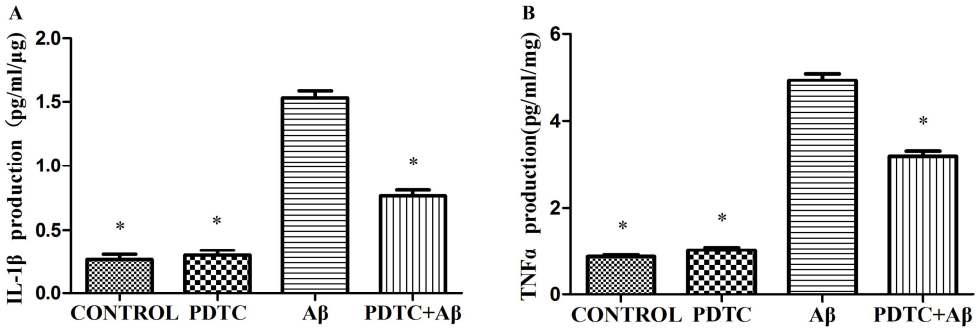


Fig 1 ELISA assay for IL-1β and TNFα protein after the tissue of cortex and hippocampus of mice were treated with or without Aβ in the absence or presence of PDTC. Aβ markedly enhanced the protein expression of IL-1β and TNFα, PDTC alone did not affect protein expression. PDTC significantly inhibited the protein expressions of IL-1β and TNFα induced by Aβ (Fig 1 A, B), Data are presented as means±S.D. *P < 0.05, **P < 0.01 compared with Aβ group.

3.2 PDTC attenuated the gene expressions of IL-1β and TNFα stimulated by Aβ in BV2 cells and tissue in the cortex and hippocampus of mice

We next analyzed the influence of PDTC on the gene expression of IL-1β and TNFα (Fig 2). Aβ markedly up-regulated the IL-1β and TNFα mRNA level, while PDTC significantly down-regulated the mRNA level of IL-1β and TNFα induced by Aβ. PDTC alone did not affect the IL-1β and TNFα mRNA level. These observations are consistent with the expression of IL-1β and TNFα proteins, and the results imply that PDTC inhibits IL-1β and TNFα protein expression by directly modulating gene transcription.

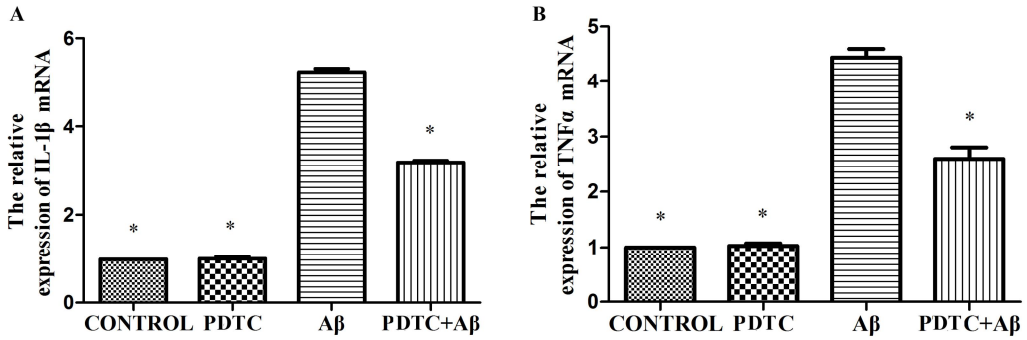


Fig 2 RT-PCR assay for IL-1β and TNFα mRNA after the tissue of cortex and hippocampus of mice were treated with or without Aβ in the absence or presence of PDTC. Aβ markedly enhanced the gene expression of IL-1β and TNFα, PDTC alone did not affect gene expression. PDTC significantly attenuated the gene expressions of IL-1β and TNFα induced by Aβ (Fig 2 A, B), Data are presented as means±S.D. *P < 0.05, compared with Aβ group.

3.3 PDTC blocked IκBα degradation and NF-κB p65 subunit translocation into the nucleus stimulated by Aβ in tissue in the cortex and hippocampus of mice

This part of the study investigated whether PDTC acts to block the activation of the NF-κB pathway. The effects were determined by western blot (Fig 3) The results indicate that the degradation of IκBα , which is a key step in the regulation of the NF-κB pathway, can be inhibited by PDTC, and the following translocation of NF-κB p65 subunit was decreased, which means that only a few DNA binding forms of NF-κB could translocate into the nucleus where it binds to its recognition sites in the upstream region of genes of IL-1β and TNFα. Thus, the gene expressions of IL-1β and TNFα were attenuated.

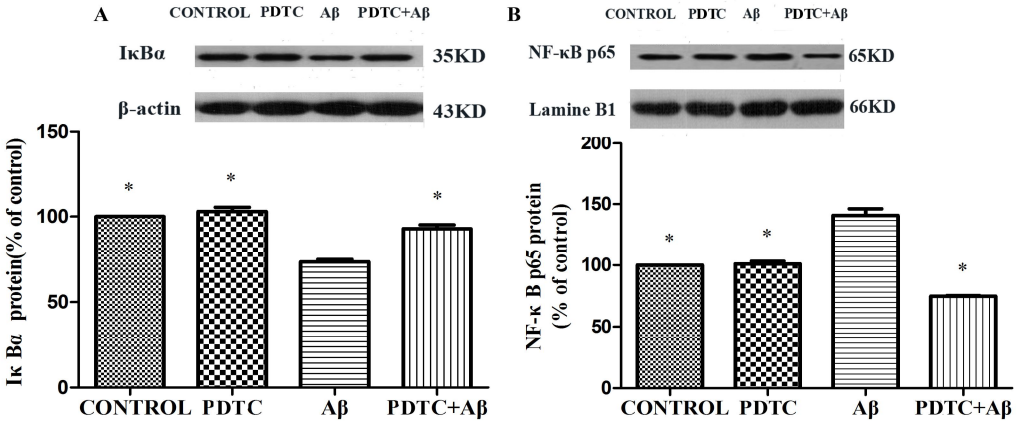


Fig 3 Western blot analyses of IκBα and NF-κB p65 after the tissue of cortex and hippocampus of mice were treated with or without Aβ in the absence or presence of PDTC. Plasmic extracts or nuclear extracts were separated and blotted sequentially with the indicated antibodies to evaluate both IκBα protein contents in cytoplasm and NF-κB p65 protein contents in nucleus. Aβ caused a marked degradation of IκBα, and then induced the NF-κB p65 subunit to enter the nucleus, these reactions could be inhibited by PDTC (Fig 3 A, B).The data represent the mean±SD of at least three independent experiments. *P < 0.01 compared with Aβ group.

4. Discussion

AD, a progressive neurodegenerative disease, is the most common causes of dementia, affecting a large number old people in the world. With the aging population increasing promptly, patients, families and society are facing more and more problems associated with AD in China. The presence of senile plaques and neurofibrillary tangles is the pathological hallmarks in AD. The plaques contain extracellular deposits of β-amyloid (Aβ) surrounded by damaged neuronal processes and reactive glia, and the hyperphosphorylated tau proteins accumulates intracellular as neurofibrillary tangles. Multiple studies suggest that pathological changes of AD is the result of cumulative damage of inflammation (Wyss-Coray T, 2006). Some clinical study indicate that the levels of IL-1β、IL-6 and TNFα elevate in the AD brain (Candore G, 2007; Candore G, 2004; Tarkowski E, 1999; Frei K, 1989; Griffin WS, 1995)，and the anti-inflammatory drug down-regulates the expression of IL-1β and TNFα (Jun Wu , 2011). Some study report that nonsteroidal anti-inflammatory drugs (NSAIDs) may reduce the risk for AD, the relative risk of AD was 0.95 in subjects with 1 month use of NSAIDs, 0.83 in those with 1 month - 1 year use, and 0.20 in those with 2 years use (Bas A. in t' Veld BA, 2001).；Other studies indicate that the incidence of AD decreased after taking NSAIDs (Breitner JC, 2003; Etminan M, 2003; McGeer PL, 1996). These provide very strong evidence that that inflammation contributes to the progression of AD. Being part of the complex biological response of tissues to pathogens, inflammation exists in the pathological process of variety of diseases. They are an important and necessary part of the normal host responses to harmful stimuli, but persistent inflammatory response and the overproduction of inflammatory molecules causes severe damage to organs and tissues (Licastro F, 2005), and may aggravate immune-inflammatory-related disease. Compelling evidence from basic and clinical research studies has supported the conclusion that neuroinflammation induced by Aβ is associated with the development of AD neuropathology (Wyss-Coray T, 2006)，Aβ self-aggregate to form insoluble fibrils, deposit as plaques (Pereira C, 2005), activate microglia, the resident macrophages of the brain, and these activated microglia (Rogers J, 2001) may then promote neuronal injury through the release of proinflammatory and cytotoxic factors, including IL-1, IL-6 and TNFα (Candore G, 2007; Hanisch UK, 2002; Minghetti L, 1998; McGeer EG, 1998; Mrak RE, 1995; Griffin WS, 1998; Akiyama H, 2000; Town T, 2005; Combs CK, 2001; Meda L, 2001). IL-1 is an early response cytokine which is overexpressed in brains of AD and associated with neuritic plaque (Griffin WS, 1998; Griffin WS, 2002; Sheng JG, 1996). A large number of IL-1 increased the production and enhance the activity of neuronal acetylcholinesterase, leading to dysfunction of the cholinergic system and memory impairment of AD (Li Y, 2000). IL-6 promote enhanced Aβ1-40 and Aβ1-42 production，reverse the neuroprotective effect of soluble amyloid precursor protein (sAPP) (Ringheim GE, 1998; Del Bo R, 1995). High levels of IL-6 induce hippocampal neuronal degeneration and damage cholinergic neural pathways seriously, resulting in impairments in learning and memory in the central nervous system of transgenic mice (Campbell IL, 1997). TNF α up-regulate the expression of COX-2 , leading to free radicals generation and subsequent nerve cells damage (Culpan D, 2003). TNFα enhanced the sensitivity of neurons to free radicals, leading to neuronal degeneration (Combs CK, 2001). TNFα also stimulate the synthesis of Aβ (Blasko I, 1999). In summary, inflammatory molecules play an important role in the process of amyloid plaques and the neurofibrillary tangles formation，neuronal damage，memory and cognitive impairment.

Our study found that，the degradation of IκBα and NF-κB p65 subunit translocation into the nucleus stimulated by Aβ, and the following expression of inflammatory factors in tissue in the cortex and hippocampus of mice can be inhibited by PDTC, the specific inhibitors of NF-κB signaling. These findings suggest that the IL-1β, TNFα protein and mRNA were induced by Aβ via the NF-κB signal pathway in the tissue of cortex and hippocampus of mice.

NF-κB is a nuclear factor that bound selectively to the κ enhancer and was found in extracts of B-cell tumors, it bound only to the κ light-chain enhancer, and it covered the sequence GGGACTTTCC (Sen R, 1986). NF-κB plays a key role in inflammatory processes. IkBα is the inhibitor of NF-κB (Baldwin AS, 1996; Ghosh S, 1998), and it bind to NF-κB (p65/p50 dimer) retained within the cytoplasm, preventing NF-κB importing into the nucleus. IkBα (Ghosh S, 1998; Karin M, 2000; Silverman N, 2001; Ghosh S, 2002; Huxford T, 1998; Jacobs MD, 1998). Multiple studies have found that a variety of inflammatory cytokines, including TNF and IL-1 family members can activate NF-κB signal pathway (Ghosh S, 1998; Siebenlist U, 1994; Pahl HL,1999), thereby, Aβ can active NF-κB signaling pathway, and induce further activation, resulting in a detrimental cycle, thus, that would exacerbate nerve cells and tissue damage. Some studies show that, the senile plaques and neurofibrillary tangles, as the pathological hallmarks of AD, are all related to activation of NF-κB (Yamamoto Y, 2001; Kaltschmidt B, 1999). This imply that inhibition of NF-κB activation, not only significantly suppressed the Aβ-induced inflammatory response, but also reduced

the intracellular neurofibrillary tangles and extracellular deposition of Aβ, and reduced the risk of cognitive decline. As the main therapeutic target, inhibition of NF-κB activation has become a key therapeutic target for treatment of AD, and is likely to have far-reaching consequences.

5. Conclusions

IL-1β, TNFα protein and mRNA were induced by Aβ via the NF-κB signal pathway in the tissue of cortex and hippocampus of mice and BV2 microglial cells.

Acknowledgements:

This work was supported by the Grant from The National High Technology Research and Development Program of China (NO. 2007AA03Z312).

Co corresponding authors:

Pro. Yuming Xu.
Department of Neurology
The First Affiliated Hospital of Zhengzhou University
Zhengzhou 450052, China
Email: xuyuming@zzu.edu.cn

Pro. Suming Zhang.
Department of Neurology
Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology
Wuhan 430030, China
Email: suming_zhang@hotmail.com.

References

1. Hoozemans JJ, Veerhuis R, Rozemuller JM, Eikelenboom P. Neuroinflammation and regeneration in the early stages of Alzheimer's disease pathology. *Int J Dev Neurosci*. 2006;24:157-165.
2. Eikelenboom P, Veerhuis R, Scheper W, Rozemuller AJ, van Gool WA, Hoozemans JJ. The significance of neuroinflammation in understanding Alzheimer's disease. *J Neural Transm*. 2006;113:1685-1695.
3. Graeber MB, Streit WJ. Microglia: Biology and pathology. *Acta Neuropathol*. 2010;119:89-105.
4. Moore AH, O'Banion MK. Neuroinflammation and anti-inflammatory therapy for Alzheimer's disease. *Adv Drug Deliv Rev*. 2002;54:1627-1656.
5. Wang MJ, Lin WW, Chen HL, Chang YH, Ou HC, Kuo JS, Hong JS, Jeng KC. Silymarin protects dopaminergic neurons against lipopolysaccharide-induced neurotoxicity by inhibiting microglia activation. *Eur J Neurosci*. 2002;16:2103-2112.
6. Laursen SE, Belknap JK: Intracerebroventricular injections in mice. Some methodological refinements. J Pharmacol Methods, 1986; 16 (4): 355-357.
7. Wyss-Coray T. Inflammation in alzheimer disease: Driving force, bystander or beneficial response? *Nat Med*. 2006;12:1005-1015.
8. Candore G, Balistreri CR, Grimaldi MP, Listi F, Vasto S, Chiappelli M, Licastro F, Colonna-Romano G, Lio D, Caruso C. Polymorphisms of pro-inflammatory genes and Alzheimer's disease risk: A pharmacogenomic approach. *Mech Ageing Dev*. 2007;128:67-75.
9. Candore G, Balistreri CR, Colonna-Romano G, Lio D, Caruso C. Major histocompatibility complex and sporadic Alzheimer's disease: A critical reappraisal. *Exp Gerontol*. 2004;39:645-652.
10. Tarkowski E, Blennow K, Wallin A, Tarkowski A. Intracerebral production of tumor necrosis factor-alpha, a local neuroprotective agent, in alzheimer disease and vascular dementia. *J Clin Immunol*. 1999;19:223-230.
11. Frei K, Malipiero UV, Leist TP, Zinkernagel RM, Schwab ME, Fontana A. On the cellular source and function of interleukin 6 produced in the central nervous system in viral diseases. *Eur J Immunol*. 1989;19:689-694.
12. Griffin WS, Sheng JG, Roberts GW, Mrak RE. Interleukin-1 expression in different plaque types in Alzheimer's disease: Significance in plaque evolution. *J Neuropathol Exp Neurol*. 1995;54:276-281.
13. Jun Wu , Aitao Wang, Zhe Min, Yongjie Xiong, Qiuyue Yan, Jinping Zhang, Jie Xu, Suming Zhang. Lipoxin A4 inhibits the production of proinflammatory cytokines induced by b-amyloid in vitro and in vivo. *Biochemical and Biophysical Research Communications*. 2011; 408:382-387.
14. Bas A. in t' Veld BA, Ruitenberg A, Hofman A, Launer LJ, van Duijn CM, Stijnen T, Breteler MM, Stricker BH. Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. *N Engl J Med*. 2001; 345:1515-1521.
15. Breitner JC. Nsaids and Alzheimer's disease: How far to generalise from trials? *Lancet Neurol*. 2003;2:527.
16. Etminan M, Gill S, Samii A. Effect of non-steroidal anti-inflammatory drugs on risk of Alzheimer's disease: Systematic review and meta-analysis of observational studies. *BMJ*. 2003;327:128.
17. McGeer PL, Schulzer M, McGeer EG. Arthritis and anti-inflammatory agents as possible protective factors for Alzheimer's disease: A review of 17 epidemiologic studies. *Neurology*. 1996;47:425-432.
18. Licastro F, Candore G, Lio D, Porcellini E, Colonna-Romano G, Franceschi C, Caruso C. Innate immunity and inflammation in ageing: A key for understanding age-related diseases. *Immun Ageing*. 2005;2:8.
19. Pereira C, Agostinho P, Moreira PI, Cardoso SM, Oliveira CR. Alzheimer's disease-associated neurotoxic mechanisms and neuroprotective strategies. *Curr Drug Targets CNS Neurol Disord*. 2005;4:383-403.
20. Rogers J, Lue LF. Microglial chemotaxis, activation, and phagocytosis of amyloid beta-peptide as linked phenomena in Alzheimer's disease. *Neurochem Int*. 2001;39:333-340.
21. Hanisch UK. Microglia as a source and target of cytokines. *Glia*. 2002;40:140-155.
22. Minghetti L, Levi G. Microglia as effector cells in brain damage and repair: Focus on prostanoids and nitric oxide. *Prog Neurobiol*. 1998;54:99-125.
23. McGeer EG, McGeer PL. The importance of inflammatory mechanisms in alzheimer disease. *Exp Gerontol*. 1998;33:371-378.

24. Mrak RE, Sheng JG, Griffin WS. Glial cytokines in Alzheimer's disease: Review and pathogenic implications. *Hum Pathol*. 1995;26:816-823.
25. Griffin WS, Sheng JG, Royston MC, Gentleman SM, McKenzie JE, Graham DI, Roberts GW, Mrak RE. Glial-neuronal interactions in Alzheimer's disease: The potential role of a 'cytokine cycle' in disease progression. *Brain Pathol*. 1998;8:65-72.
26. Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffin WS, Hampel H, Hull M, Landreth G, Lue L, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, Rogers J, Rydel R, Shen Y, Streit W, Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, Wyss-Coray T. Inflammation and Alzheimer's disease. *Neurobiol Aging*. 2000;21:383-421.
27. Town T, Nikolic V, Tan J. The microglial "Activation" Continuum: From innate to adaptive responses. *J Neuroinflammation*. 2005;2:24.
28. Combs CK, Karlo JC, Kao SC, Landreth GE. Beta-amyloid stimulation of microglia and monocytes results in tnfalpha-dependent expression of inducible nitric oxide synthase and neuronal apoptosis. *J Neurosci*. 2001;21:1179-1188.
29. Meda L, Baron P, Scarlato G. Glial activation in Alzheimer's disease: The role of abeta and its associated proteins. *Neurobiol Aging*. 2001;22:885-893.
30. Griffin WS, Mrak RE. Interleukin-1 in the genesis and progression of and risk for development of neuronal degeneration in Alzheimer's disease. *J Leukoc Biol*. 2002;72:233-238.
31. Sheng JG, Mrak RE, Rovnaghi CR, Kozlowska E, Van Eldik LJ, Griffin WS. Human brain s100 beta and s100 beta mrna expression increases with age: Pathogenic implications for Alzheimer's disease. *Neurobiol Aging*. 1996;17:359-363.
32. Li Y, Liu L, Kang J, Sheng JG, Barger SW, Mrak RE, Griffin WS. Neuronal-glial interactions mediated by interleukin-1 enhance neuronal acetylcholinesterase activity and mrna expression. *J Neurosci*. 2000; 20:149-155.
33. Ringheim GE, Szczepanik AM, Petko W, Burgher KL, Zhu SZ, Chao CC. Enhancement of beta-amyloid precursor protein transcription and expression by the soluble interleukin-6 receptor/interleukin-6 complex. *Brain Res Mol Brain Res*. 1998;55:35-44.
34. Del Bo R, Angeretti N, Lucca E, De Simoni MG, Forloni G. Reciprocal control of inflammatory cytokines, il-1 and il-6, and beta-amyloid production in cultures. *Neurosci Lett*. 1995;188:70-74.
35. Campbell IL, Stalder AK, Chiang CS, Bellinger R, Heyser CJ, Steffensen S, Masliah E, Powell HC, Gold LH, Henriksen SJ, Siggins GR. Transgenic models to assess the pathogenic actions of cytokines in the central nervous system. *Mol Psychiatry*. 1997;2:125-129.
36. Culpan D, MacGowan SH, Ford JM, Nicoll JA, Griffin WS, Dewar D, Cairns NJ, Hughes A, Kehoe PG, Wilcock GK. Tumour necrosis factor-alpha gene polymorphisms and Alzheimer's disease. *Neurosci Lett*. 2003;350:61-65.
37. Blasko I, Marx F, Steiner E, Hartmann T, Grubeck-Loebenstein B. Tnfalpha plus ifngamma induce the production of alzheimer beta-amyloid peptides and decrease the secretion of apps. *FASEB J*. 1999;13:63-68.
38. Sen R, Baltimore D. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell*. 1986;46:705-716.
39. Baldwin AS, Jr. The nf-kappa b and i kappa b proteins: New discoveries and insights. *Annu Rev Immunol*. 1996;14:649-683.
40. Ghosh S, May MJ, Kopp EB. Nf-kappa b and rel proteins: Evolutionarily conserved mediators of immune responses. *Annu Rev Immunol*. 1998;16:225-260.
41. Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: The control of nf-[kappa]b activity. *Annu Rev Immunol*. 2000;18:621-663.
42. Silverman N, Maniatis T. Nf-kappab signaling pathways in mammalian and insect innate immunity. *Genes Dev*. 2001;15:2321-2342.
43. Ghosh S, Karin M. Missing pieces in the nf-kappab puzzle. *Cell*. 2002;109 Suppl:S81-96.
44. Huxford T, Huang DB, Malek S, Ghosh G. The crystal structure of the ikappabalpha/nf-kappab complex reveals mechanisms of nf-kappab inactivation. *Cell*. 1998; 95:759-770.
45. Jacobs MD, Harrison SC. Structure of an ikappabalpha/nf-kappab complex. *Cell*. 1998; 95:749-758
46. Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of nf-kappa b. *Annu Rev Cell Biol*. 1994;10:405-455.
47. Pahl HL. Activators and target genes of rel/nf-kappab transcription factors. *Oncogene*. 1999;18:6853-6866.
48. Yamamoto Y, Gaynor RB. Role of the nf-kappab pathway in the pathogenesis of human disease states. *Curr Mol Med*. 2001;1:287-296.
49. 49. Kaltschmidt B, Uherek M, Wellmann H, Volk B, Kaltschmidt C. Inhibition of nf-kappab potentiates amyloid beta-mediated neuronal apoptosis. *Proc Natl Acad Sci U S A*. 1999;96:9409-9414.

2/21/2012

Construction and identification of a recombinant adenovirus vector expressing His-tag-ICP47 fusion gene

WANG Peng[1] , KAN Quan-cheng[2], YU Zu-jiang[2], LI Ling[3] , ZHANG Zhenxiang[1] and PAN Xue[1]

1.Nursing College, Zhengzhou University, Zhengzhou, Henan 450052, China (Wang P, ZHANG ZX and Pan X)
2.Clinical Pharmacology Base (Kan QC), Department of Infectious Disease (Yu ZJ), 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 (Li L)

Abstract: Background: 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) expressesd 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 reduces the rate of cytolysis of infected cells by sensitized CTL and evades the host immune clearance. The aim of this study is to construct and identify a recombinant adenovirus expressing His-tag-ICP47 fusion gene for the following studies of its immunological activities. **Methods:** The adenoviral vector system of AdEasy-1was used to prepare the recombinant adenovirus expressing His-tag-ICP47 fusion gene or the control empty recombinant adenovirus r-Track. His-tag-ICP47 fusion gene was firstly cloned into the pAdTrack-CMV vector. The gene fragments digested by Pme Ⅰ were co-transformed with adenoviral backbone vector pAdEasy-1 in E.coli BJ5183 cells to produce recombinant adenoviral vector pAdEasy-H-ICP47. Linearized with *Pac Ⅰ*, recombinant adenoviral vector was subsequently transfected into 293 cells to product r-H-ICP47. Meanwhile, the control empty recombinant adenovirus r-Track was built in the same way. Finally, The viruses were amplified and virus particle titres were determined. Purified virus was analyzed for the presence and expression of His-tag-ICP47 fusion gene by PCR and Western-blot analysis. **Results:** The recombinant adenoviruses of r-H-ICP47 and r-Track were successfully constructed and successfully transfected into 293 cells and virus granules appeared. The virus particle titres of r-H-ICP47 and r-Track were determined with the resulting of 3.7×10^{10}efu/mL and 4.4×10^{10}efu/mL The proteins producted by recombinant adenovirus-infected cells were confirmed by PCR and Western-blot analysis. **Conclusion:** The replication-defective recombinant adenoviruses of r-H-ICP47 and r-Track are successfully constructed, and the virus particle titres are highly enough to infect and express relevant genes in cells at a desired level, respectively.
[WANG Peng , KAN Quan-cheng, YU Zu-jiang, LI Ling , ZHANG Zhenxiang and PAN Xue. **Construction and identification of a recombinant adenovirus vector expressing His-tag-ICP47 fusion gene.** Life Science Journal. 2012;9(1):756-763] (ISSN:1097-8135). http://www.lifesciencesite.com. 109

Key Words: recombinant adenovirus; infected cell protein 47; fusion gene

Introduction

Adenoviral 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 [1]. But the response of host immune systems against gene products expressed by genetically modified cells is a major obstacle to successful gene therapy[2]. Immuno-suppressants are not always effective and associated with well-known toxic effects [3-4]. Therefore, prevention of host immune response against transplanted cells and products of introduced genes could be a critical subject for the long-term success of gene therapy[5].

In particular, 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[6]. Infected cell protein 47 (ICP47), 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 reduces the rate of cytolysis of infected cells by sensitized CTL and evades the host immune clearance[7-8].

Based on these studies，this subject was designed to construct a recombinant adenovirus vector expressing His-tag-ICP47 fusion gene and identify its immunological activity. We expect this finding should have important implications for analyzing the mechanisms of immune tolerance as well as human gene therapy.

METHODS

DNA manipulation and PCR amplification

The template DNA of ICP47 was extracted from HSV-1-positive CSF specimens (Axygen Biosciences CO., Ltd), ICP47 primers were: sense 5′-GGCAAGCTTATGTCGTGGGCCCTGGA-AATG-3′, antisense 5′-GGCCGATATCTCAACGGGTTACCGGATTACG ‐ 3′, yielding a 286bp. The restriction sites of *Hind Ⅲ* and *EcoR V* were added at the 5' termini of forward and reverse primers, respectively.

PCR amplification was subsequently carried out for the target sequences by using a S1000 PCR cycler (Bio-Rad) in 200μl PCR tubes containing the following reaction mixture: 5 μl template DNA, 5 μl Pfu DNA Polymerase 10× Buffer, 0.5μl Pfu DNA Polymerase (Promega Corporation), 4 μl dNTPs (2.5 mM), and 20 pmol each primer in 50 μl final volume. The amplification cycling profile was as follows: initial denaturation 95°C for 2 minutes; cycling: 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 2 minute (35 cycles); final extension at 72°C for 5 minutes. After reaction completion, 5 μl of PCR products was run on 1.5% agarose gel electrophoresis stained with ethidium bromide, and then, images were scanned by a Transilluminator JY04S Gel analysis system under UV light (Beijing Junyi-Dongfang electrophoresis Equipment CO., Ltd). Finally, PCR products were recovered and purified.

Construction of Adenovirus Vector pAd-H-ICP47

Purified ICP47 DNA segments and pAdTrack-CMV vector were digested with restriction endonuclease *Hind Ⅲ* and EcoR V (New England Biolabs), respectively. Recovered products was connected with *T4* DNA ligase (New England Biolabs), and then the ligation mixture was transformed into DH5α chemically competent cells. Kana-resistant transformants were selected by plating the transformation mixture on LB agar plates supplemented with 50μg/mL Kana. After incubation overnight at 37°C, the recombinant plasmid DNA was isolated and further confirmed via *Hind Ⅲ* and *EcoR V* double digestion, Finally, the confirmed recombinant adenoviral vector was named pAdTrack-ICP47.

A segment containing a 6×His tag was synthesized and directionally inserted pAdTrack-ICP47 at the *Kpn I* and *Hind*Ⅲ sites, resulting in pAdTrack-H-ICP47. After identified with enzyme digestion assay, the His-tag-ICP47 fusion gene nucleotide sequence of final constructs was determined to confirm that no mutation was introduced by the amplification step.

Homologous recombination of pAdEasy-H-ICP47

pAdTrack-H-ICP47 was linearized by restriction endonuclease *Pme I* (New England Biolabs), and subsequently was co-transformed with adenoviral backbone vector pAdEasy-1 in E.coli BJ5183 cells. Kana-resistant transformants were selected by plating the transformation mixture on LB agar plates supplemented with 50μg/mL Kana. Positive clones were selected and enzyme identified, and the recombinant adenoviral vector was obtained and named pAdEasy-H-ICP47. Meanwhile, the control empty recombinant adenoviral vector pAdEasy-Track was constructed in the same way.

Package of recombinant adenovirus

Human embryonic kidney 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100μg of penicillin/ml, and 100 U of streptomycin/ml. 293 cells were cultured and plated at a density of 1×10^6 cells/ml for 24 h before transfection, and cells were transfected at about 70% confluence on the next day. pAdEasy-H-ICP47 was digested by *Pac I* (New England Biolabs, Inc) and transfected into 293 cells with Lipofectamine 2000 (Invitrogen) according to the transfection agent instruction to generate adenovirus r-H-ICP47. Seven-ten days later, green fluorescence could be seen under a fluorescence microscope. The supernatant and cells were collected and repeatedly freezed-thawed three times at 37°C/-80°C. The supernatant containing virus was taken in centrifugation and stored at -80°C. Similarly, the control empty recombinant adenovirus r-Track was generated.

Amplification, concentration and purification of recombinant adenovirus

Amplified, concentration and purification of the recombinant adenovirus were carried out as previously described [9], Briefly, the recombinant adenoviruses -infected cells were harvested and washed three times with PBS, and the virus particles were released from the 293 cells by three freeze-thaw cycles and purified by ultracentrifugation on a cesium chloride (CsCl) step gradient and dialysis in the buffer containing 5% sucrose, 10 mM Tris pH 8.0 and 2 mM $MgCl_2$.

Determination of virus particle titres

Purified virus particle titres were determined by counting the number of green fluorescence protein (GFP)-positive cells as previously described[9]. Briefly, 293 cells of a final density of 1×10^5 cells/mL were infected by recombinant adenovirus in a 12-well dish with 10μL aliquots of 1:10 geometric dilution. After 24 h of incubation in 5% CO_2 at 37°C, the GFP-positive cells were counted with a fluorescent light microscope, and the titer was defined in expression- forming units per milliliter (efu/mL) as follows: (efu/mL =number of GFP-positive cells$\times10^{n+1}$/mL).

PCR Analysis

To test whether the His-tag-ICP47 fusion gene were preserved in transduced target cells, total DNA was prepared by lysing transfected 293 cells with 400 mg of proteinase K per milliliter at 55°C for 1 h , boiling for 5 min and then centrifuging to pellet cell debris. PCR amplification was subsequently carried out as described previously. The oligonucleotide primers specific to His-tag-ICP47 fusion gene sequence were as follows:

Sense 5'-ATGCATCATCATCATCATCATA-3' , antisense 5′-GGCCGATATCTCAACGGG-TTACCGGATTACG‐3′, yielding a 304bp. After reaction was completed, 5 μL of the amplified DNA fragments was analyzed by agarose gel electrophoresis.

Western blot analysis

Expression of the proteins produced by recombinant adenovirus was analyzed by Western blot analysis. In brief, the total proteins were extracted from 293 cells infected by r-H-ICP47 or r-Track, and separated by SDS-PAGE on 0.1% SDS in 10% polyacrylamide gels. At the end of the run, polypeptide bands in the gel were electrophoretically transferred to a PVDF membrane (Bio-Rad). The membrane was incubated at room temperature for 1 h with rabbit anti-β-tubulin antibody, rabbit anti-GFP antibody, or rabbit anti-6×His antibody (Bioss Inc.), respectively. The binding of antibody to the specific protein band on the membrane was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bioss Inc.) and was analyzed with an ECL Western blotting detection system (Beyotime Institute of Biotechnology).

RESULTS

Identificantion of pAdTrack-ICP47 with endonuclease digestion assay

The results of electrophoresis showed that there were two bands at approximately 267bp and 9200bp after pAdTrack-ICP47 was digested with *Hind Ⅲ* and *EcoR V*. The both bands were in the right range consistent with the expected length. On the contrary, there was only one band at approximately 9200bp after the control empty adenoviral vector pAdTrack-CMV was digested with *Hind Ⅲ* and *EcoR V*. Similarly, one band was detected at approximately 9200bp after pAdTrack-ICP47 was digested with *Hind Ⅲ* or *EcoR V*, respectively. The result of the recombinant shuttle plasmid pAdTrack-ICP47 was identified by endonuclease digestion of *Hind Ⅲ* and *EcoR V* was shown in Fig. 1.

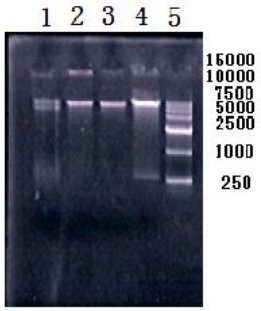


Fig. 1. Identification of pTrack-ICP47 by endonuclease digestion with *Hind Ⅲ* and *EcoR V*

Lane l：pTrack-ICP47；Lane 2：pTrack-CMV/ *Hind Ⅲ* and *EcoR V*；Lane 3：pTrack-ICP47/ *Hind Ⅲ* or *EcoR V*；Lane 4：pTrack-ICP47/ *Hind Ⅲ* and *EcoR V*；Lane 5：DNA Marker DL15000.

Identificantion of pAdTrack-H-ICP47 with endonuclease digestion assay

There were two bands at approximately 294bp and 9200bp after pAdTrack-H-ICP47 was digested with *Kpn I* and *EcoR V*. The both bands were in the right range consistent with the expected length. On the contrary, there was only one band at approximately 9200bp after the control empty adenoviral vector pAdTrack-CMV was digested with *Kpn I* and *EcoR V*. Similarly, one band was detected at approximately 9200bp after pAdTrack-H-ICP47 was digested with *Kpn I* or *EcoR V*, respectively. The result of the recombinant shuttle plasmid pAdTrack-H-ICP47 was identified by endonuclease digestion of *Kpn I* and *EcoR V* was shown in Fig. 2.

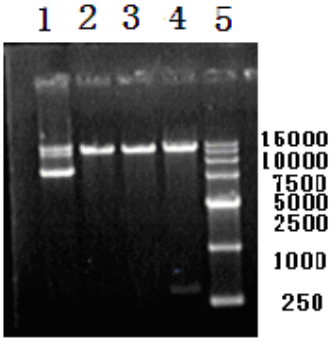


Fig. 2 Identification of pTrack-H-ICP47 by endonuclease digestion with *Kpn I* and *EcoR V*

Lane l：pAdTrack-H-ICP47；Lane 2：pAdTrack-CMV/ *Kpn I* and *EcoR V*；Lane 3：pAdTrack-H-ICP47 / *Kpn I* or *EcoR V*；Lane 4：pAdTrack-H-ICP47 / *Kpn I* and *EcoR V*；Lane 5：DNA Marker DL15000.

Antisense-sequencing identification of pAdTrack-H-ICP47

The result of antisense-sequencing identification of pAdTrack-H-ICP47 was shown in Fig. 3（from site 57 to site 350, 294bp）. Sequencing results verified that His-tag-ICP47 fusion gene fragment had been correctly cloned into pAdTrack-CMV.

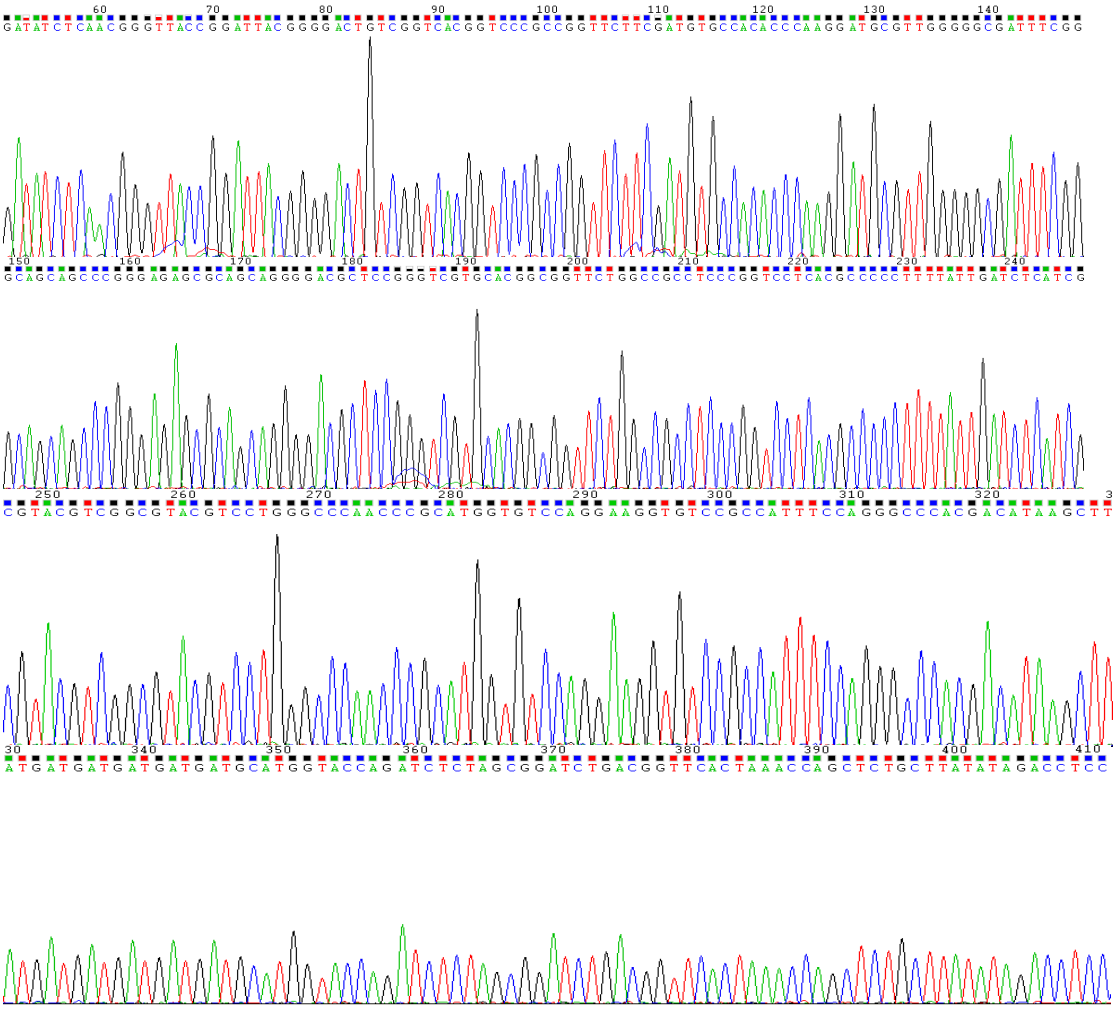


Fig.3. The antisense-sequencing identification of pAdTrack-H-ICP47

Identification of recombinant adenovirus vector pAdEasy-H-ICP47

The recombinant shuttle plasmid pAdTrack-H-ICP47 and the bone structure pAdeasy-1 were homologously recombined into BJ5183 cells. There were two bands at approximately 30 kb and 4.5 kb which were corresponded with the expectation after pAdTrack-H-ICP47 was digested with *Pac I*. The result of recombinant adenoviral vector pAdEasy-H-ICP47 identified by digestion of *Pac I* was shown in Fig. 4.

Fig.4. Identification of pAdEasy-H-ICP47 by endonuclease digestion with *Pac I*

Lane l：pAdEasy-H-ICP47；Lane 2：pAdEasy-H-ICP47/ *Pac I*； Lane 3： DNA Marker DL15000

Fluorescence photomicrograph observation

The verified pAd Easy-H-ICP47 was linearized with *Pac* Ⅰ and then transfected into 293 cells to generate adenovirus. Seven-ten days later, the adherent cells rounded and ablated. The green fluorescence could be seen under a fluorescence microscope (Fig. 5). High fluorescence intensity potentially correlated with high expression of His-tag-ICP47 fusion protein in 293 cells. The results of fluorescence photomicrograph observation verified that the recombinant adenoviruses of r-H-ICP47 and r-Track were successfully constructed and transfected into 293 cells. The viruses were amplified and the virus particle titres of r-H-ICP47 and r-Track were determined with the resulting of 3.7×10^{10}efu/mL and 4.4×10^{10}efu/mL.

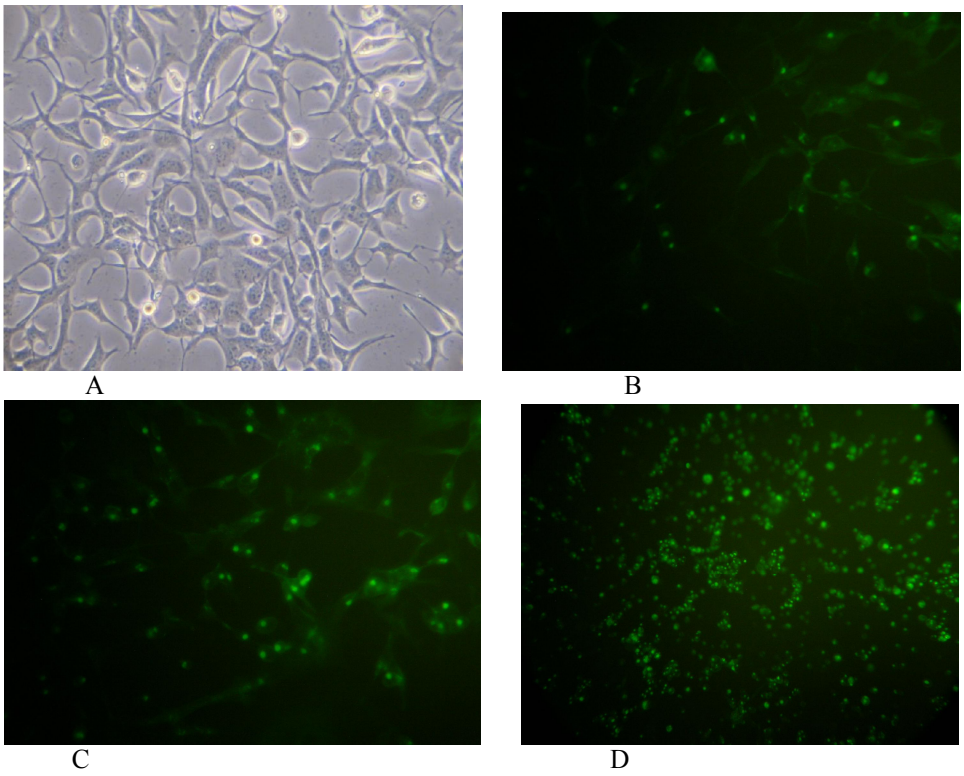


Fig.5 Transfection of recombinant adenovirus pAdEasy-H-ICP47 in 293 cells

A. Normal 293 cells before transfection of recombinant adenovirus（200×）; B Transfection of recombinant adenovirus cell at the 7th d（200×）; C Transfection of recombinant adenovirus at the 8th d（200×）; D Transfection of recombinant adenovirus at the 10th d（100×）.

PCR analysis of the expression of His-tag-ICP47 fusion gene

The result of PCR analysis showed that one band was detected at approximately 304bp in pAdEasy-H-ICP47 and pAdTrack-H-ICP47 samples and the band was in the right range consistent with the expected length, but no band was detected at approximately 304bp in the control empty adenovirus r-Track sample. The result of PCR analysis was shown in Fig. 6.

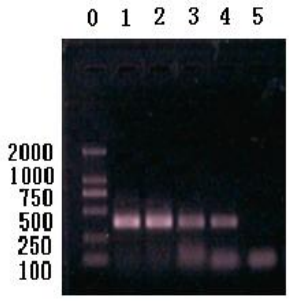


Fig. 6. Analysis of PCR amplification assay

Lane 0：DNA Marker DL2000；Lane 1：pAdEasy-H-ICP47；Lane 2：pAdEasy-H-ICP47；Lane 3：r-H-ICP47；Lane 4：r-H-ICP47；Lane 5：r-Track.

Western blot analysis of recombinant adenovirus -infected 293 cells

To confirm the proteins producted by r-H-ICP47-infected, r-Track-infected or mock-infected 293 cells, extracts were reacted with three kinds of antibodies in Western blot analysis (Fig. 7). The blots probed with anti-β-tubulin antibody were recognized in all cells extracts at approximately the same molecular mass (55 kDa). When an blot was probed with anti-GFP antibody, bands of extracts of r-H-ICP47-infected and r-Track-infected 293 cells were detected at approximately the same molecular mass (27 kDa), but no bands was detected in the mock-infected 293 cells. When an identical blot was probed with anti-6×His antibody, band of His-tag-ICP47 fusion protein was detected in extracts of r-H-ICP47-infected 293 cells (11kDa), but no band was detected in r-Track-infected and mock-infected 293 cells.

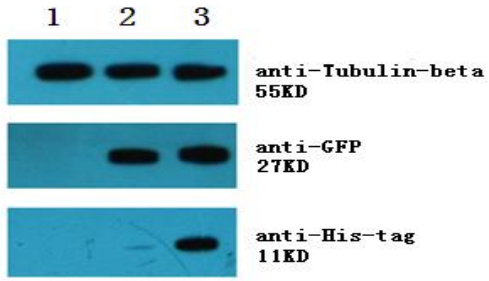


Fig. 7 Analysis of Western-blot assay
Lane1: mock-infected 293 cells; Lane 2: r-Track-infected 293 cells; Lane 3: r-H-ICP47-infected 293 cells

DISCUSSION

Adenovirus vectors offer many advantages, including high titer, broad range of infectivity, efficient gene transfer, limited pathological potential, and feasibility for delivery in vivo compared to plasmid vector, as has been reviewed previously[1,10]. They have become versatile tools for gene delivery and expression in the clinical trials and they have been used extensively in early clinical trials aimed at treatment of genetic disease[11] and cancer[12].

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. For these reasons, transient expression of the transgene has been reported in the majority of immunocompetent animal models, which is associated with problems with repeat administration of the vector and vector-induced inflammatory response [2].

Long-term persistence of gene-modified cells could potentially be achieved by using immunosuppressive regimens, which are commonly used in preventing of autoimmune disorders, solid organ graft rejection and graft-versus-host disease [13]. However, immunosuppressive regimens are not always effective and associated with the risk of infectious complications, and other regimen-related toxicities[3-4].

Survival of vertebrates is strongly dependent on the adaptive immune system and prevention of host immune response against transplanted cells and products of introduced genes could be a critical subject for the long-term success of gene therapy [14]. Inhibition of costimulatory interactions between T cells and antigen- presenting cells, such as blocking monoclonal antibody (MAb) against CD40 ligand and CD28-CD40 signaling with CTLA4Ig, has been reported in many studies of adenovirus-mediated gene therapy [15-17]. However, cellular and humoral immune responses were markedly reduced but not completely abrogated in these studies. On the cellular level, MHC class I–restricted pathway of antigen processing is critical for elimination of most virus infections, tumor surveillance, transplantation rejection, graft-versus-host reactions, and has been an attractive target for immune rejection. Blocking MHC Ⅰ antigen expression is becoming a research hotspot of inducting immune tolerance[6], and efficient antigen presentation restricted by MHC class I is associated with TAP[18]. As 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 assembly with MHC class I heavy chain and β_2-microglobulin (β_2m). Endogenous peptide presentation is affected by inhibition of TAP[19-20]. In the absence of functional TAP and antigenic peptides, most MHC class I molecules are eventually redirected to the cytosol and degraded by proteasomes [21-24].

ICP47, which is an immediate-early protein expressed by HSV-1 and an 88–amino acid cytosolic polypeptide, binds to the TAP1–TAP2 heterodimer in human but not in mouse cells and inhibits transport of proteasomally generated antigenic peptides from mostly cytosolic proteins into the endoplasmic reticulum (ER), where they would be loaded onto freshly synthesized HLA class I molecules. As a consequence, MHC class I molecules fail to be loaded with peptides, and the empty MHC class I molecules are retained in the ER and presentation of epitopes to $CD8^+$ T cells is abolished in HSV-infected human cells [7-8].Thereby the HSV-infected human cells are not lysed by cytotoxic T lymphocytes (CTL) and effectively evade the immune response in humans just as during HSV infection in vivo[25-27].

To investigate further the role of ICP47 in the elimination of transgene expression, we constructed an adenovirus vector expressing the His-tag-ICP47 fusion protein. In our study, AdEasy-1 system shuttle plasmid

contains a GFP gene incorporated into the adenoviral backbone, allowing direct observation of the efficiency of transfection and infection, which is more convenient for operation[28], and 6×His tag also facilitates detection by using biotinylated anti-6×His antibody and enables purification and detection of recombinant adenovirus without affecting tropism or production[29]. Furthermore the His-tag-ICP47 fusion gene in the E1/E3-deleted construct which is under the control of the cytomegalovirus promoter could block the expression of other viral proteins and minimizing unwanted effects on antigen processing.

In conclusion, a recombinant vector expressing the His-tag-ICP47 fusion protein is successfully constructed and the proteins producted by r-H-ICP47-infected cells are confirmed by PCR and Western blot analysis. In the following studies, we should verified the immunological activities of the His-tag-ICP47 fusion protein by cells and animal experiments and 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.

Correspondence to: Dr. KAN Quan-cheng, Clinical Pharmacology Base, First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan 450052, China (Email: upliz@163.com)

This work was supported by a grant from National Natural Science Foundation of China (No.30472031) and Scientific and Technological Projects, Zhengzhou, Henan Province, China (No. 2001123).

Acknowledgments

This work was supported by a grant from National Natural Science Foundation of China (No.30472031) and Scientific and Technological Project of Innovative personnel projects,Henan Province, China (No. 2001123).

References

[1] AC Silva, C Peixoto, T Lucas, C Kuppers, PE Cruz, PM Alves, and S Kochanek. Adenovirus vector production and purification [J]. Curr Gene Ther, 2010, 10(6): 437-455.

[2] Peter Johannes Holst, Cathrine Ørskov, Allan Randrup Thomsen, 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]. J. Immunol., 2010, 184 (8): 4431- 4439.

[3]U Christians, V Schmitz, W Schoning, J Bendrick-Peart, J Klawitter, M Haschke, and J Klawitter. oxicodynamic therapeutic drug monitoring of immunosuppressants: promises, reality, and challenges [J]. Ther Drug Monit, 2008, 30(2): 151-158.

[4] Albert MH, Anasetti C, Yu XZ. Cozza. T regulatory cells as an immunotherapy for transplantation [J]. Expert Opin Biol Ther. 2006, 6(4):315-324.

[5]Christopher Aisenbrey, Christina Sizun, Joachim Koch, 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]. J. Biol. Chem., 2006; 281(41): 30365 - 30372.

[6]Nathan J. Felix, W. June Brickey, Robert Griffiths, et al. H2-DM$\alpha^{-/-}$- Mice Show the Importance of Major Histocompatibility Complex–bound Peptide in Cardiac Allograft Rejection [J]. J. Exp., 2000.192(1):192: 31-40

[7] Margit H. Lampen, Marieke C. Verweij, Bianca Querido, et al. $CD8^+$ T Cell Responses against TAP-Inhibited Cells Are Readily Detected in the Human Population [J]. J. Immunol., 2010, 185(11): 6508 -6517.

[8] Christopher Aisenbrey, Christina Sizun, Joachim Koch, et al. Structure and Dynamics of Membrane-associated ICP47, a Viral Inhibitor of the MHC I Antigen-processing Machinery [J]. J. Biol. Chem., 2006, 281 (41): 30365-30372.

[9] Huang-Ge Zhang, Jinfu Xie, Igor Dmitriev, Elena Kashentseva, David T. Curiel, Hui-Chen Hsu, and John D. Mountz. Addition of Six-His-Tagged Peptide to the C Terminus of Adeno- Associated Virus VP3 Does Not Affect Viral Tropism or Production [J]. J VIROL, 2002, 76(23):12023–12031.

[10] Raviprakash K, Wang D, Ewing D, Holman DH, Block K, Woraratanadharm J, Chen L, Hayes C, Dong JY, Porter K. A tetravalent dengue vaccine based on a complex adenovirus vector provides significant protection in rhesus monkeys against all four serotypes of dengue virus. [J]. J Virol. 2008, 82 (14):6927-6934.

[11] Eto Y, Yoshioka Y, Asavatanabodee R, Mizuguchi H, Mukai Y, Okada N, Nakagawa S. Development of pegylated adenovirus vector for cancer gene therapy. [J]. Yakugaku Zasshi. 2008, 128(12):1733-1742.

[12]Furukawa K, Ohashi T, Haruki K, Fujiwara Y, Iida T, Shiba H, Uwagawa T, Kobayashi H, Yanaga K. Combination treatment using adenovirus vector-mediated tumor necrosis factor-alpha gene transfer and a NF-κB inhibitor for pancreatic cancer in mice [J]. Cancer Lett. 2011, 306(1):92-98.

[13]Liu QF, Fan ZP, Zhang Y, Jiang ZJ, Wang CY, Xu D, Sun J, Xiao Y, Tan H. Sequential intensified conditioning and tapering of prophylactic immunosuppressants for graft-versus-host disease in allogeneic hematopoietic stem cell transplantation for

refractory leukemia [J]. Biol Blood Marrow Transplant. 2009, 15(11):1376-1385.
[14]Chaanine AH, Kalman J, Hajjar RJ. Cardiac gene therapy [J]. Semin Thorac Cardiovasc Surg. 2010, 22(2):127-139.
[15]Xiao B, Xia W, Zhao K, Yang Y, Fu S, Li Y, Liu B, Guo S. Ex vivo transfer of adenovirus- mediated CTLA4Ig gene combined with a short course of rapamycin therapy prolongs free flap allograft survival [J]. Plast Reconstr Surg. 2011, 127(5):1820-1829.
[16] Iida T, Shiba H, Misawa T, Ohashi T, Eto Y, Yanaga K. Immunogene therapy against colon cancer metastasis using an adenovirus vector expressing CD40 ligand [J]. Surgery. 2010, 148(5):925-935.
[17] Wang X, Hao J, Metzger DL, Mui A, Ao Z, Verchere CB, Chen L, Ou D, Warnock GL. Local expression of B7-H4 by recombinant adenovirus transduction in mouse islets prolongs allograft survival[J]. Transplantation. 2009, 27;87(4):482-490.
[18]L Lybarger, X Wang, M Harris, et al. Viral immune evasion molecules attack the ER peptide- loading complex and exploit ER-associated degradation pathways [J]. Curr Opin Immunol, 2005, 17 (1): 71-78.
[19]J Koch, R Tampe. The macromolecular peptide-loading complex in MHC class I-dependent antigen presentation [J]. Cell Mol Life Sci, 2006, 63 (6): 653-662.
[20]T Elliott, A Williams. The optimization of peptide cargo bound to MHC class I molecules by the peptide-loading complex [J]. Immunol Rev, 2005, 207 (10): 89-99.
[21]Maaike E. Ressing, Sinéad E. Keating, Daphne van Leeuwen, et al. Impaired Transporter Associated with Antigen Processing-Dependent Peptide Transport during Productive EBV Infection [J]. J. Immunol., 2005, 174 (11): 6829-6838.
[22]Danijela Koppers-Lalic, Eric A. J. Reits, Maaike E. Ressing, et al. Varicelloviruses avoid T cell recognition by UL49.5-mediated inactivation of the transporter associated with antigen processing [J]. PNAS, 2005, 102 (14): 5144- 5149.
[23]N Nozawa, N Inoue. Mechanisms of congenital CMV infection [J]. Nippon Rinsho, 2006, 64 (Suppl 3): 446-450
[24]Nupur T. Pande, Colin Powers, Kwangseog Ahn, et al. Rhesus Cytomegalovirus Contains Functional Homologues of US2, US3, US6, and US11 [J].J. Virol., 2005, 79 (9): 5786 - 5798.
[25]M Hong, WZ Li, QH Li. The immune escape mechanism of herpes simplex virus type 1 suppression of transporter associated with antigen processing (TAP) by ICP47 [J]. Bing Du Xue Bao, 2007, 23 (1): 72-75.
[26]Liesbeth E. M. Oosten, Danijela Koppers-Lalic, Els Blokland, et al. TAP-inhibiting proteins US6, ICP47 and UL49.5 differentially affect minor and major histocompatibility antigen-specific recognition by cytotoxic T lymphocytes [J]. Int. Immunol., 2007, 19 (9): 1115 - 1122.
[27]Christopher Aisenbrey, Christina Sizun, Joachim Koch, et al. Structure and Dynamics of Membrane-associated ICP47, a Viral Inhibitor of the MHC I Antigen-processing Machinery [J]. J. Biol. Chem., 2006, 281 (41): 30365 - 30372.
[28]Zhang XW, Meng ZH, Zhao JS, Zhang D, Xie YJ, Gao SH, Wang CY. Gene therapy targeting for carcinoma regulated by E2F-1 promoter [J]. Zhonghua Wai Ke Za Zhi. 2006, 44(23):1636- 1639.
[29]Wu Q, Fu Q, Chen Q, Cai Q, Fan Z, Zhan Z, Niu L, Pei H, Zhou P. Prokaryotic expression, identification and bioinformatics analysis of fbpB-esxA fusing gene from Mycobacterium tuberculosis [J]. Asian Pac J Trop Med. 2011, 4(7):530-534.

2/25/12

The Theoretical Framework and Application on the Relationships between Family Functioning, Alexithymia and Emotional Intelligence among Early Adolescents

Fataneh Naghavi[1], Ma'rof Redzuan[2]

[1]School of Humanities and Social Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Social and Development Sciences, Faculty of Human Ecology, University Putra, Malaysia.
[1]E-mail: ahlo_1359@yahoo.com, [2]E-mail: marof@putra.upm.edu.my

Abstract: With respect to the relationship between family functioning, alexithymia, and early adolescent's emotional intelligence construct is still being developed with empirical support. Hence, our the perivious researches showed that how family functionig, alexithymia and several family's characters could effect on early adolescent's emotional intelliegnce. This study defines and discusses the theoretical framework in terms of the relationships between family functionig, alexithymia and several families' factors that influece on early adolescent's emotional intelliegence, specifically.

[Fataneh Naghavi, Ma'rof Redzuan. **The Theoretical Framework and Application on the Relationships between Family Functioning, Alexithymia and Emotional Intelligence among Early Adolescents.** Life Science Journal 2012;9(1):764- 770]. (ISSN:1097-8135). http://www.lifesciencesite.com.110

Keywords: Family Functioning, Early Adolescent's Emotional Intelligence, , Alexithymia, Children's Alexithymia, Emotional Quotient, Emotional Intelligence Theory, Social Cognitive Theory, McMaster Model

1. Introduction

As emotional intelligence is acquisitive and of social origin (Goleman 1995, Mayer and Salovey 1990), parents and children, in their interactions, expose their emotions expression way to one another consciously or unconsciously. The family has the highest effect on the individuals and it can mould their behaviors at any moment. A behavior which is created in relation to other family members is not limited to a normal agreeable behavior. The family can form abnormal behaviors, too (Sanaei, 2000). Considering, alexithymia is not classified as a mental disorder in the DSM-IV. It refers to the difficultly to identify, describe feelings to other people and it is a dimensional personality characteristic that varies in severity from person to person thus, family as a first circumstance can create this kind of characters. In addition, the treatment by parents to their children and how they react to their interests and activities, as well as children treatment to one another, emotion and information exchange among them, emotional protection to one another, and the relationships of the family members' with outsiders may also influence the children's emotional intelligence (Naghavi, 2010).

The family functioning construct is a relatively new concept with little empirical research, particularly related to the link between seven specific sub-components of the family function (dysfunction) and their emotional intelligence's early adolescent.

There has been a growing interest in the family functioning and emotional functioning of early adolescence (Goodyer, & Herbert, 1998; Walsh, 1993, Patterson, 2002, Ozbaci , 2006) and the factors that influence it (Goleman 1996, Mayer and Salovey, 1990; Carlson, 1999; Palmer et al., 2007; Bar-On, 1997; Martinez-Pons, 1997; Schutte's, 1998) in order to develop more integrated theories of development (McMaster's, 1995; Epstein, Bishop, & Levin, 1960; Goleman, 1995). There are many assumptions about family functioning and emotional intelligence. Ozbaci (2006) has assessed the relationship between and family environment and emotional intelligence EQ. The sample of the study was selected as 274 parents who live in Istanbul including 152 female, 122 male. Data were collected by EQ-NED and "Family Environment Scale" to determine family characteristics and the EQ. The results of the study indicate that there was a relationship between family cooperation and EQ.

A conceptually similar emotional intelligence construct is alexithymia. A comparison of the definitions of emotional intelligence and alexithymia suggests that the two constructs are closely related (Parker *et al*., 2001). Meanwhile, the emotional intelligence construct emerged from an integration of an array of research findings on how people appraise, communicate and use emotion (Salovey & Mayer, 1989, 1990). Although psychological systems have negatively looked into emotions, the attention given to emotions and feelings can be regarded as the core and basis of psychology and one can therefore look for mental disturbances roots in emotional perturbations like fear, anxiety, depression and alexithymia (Naghavi et al., 2010). Moreover, there is empirical evidence indicating that alexithymia is associated with the difficulties in discriminating among different emotional states (Bagby *et al.,* 1993). In research studies by Salovey and Mayer (1989, 1990), the overlapping emotional intelligence and alexithymia constructs were

acknowledges, and the researchers made attempts to empirically evaluate the relationships between the two constructs. One possible explanation for this is that these investigators have yet to introduce a standardized method for assessing emotional intelligence.

It is understood from the previous studies that emotional intelligence is associated with some factors, such as family function and some personality characters like alexithymia. This research studied the relation between family functioning and emotional intelligence so as to develop and expand the concept of emotional intelligence in the family. In other words, the importance of family functioning on alexithymia and emotional intelligence has been found to be very significant. It is expected that this research would identify different family functioning dimensions have influences on early adolescents' emotional intelligence.

2. Difinations of Family functioning, Alexithymia and Emotional intelligence

The family functioning refers to a set of tasks, roles, and expectations members of a family have for one another (Najarian, 1995). Patterson (1995) has shown that family functioning refers to performing the functions of the family and the pattern of relationships connecting members of a family system. Note that there are different patterns of relationships, such as patterns for showing affection, for problem solving in daily tasks.

The alexithymia is considered as a personality trait that places individuals at risk of other medical and psychiatric disorders while reducing the likelihood that these individuals will respond to conventional treatments for other conditions (Haviland, Warren & Riggs, 2000). Alexithymia is not classified as a mental disorder in the DSM-IV. It is a dimensional personality trait that varies in severity from one person to another. A person's alexithymia score can be measured using questionnaires, such as the Toronto Alexithymia Scale (TAS-20), Rieffe's Children's Alexithymia scale (2006) and the Bermond-Vorst Alexithymia Questionnaire (BVAQ) (Vorst & Bermond, 2001) or the Observer Alexithymia Scale (OAS) (Havilland *et al*., 2000). Based on the definition given by Taylor, Parker & Bagby (1990), in the context of this study, alexithymia is defined as:

1. Difficulty in identifying feelings and distinguishing between these feelings and the bodily sensations of emotional arousal.
2. Difficulty in describing feelings to other people.
3. Constricted imaginably processes, as evidenced by a paucity of fantasies.
4. A stimulus-bound, externally oriented cognitive style.

The emotional intelligence is defined as the ability to understand feelings in the self and others, and to use these feelings as informational guides for thinking and action (Salovey & Mayer, 1990). Bar-On (1997) defines emotional intelligence as the emotional, personal, social, and survival dimensions of intelligence, which are often more important for daily functioning than the more cognitive aspects of intelligence. Martinez-Pons (1997) stated that emotional intelligence refers to the ability to monitor one's own and others' emotions, to discriminate among them, and to use the information to guide one's thinking.

3. Theoretical Framework

Emotional intelligence is acquisitive and of social origin. Thus, the family with multi-functional aspects serves as a fundamental dimension in the development of early adolescents' emotional intelligence. According to Epstein, Bishop, and Levin (1960), family structure and its appraisal method is problem-oriented. These emphasize family functioning in three areas:

(1) Basic tasks such as supply of foodstuff, clothes, as well as transportation and accommodation expenses

(2) Developmental task area (i.e. how a family copes with the transition stage problems such as adolescents going out of childhood or their leaving home

(3) Jeopardizing task area (i.e. crises may happen to the family, such as job losing, accidents, etc.) (Epstein, Baldwin, Bishop, 1983 cited in Tamplin, Goodyer & Herbert, 1998).

In a family with high functioning, members inform one another of their thoughts and feelings through interaction and communication. Communication is one of the main family functions that improve adolescents' emotional intelligence. In fact, effective communication is an important requirement of any personal relationship. In the context of this study, the researcher mentioned the role of communication in the family and the improvement of early adolescents' emotional intelligence.

The theoretical framework in this study is organized in three sections. (i)The first section includes the emotional intelligence theory. This particular section is an attempt to reveal the effects of several factors that influence emotional intelligence. (ii)The second section highlights the importance of social cognitive theory in the emotional processing of early adolescents' emotional intelligence improvement and vas versa can cause alexithymia. The purpose of this particular study was to examine the associations or relationships between family functioning and emotional intelligence in early adolescents' emotional intelligence and alexithymia. In addition, social cognitive can be related of the role of early family relationships or early mother-child relationships in

affecting the development and in the acquisition of self-regulatory capacities. It is needed because of the important concepts in social cognitive theory, which are interaction, reciprocal process through person, environment and behaviour. According to social cognitive theory, family as the first unit with communication functioning, can develop early adolescence's lives, and thus, social cognitive theory refers to learning by observing others, with the environment, behaviour, and cognition as all the key factors influencing the development of early adolescents. Therefore, it seems that family with dysfunctioning, according to this theory, can cover alexithymia. (iii) Finally, this section also included family functioning model, and attempted to reveal the role of family via multi functioning aspects.

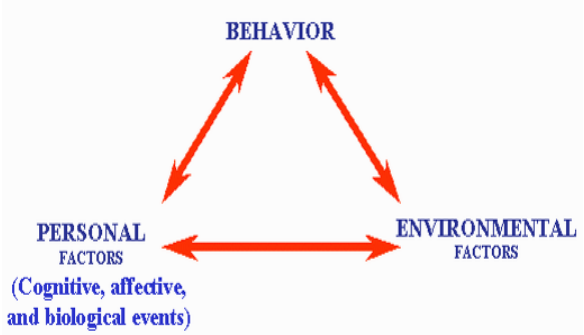
Emotional Intelligence Theory

Emotional intelligence is conceptualized as comprising five primary domains, which include knowing one's emotions, recognizing emotions in others, handling relationships, and motivating oneself (Goleman, 1995). The emotional intelligence theory was introduced by Goleman (1995). These domains, as the main factors in emotional intelligence, can support this area of the current study. The framework of emotional intelligence was used to bring, in an organized whole, the five related components comprising the abilities related to the understanding, managing, and use of emotions. The first of these domains, i.e. knowing one's own emotions, involves the ability to accurately recognize, identify, and label feeling and emotions as they arise in oneself. It entails removing oneself from the experience of an emotion that is enough to develop a self-awareness of what one is feeling or one's mood. The second emotional intelligence, i.e. managing one's emotions, is built largely on self-awareness, and it involves the ability to regulate emotions so that they are experienced appropriately. Individuals who are skilful at intensity and duration of feelings such as persons are able to cope more effectively with emotionally-laden situations in their lives. Similarly, the third domain of emotional intelligence is also built on self-awareness. Synonymous with empathy, it involves the ability to read the feelings in others and to become emotionally attuned to subtle social cues. Individuals who are deficient in identifying their own emotions are also necessarily deficient in knowing what others are feeling. The fourth of emotional intelligence comprises the ability to appropriately and effectively handle interpersonal relationships, a skill more generally known as social competence. It is important to note that a core aspect of social competence involves the skill of managing emotions in others, and is contingent on having developed the ability to accurately identify how others are feeling and the ability to exert self-control over one's own emotions. The fifth domain of emotional intelligence is unique as it emphasizes on the utilization of emotions, as opposed to the appraisal, expression, or regulation of emotions which are emphasized by the other four domains. Mayer and Salovely (1997) conceptualized this domain as comprising four independent abilities, including flexible planning, creative thinking, mood redirected attention, and motivation. They further proposed that emotionally intelligent individuals are able to adaptively utilize and modify their emotional states to solve problems more effectively and achieve goals. For the purpose of the proposed study, the fifth domain of emotional intelligence is broadly defined as "achievement orientation". In keeping with Goleman's description of this particular domain, achievement orientation is viewed as encompassing the following components: motivation, optimism, delay of gratification, and self-efficacy. In addition, a high achievement orientation was also proposed to include an internal locus control or attribution style and not controlled by outside forces. According Naghavi & Ma'rof, early adolescence whose parents consistently practice emotion coaching have better physical health and score higher academically than early adolescence whose family do not offer such guidance (2012). On the other hand, the role of families' performance in all individual's behaviours, normal and abnormal behaviours, personality, and development of his different talents is important. Family functioning of their early adolescents and how they react to their interests and activities, early adolescents' treatment to one another, emotion and information exchange among them, emotional protection for one another, and the family members' outside relations may influence the early adolescents' emotional intelligence. Whence, the alexithymia is an antonym to emotional expression, alexithymia is utilized to explain the lack of ability to express and regulate emotions (Apfel & Sifneos, 1979). In fact, as an individual difference, alexithymia overlaps conceptually with the emotional intelligence construct, which encompasses the ability to perceive and appraise one's own and others' and emotions, the ability to access and use the feelings to guide one's thinking and actions, and the ability to regulate emotions to promote emotional and intellectual growth (Mayer & Salovey, 1997).

Social Cognitive Theory

Social cognitive theory is a learning theory based on the ideas that people learn by watching what others do and that human thought processes are central to understanding personality. People learn by observing others, with the environment, behaviour, and cognition as the chief factors in influencing their development.

These three factors are not static or independent; rather, they are all reciprocal. This is the foundation of Bandura's (1986) conception of reciprocal determinism, i.e. the view that (a) personal factors in the form of cognition, affect, emotion, and biological events, (b) behaviour, and (c) environmental influences, such as parent, sibling relations in family create interactions that result in a triadic reciprocality. The reciprocal nature of the determinants of human functioning in social cognitive theory makes it possible for therapeutic and counselling efforts to be directed at personal, environmental, or behavioural factors (Pajares, 2002). Figure.1 indicates the Conceptual Model of Social Cognitive theory. More examples can be cited to clarify each witness that can change a person's way of thinking and interaction with family members. Similarly, the environment every person is raised in may influence his or her later behaviours, just as a father's or mother's mindset (also cognition) will determine the environment in which his or her children are raised (Santrock, 2008).



Figur 1: The Conceptual Model of Social Cognitive theory (Source: Pajares, 2002).

On the other hand, several theories have hypothesized that personal characters, whether positive or negative, stem from adaptive or maladaptive early life experiences, and are usually associated with family functioning. According to Lumley, Mader, Gramzow & Papineau (1996), trauma, excessive parent's attention to a child's bodily rather than emotional needs, and family's emotional unavailability or inconsistency have been posited as leading to disturbed effect representation and self-regulation of alexithymia. Although empirical studies have also demonstrated that a family's behaviour influences early adolescent functioning (Taylor, 2000; McElwain, Halberstadt, & Volling, 2007). These researchers found young adults' reports of diminished family expressiveness that specifically limit positive communication are related to their own affective identification and communication deficits. Based on the alexithymia's aetiology (Taylor & Taylor, 1997; Taylor, 2000) some researches demonstrating that pathological parenting it was hypothesized that early adolescent's level of alexithymia would be positively related to the levels of general pathology in their families. Furthermore, based on the social cognitive theory's predictions of specificity in learning and the findings of Berenbaum and James (1994) of a unique relationship between the type of alexithymia deficit and specific family dysfunction, it was hypothesized that dysfunction in a family member's emotional involvement would be associated with affective alexithymia deficits, whereas a lack of rules governing family behaviour and deficient family solving ability would be related to externally oriented thinking.

However, there seems to be a contradiction for alexithymia individuals as they can be socially adaptive. The key is that they are able to anticipate and interact with others so as to form functional, but not close relationships. Their ability to understand and deal with the subtleties of intimate relationships is very poor. It appears that alexithymic individuals can predict beliefs and actions at a macroscopic social level but have difficulty at the intimate individual level. Hence, social cognitive theory is at the base of children's social understanding, and thus, in all cultures, family is the first of social unit and the centre of the individual's identity formation. Early adolescent in family environment learn to express, comprehend, understand, and regulate emotions by exchanging their emotions with parents and coevals. Some of them take control of their own and others' emotions more skilfully due to the big differences in the individual abilities and their social world. However, some children lack adequate skills to interpret emotions for their own achievement in the social world (Scharfe, 2000). Emotional intelligence enables an individual to correctly exhibit suitable amount of different emotions, such as rage, fear, love, happiness, etc., in his/her behaviour, proportional to the situation and time. In addition, it also enables them to understand others' emotions and react accordingly (Goleman. 1996; Mayer, Salovey, & Caruso, 2000). In contrast, alexithymia overlay emotional intelligence in the field of emotion identification to some extent, into feelings expression. In other words, an alexithymia adolescent cannot express his/her emotions orally due to his/her inability to identify these feelings, whereas preliminary emotional abilities have specific importance because skilfulness in appraisal and quick precise expression of emotions bring about suitable compatibility in relation to the environment and others (Taylor & Bagby, 2000). As a result, as emotional intelligence is acquisitive and of social origin (Goleman, 1996; Mayer & Salovey, 1990), parents and children, in their interactions, expose the expression of their emotions to one another consciously or unconsciously. Therefore, regarding to this reciprocal process, the theory of social cognitive can support relationships between family functioning, alexithymia and emotional intelligence. Consistent with

the social cognitive perspective, prior studies have demonstrated that the development and interaction among each domain have significant relationships with child psychological development (e.g., emotional intelligence, problem behaviours, alexithymia). In addition, Naghavi (2011), demonstrated that early adolescent develop specific alexithymia characteristics as a result of family dysfunction in emotional or cognitive domains in her study. These findings are well agreed with theories of the social cognitive theory. Due to according social cognitive theory early adolescents learn to express, understand, and regulate their emotions in interactions with their family, siblings. Family is strong shapers to early adolescent's behavior (Stover, 2003). Forthermore, parental emotion affect on early adolescent's emotion and social behaviors by its emotional regulation.

McMaster's Model of Family Functioning Appraisal

This model was introduced by Epstein, Bishop, and Levin in the early 60's during a prolonged study. It concentrates on family structure and its appraisal method is problem-oriented. This model emphasizes family functioning in three different areas, namely: Basic tasks such as supply of foodstuff, clothes, transportation and accommodation expenses. Developmental task area: how a family copes with the transition stage problems, such as adolescents' going out of childhood or their leaving home. Jeopardizing task area: crises may happen to the family, such as losing jobs, accidents, etc. (Epstein, Baldwin, Bishop, 1983 cited in Goodyer, & Herbert, 1998).

There are six dimensions of family functions assessment in the Family appraisal model. These functions include: (1) problem solving; (2) communication; (3) roles, (4) affective responsiveness; (5) affective involvement; and (6) behavioural control.

Problem-solving: This dimension deals with the family's ability to solve problems to the extent that it becomes efficient.

Communication: This dimension deals with the family's ability to communicate information. The main function is communication, which in this research was more emphasized for its efficacy on early adolescents' emotional intelligence improvement.

Roles: This structure describes family efficiency based on tasks allocation to members and their accomplishing these tasks.

Affective Responsiveness: This refers to family members' ability in making suitable responses to positive and negative emotional situations.

Affective Involvement: A reaction each member shows to others' interests and attention. In fact, this describes family members' interests and views toward one another.

Behaviour Control: This dimension is associated with the rules based on which the family passes training. It includes the amount of influence members leave on one another.

The McMaster Model emphasizes speech communication. Four kinds of communication are specified in this model, namely; (1) clear and explicit, (2) explicit, (3) clear and inexplicit, and (4) masked and incorrect. The most effective form of communication is the clear and explicit kind, whereas the most ineffective one is the masked incorrect kind because unclear and incorrect messages confuse addressees due to their ambiguity, equivocalness, and conflict, clear and inexplicit, masked and incorrect. It is preferred that messages be sent directly by the messenger, not via a medium. In such conditions, misunderstanding or even misuse by the messenger is likely. In a family with high functioning, members inform one another of their thoughts and feelings in delicate ways. They express their interests and concerns and talk about important issues. They can talk with other members about themselves and their life; they know that they are listened to and understood. Thus, effective communication is an important requirement for intimate relations. Communicative models in a family have effects on the models which will be applied by children in their future families. Communication is not limited to words; it includes listening, silence, looks exchange, facial state, poses, touching, body state, and other non-vocal symbols and codes used to send and receive meanings (Nowruzi, 1998).

Stern, Yuen & He (2004), believe that effective family communication play a critical role in maintaining positive, healthy relationship, problem solving and managing stress. According this point of view, communication in family is as a key factor to improve the emotional intelligence of early adolescents. Communication is one of the family functions that incorporate the interaction between the parents and children. Families that use clear and positive communication in their interaction with each other are always consistent in what is said and what is done. Furthermore, family members will discuss personal fears, stresses, criticisms, complaints, and other feelings with each other rather than censoring such topics from conversation. Such families moreover have the adults clarifying ambiguous situations to children, explaining their own expectations or feelings in terms that the children can recognize, and encouraging children to explain their own fears and feelings and to have a voice in family decision making and problem solving (Conger & Conger, 2002). These types of family communication have shown to promote better emotional regulation, coping skills and cognitive ability (Cox & Davis, 1999; Conger & Conger, 2002;

Wagner, Cohen, Brooks, 1996). In addition, family functioning research suggests that family environment has a significant influence on developing empathy and identification of emotions (Saarni, 1999, Honig, 1981). Baumrind (1993) and Harris (1995) believe that the importance of socialization and family functioning on children's subsequent emotional expression as they mature. Contrary to the alexithymia is an antonym to emotional expression. Alexithymia is used to describe the inability to express and regulate emotions (Apfel & Sifneos, 1979).

4.Conclusion

Regarding in relation to the above discussion, the related literature has indicated that early adolescents' emotional intelligence is influenced by some factors such as the different tasks, communications, roles and aspects of family and some personal characters. Hence, this study attempted to, firstly describe these factors, and secondly examine the relationship between these factors and early adolescents emotional intelligence so as to understand some aspects of emotional intelligence related to alexithymia, family functioning and their early adolescents to provide the existing evidence and criteria for future programmes.

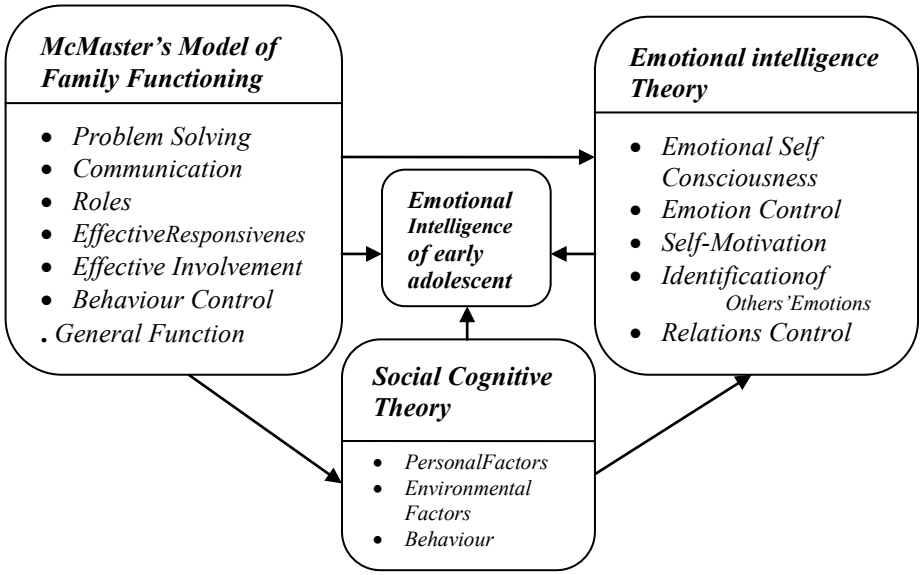


Figure 2: A Diagram of the Theoretical Framework

References

1. Goleman, D. *Emotional Intelligence: Why It Can Matter More Than IQ*. New York: Bantam, 1995.
2. Sanaei, B.C.D. *50 Great Philosophers: from Thales to Sartre* (M. R. Mehrabadi, Trans.). Tehran: Ataei Publications, 2000.
3. Naghavi, Fataneh. The relationship between family functioning and alexithymia. Canada International Conferenc on Education(CICE-2010).Canada, 2010.
4. Goodyer , T.I.M. & Herbert, J. Family functioning and parent general health in families of adolescents with major depressive disorder. *Journal of Affective Disorders*, *48, 1998*. 1-14.
5. Walsh, F. *Normal family process*. New York: The Guilford Press, 1993.
6. Patterson, J.M. *Promoting Resilience in Families Experiencing Stress, 2002.*
7. Ozbaci, N. Emotional intelligence and family environment. *Sosyal Bilimler Dergisi*, *16,2006*.169-175.
8. Goleman, D. *Emotional Intelligence: Why It Can Matter More Than IQ*. Learning, 24,1996. 49-50.
9. Mayer, P.Salovey a. J. D. Emotional intelligence. *Imagination, Cognition and Personality, 9,* 1990. 185-211.
10. Carlson, G. L. *Family Treatment: Efficient Therapy Guarantee* (S. Navabinejad, Trans.). Tehran: Teachers and Parents Association Publications, 1999.
11. Palmer, C.D. a. C. S. Emotional intelligence and life satisfaction. *Personality and Individual Differences, 33,2007*. 1091-1100.
12. BarOn, R. *Emotional Quotient Inventory Technical Manual*. Toronto: Multi-Health Systems, lnc, 1997.
13. Martinez, P.M. The relation of emotional intelligence with selected areas of personal functioning. *Imagination, Cognition, and Personality, 17*, 1997. 3-13.
14. Schutte, N., Malouff, J.M., Hall, L.E., Haggerty, D.J., Cooper, J.T., Golden, C.J., & Dornheim, L. Developmet and validation of a measure of emotional intelligence. *Personality and Individual Differences, 25,* 1998. 167-177.
15. McMaster, A. *The Intelligence Advantage: organizing for complexity, Knowledge Based Development*. IOM: Douglas, 1995.
16. Epstein, N.B., Baldwin, L.M. & Bishop, D.S. The McMaster family assessment device. *Journal of Marital and Family Therapy, 9*, 1983, 171-180.
17. Parker, J.D.A., Taylor, G. & Bagby, R.M. The relationship between emotional intelligence and

alexithymia. *Personality and Individual Differences 30,* 2001. 107-115.
18. Salovey, P. & Mayer, J.D. Emotional intelligence. *Imagination, Cognition, and Personality*, *9, 1989/1990.* 185-211.
19. Naghavi, Fataneh, R. Ma'rof., and Mariani, M. The relationship between alexitymia and emotional intellience. Asian Social Science, Published by Canadian Center of Science and Education, 2010; (6), 2010. 166-170.
20. Bagby, R. M., Parker, J.D.A., Taylor, G.J., & Acklin, M.W. *Alexithymia and the ability to distinguish different emotional states*. Poster Presentation at the Annual Meeting of the American Psychosomatic Society, Charleston, S.C.,1993, March.
21. Najrian, F. Factors Influencing Family Efficiency: The study of Psychometric Characteristics of FAD. Rudhen: Islamic Azad University, Rudhen Beranch, 1995 .
22. Haviland, M.G., Warren, W.L. & Riggs, M.L. An observer scale to measure alexithymia *Psychosomatics, 41*, 2000, 385-392.
23. Carolien Rieffe, P.O. & Terwogt, M.M. An alexithymia questionnaire for children: Factorial and Concurrent Validation results. *Personality and Individual Differences, 40*, 2006. 123-133.
24. Baumrind, D. The average expectable environment is not good enough: A response to scar. *Child Development, 64,1993. 1299-1317.*
25. Vorst, H.C.M. & Bermond, B. Validity and reliability of the Bermond-Vorst Alexithymia Questionnaire. *Personality and Individual Differences, 22,* 2001. 413-434.
26. Tamplin, A., Goodyer , I.M., & Herbert, J. Family functioning and parent general health in families of adolescents with major depressive disorder. *Journal of Affective Disorders*, *48,* 1998. 1-14.
27. Mayer, J. D. & Salovey, P. *What is Emotional Intelligence? Emotional Development and Emotional Intelligence: Implications for educators.* New York: Basic Books, 1997.
28. Naghavi, F. & Mar'of, R. Relationship between family environment and emotional intelligence: Examination of the moderating factor. *Life Science Journal*, 2012, 9. 391-395.
29. Apfel, R. J., & Sifneos, P. E. (1979). Alexithymia: Concept and measurement. *Psychotherapy & Psychosomatics*, 32. 180-191.
30. Bandura, A. Social Foundations of Thought and Action. Englewood Cliffs, NJ: Prentice-Hall, 1986.
31. Pajares, F. *Overview of social cognitive theory and of self-efficacy*. Retrieved from http://www.emory.edu/EDUCATION/mfp/eff.html, 2002.
32. Santrock, J.W. A Topical Approach to Lifespan Development. In M. Ryan (Ed.), 4th ed.). New York, NY: McGraw-Hill Companies, Inc, 2008.
33. Lumley, M., Mader, J., Gramzow, A. and Papineau, K. Family factors related to alexithymia characteristics. *Psychosomatic Medicine, 58,* 1996.211-216.
34. Taylor, G.J. Recent developments in alexithymia theory and research. *Canadian Journal of Psychiatry, 45*, 2000. 134-142.
35. McElwain, N., Halberstadt, A. G., & Volling, B. Mother- and father-reported reactions to children's negative emotions: Relations to young children's emotional understanding and friendship quality. *Child Development, 78, 2007.*1407-1425.
36. Taylor, G.J. a. Taylor, H. L. Alexithymia. In: McCallum. *Psychological Mindedness*, *2,* 1997. 77-104.
37. Berenbaum, H. a. James, T. Correlates and retrospectively reported antecedents of alexithymia. *Psychosomatic Medicine, 56*, 1994. 353-359.
38. Scharfe, E. *Development of Emotional Expression, Understanding, and Regulation in Infants and Young Children*. In R. Bar-on & J. D. A. Parker, Handbook of Emotional Intelligence. (pp. 244-262). San Francisco: Jossy-Bass Inc, 2000.
39. Mayer, J. D., Salovey. P. a. Caruso. D. R. Emotional Intelligence as Zeitgeist, as personality, and as a mental ability. In R. Bar-On & J.D.A. Parker (Eds.). The handbook of emotional intelligence: Theory, development, assessment, and application at home, school, and in the workplace. San Francisco, CA: Jossey-Bass, 2000.
40. Taylor, G.J., Bagby, R. M. *An Overview of the Alexithymia Construct*. San Francisco: Jossy-Bass Inc, 2000.
41. Naghavi, Fataneh. Family functioning and early adolescens' psychopathology. World Applied Sciences Journal 15, 2011.1512-1517.
42. Stover, J. Fathers' Meta-Emotion and children's social status, Doctoral dissertation, Seattle Pacefic University, USA, 2003.
43. Nowruzi, M. *Study of Family Functioning Effect on Psychic Disorders of Tehran Adolescents of 15-18 Age Group.* Rudhen: Islamic Azad University, Rudhen Beranch, 1998.
44. Conger, R. D., & Conger, K. J. Resilience in Midwestern families: Selected findings from the first decade of a prospective, longitudinal study. *Journal of Marriage and Family,* 64, 2002. 361-373.
45. Cox, R. P., & Davis, L. L. Family problem solving: Measuring an elusive concept. *Journal of Family Nursing*, 5, 1999. 332-360.
46. Wagner, B. M., Cohen, P., Brooks, J. S. Parent/adolescent relationship: Moderators of the effects of stressful life events. *Journal of Adolescent Resrach*, 11, 1996.347-374.
47. Saarni, C. *The Development of Emotional Competence*. New York: The Guilford Press, 1999.
48. Honig, A. S. The gifts of families: Caring, courage and competence. ERIC Document Reproduction Service No. ED 1981. 208 972.
49. Harris, J.R. Where is the child's environment? A group socialization theory of development. *Psychological Bulletin, 102*, 1995. *458-489.*

2/25/12