CONTENTS

Securing Computerized Personal Identification Data with Confidentiality and Non-repudiation Capabilities Based on Programmable System on Chip (PSoC) Technology

Sung-Tsun Shih[1], Chian-Yi Chao [2], Chin-Ming Hsu [3]

[1]Department of Electronic Engineering, Cheng Shiu University, Kaohsiung, Taiwan
[2]Department of Electronic Engineering, Kao Yuan University, Kaohsiung, Taiwan
[3]Department of Information Technology, Kao Yuan University, Kaohsiung, Taiwan
stshih@csu.edu.tw

Abstract: In this study, a cipher/decipher based on programmable system-on-chip (PSoC) technology is proposed to prevent unauthorized people from revealing and abusing users' computerized personal identification data (PID) during transit and at rest. The proposed PSoC-based cipher/decipher consists of a Public Key Infrastructure (PKI) authentication protocol, a programmable microcontroller named AT89S51 single chip, and an application specific integrated chip (ASIC) named EPF10K Field Programmable Gate Array (FPGA) device. The PKI authentication protocol with the registration and transaction processes for verification/identification is used to secure the encrypted PID data transmitted on the Internet. The programmable microcontroller mainly programs the encrypting/decrypting codes and the ASIC device is implemented with the stream cipher's logical operations, including, segmentation, exclusive or (XOR), and rotation. This design supports off-line encryption of numerous users' PID. The proposed approach has been simulated using C programming language running on a 1,500MHz Pentium PC with 512 MB of RAM. From the experimental results, the size of the ciphertext, the size of the key stream, and the encryption time are dependent on the secret key, nonce, and plaintext; the design of ASIC hardware device is of low hardware cost and low design complexity; and the software of PSoC-based cipher/decipher could provide better code density. Conclusively, using certificates and dynamic cookies for verification/identification ensures that only authorized users can obtain the access to their personnel record.
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Keywords: Computerized personal identification data, cipher/decipher, PKI, and authentication.

1. Introduction

Privacy means an ability to protect personal secrets and/or a state of being hidden from public view [1-2]. Before the advent of computers, privacy protection was usually achieved by using key locks to protect physical resources. With the developed computer and Internet technologies, the traditional key locks are not effective for privacy protection because most organizations, including commercial and social organizations, store data on computers. Specifically, data is shared among different institutions via the Internet and a large amount of commerce is electronically conducted. These advanced technologies are able to bring more convenience to life but also bring an increased risk of abusing or disclosing personal data, thereby resulting in the privacy threat: identity theft.

Identity theft is usually initiated by disloyal insiders or adversaries who steal one's identifying data stored in a database or transmitted on a network [3]. Currently, identity theft is one of the fastest growing crimes in the United States. An estimated 500,000 to 700,000 Americans fall victims to identity theft every year, and losses resulting from identity theft cost around 3.5 billion dollars in 2002 and the losses rose to over 8 billion dollars in 2006 [4-5]. In other countries, this problem is just as urgent as in the United States. One risk of abuse or improper disclosure of personal data may occur in e-commerce services. For example, an individual may register his identity information including name, address, social security number, and account details in an institution's database. The administrator of the institution will use the registration information to make decisions on supplying data in response to service requests from the user. In the absence of effective protection on users' personal data, their identity information is susceptible to being misused by unauthorized users or organizations. Consequently, an individual may acquire a bad reputation due to improper disclosure of his personal data, thus requiring extra money, time, and effort to recover one's reputation and credit standing. Benner, Givens, and Mierzwinski [6] have investigated that an average of $808 and 175 hours of effort is needed for a person to put things right.

Cryptography is commonly classified into three parts: un-key, secret key, and public key cryptography. Un-key cryptography uses a function to map, hash, or transform a message into different

presentation by mathematical operations, such as permutations and involutions, for data integrity. Secure hash algorithm (SHA) [7] and message digest (MD5) [8] are two typical researches which usually condense a message of any size down to a small fixed-size block of messages by using bitwise logical AND, inclusive-OR, eXclusive-OR, and complement operations.

Secret key cryptography, symmetric cryptography, encrypts/decrypts a message using a secret key shared between two communicating parties. A typical example named data encryption standard (DES) uses a secret key to transform a plaintext which is grouped into contiguous fix-length blocks via substitution and permutation operations. The length of the ciphertext is the same as that of the plaintext. However, it is insecure because a DES key can be recovered in 56 hours [9]. Niinimaki and Forsstron [10] proposed an electronic prescription system using this cryptography, which stored a secret key in a smart card to prevent hackers or unauthorized people from disclosing users' passwords. In general, such type of cryptography performs efficiently but it has key distribution problem. Several researches [11-15] have proposed key-exchange-based authentication protocols coupled with symmetric-key techniques to solve the key distribution problem. This kind of password- and PKI-based key-exchange protocol technology mainly uses a shared session key to prevent the password from guessing attack with the computation-efficiency capability. However, it requires periodically changing passwords for better security.

Public key cryptography, asymmetric cryptography, encrypts/decrypts a message by using a mathematically related key pair, a public key and private key, in which the key used for encryption is different from decryption. The public key is open publicly and the private key needs to be held only by its owner securely. This cryptography is usually used for key encryption/decryption, digital signature generation, and key exchange. RSA [16], DSA [17], and Diffie-Hellman [18] use this cryptography for data encryption/decryption, data authentication, and key exchange. RSA is based on integer factorization operations whereas DSA and Diffie-Hellman are based on discrete logarithm operations. Blobel and Pharow [19] proposed a public key cryptosystem that stored the keys in a smartcard to protect medical data from network intruders. Meyer and Lundgren [20] produced the digital signature of an individual's authentication code to increase the security of healthcare networks. Ohta and Okamoto [21] introduced multi-signature schemes based on elliptic curve mathematics to ensure the security of digital signatures. However, this cryptography requires time-consuming computations in generating keys.

Menasce [22] examined the security performance of cryptographic algorithm/key-length combinations. As Menasce points out, the security methods based on cryptographic technology have constraints on the key size used to meet certain security requirements. In general, the processing time of the symmetric key encryption is exponentially faster than that of the public key encryption. The symmetric key encryption with a secret key shared between two certified users has key management and distribution problems because the quantity of keys increases with the square of the number of communicating parties. In addition, the use of cryptography-based security mechanism usually needs intensive mathematical computation in deriving or generating keys. A great quantity of memory used to store public parameters is needed when the number of classes or nodes increases. For the conventional authentication techniques based on hard mathematical operations, the use of larger key sizes results in better security but the processing time will be increased greatly.

In order to improve the disadvantages mentioned above, this study proposes a PSoC-based security mechanism to prevent unauthorized disclosure and improper modification of one's computerized personal data during transit and at rest. The proposed privacy technology not only minimizes the quantity of RAM usage to store temporary parameters but also implements simple mathematical operations at a small expense of hardware to process data efficiently.

2. Proposed Research

Figure 1 shows the PKI-based system overview of securing computerized personal data during transit and at rest. It consists of four components: E-commerce certificate authority (ECA), affiliated E-commerce merchant database, users' authentication server, and PSoC-based cipher/ decipher at user terminal. The ECA manages the certificates and public-private key pairs. The database stores users' encrypted personal identification data (PID). The authentication server interacts with the ECA, encrypted PID database, and PSoC-based user terminal via the authentication protocol. It handles verification/identification processes and stores temporary processed data in the PID buffer. An individual can utilize an IC token or a keyboard to access his PID. The principles of the proposed PSoC-based cipher/decipher and PKI-based authentication protocol are detailed below.

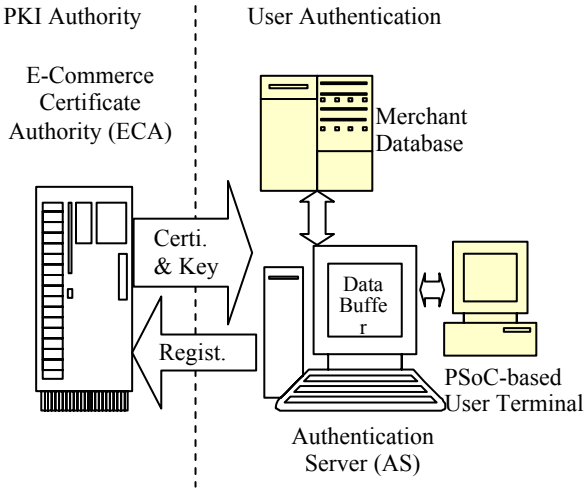


Figure 1. PKI-based system overview of securing computerized personal data during transit and at rest

2.1. PSoC-Based Cipher/Decipher

As shown in Figure 2, the proposed PSoC-based cipher/decipher consists of three components: a database system, an 89S51-based programmable microcontroller, and an FPGA device for encryption/decryption. The database system stores uses' computerized personal data; the 89S51-based programmable microcontroller is used to program the encryption/decryption codes; and the FPGA device allows users to implement encryption/decryption algorithm at a small expense of hardware such as EPF10K20RC240-4.

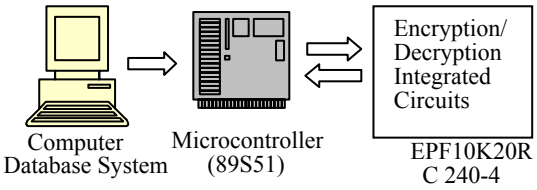


Figure 2. PSoC-based cipher/decipher

2.1.1. Database System

In this study, we assume that the database system stores users' personal identification information and order information for E-commerce application. The format of personal identification information contains an individual's user name, password, sex (female/male), date of birth, social security number, address, and E-mail. The format of the order information contains product name, product identification, date of order, quantity, price, payment method, status, and total price.

2.1.2. AT89S51 Programmable Microcontroller

Figure 3 shows the circuit schematic of 89S51-based programmable microcontroller. As shown in Figure 3, the user's personal identification is transmitted from the computer database system to the 89S51 microcontroller byte by byte at first. The 89S51 stores the received PID and then passes the received data to the input of the FPGA. After all the PID data are stored in the ROM of the 89S51, the 89S51 sends the encrypting/decrypting codes based on the algorithm as given in the authors' previous study, stream cipher [23], byte by byte to the input of the FPGA device via the PORT 0 of the 89S51. The FPGA device processes the received machine codes to generate the encrypted PID; the encrypted PID are then sent byte by byte back to the 89S51 via PORT 2, stored in the 89S51's ROM, and transmitted back to the computer via the RS232.

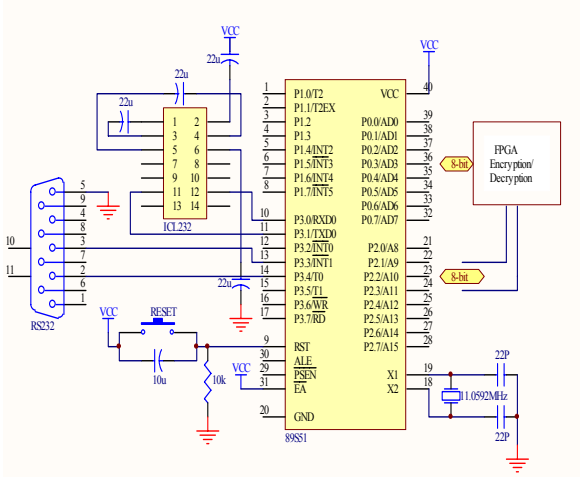


Figure 3. Circuit schematic of AT89S51 programmable microcontroller

2.1.3. The Design of FPGA Device

The hardware structure of the encryption/ decryption FPGA device is shown in Figure 4, which includes 6 units: decoding, addressing, register, ALU, memory, and buffers. The decoding unit decodes 8-bit input machine code into three parts: 2-bit instruction, 2-bit addressing mode, and 4-bit operand. Table 1 illustrates the instruction sets used in the FPGA device. There are four kinds of instruction sets: MOV, XOR, Shifting, and Rotating and four kinds of addressing modes: register, register/memory, memory /register, and direct. In addition, there are seven different registers: A, B, C, D, MBR (data buffer register), MAR (address buffer register), and MOR (output buffer register). The 4-bit operand can be either register or address. The memory has 64 bytes. The ALU typically can execute a range of standard arithmetic and logic instructions.

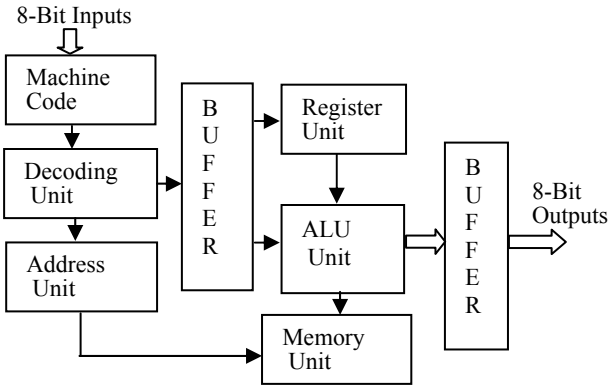


Figure 4. Hardware structure of encryption/ decryption FPGA

Table 1. Instruction sets

Instructions	Modes	Machine Codes in Hex
MOV	R, R Add R/Add Add/R	00 - 0F 10 - 1F 20 – 2F 30 – 3F
XOR	R, R Add R/Add Add/R	40 - 4F 50 - 5F 60 – 6F 70 – 7F
LS LSC LR LRC	R R R R	80– 8F 90– 9F A0 - AF B0 - BF
RS RSC RR RRC	R R R R	C0– CF D0– DF E0 - EF F0 – FF

2.2. PKI-Based Authentication Protocol

As illustrated in Figure 5, the proposed online PKI-based E-commerce transaction protocol consists of the registration process and transaction process. The definitions of the notations used throughout in this subsection are listed below.

U, M : user, merchant

ECA : E-commerce certificate application

C_U, C_M : user's certificate, merchant's certificate

$E_{K_{UM}}(PID_i)$: the PID_i ciphertext, where $E_{K_{UM}}$ denotes a secret key encryption algorithm (e.g. DES) with the secret key K_{UM} shared by the U and the M

$P_{d_C}[K]$: the CA's signature, where P_{d_C} denotes a public key encryption algorithm with the CA's private key d_C; K is an individual's public key

O_I, C_I, $D(O_I)$, $D(C_I)$: order information, card information (e.g. Name, N_C, and valid date), digest of O_I, digest of C_I

PID_i, PID_{i+1} : i^{th} transaction identifier, $i+1^{th}$ transaction identifier

Note that the PID_i and PID_{i+1} are automatically generated by taking the PID_0 of the U as the initial value through the random number generator stored in the U's and M's database.

As shown in Figure 5(a), the U (M) sends the paper application to the ECA to initialize a registration process. The U (M) will receive the PSoC-based cipher/decipher, the C_U (C_M), and the public-private key pair from the ECA, where the C_U (C_M) basically includes a K, $P_{d_C}[K]$, and other parameters. The PSoC-based cipher/decipher contains the user information, a session secret key generator, encryption/decryption algorithm, and a dynamic personalized identifier generator. The U (M) uses it to produce the $D(O_I)$, $D(C_I)$, and $E_{K_{UM}}(PID_i)$. The procedures of authorizing an online e-transaction, as shown in Figure 5(b), are described in the following.

Step 1: Requiring an online E-commerce transaction

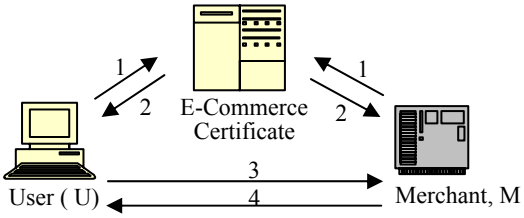
The U sends the C_U, O_I, $D(O_I)$, $D(C_I)$, $P_{K_G}(C_I)$, and $E_{K_{MB}}(PID_i)$ to the M's website via the Internet to request an e-transaction.

Step 2: Verifying the U's identity

Upon the confirmation of the validity of the C_U, if the U is identified and the decrypted C_I of the U is legitimate and the decrypted PID_i is valid, the M sends the acknowledgement back to the U.

Step3: Updating PID_{i+1} in the U's and M's database

Upon the reception of the acknowledgement from the M, the U updates the PID_{i+1} in its database system and the M updates the PID_{i+1} in its database, respectively.



Note. 1. The paper application form;
2. The PSoC-based cipher/decipher, certificate, and public-private key.
3. The application form via the Internet
4. Acknowledgement

(a)

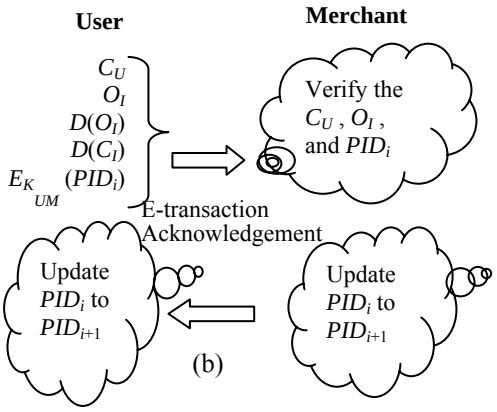


Figure 5. Proposed PKI-based E-commerce transaction; (a) the registration; (b) the transaction process.

3. Experimental Results

The proposed approach described above has been simulated using C programming language running on a 1,500MHz Pentium PC with 512 MB of RAM. Figure 6(a) shows a part of a user's personal identification data. Figure 6(b) shows the results of the encrypted *PID* by means of the *PW* = #qF!Lm2!, where

***P*$_{SSN}$ = A220F70346 (10 bytes)**
***PID* = TRACEY LI|M|1453 THUNDERBAY BROADWAY LOSANGELAS, USA|19900819|(042)22642993 (74 bytes)**

The author observes that the total size of the encrypted *PID* is 98 bytes, whereas that of the original *PID* is 84 bytes. This indicates that there is no overhead memory usage. One important feature is that the size of the ciphertext, the size of the key stream, and the encryption time are dependent on the secret key, nonce, and plaintext. This feature indicates that the proposed cipher is much more difficult for the statistical and differential cryptanalysis because the relationship among ciphertexts and key-stream numbers are dynamically associated for each encryption. Additionally, there is no influence on frequent record access because the encryption of numerous users' PID is done off-line.

Table 2 summarizes the definitions and countermeasures of four attacks for an opponent from inside or outside the system. One such type of attack is the brute-force attack. In the method proposed, it would take a hacker about 42,000 years to search the entire space of the key *PW* with no less than 8 keyboard characters [24]. Thus, it is difficult for hackers to break the keys. Another type of attack is the collaborative attack. In order to cooperatively reveal a user's identity, the function *F* has the security complexity of *O* (2^n) [25] with zero knowledge transmission of the key and *PID*. Thus, it is difficult to achieve collaborative attack. Moreover, because a random number, which is updated for each authentication trail, is used to produce a variant *PID* ciphertext and to protect the key *PW*. It is almost impossible to use the information from previous execution to disclose a user's PID and break the key to find out the *PID* by means of a large number of samples *PID* ciphertexts.

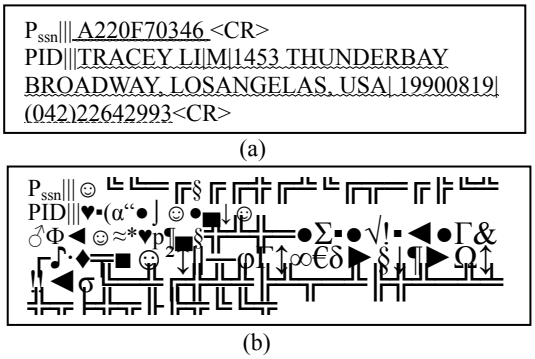


Figure 6. (a) Part of a user's PID; (b) results of the encrypted *PID* with the *PW* = #qF!Lm2!

Table 2. Definitions and countermeasures of four attacks for an opponent from inside or outside the system

Types of attacks	Definition	Principles to avoid attacks
Brute-force	An adversary searches the space of all possible keys.	Using the *PW* that is composed of keyboard characters
Collabo-rative	An adversary decrypts pieces of the ciphertext to extract the key without additional information.	Using several parameters to encrypt an EMR and zero knowledge transmission of the *PID* during transit and at rest.
Replay	Two adversaries are cooperatively trying to reveal the EMR without the patient's consent.	Using a random number, which is updated for each process, to produce a variant *PID* ciphertext and prevent the key *PW* from being easily disclosed.
Crypto-graphic	An adversary purports to be another using the information obtained from previous executions.	

4. Conclusions

This study proposes a programmable system-on-chip (PSoC)-based security technology to prevent unauthorized users from abusing or disclosing an individual's computerized personal identification data during transit and at rest. The proposed security technology includes two clams: One clam is that end-users and data owners must have more hands-on participation as safeguards. The other clam is that the protection technology must use trusted third parties to support origin verification, message integrity, and non-repudiation capabilities. In this study, the PKI verification/identification protocol is mainly used to secure the encrypted PID data transmitted on the Internet and support non-repudiation capability. The programmable microcontroller and ASIC technology are used for encryption/decryption and for stream cipher's implementation, which provides the confidentiality capability and supports the characteristic of off-line encryption of numerous users' PID. The proposed technology not only minimizes the quantity of RAM usage to store temporary parameters but also implements simple mathematical operations at a small expense of hardware to process data efficiently.

Conclusively, the advantages of the technology are three-fold. First, an agent can install a large number of encrypted users' personal identification data in their databases without excessive overhead processing time. Second, the hardware structure is of low hardware cost and low design complexity; the use of 8-bit instruction length allows better code density. Third, using certificates and dynamic cookies for verification/identification ensures that only authorized users can obtain the access to their personnel record. This work can be applied in E-commerce activity. An agent can store the keys in both the PSoC system and the agent's database for encryption/ decryption.

Corresponding Author:
Dr. Sung-Tsun Shih
Department of Electronic Engineering,
Cheng Shiu University,
Kaohsiung , Taiwan, R.O.C.
E-mail: stshih@csu.edu.tw

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A Tea Brewing Service Device with the Added Value of Increasing Tea Drinking Quality in Our Leisure Life

Sung-Tsun Shih[1], Chin-Ming Hsu[2], Chian-Yi Chao[3]

[1]Department of Electronic Engineering, Cheng Shiu University, Kaohsiung, Taiwan
[2]Department of Information Technology, Kao Yuan University, Kaohsiung, Taiwan
[3]Department of Electronic Engineering, Kao Yuan University, Kaohsiung, Taiwan
stshih@csu.edu.tw

Abstract: This study proposes a loose-leaf tea brewing service device with the added value of increasing tea drinking quality in our leisure life. The proposed device with multi-infusion timing control and user friendly interface characteristics utilizes AT89S51 programmable microcontroller to detect the input signals and trigger the corresponding outputs. The input signals come from a power switch for turning the controller on/off, three push buttons for setting the brewing time, and two other push buttons for controlling initial/extra brewing time setting and start/stop time control. The corresponding outputs include a power LED for indicating whether the controller is working or nonworking, a buzzer for reminding the completion of timing, and three 7-segment displays for showing the remaining time. Conclusively, the proposed microcontroller-based tea brewing service device can remind users remembering pour the water out timely, allow users to reset brewing time based on personal favorite tastes, and allow users to make the second or third rounds of tea infusion time for each individual setting. Therefore, the tea taste quality can be guaranteed.

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Keywords: Leisure life quality, loose leaf tea, infusion, timing controller, and microcontroller.

1. Introduction

With the increased life expectancy and the developed technology, diet and health are intrinsically linked for healthier aging based on a scientific understanding of the health benefits related to the diet. Tea, a non-alcoholic caffeine-containing beverage, has replaced high-calorie beverages and is one of the most popular beverages in the world. Nowadays, it is widely cultivated in China, England, India, Indonesia, Japan, Korea, Taiwan, et al. According to the literatures stated by Lian and Astill [1] and Hamer [2], drinking tea can provide many beneficial effects, such as relieving thirsty, maintaining clear mind, keeping clear vision, enhancing judgment ability, eliminating toxins, helping digestion, and prolonging the life. Some scientific evidences proved that tea leaves has catechins and flavonoids dietary resources and can against coronary heart disease [3-4]. In addition, Epidemiologic and experimental studies have shown that tea leaves have protective effects on chemically induced tumors in rats' gastrointestinal tract, liver, and mammary epithelium [5-6]. Ramos [7], Doss et al. [8], and Marchand [9] have proved that tea catechins and flavonoids can reduce the risk of chronic diseases, such as lung cancer, breast cancer and prostate cancer, and can provide health benefits on immune function with potential resistance against infections.

Commonly, there are three main types of teas, Oolong (half fermented), Green (not fermented), and Black (entire fermented) teas, consumed in the world and two kinds of tea packages, in bags or in loose leaf forms, sold in the markets. Tea in teabags usually referred to as "dust" does not consist of leaves and is generally made from low quality leaf. The bagged tea gives users the advantage of a quick cup of tea but loses its flavor quickly. Because of its fine siftings, bagged tea produces the infusion with more caffeine than loose leaf tea; and brewing bagged tea is cloudier and far more quickly than brewing loose leaf tea. In addition, Peterson et al. [10] stated that tea variety, weight of tea, and brewing techniques all affect estimates of flavonoids intake. Malinowska et al. [6] also found that brewing time (5, 10 and 30 min) does increase the fluoride content and most of the antimutagenic components were released from teas within 1–2 min of brewing. Under the identical brewing condition, green tea is significantly more effective than black tea for their anti-oxidant properties. Hicks et al. [11] indicated that the overall average caffeine released in the first through third tea brews were 69%, 23%, and 8%, respectively. The literature on the website [12] stated that tea brewing time and temperature affect their taste and smell; the tea quantity and the immersion time are generally in reverse proportion. Moreover, teas are better when infused 2-3 times; if the water is too hot, it will "cook" the leaves and make the tea taste bitter.

Conclusively, as stated by Heinrich and Prieto [13], a mentally and physically healthy ageing will require us to look at the dietary habits and how this impacts on health, to integrate this knowledge and to apply it in all sectors of food production and preparation.

Several references [14-19] have discussed the influences and effects of tea drinking based on the tea types and different preparing methods. Keenan et al. [14] discussed the L-theanine contained in cups of tea, such as green tea and black tea, and the effects of various preparation factors on the amounts of L-theanine extracted. The preparation factors include water temperature, brewing time, and materials added like sugar or milk. The research found that brewing time was found to be a major determinant of the amount of L-theanine extracted, while the addition of small amounts of milk and sugar made no significant difference. Moreover, a standard (200 ml) cup of black tea was found to contain the most L-theanine (24.2 ± 5.7 mg) while a cup of green tea contained the least (7.9 ± 3.8 mg). In the El-lethey and Shaheed [15] experimental report, it stated that drinking black tea definitely has a beneficial influence in impeding Na-F-induced reproductive toxicity in rats. Karak and Bhagat [16] traced elements in tea leaves, made tea and tea infusion. The trace elements in different tea infusions are such as aluminium (Al), arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), fluoride (F) manganese (Mn), and nickel (Ni). The experimental results showed that the presence of trace elements in green tea is lower than the black tea in most cases. Komes et al. [17] investigated the effect of different extraction conditions and storage time of prepared infusions on the content of bioactive compounds of green teas. The findings of this investigation suggest that maximum extraction efficiency of studied bioactive compounds from green tea is achieved during aqueous extraction at 80℃, for 50′ (powder), 15′ (bagged) and 30′ (loose leaf). Moreover, Suteerapataranon et al. [18] discussed the Caffeine in Chiang Rai tea infusions based on the considerations of tea variety, type, leaf form, and infusion conditions. Caffeine in Chiang Rai tea infusions was found to be dependent on infusion conditions (water temperature and infusion time), and leaf form but independent to different tea types. For tea leaf samples, the higher the water temperature and the longer the infusion time, the higher the caffeine concentrations in tea infusions. Lin, Liu, and Mau [19] investigated the effects of different brewing methods on antioxidant properties of steaming green tea. The extracts were prepared from cold or hot brewed steaming green tea at different concentrations (2, 6, and 10%). The experimental results showed that the hot water extracts were significantly more effective than the cold water extracts in antioxidant activity.

By examining current tea brewing techniques, two main disadvantages exist: (1) tea brewing depends on personal experience to make a pot of tea; therefore, the tea quality is unguaranteed. (2) the existing alarm for tea brewing time control only can be set once for each individual setting and there is no human friendly device specifically used for loose tea brewing service. In order to achieve the wishes in demanding good tea quality and keeping the body and life in good health and joyfulness, people nowadays would drink a pot of good tea while at works, self meditation, or chatting with friends. Therefore, this paper aims to build a multi-infusion timing controller used for loose leaf tea brewing service. The proposed microcontroller-based tea brewing service device can remind users remembering pour the water out timely and allow users to make three rounds of tea infusion time for each individual setting. In the following, Section 2 describes loose leaf tea brewing controller. The experimental results are shown in Section 3. Finally, the conclusions and future works are summarized in Section 4.

2. Loose Leaf Tea Brewing Controller

A multi-infusion timing controller with user friendly interface for loose-leaf tea brewing service consists of two parts: designing hardware control circuitry and programming software control codes. The hardware control circuitry includes an AT89S51 microcontroller, five push buttons, and three 7-segment displays, which provides the advantages of small size, easy use, good extendibility, and low cost. The software control codes are programmed and downloaded into the AT89S51 chip to control the hardware circuitry. The following describes their detail functions and design methodologies.

2.1. Hardware Control Circuitry

Figure 1(a) shows AT89S51 pin functions. Figure 1(b) is the fundamental circuitry connections for activating an AT89S51 chip. Figure 1(c) gives the block diagram of loose-leaf tea brewing controller. As shown in Figure 1(a), an AT89S51 with total 40-pins has four 8-pin I/O ports named P0, P1, P2, and P3. As shown in Figure 1(b), there are four parts of basic circuitry connections to activate AT8951 single chip: (1) connecting the 20th pin to ground and the 40th pin to power +5 Volts; (2) connecting the 31th pin to power +5 Volts; (3) resetting circuitry (pin 9); and (4) oscillating circuitry (pin 18 and 19). As shown in Figure 1(c), three I/O ports, P0, P1, and P3, are connected to three 7 segments for displaying the remaining time; P2 is connected to a buzzer (P2.0) for reminding the completion of timing; three push buttons are used for time setting (P2.1 for minute

digit, P2.2 and P2.3 for second digits) ; one push button (P2.4) is used for controlling extra infusion time setting; another push button (P2.5) is used for controlling start/stop timing process.

2.2. Software Control Codes

Figure 2 shows the flow chart of the software control codes. The main functions of the software control codes include:

- Setting parameters: P0 and P1 are used for displaying second digits and P3 is used for displaying minute digit. P2.0 controls buzzer; P2.1, P2.2, and P2.3 control the time setting of three digits; P2.4 controls the extra infusion time setting; P2.5 controls start/stop timing processes.

- Setting infusion time: three 7-segments displays one increment whenever P2.1, P2.2, P2.3 push buttons are pushed, respectively.

- If P2.4 is trigged, the timing controller starts counting down. When it counts to zero, the buzzer sounds. If P2.5 is triggered, the buzzer shut off and the controller resets to the initial time setting in which the initial time setting is stored in the memory for the next brewing process.

The following is the procedures of brewing loose leaf tea in a teapot.

Step 1: Power on the timing controller and set the initial brewing time *Ti* and extra infusion time *Te*.
Step 2: Add loose leaf tea in the tea pot.
Step 3: Fill the teapot with hot brewing water.
Step 4: Cover the teapot and let the tea be steeped
Step 5: Start tea infusion by push the start/stop button of the timing controller.
Step 6: When buzzer sounds, stop tea infusion by pushing the start/stop button of the timing controller.
Step 7: Pour the brewed tea into your teacup through a strainer to filter out any tea leaves.
Step 8: If your brew is particularly strong, you may try a second or even a third infusion by repeating step 3 to step 7 processes.

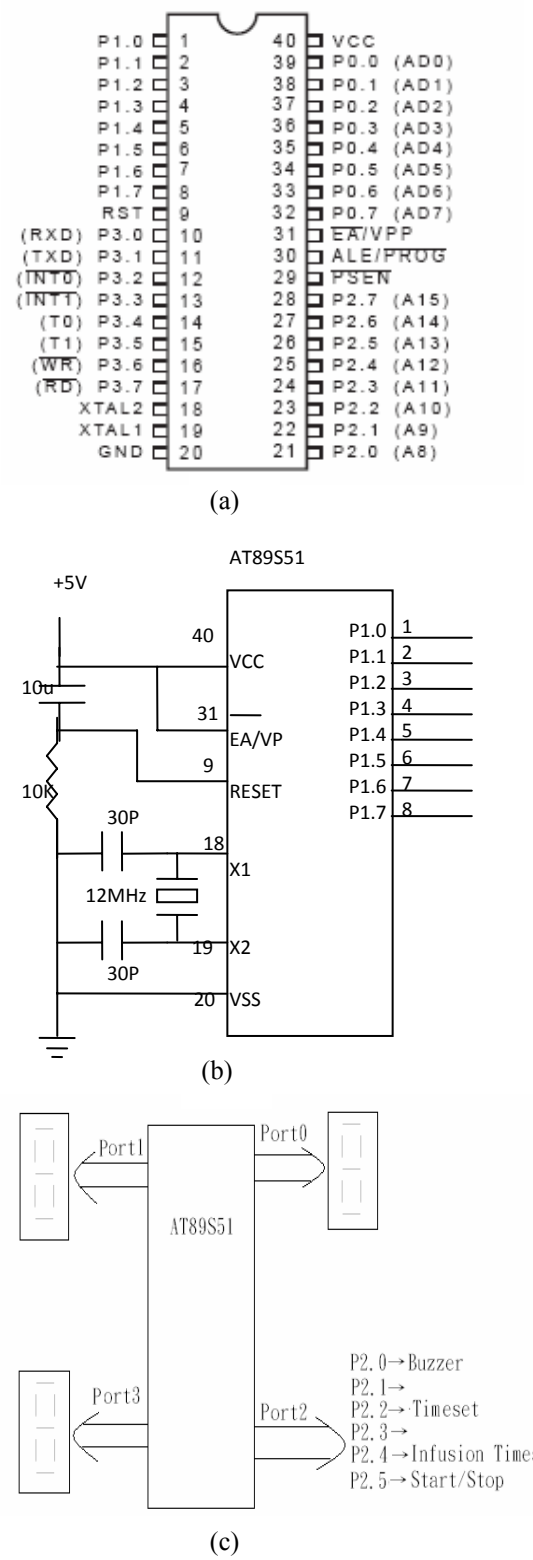


Figure 1. (a) AT89S51 pin functions; (b) basic activating circuitry connections; (c) block diagram of loose leaf tea brewing controller.

3. Experimental Results

Table 1 shows the investigation of people's favorites on oolong, green, and black loose leaf teas. From Table 1, teenagers usually don't drink loose leaf teas; about 50% of 50 people at 15-30 years old would drink black tea and green tea. For the people at the age between 31 and 50 years old, 60% of 50 people would like to drink green tea and 40% of them would like to have oolong tea. As for the people at the age from 51 to 65 years old, 20% of 50 people would choose green tea and 80% of them would choose oolong tea. Table 2 gives the recommended brewing time for oolong, green, and black loose-leaf teas.

The experiments use 12 ounce teapot and 4 ounce of tea weight. From Table 2, the first infusion time is one and half minutes, one minute, and forty seconds for black, green, and oolong teas, respectively. For the second infusion time, it takes extra 15 seconds for all three types of tea and 20 more seconds for the third infusion time. In addition, according to the experiments on teas' brewing time, we can conclude that brewing the tea for too long will "stew" the tea and produce a bitter brew. Fig. 3 illustrates the timing controller using (a) 40-pins and (b) 20-pins AT8951, respectively. The main difference of two devices is on the hardware structure. As shown in Figure 3(a), five push buttons are used for time setting and system start/stop controls whereas Figure 3(b) uses two push buttons because 20-oin AT8951 only has 3 I/O ports. Since 40-pins 89S51 has 4 I/O ports, the proposed timing controller uses three 8-pins I/O ports to control three 7-segment displays instead of using three more 7447 IC for 7-segment displays' decoding.

Table 1. Investigation of different people's favorites on oolong, green, and black loose leaf teas

Teas Ages(yrs. old)	Black Tea	Green Tea	Oolong Tea
under 15	rare	rare	
15-30	50%	50%	
31-50		60%	40%
51-65		20%	80%

Table 2. Recommended brewing time for oolong, green, and black loose leaf teas

Brewing Time Rounds	Black Tea	Green Tea	Oolong Tea
Initial	1-2min.	1 min.	40 sec.
Second (Extra)	10 sec.	10 sec.	10 sec.
Third (Extra)	15 sec.	15 sec.	15 sec.

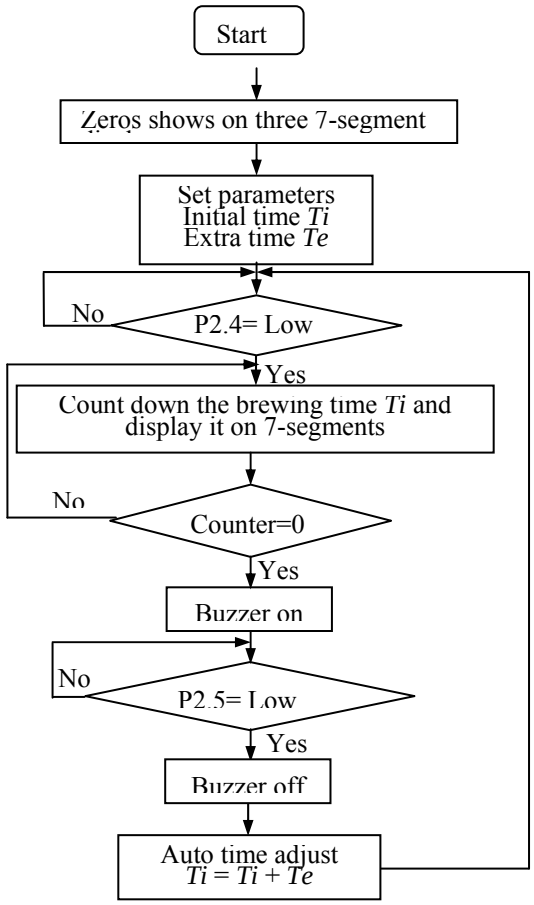
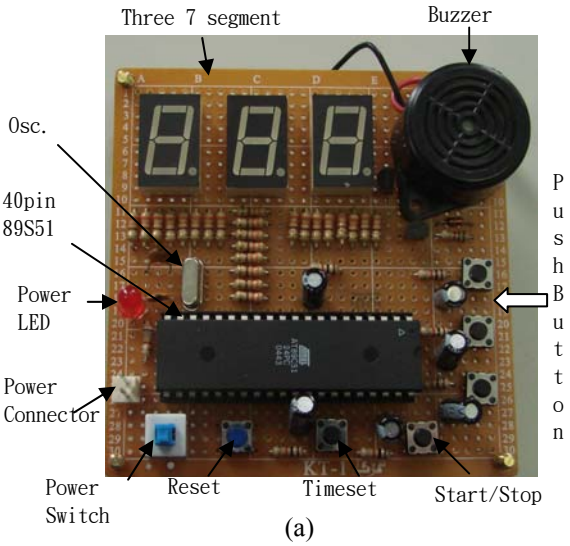


Figure 2. Flow chart of the software control codes.



(a)

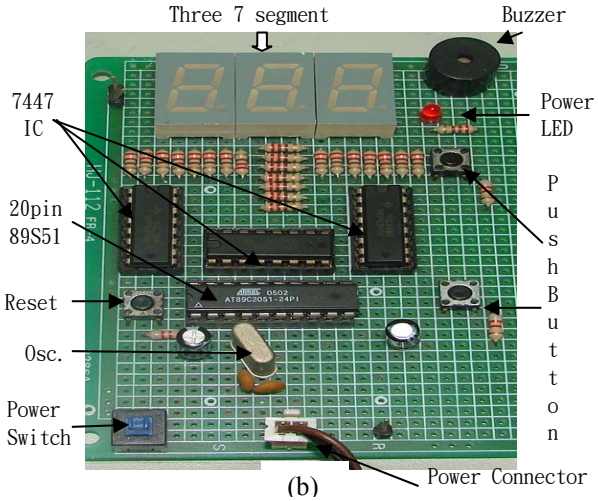


Figure 3. Timing controller using (a) 40-pins and (b) 20-pins AT8951.

4. Conclusions and Discussions

In order to satisfy people's demanding on good tea drinking quality and keeping the body and life in good health and joyfulness, this paper proposes a multi-infusion timing controller with user friendly interface for loose-leaf tea brewing service. The proposed device provides three advantages: (1) Users are allowed to adjust the brewing time and multi-round of infusion time based on personal favorite tastes. Therefore, tea quality is ensured. (2) The proposed timing controller provides user friendly hardware interfaces with the capabilities of indicating whether the controller is working or nonworking (the power LED), displaying the remaining time(three 7-segments), and reminding users to pour the brewed tea into the teacup through a strainer to filter out any tea leaves timely (the buzzer). (3) Because of its simplicity and applicability, people with fundamental microcontroller knowledge are capable of building such device with low expenses. In addition, users can refer to the authors' previous research [20] with the basic knowledge of microcontroller system designing and debugging techniques and try to built the device by selves.

Nowadays, people would drink a pot of good tea while at works, self meditation, or chatting with friends in their leisure time. Therefore, the proposed tea service device can be embedded in a tea table or a tea plate and can be developed as a consumer product in advance.

Corresponding Author:
Dr. Sung-Tsun Shih
Department of Electronic Engineering,
Cheng Shiu University,
Kaohsiung , Taiwan, R.O.C.
E-mail: stshih@csu.edu.tw

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Prevalence and Factors Associated with Aggression among Preschool Age Children at Baraem Bader Nursery School in Al-Asher 10th of Ramadan city, Egypt

Fatma M. Amin[1] ; Sahar G. Behalik[2] and Wafaa H. El Soreety[3].

[1]Department of Pediatric Nursing, Faculty of Nursing, Mansoura University, [2]Department of Psychiatric Nursing, Faculty of Nursing, Mansoura University, [3]Department of Pediatric Nursing, Faculty of Nursing, Tanta University. behilak_s@hotmail.com

Abstract: Childhood aggression is a major public health problems leading to children at a significant risk for continued behavior problems and other social and emotional challenges throughout their lifetime. This study aimed to study the prevalence and factors associated with aggression in preschool age children. Baraem Bader nursery school of Al-Asher 10th of Ramadan city was chosen as the study setting. The study sample compromised 50 preschool age children from both sex. The validated observational checklist sheet which is designated to detect developing behavior problems of the preschool age children was used to collect required data. The study confirms that the prevalence of preschool age aggression were 68% who occasionally suffering from aggression and 32 % who always suffering from aggression. The associated factors of aggression are increased with age category, male, and first birth child, also they tend to be from mothers who are working and less than university education. The study recommended that professional nurse play a critical role in partnering with parents, educators, school psychologist for early identification, prevention and intervention of aggression in preschool age children, which are essential in the prevention of serious and costly problems in later childhood.

[Fatma M. Amin; Sahar G. Behalik and Wafaa H. El Soreety **Prevalence and Factors Associated with Aggression among Preschool Age Children at Baraem Bader Nursery School in Al-Asher 10th of Ramadan city, Egypt]** Life Science Journal, 2011; 8(4):929-938] (ISSN: 1097-8135). http://www.lifesciencesite.com.

Key words: prevalence, aggression, aggressive behavior, preschool and risk factors.

1. Introduction

Childhood Aggression is a major public health problem worldwide especially in recent years [1,2]. Globally the prevalence of childhood aggression was 5-10% in both male and female [3] . In the United States the prevalence of preschool age children aggression was 22% for both male and female [4]. In a Canadian nationwide longitudinal study the estimated prevalence of childhood physical aggression was 16.6% [5]. In A Vietnam veterans study the prevalence of reported childhood aggression was 19.4% among childhood [6].

In Egypt there is no national figure available regarding the prevalence of aggression in the preschool age children. Also study carried out at Pittsburgh showed that the prevalence of childhood aggression was ranged from 3% to 40% [7]. Further evidence was provided by A 2006 Trenton and Philadelphia study in which it was estimated that 32% overlap in children identified as higher in aggression by both mothers and teachers [8].

The reason behind this major pediatric health problem could be attributed, on one hand to a complex set of biologic, psychosocial, cultural, and familial variables [7,8] . On the other hand, gender, frustration, modeling and reinforcement are other factors that may play a role in increasing aggressive behavior in childhood [9,10] .

Childhood aggression is a serious problem particularly among children who exhibit aggression across time and contexts [11-12]. Preschool-aged children displaying high levels of aggression are known risk factors for increased social and emotional problems, as they are more likely to exhibit attention deficit disorder, conduct disorder, future juvenile delinquency, and later antisocial behaviors [13-15]. Aggression is also associated with alcohol, drug abuse, accidents, violent crimes, depression, suicide attempts, spouse abuse, and neglectful and abusive parenting [16-18]. Furthermore, violence commonly results in serious injuries to the perpetrators themselves [19,20]. Indeed, young children who manifest severe and pervasive forms of aggression demonstrate significant levels of social impairment and are therefore significantly more likely to develop subsequent mental health problems [21,22]. **Whitaker & Blake & Hamrin,** have shown that such problematic levels of aggression in adolescence or adulthood can be identified in the preschool years and can be traced to events occurring early in life [23-26] .

Childhood aggressive behavior prevention has become one of the most pressing issues facing our society today [27,30]. While preventing aggressive behavior are increasingly receiving international attention, the nursing profession play potential role to reduce this major problem in society [31]. Ironically, the nursing profession had its origins in warfare [7] . Together nurse, parents, educators and school psychologist play a major role for early identification, prevention and intervention of aggression in

preschoolers which leads to prevention of serious and costly problems in later childhood [32-36].

2. Subject and Methods

Aim of the study:

To study the prevalence and factors associated with aggression in preschool age children.

Sample:

50 children from both sexes were chosen by systematic random sample their age ranged from 3 to less than 6 years.

Type of the study:

Cross sectional study.

Setting:

Baraem Bader nursery school at Al-Asher 10th of Ramadan City .

Study tool:

Data was collected through using: Modified Observation checklist sheet: It is a screening test designated to detect developing behavior problems of the preschool children from 3-6 years. It was developed by **Behar**, [37] preschool questionnaire, translated and modified by **Abdelatif**, [38]. The checklist contain 30 items pertaining for the following categories; Hostile aggressive, anxious fearful, hyperactive distractible in addition to nervous mannerisms thumb sucking, nail biting, wet or soiled self, speech problems, unusual sexual behavior and over activity

Methods:

- Required letters were adopted from the administrator of the nursery school and Permission were obtained from the principles of the schools to facilitate the required data collection.
- The modified observational checklist sheet which is designated to detect developing aggressive behavior was used to study children aggressive behavior.
- Data were collected from 1 August to 30 September 2009. During the initial introductory period an attempt was made to establish an informal and friendly atmosphere to facilitate communication between the child and the researcher and to help children to be more relaxed. Three children were observed daily for 3 consecutive days from 9 am to 1 p.m.

Statistical analysis:

The collected data were organized tabulated and statistically analyzed using SPSS software statistical package version 17. The variables were presented in number and percentage. The children's behavior was observed three times at different occasions. The frequency of behavior was rated at which the child was aggressive along a three-point scale ranging from rarely, occasionally and always. This rating was scored as 1 for rarely, 2 for occasionally and 3 for always. The score of the three observations was summated for each item of behavior and the average calculated. The total score was calculated by summation of the score of the nine questions.Total score of each behavior ranged as rarely (1-8), occasionally (9-17) and always (18-27).The mean and standard deviation of total score was calculated and T test was used to compare between subgroups. The chi square test was used for statistical significance of observed differences of behavior score in reaction to explanatory variables studied. Whenever the chi square was found not suitable, the Fisher exact test was used. The interpretation of significance was done using a p value of < 0.05.

3. Results

From Table (1) It was found that 34.0% of preschool children were always not considering to other's feelings, and biting or pinches others and 42.0 % of them were occasionally fight with other children ,and disobedient .The result also reveals that 26.0% ,24.0% , and 22,0% of children were always blames others, opposing with other children , and destroying their and other's things respectively. In addition it was illustrated that 68% of the preschool age children were occasionally suffering from aggression and 32% them were always suffering from aggression.

Table (1) Distribution of preschool age children in relation to their aggressive behavior.

Items of behavior	Rarely		Occasionally		Always	
	N	%	N	%	N	%
Destroying his and others' things	30	60.0	9	18.0	11	22.0
Fight with other children	20	40.0	21	42.0	9	18.0
Disobedient	22	44.0	21	42.0	7	14.0
Tells lies	32	64.0	11	22.0	7	14.0
Opposing with other children	30	60.0	8	16.0	12	24.0
Not sharing playing with others	47	94.0	1	2.0	2	4.0
Blames others	21	42.0	16	32.0	13	26.0
Not considering to others' feelings	14	28.0	19	38.0	17	34.0
Bites or pinches others	24	48.0	9	18.0	17	34.0
Total score	0	0.0	34	68.0	16	32.0

Table (2) shows that less than half (42.4%) of children whose age ≥5 years were always bites or pinches others compared to 17.6% of children whose age <5 years. On the other hand the preschool age children whose' age ≥5 years suffering from aggression than those aged <5 years and the significant differences were not found.

Table (2): Children's aggressive behavior in relation to their age in years

Items of behavior	Boys (n=23)		Girls (n=27)		P
	N	%	N	%	
Destroying his and others' things					
Rarely	11	47.8	19	70.4	0.234
Occasionally	6	26.1	3	11.1	
Always	6	26.1	5	18.5	
Fight with other children					
Rarely	6	26.1	14	51.9	0.189
Occasionally	12	52.2	9	33.3	
Always	5	21.7	4	14.8	
Disobedient					
Rarely	7	30.4	15	55.6	0.134
Occasionally	11	47.8	10	37.0	
Always	5	21.7	2	7.4	
Tells lies					
Rarely	12	52.2	20	74.1	0.241
Occasionally	6	26.1	5	18.5	
Always	5	21.7	2	7.4	
Stupor with other children					
Rarely	11	47.8	19	70.4	0.214
Occasionally	4	17.4	4	14.8	
Always	8	34.8	4	14.8	
Not sharing playing with others					
Rarely	22	95.7	25	92.6	0.344
Occasionally	1	4.3	0	0.0	
Always	0	0.0	2	7.4	
Blames others					
Rarely	6	26.1	15	55.6	0.096
Occasionally	10	43.5	6	22.2	
Always	7	30.4	6	22.2	
Not considering to others' feelings					
Rarely	5	21.7	9	33.3	0.180
Occasionally	7	30.4	12	44.4	
Always	11	47.8	6	22.2	
Bites or pinches others					
Rarely	9	39.1	15	55.6	0.149
Occasionally	3	13.0	6	22.2	
Always	11	47.8	6	22.2	
Total score (mean±SD)	16.7	±5.1	13.8	±3.9	0.028*

Table (3) shows that less than half (47.8 %) of boys were always bites or pinches others, and not considering to others' feelings compared to, 22.2% of girls respectively. On the other hand, 21.7% of boys were always fight with other children, disobedient, and tells lies compared to 14.8%, and 7.4% of girls respectively. In addition the aggressive score was more in boys (±5.1) than in girls (±3.9) and there was a significant difference between the aggressive score in boys and girls.

Table (3): Children's' aggressive behavior in relation to their gender.

Items of behavior	First birth (n=21)		Second and more (n=29)		P
	N	%	N	%	
Destroying his and others' things					
Rarely	12	57.1	18	62.1	1.000
Occasionally	4	19.0	5	17.2	
Always	5	23.8	6	20.7	
Fight with other children					
Rarely	9	42.9	11	37.9	0.521
Occasionally	7	33.3	14	48.3	
Always	5	23.8	4	13.8	
Disobedient					
Rarely	10	47.6	12	41.4	0.494
Occasionally	7	33.3	14	48.3	
Always	4	19.0	3	10.3	
Tells lies					
Rarely	13	61.9	19	65.5	0.768
Occasionally	4	19.0	7	24.1	
Always	4	19.0	3	10.3	
Stupor with other children					
Rarely	13	61.9	17	58.6	1.000
Occasionally	3	14.3	5	17.2	
Always	5	23.8	7	24.1	
Not sharing playing with others					
Rarely	20	95.2	27	93.1	1.000
Occasionally	0	0.0	1	3.4	
Always	1	4.8	1	3.4	
Blames others					
Rarely	10	47.6	11	37.9	0.245
Occasionally	4	19.0	12	41.4	
Always	7	33.3	6	20.7	
Not considering to others' feelings					
Rarely	5	23.8	9	31.0	0.934
Occasionally	8	38.1	11	37.9	
Always	8	38.1	9	31.0	
Bites or pinches others					
Rarely	8	38.1	16	55.2	0.517
Occasionally	4	19.0	5	17.2	
Always	9	42.9	8	27.8	
Total score (mean±SD)	15.5	±5.1	14.8	±4.4	0.588

From table (4) It was observed that 23.8 of the first birth were always destroying their and others' things and fight with other children compared to 20.7% and 13.8 % of the second birth respectively and the significant difference were not found.Although the significant difference were not found regarding Children's' aggressive behaviour in relation to birth order, the preschool age children whose first birth suffering from aggression than those whose second and more rank.

Table (4)Children's' aggressive behavior in relation to their birth order.

Items of behavior	First birth (n=21)		Second and more(n=29)		P
	N	%	N	%	
Destroying his and others' things					
Rarely	12	57.1	18	62.1	1.000
Occasionally	4	19.0	5	17.2	
Always	5	23.8	6	20.7	
Fight with other children					
Rarely	9	42.9	11	37.9	0.521
Occasionally	7	33.3	14	48.3	
Always	5	23.8	4	13.8	
Disobedient					
Rarely	10	47.6	12	41.4	0.494
Occasionally	7	33.3	14	48.3	
Always	4	19.0	3	10.3	
Tells lies					
Rarely	13	61.9	19	65.5	0.768
Occasionally	4	19.0	7	24.1	
Always	4	19.0	3	10.3	
Stupor with other children					
Rarely	13	61.9	17	58.6	1.000
Occasionally	3	14.3	5	17.2	
Always	5	23.8	7	24.1	
Not sharing playing with others					
Rarely	20	95.2	27	93.1	1.000
Occasionally	0	0.0	1	3.4	
Always	1	4.8	1	3.4	
Blames others					
Rarely	10	47.6	11	37.9	0.245
Occasionally	4	19.0	12	41.4	
Always	7	33.3	6	20.7	
Not considering to others' feelings					
Rarely	5	23.8	9	31.0	0.934
Occasionally	8	38.1	11	37.9	
Always	8	38.1	9	31.0	
Bites or pinches others					
Rarely	8	38.1	16	55.2	0.517
Occasionally	4	19.0	5	17.2	
Always	9	42.9	8	27.8	
Total score (mean±SD)	15.5	±5.1	14.8	±4.4	0.588

From Table 5 we found that 23.7 of preschool age children whose mother less than university were always destroying their and others' things and fight with other children compared to 16.7% and none of them whose mother have university education respectively. In addition the aggressive score level in children whose mother less than university were higher (±4.8) than those of children whose mothers have university education (±3.8) and significant differences were not found.

Table (5): Children's' behavior in relation to mothers' education

Items of behavior	Less than university (n=38)		University (n=12)		P
	N	%	N	%	
Destroying his and others' things					
Rarely	24	63.2	6	50.0	0.380
Occasionally	5	13.2	4	33.3	
Always	9	23.7	2	16.7	
Fight with other children					
Rarely	12	31.6	8	66.7	0.060
Occasionally	17	44.7	4	33.3	
Always	9	23.7	0	0.0	
Disobedient					
Rarely	16	42.1	6	50.0	0.360
Occasionally	15	39.5	6	50.0	
Always	7	18.4	0	0.0	
Tells lies					
Rarely	23	60.5	9	75.0	0.720
Occasionally	9	23.7	2	16.7	
Always	6	15.8	1	8.3	
Stupor with other children					
Rarely	24	63.2	6	50.0	0.740
Occasionally	5	13.2	3	25.0	
Always	9	23.7	3	25.0	
Not sharing playing with others					
Rarely	36	94.7	11	91.7	0.520
Occasionally	1	2.6	0	0.0	
Always	1	2.6	1	8.4	
Blames others					
Rarely	13	34.2	8	66.7	0.220
Occasionally	13	34.2	3	25.0	
Always	12	31.6	1	8.3	
Not considering to others' feelings					
Rarely	11	28.9	3	25.0	0.280
Occasionally	12	31.6	7	58.3	
Always	15	39.5	2	16.7	
Bites or pinches others					
Rarely	19	50.0	5	41.7	0.320
Occasionally	5	13.2	4	33.3	
Always	14	36.8	3	25.0	
Total score (mean±SD)	15.5	±4.8	13.9	±3.8	0.316

Table 6 illustrates that there is a significant difference in relation to preschool age children of working mothers and aggressive score. It was found that 20% of children whose mothers were working always disobedient and 48% of them were always bites or pinches others compared to 8% and 20% of children whose mothers were housewife respectively, there was statistically significant differences.

Table (6):Children's' aggressive behavior in relation to their mothers' occupation

Items of behavior	Housewife (n=25)		Working (n=25)		P
	N	%	N	%	
Destroying his and others' things:					
Rarely	18	72.0	12	48.0	0.062
Occasionally	5	20.0	4	16.0	
Always	2	8.0	9	36.0	
Fight with other children:					
Rarely	10	40.0	10	40.0	1.000
Occasionally	10	40.0	11	44.0	
Always	5	20.0	4	16.0	
Disobedient:					
Rarely	16	64.0	6	24.0	0.021*
Occasionally	7	28.0	14	56.0	
Always	2	8.0	5	20.0	
Tells lies:					
Rarely	17	68.0	15	60.0	0.598
Occasionally	6	24.0	5	20.0	
Always	2	8.0	5	20.0	
Stupor with other children:					
Rarely	18	72.0	12	48.0	0.180
Occasionally	2	8.0	6	24.0	
Always	5	20.0	7	28.0	
Not sharing playing with others:					
Rarely	24	96.0	23	92.0	1.000
Occasionally	0	0.0	1	4.0	
Always	1	4.0	1	4.0	
Blames others:					
Rarely	12	48.0	9	36.0	0.767
Occasionally	7	28.0	9	36.0	
Always	6	24.0	7	28.0	
Not considering to others' feelings:					
Rarely	10	40.0	4	16.0	0.204
Occasionally	8	32.0	11	44.0	
Always	7	28.0	10	40.0	
Bites or pinches others:					
Rarely	17	68.0	7	28.0	0.021*
Occasionally	3	12.0	6	24.0	
Always	5	20.0	12	48.0	
Total score (mean±SD)	13.7	±4.3	16.5	±4.7	0.034*

* Significant

4. Discussion

Aggression is widely prevalent among preschool age children in Al-asher 10th of Ramadan City Bader nursery school. Results of present study revealed that the overall prevalence of preschool age children who always suffering from aggression was 32% the same results was reported by **Bendersky *et al.*** [8] who indicated that 32% overlap in children identified as higher in aggression by both mothers and teachers. Our study finding was higher than that reported by **Crick *et al.*** (22%) [4] and **Cote *et al.*** [5] (16.6%), this may be attributed to adverse family background and

family environment as compared to all other children.

Regarding child aggressive behavior in relation to their age in years, results of the present study showed that the preschool age children ≥5 years suffering from aggression than those aged less < 5 years. These findings are in line with the **NICHD** [36] and **Tremblay, *et al.*** [22] who indicated that children who exhibit high levels of aggression during the preschool remain on a relatively high throughout childhood. Also **Cote *et al.*** confirmed that some children become aggressive in kindergarten as a result of the stress generated by the transition from preschool to formal schooling [5] .

Results of present study revealed that the gender of the child was an important factor associated with aggression in the preschool age children as the boys had aggressive behavior than girls. This may be due to the biology in particular to male sex hormones, at the same time the development of gender typing that males and female are expected to behave differently. In addition parents tendency to discipline boys more harshly magnifies this effect. This results also go in line with recent large US study and Canadian sample of children **NICHD, 2004** & **Cote, *et al.*** who found that children in a high level of aggression were likely to be boys from families whose using a hostile ineffective parenting strategies [36,5]. Moreover the differences in gender possibly are explained as children learn aggressive behavior tactics from their families cultural or media influence also our finding agree with **Keller *et al.*** [39] **Ostrov *et al.*** [40] and **Adams,** [41] they emphasized that gender is blamed by the general public as a reason for aggressive behavior, which indicated that males were more physically aggressive than female where males were likely to engage in physically and avoidance aggression.

Concerning children aggressive behavior in relation birth order, results showed that first birth children suffering from aggression than those whose second and more rank this may be due to children learn to be aggressive from all around them including parents, teachers and peers. The same was reported by **Gershhoff, in 2002** [42] and **Breitenstein *et al.*** [43] who stated that children who see their parents as physically abusive are observing that they come to know as acceptable and therefore may exhibit this behavior with others, also early harsh discipline may lead to aggressive behavior. This is parallel with **Adams**,[41] who revealed that first born children were most likely to exhibit more aggression than third or later born children.

Regarding mother education, findings of this study revealed that the aggressive score level in children whose mother less than university were higher than those of children whose mothers have university education. The same was reported by **Cote *et al.*** [5] and **Adam**, [41] who reported that a child's mothers with less than a high school diploma significantly predicted aggression in preschool age children. **Tremblay, *et al.*** emphasized that children who remain aggressive throughout childhood are tend to be from mothers who have low educational level [22].

As regards children's aggression in relation to their mothers' occupation, results showed that children whose mothers were working always aggressive than whose mother housewife, this may be due to mothers exposed to life stress, low social support may provide a mother experience characterized by inconsistency of rule setting, harsh discipline and low supervision and lack of warmth, all found relate to aggressive behavior in children. Results are consistent with **Campbell *et al.*,** [13] **Dodge and Pettit**, [44] and **Bendresky *et al.*,** [8] who found that children whose mothers are working remain aggressive throughout childhood.

Conclusion

The study concluded that prevalence of preschool age aggression were 68% who occasionally suffering from aggression and 32 % who always suffering from aggression. The associated factors of aggression are increased with age category, male, and first birth child. They tend to be from mothers who are working and less than university.

Recommendations

Based on the findings of the present study, the following can be recommended

1. Provide early identification and appropriate referral of children who are suffering from aggression in the nursery school.
2. Incorporate mental health assessment in the daily pediatric practice.
3. Conduct early aggression prevention program to make school safer for today's young children.
4. Provide a consistent school nurse in the nursery school.

Corresponding author
Sahar G. Behalik. Mansoura University, Department of Psychiatric Nursing. behilak_s@hotmail.com.

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Effect of Dopamine Antagonist (Resperdal)® on Ovarian Activity of Egyptian Baladi Goats Out-Off Breeding Season

Sabra, H. A and Hassan, S. G

Department of Animal Reproduction & A.I, National Research Center, Cairo, Egypt
Sabra_hussein@yahoo.com

Abstract: The present investigation aimed to treat the female goats out – off breeding season by means of one of dopamine antagonist preparation which block dopamine receptors in the reproductive system. Two groups of mature non cyclic Baladi does (experimental and control) fed and managed under the natural Egyptian environmental conditions were used at the end of the breeding season. The experimental animals were treated orally by 16 mg. dopamine receptor antagonist drug (Resperdal)® / doe divided into two doses with three days interval. The estrous signs and ovarian activity were investigated by ultra- sonography and the results were confirmed by blood plasma progesterone assay. The results indicated more number of does showing estrus signs and improvement in the ovarian activity as well as blood plasma progesterone in experimental animals than in control ones. It was concluded that administration of dopamine receptor antagonist (Resperdal)® can be used for treating more than 50% of does out – off breeding season which reflect positively on the kidding crop.
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Key words: (Resperdal)®; ovarian activity; goats; dopamine antagonist

1. Introduction

Seasonal breading in sheep, goat and horse is governed by photoperiod. The signal requires a euro-endocrine transduction (**Herbison, 1995**). Dopamine, opioids and melatonin are the wide known mediators between brain and gonadal functions (**Parvizi, 2000**). Dopamine is a hormone, derived from amino acid tyrosine, synthesized from hypothalamus and ventrolateral medulla, act as a neurotransmitter signal for reproduction in seasonal breeding animals (**Simonian *et al.*, 1998**). Its function in male differ than in female animals, however, it was recorded that, central dopamine is a neurotransmitter in the control of the sexual function in male, its stimulation enhances erection, increases testosterone level and increases sexual desire (**Francois and Juleen, 2001 and Juan *et al.*, 2005**). In female, cerebrospinal dopamine, inversely correlated with plasma LH (**Besogent *et al.*, 1996**), inhibits gonadotropine releasing hormones and or luteinizing hormone (GnRH/LH) in an anoestrus ewes (**Anderson *et al.*, 2001**). Moreover, dopamine inhibits the release of prolactin and interferes with mammary gland development and lactation (**Serafim and Felicio, 2001**).

Dopamine antagonists are drugs which blocks dopamine receptors. In this respect, it was demonstrated that, the application of dopamine receptors antagonists (Sulpride, Resperdal,….®) can be used for improving reproductive performances of seasonal breeding animals and treating the animals out-off season. Dopamine D2 antagonist was used to induce ovarian activity in seasonal anoestrus mare, increase prolactin, FSH and LH concentrations, the estrous cycle length, ovulation and luteal progesterone were as normal estrus animals (**Besognet *et al.*, 1996**). In ewe, dopamine antagonists enhance the amplitude of LH pulses during non breeding season (**Gallegos *et al.*, 1998**). On the other hand, administration of dopamine antagonist (sulpride) to seasonally anovulatory mares increased daily prolactin levels but did not stimulate gonadotropin secretion or ovarian activity (**Donadeu and Thompson, 2002**). Moreover, it was postulated that dopamine D2 antagonist had been used for induction of lactation in intact mares during birth season with the presence of gonadal hormones but not in ovarictomized animals (**Guillaume *et al.*, 2003**). In goats, **Luis *et al.* (2011)** Stated that dopamine antagonist increased LH secretion under high plane of nutrition during short day light. Therefore, the aim of the present investigation was a trial to improve the ovarian activity of Egyptian Baladi goats out-off breeding season by administration of dopamine antagonist (Resperdal)®.

2. Materials and Methods

A total number of 14 mature non-cyclic Baladi goats (14 -16 months age and 26±2.5 kg. life body weight.) were used during June (The end of the breeding season) after ultrasonic (U, 1000, scanning probe, 3.5 M.Hz) examination of their ovaries. The animals were divided into 2 groups (experimental and control) and were fed on concentrated mixture

(250 g/goat) beside rice straw *ad. Libdium*.

The animals were housed and managed under Egyptian environmental conditions. The experimental animals were orally administered 16 mg dopamine receptor antagonist drug (Resperdal® 4 mg/capsule manufactured by Janssen-cilag pharm – Beerse, Belgium.) divided into 2 doses with 3 days interval. The estrus signs were detected by vasectomized buck. The ovarian activity was examined weekly by ultrasonography for three weeks as well as heparin blood samples were collected. Plasma of the blood samples were separated by centrifugation (3000 g/ 15 min. 4°c) then kept at – 20°c till progesterone assay. Progesterone assay was carried out in order to confirm the functional corpus luteum. The assay was run by radioimmunoassay (**Abraham, 1981**) with using kits from diagnostic product corporation (Los Angles, USA). Sensitivity of assay was 0.02 ng/ml. the results were analyzed statistically according to **Snedecor and Cochran (1980)** using "t" test.

3. Results

The present results (Table 1) showed that administration of dopamine receptors antagonists (Resperdal®) to does with smooth ovaries activated them in the form of increase the number of does showing estrus signs (71.43 vs. 14.28% in non treated does). The sonographic examination showed more does number with mature Graffian follicles (71.43 vs. 14.28%) and functional corpus luteum (57.14 vs. 0.0%) in experimental does than in control ones. Moreover, Resperdal® treatment induced regular estrus cycle length averaged 19 ± 2.27 days in comparing to more than 21 days in control non-cyclic does.

The present results in table(2) showed that the level of progesterone in experimental does was significantly higher than in the control ones, the highest level was during 2nd week (4.89±1.08 ng/ml) however, the level was below 1 ng/ml in control does. The ultrasonographic examination showed the state of the ovaries (Figs. 1,2 and 3).

Table (1) Effect of dopamine receptors antagonist (Resperdal)® on estrus activity in Baladi does

	Experimental	Control
No of does used	7	7
No of does exhibited estrus signs	5/7 (71.43 %)	1/7 (14.28)
No of does showed smooth ovaries	2/7 (28.58%)	6/7(85.27%)
No of does showed follicles	5/7 (71.43%)	1/7(14.28%)
No of does showed corpus luteum	4/7 (57.14%)	-
Estrus cycle length (days)	19±2.27	‹ 21

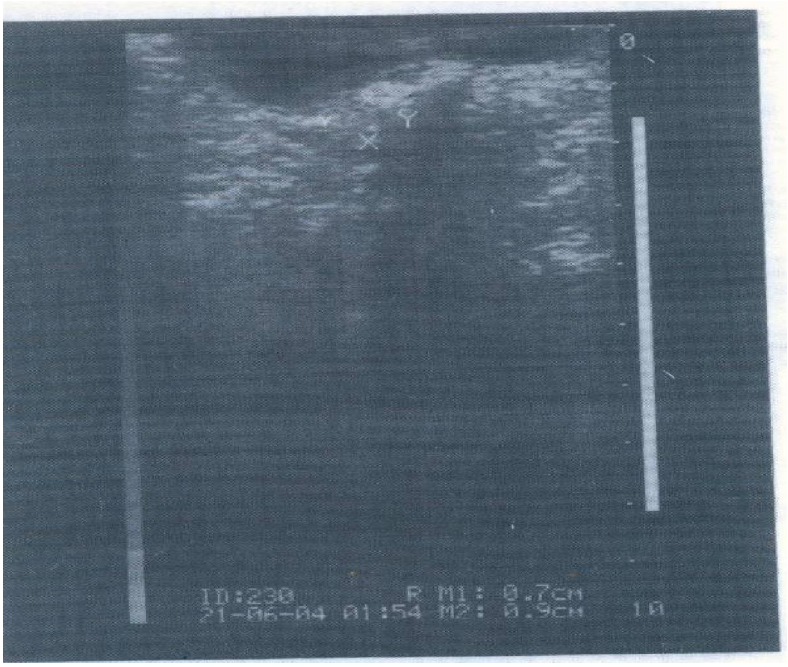

Figure 1: Ovary of doe showing mature follicle.

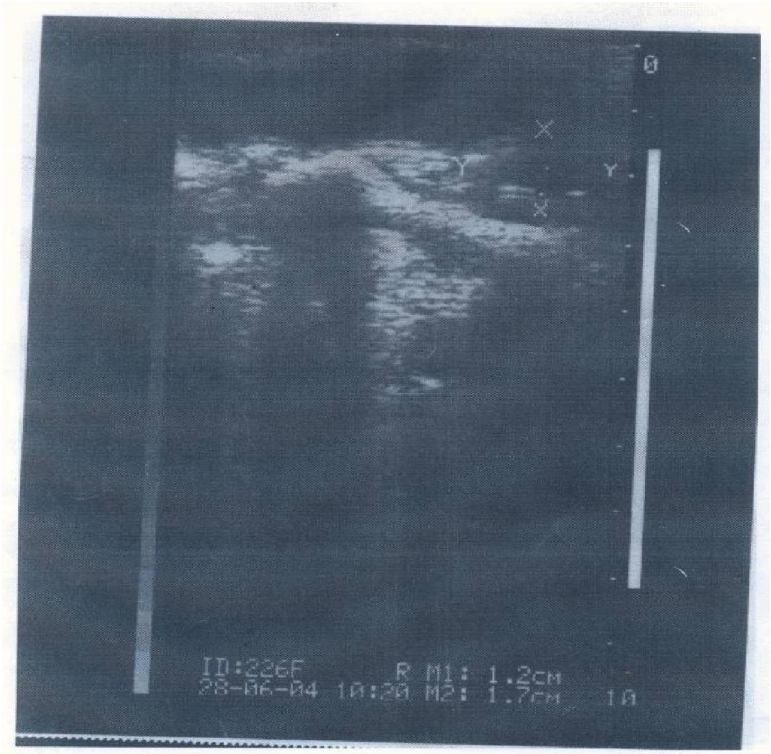

Figure 2: Smooth Ovary of doe.

Table (2) Effect of dopamine receptor antagonist (Resperdal)® on progesterone levels of Baladi does

Progesterone(ng/ml)	Experimental	Control
1st week	2.65±0.93 **	› 1
2nd week	4.89±1.08 *	1.02±0.13
3rd week	3.12±1.01 **	› 1

Mean ± S.D * P ‹ 0.05 ** P ‹ 0.01

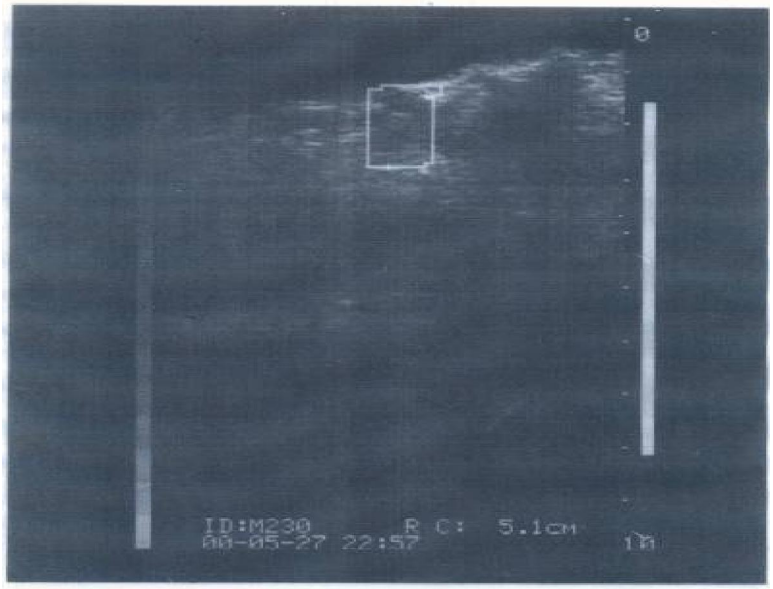

Figure 3: Ovary of doe showing corpus luteum.

4. Discussion

The present results (Table 1) showed that the treatment of smooth ovaries of does out-off the breeding season with dopamine receptor antagonist (Resperdal)® was associated with higher incidence of estrus and their ovaries showed higher shedding of both mature, Graffian follicles and corpus luteum. It can be suggested that Resperdal inhibit / antagonize the release of dopamine hormone which inhibit the synthesis and release of gonadotropins (**Anderson *et al.*, 2001**). Resperdal may be act directly on the ovarian function (**Besognet *et al.*, 1996**) or directly on the hypothalamus and pituitary gland to increase prolactin, FsH and LH concentration (**Besognet *et al.*, 1997**). Moreover, Resperdal may be act by blocking the receptors of dopamine hormone on reproductive system, however, it was recorded that in ewes and goats, the seasonal changes in the hormonal LH pattern mainly reflect an increase in the negative feedback exerted by estradiol under long days on the frequency of pulsitile LH secretion. However, the resulting seasonal inhibition of LH secretion involves the activation of monoaminergic and specially dopaminergic systems by estradiol (**Thiery *et al.*, 2002**).

The present investigation resulted (Table 2 and Figs,1,2 and 3) in regular regthem of progesterone level and estrus cycle in does treated with Resperdal with mature functional corpus luteum, may be due to the positive effect of Resperdal on LH release and ovulation, however, it was recorded that dopamine antagonists increased LH secretion in goats under high plane of nutrition during short day light (**Luis *et al.*, 2011**).

Therefore, it can be concluded that administration of dopamine receptor antagonist (Resperdal)® can be used for treating more than 50 % of does out-off breeding season which reflect positively on the kidding crop.

Corresponding author
Sabra, H. A
Department of Animal Reproduction & A.I, National Research Center, Cairo, Egypt
Sabra_hussein@yahoo.com

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Measurement of Monocyte CD86 Expression as Prognostic markers of Post Inflammatory Immunodeficiency in Critically Ill Patients

F. Ragab, M. Khaled, A. Mahmoud Kamel*, A. Abd El Bary, M.Abd El Monem

Critical Care Department, *National Cancer Institute, Faculty of Medicine, Cairo University, Egypt
mkhicu@yahoo.com

Abstract: Post inflammatory immunodeficiency frequently becomes life threatening since patients are predisposed to nosocomial infection. MHC-II molecules are essential for the activation of CD4+ cells and therefore for the initiation of any adaptive immune response and enhancement of the innate immunity. Aim Of The Work: The aim of this work is to study the prognostic effect of the level of monocyte CD86 expression as an indicative of post inflammatory immunodeficiency states in critically ill patients. Also to study the relation of the level of monocyte CD86 to patient outcome. Study Design: This is a prospective non randomized control trial conducted in Critical Care Department, Faculty of medicine Cairo University, Egypt. Inclusion criteria: Twenty critically ill patients who were admitted to critical care department. Exclusion criteria was age more than 80 years, Age less than 18 years, Disseminated malignancy and Co-morbid severe organ dysfunction. All patients subjected to:1. History taken, 2. Complete detailed clinical examination, 3. vital signs 4. Complete blood count (CBC), Liver profile, Coagulation profile & Daily arterial blood gases. 5. Measurement of monocyte expressive co-stimulatory factor CD86 using systematic flow cytometry analysis technique starting from day 1 to day 4. Results: Out of the twenty patients 7 survivors and 13 non survivors. Age of the survivor group ranged from 30-60 years, non survivors age ranged from 35 to 70 years. Five out of 14 males (35.7%) were survivors as compared to 2/6 females (33.3%). There were statistically significant difference between both groups as regards higher mean of arterial blood pressure and central venous pressure in survivors, and a highly significant difference was encountered as regard higher hear rate, temperature and respiratory rate in non survivors. A highly statistically significant difference was encountered also as regards total leucocytic count, serum glutamic pyruvate transaminase, serum glutamic oxaloacetic transaminase, serum creatinine, prothrombin time and international normalized ratio which was higher in non survivors (P<0.001). Of the 7 surviving patients, only 30% showed positive blood culture; while in non survivors 70% of pts showed positive blood C/S and there was no statistically significant difference (P: 0.089). Positive sputum culture was encountered in 43% of the 7 surviving patients, and it was +ve in 70% of non survivors with borderline significance statistically (P: 0.05). In day 1 CD86 monocytes expression by mean fluorescent ratio showed statistically significant higher level in non survivors, in day 2 there were no statistically significant difference. In day 3 CD86 monocytes expression was higher in survivors and in day 4 both CD86 were statistically significant higher in the survivor group. Survivors vs non survivors mean fluorescent (4+2 vs 7+2.5) (4+2.4 vs 5+2.2, 6.3+2.1 vs 4.2+1.5 & 7+2.5 vs 3.5+1.6) with P value 0.01, 0.4, 0.0 & 0.001 respectively). The trend of CD86 expression change over the 4 days is presented as CD86 mean showed an increasing pattern in survivors. Conclusion: Semiquantitative measurement of CD86 level expressed by mean fluorescent ratio is a good and valid prognostic test of mortality in post inflammatory immuno deficiency patients.

[F. Ragab, M. Khaled, A. Mahmoud Kamel, A. Abd El Bary, M.Abd El Monem **Measurement of Monocyte CD86 Expression as Prognostic markers of Post Inflammatory Immunodeficiency in Critically Ill Patients]** Life Science Journal, 2011; 8(4):943-950] (ISSN: 1097-8135). http://www.lifesciencesite.com. 121

Key words: Monocyte CD86, Nosocomial infection, MHC-II molecules

1. Introduction

Major surgery, poly trauma, burns, stroke and pancreatitis are often accompanied by a massive activation of the immune system called systemic inflammatory response syndrome ***(1).***

Due to counter regulatory mechanisms such as endocrine, paracrine or autocrine actions along with intracellular alterations this hyperinflammation is followed by a temporary immunodeficiency called compensatory anti-inflamamtory response syndrome. In its most severe form it is also referred to as immune paralysis state ***(2).***

Post inflammatory immunodeficiency frequently becomes life threatening since patients are predisposed to contract nosocomial infection. However, these infections are difficult to identify since they are scarcely associated with any clinical signs. Moreover, these infections can not be fought by the enfeebled immune system of such patients and may evolve into sepsis. It is therefore not surprising that sepsis and resultant multiple organs failure are the most common causes of death in intensive care units (ICUs) ***(2).*** In fact, in the United States alone more than 20.000 patients die of sepsis each year ***(3).***

The mechanisms responsible for post inflammatory immuno deficiency are not clear, which is the reason why no causal therapy has been established to date ***(4).*** Most probably, monocytic cells play a key role in the development and maintenance of this state. This monocytic cells seem to be impaired in their antigen presentation and inflammatory capacity. In fact, blood monocytes show a strongly reduced expression of major histocompatibility complex class II (MHC-II) and produce only minor amounts of preinflammatory cytokines in response to bacterial lipo-polysacchafrides (LPs) ***(4).*** The magnitude of MHC-II reduction correlates with increased susceptibility to infection and subsequent mortality and is used for diagnosis of post inflammatory immunodeficiency ***(2).***

MHC-II molecules are essential for the activation of CD4+ cells and therefore for the initiation of any adaptive immune response and enhancement of the innate immunity ***(5).***

In fact, the engagement of the T-cell receptor (TCR) with MHC-II complexes with antigenic peptides delivers a stimulatory signal to CD4+ cells ***(6).***

However, naïve CD4+ cells in particular need to receive a second signal set from Co-stimulatory molecule is blood antigen presenting cells from ICU patients is CD86 ***(2).***

Aim of the Work

The aim of this work is to study the prognostic effect of the level of monocyte CD86 expression as an indicative of post inflammatory immunodeficiency states in critically ill patients.

Also to study the relation of the level of monocyte CD86 to patient outcome.

2. Patients and Methods

Study Design:

This is a prospective non randomized control trial was conducted in Critical Care Department, Faculty of Medicine Cairo University, Egypt, Which is a tertiary critical care centre that contains surgical, medical and coronary care units of total capacity 52 beds. Patients were managed by the ICU team, which were available 24 hours per day. The Ethics Committee of the Faculty of Medicine Cairo University Approved the study.

Inclusion criteria:

Twenty critically ill patients who were admitted to critical care department.

Exclusion criteria

Age more than 80 years, Age less than 18 years, Disseminated malignancy and Co-morbid severe organ dysfunction.

Data collection and classification:

1. History taken, 2. Complete detailed clinical examination was performed for all patients, 3. vital signs composed of arterial blood pressure, mean arterial blood pressure, heart rate, temperature, respiratory rate and central venous pressure, 4. Length of stay, 5. Investigation: Complete blood count (CBC), Liver profile, Coagulation profile & Daily arterial blood gases. 6. Measurement of monocyte expressive co-stimulatory factor CD86 using systematic flow cytometry analysis technique starting from day 1 to day 4. Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from particles and cells in the size range of 0.5 um to 40um diameter. Cells are hydro-dynamically focused in a sheath of PBS before intercepting an optimally focused light source. Lasers are most often used as a light source in flow cytometrty.

As your cells or particles of interest intercept the light source they scatter light and fluorochromes are excited to a higher energy state. This energy is released as a photon of light with specific spectral properties unique to different fluorochromes for a listing of commonly used fluorescent dyes and their excitation and emission spectra.

One unique feature of flow cytometry is that it measures fluorescence per cell or particle. This contrasts with sepctrophotometry in which the percent absorption and transmission of specific wave lengths of light is measured for a bulk volume of sample.

Scattered and emitted light from cells and particles are converted to electrical pulses by optical detectors. Collimated (parallel light waveforms) light is picked up by confocal lenses focused at the intersection point of cells and the light source. Light is send to different detectors by using optical filters. For example a 525 nm band pass filter placed in the light path prior to the detector will only allow "green" light into the detector. The most common type of detector used in flow cytometry is the photomultiplier tube (PMT).

The electrical pulses originating from light detected by the PMTs are then processed by a series of linear and log amplifiers. Logarithmic amplifications most often used to measure fluorescence in cells. This type of amplification expand the scale for weak signals and compresses the scale for "strong" or specific fluorescence signals.

After the different signals or pulses are amplified they are processed by an Analog to Digital Converter (ADC) which in turn allows for events to be plotted on a graphical scale (one parameter, two parameter Histograms).

Flow cytometry analysis of a single cell suspension yields multiparameter data corresponding to Forward Light scatter (FLS), 90^{o} Light Scatter (90 LS), and FL1-FL4. By the Beckman-Coulter XL instruments are bench-top, flow cytometer, analyzers.

This information allows researches to identify and characterize various subpopulations of cells. The process of separating cells using flow cytometry multiparameter data, is referred to as sorting.

Outcome and mortality: According to outcome and mortality patients were classified into 2 groups: Survivors, Non survivors

Data Analysis:

All data were collected prospectively, Categorical data were displayed as absolute and relative frequencies. Continuous data were reported as mean values ± standard deviation (SD), or as median and range according to presence or not of a normal data distribution. Comparisons were performed with an unpaired student's t test for continuous, normally distributed data, and with a Mann Whitney U test, or Wilcoxon rank sum test for continuous non normally distributed data. Comparisons between categorical variables were performed with Chi square X2 test. Yates correction equation, or Fisher exact test was used instead of any frequency was <5. A two sided probability value< 0.05 was considered as significant. Professional statistical Package for Social Science version 15 (SPSS Incorporation, Chicago, IL, USA) computer software was used for data analysis.

Receiver operator curve (ROC) was used to estimate the cut-off value for the different predictor and assessment of these predictors was madder using the area under curve (AUC) and for each cut off sensitivity and specificity was calculated.

3. Results

Demographic data of critically ill patients classified into survivors and non survivors:

Age: The age of the survivor group ranged from 30-60 years with a mean of 45±12 , and a median of 44 years, non survivors age ranged from 35 to 70 years with a mean of 52.4+13 and a median of 53 years.

Gender: Five out of 14 males (35.7%) were survivors as compared to 2/6 females (33.3%). There was no statistically difference between both groups as regards gender.

Table (1): Hemodynamics and vital signs in 7 survivors and 13 non survivors critically ill patients

Parameter	MAP	CVP	HR	Temp	RR
Survivors					
Mean±SD	84±6	7±3.6	92.4±13	37±0.5	22.9±4.6
Range	75-92	1-12	76-109	36.6-38	17-30
Non survivors					
Mean+±SD	74±11.3	2.9±2.9	128.5±10	38.5±0.6	34.3±5.0
Range	55-90	0-8	112-151	37.8-40	25-43
	0.04	0.02	0.02	0.041	0.03

MAP: Mean arterial blood pressure **CVP:** Central venous pressure HR: Heart rate Temp**:** temperature **RR:** Respiratory rate

Statistically significant difference between both groups was encountered as regards mean arterial blood pressure and central venous pressure, and a highly significant difference was encountered as regard hear rate, temperature and respiratory rate.

Table (2): Laboratory parameters in 7 survivors and 13 non survivors critically ill patients

Parameter	Hb%	TLC	PLT	S.Alb	SGPT	SGOT	S. Creatinine	PT	INR
Survivors									
Mean±SD	11.4±1.7	12.8±3.1	254.3±93.5	3.6±0.4	30±13	42.4±6.6	0.9±0.3	13.5±0.9	1.2±0.1
Range	9-14.2	8.7-18.1	119-412	2.8-4.4	10-50	36-52	0.6-1.2	12.2-14.8	1.1-1.3
Non survivors									
Mean±SD	9.7±1.4	26.1±5.1	93.1±71	2.9±0.1	158±68	193±82	2.4±0.4	17±1.7	1.7±0.3
Range	6.9-11.2	16.1-33	21-245	2.2-3.6	63-245	79-324	1.9-3	14.4-20	1.3-2.2

Hb%: hemodglobin percentage, TLC: Total leucocytic count, PLT: Platelet count, S. Alb. Serum albumin, SGPT: serum glutamic pyruvate transaminase; SGOT: Serum glutamic oxaloacetic transaminase; S. Creatinine: serum creatinine, PT: Prothrombin time; INR: International normalized ratio.

A highly statistically significant difference was encountered as regards total leucocytic ount, serum glutamic pyruvate transaminase, serum glutamic oxaloacetic transaminase, serum creatinine, prothrombin time and international normalized ratio (P<0.001) and a statistically significant difference was encountered as regards serum albumin and

platelet count (P<0.05) and statistically insignificant difference as regards Hb% (P>0.05) (Table 2).

A highly statistically significant difference was encountered between both groups as regards the pH and pCO_2 and a statistically significant difference as regards HCO_3 and a non significant difference as regards PO_2 and oxygen saturation (Figure 1).

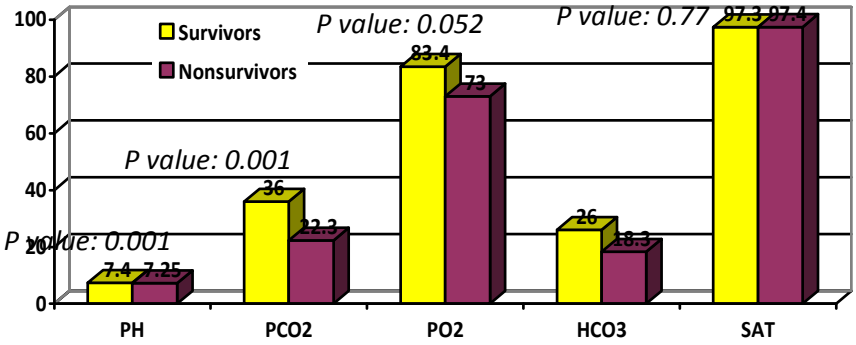

Figure (1): Arterial blood gases in 7 survivors and 13 non survivors critically ill patients

Of the 7 surviving patients, only 30% showed positive blood culture; while in non survivors 70% of pts showed positive blood C/S and there was no statistically significant difference (P: 0.089).

Positive sputum culture was encountered in 43% of the 7 surviving patients, and it was +ve in 70% of non survivors with borderlines significance statistically (P: 0.05).

Positive urine culture was encountered in 43% of the 7 surviving patients, and in 70% of non survivors and again in there were borderline statistically significant relationship (P: 0.054).

Table (3): CD86 level by Relative intensity in both groups

Parameter	Survivors	Non survivors	P value
CD86 level by relative intensity	16.25±3.5	5.4±2.7	0.004

Table (4): Monocyte CD86 mean fluorescent ratio and relative intensity in 7 survivors and 13 non survivors critically ill patients at different time results

Parameter	Outcome		
	Non Survivors	Survivors	P value
Day 1			
MFR	7±2.5*	4±2*	0.01
RI	15±6.3*	7±4.2*	0.001
Day 2			
MFR	5±2.2*	4±2.4*	0.4
RI	10±3.4*	9±5*	0.53
Day 3			
MFR	4.2±1.5*	6.3±2.1*	0.0
RI	6.4+3*	4±12.3*	0.001
Day 4			
MFR	3.5±1.6*	7±2.5*	0.001
RI	5.4±2.7*	3.5±16.25*	0.0001

There were statistically significant higher level of CD86 level by relative intensity in survivors compared to non survivors (P: 0.0004). When we compare the 7 survivors with the 13 non survivors as regards CD86 mean fluorescent (MFR) and relative intensity (RI) which were calculated by dividing mean fluorescent channel by number of he monocytes (% expression) as semi quantitative evaluation for the CD86 number of member.

In day 1 CD86 monocytes showed MFR with statistically significantly higher values in non survivors, RI showed the same statistically significant difference.

In day two there were no statistically significant difference between both groups for any of the CD86 parameters.

In day 3 CD86 monocytes parameters were higher in survivors. The difference was statically significant for mean fluorescence ration (P: 0.02) and for relative intensity (P: 0.001).

In day 4 both CD86 MFR and RI were statistically significant higher in the survivor group (P value: 0.001 and 0.0001, respectively).

The trend of CD86 expression change over the 4 days is presented as CD86 mean fluorescent ratio which showed an increasing pattern in survivors and a decreasing pattern in non survivors. The same trend was encountered with CD86 relative intensity.

Table (5): Comparison of change in CD86 level between 7 survivors and 13 non-survivors critically ill patients

Parameter		Survivors	Non survivors	P value
CD86	Increasing	7	0	0.001
	Decreasing	0	13	

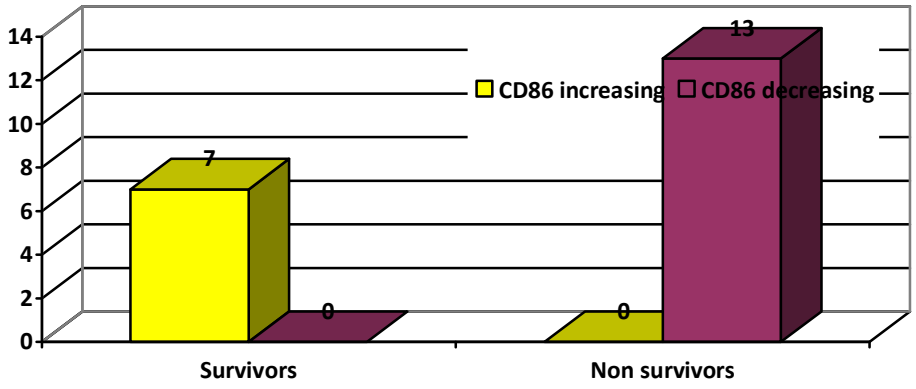

Figure (2): Comparison of change in CD86 level between 7 survivors and 13 nonsurvivors critically ill patients

ROC curves were used for parameters that showed statistical significance to determine a cut-off that can best discriminate between survivors and non survivors by using mean fluorescence ratio and relative intensity of CD86 monocytes.

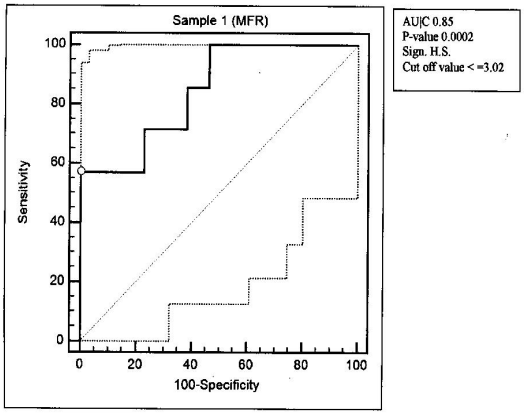

Figure (3): ROC curve of mean fluorescence ratio of CD 86 level in 7 survivors and 13 non survivors critically ill patients (day one).

Figure (3) shows that the best discriminative cut off for the mean CD86 fluorescent ratio in day 1 was 3.02 with a sensitivity of 57%, a specificity of 100%, a total accuracy of 85%, a positive predictive value of 100% and a negative predictive value of 81.2%. 5/7 survivors showed values < 3.02 as compared to 13/13 non survivors showed levels >3.02. The difference is statistically highly significant (P: 0.002).

In day 3 the best discriminative cut off for the mean CD86 fluorescent ratio was 6 with a sensitivity of 42.86%, a specificity of 100%, a total accuracy of 70%, a positive predictive value of 100% and a negative predictive value of 76.5%. Seven survivals showed values >6 while 10/13 non survivors showed levels <6 the difference was found to be statistically significant (P: 0.01), Figure (4).

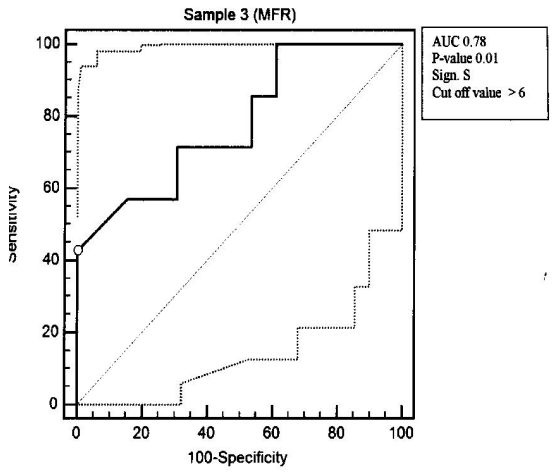

Figure (4): ROC curve of mean fluorescence ratio of CD86 level in survivors and non survivors (day three)

While in day 4 we found that the best discriminative cut off for the mean CD86 fluorescent ratio was 5.6 with a sensitivity of 71.43%, a specificity of 100%, a total accuracy of 90%, a positive predictive value of 100% and a negative predictive value of 86.7%. Seven/7 survivors showed values >5.6 while 8/13 non survivors showed levels <5.6. The difference is statistically highly significant (P: 0.0001), Figure (5).

When we studied ROC curve for relative intensity we found that the best discriminative cut off for the CD86 relative intensity in day 1 was 8 with a sensitivity of 85.71%, a specificity of 100%, a total accuracy of 95%, a positive predictive value of 100% and a negative predictive value of 92.9%. Six/7 survivors showed values <8 while 13/13 non

survivors showed levels >8. The difference is statistically highly significant (P: 0.0001), (Figure 6).

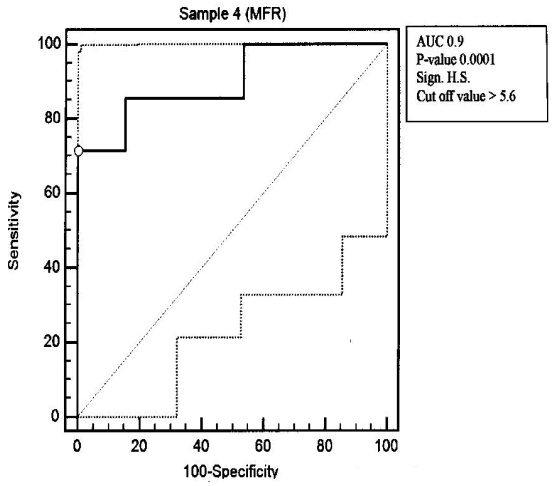

Figure (5): ROC curve of mean fluorescence ratio of CD86 level in survivors & non survivors (day four)

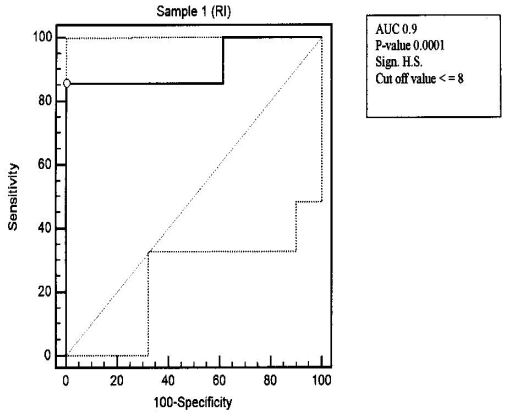

Figure (6): ROC curve of relative intensity of CD86 level in survivors & non survivors (day one).

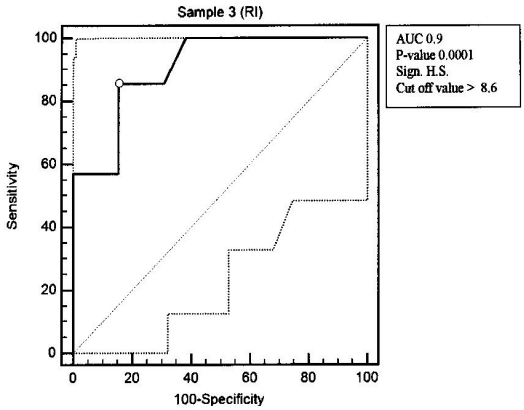

Figure (7): ROC curve of relative intensity of CD86 level in survivors & non survivors critically ill pts (day three).

While in day 3 we found that the best discriminative cut off for the CD86 relative intensity was 8.6 with a sensitivity of 85.71%, a specificity of 84.62%, a total accuracy of 85%, a +ve predictive value of 75% & a negative predictive value of 91.7%. Seven/7 survivors showed values >8.6 while 13/13 non survivors showed levels <8.6. The difference is statistically highly significant (P: 0.0001) (Figure 7).

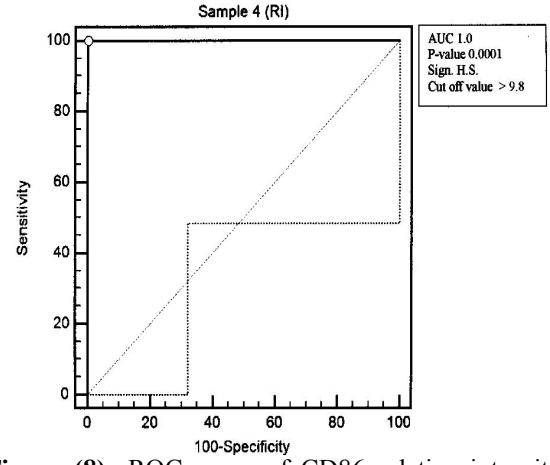

Figure (8): ROC curve of CD86 relative intensity level on monocytes in survivors & non survivors (day four).

As regards day four result we found that the best discriminative cut off for the CD86 relative intensity was 9.8 with a sensitivity of 100%, a specificity of 100%, a total accuracy of 100%, a positive predictive value of 100% and a negative predictive value of 100% and a negative predictive value of 100%. Seven/7 survivors showed values >9.8 while 13/13 non survivors showed levels <9.8. The difference is statistically highly significant (P: 0.0001). (Figure 8).

4. Discussion

Comparing laboratory parameters between both groups in our study showed a remarkable affection in the non survivors group in comparison to the survivor group. This can be attributed to the ongoing sepsis and MODS and this matches with *Nolan et al. (7)* who studied laboratory parameters together with CD40 and CD80/86 and their role to regulate inflammation and mortality in polymicrobial sepsis.

When we have compared the hemodynamics and vital signs parameters between both groups survivors and non-survivors it showed maintained, mean arterial blood pressure in the surviving group, together with the central venous blood pressure, while in the non-surviving-group, patients was vasodilated with low central venous pressure

secondary to the ongoing inflammatory response and sepsis.

Heart rate was in the normal range in the survivor group while tachycardia was evident in the non-surviving group, expressing a highly significant statistical difference between both groups. This can be attributed to the continuous production of inflammatory mediators of sepsis.

Temperature parameter was normal in survivor group while in the non-surviving group fever was evident expressing a highly statistically significant statistical difference between both groups. This can be attributed to the ongoing inflammatory response and sepsis.

Respiratory rate was in the normal range in the non survivor group while in the non-survivor group, tachypnea was evident because of fever this leads to a highly statistically significant statistical difference in comparing both groups.

All hemodynamics and vital signs matches with *Plosone, (8)* who studied the differential role for CD 80 and CD 86 in the regulation together with concomitant hemodynamic variables during this study of the innate immune response in murine polymicrobial sepsis and concluded that down regulation of CD 80 and loss of constitutive CD 86 expression on monocytes are associated with higher severity of illness and inflammation confirming the previous findings.

Our study findings as regards haemodynamices variation was also concordant with *Ludger et al., (9)* who studied the enhanced expression of CD 80 (B7-1), CD 86 (B7-2) and CD 40 and their ligands CD 28 and CD 154 in fulminant hepatic failure, and MODS. They found that CD 40 and CD 80/ CD 86 expression is upregulated before tissue damage and their increased expression leads to better prognosis while decreased expression leads to unfavorable outcome.

Arterial Blood gases parameters also showed an important role in the course of both groups with a highly statistical significant difference as regards pH and PCO_2. This is in concordance with *Newton et al. (10)*.

Blood culture positivity was studied in both groups in relation to CD 86 level showing that although there was 30% of the survivors with positive blood culture they survived because of the high level of CD 86 while in the non-surviving group there was 70% of the patients with positive blood culture leading to their death because of the low level of CD 86 *(Nolan et al.) (7)*.

The major findings of the present study were that reduced expression of CD 86 presented by MFR, and RI of expression of CD86 on blood monocytes, for four consecutive days is associated with unfavorable prognosis and this made us consider monocyte CD 86 expression as a helpful prognostic variable and enables us to postulate that reduced monocyte CD 86 expression contributes to worsened post inflammatory immunodeficiency in critically ill patients. The first study to match with our study on CD86 expression was by *Kerstin et al. (2)*; they found reduced monocytes with: long term (>3 days); had a long term reduction of CD 86 together with an unfavorable prognosis. Such patients often had infections and stayed on average three times as long in the ICU because of diminished monocyte CD 86 expression. More important, most of the patients who died in the ICU or within a month-after their stay due to multiple organ failure secondary to infection had at least a 3-day reduction of CD 86 level during their stay.

Based on these findings *Kerstin et al.,(2)* recommended that ICU patients with low CD86 expression on monoctes will be considered extremely vulnerable to infection.

This emphasizes the role of CD 86 in evaluating the prognosis of systemic inflammatory response syndrome and septic patients, it is in agreement with Nolan *et al.* who stressed on the importance of assaying the co-stimulatory molecules especially CD 86 as a biomarker for outcome in septic patients.

From these results, we have concluded that relative intensity and mean fluoresce ratio for CD 86 level from day one, day three and day four can be used as a prognostic marker in post inflammatory immunodeficiency patients and septic patients, as day one results are as highly statistically significant as day three and day four in relative intensity, while in mean fluorescence ratio showed a highly significant statistical p-value in day one and day four and significant statistical difference in day three. Accordingly to CD86 evaluation may be performed from day one and two giving early prediction of the outcome.

We can use either mean fluorescence ratio or relative intensity from day one, as their results in day one are equivalent with day three and day four. This is a new finding in prediction of the outcome in septic patients using CD86 MFR or relative intensity in day one and two this is an earlier prediction than that reported by *Kerstin et al. (2)* demonstrated that the reduction of CD 86 expression on blood monocytes for at least three consecutive days have an unfavourable prognosis but in our study we can use either MFR or relation intensity as prognostic semiqualitative method from day one in prediction of outcome.

However, it is worth mentioning that CD86 mean florescent ratio 'and relative intensity in day one were actually higher in the non survivor group.

The finding sounds paradoxical; a higher CD86 expression should reflect a better function of the monocytes as antigen presenting cells. Apparently, this was a trial from the immune system to mount up a reaction that could not face the overwhelming infection and ended up by failure. Consequently CD86 was down-regulated from day 2 with failure to control infection and ultimate death. On the other hand the survivor group mounted up their response gradually starting with CD86 levels lower in day one than the non survivors but the immune system could escalate its response with increasing CD86 levels from day 2 on.

Though the paradoxical results on day one are statistically significant and showed good predictive values, yet it may be better to include at least day 1 and 2 in our predictive model to judge according to the trend rather than the absolute values of CD86 expression.

Conclusion:

Post inflammatory immunodeficiency frequency become life threatening.

Monocytic cells play a key role in the development and maintenance of post inflammatory immunodeficiency, CD86 is an important co stimulatory molecule on monocytic cells.

Semiquantitative measurement of CD86 level expressed by relative intensity is a good and valid prognostic test in post inflammatory immuno deficiency patients.

Relative intensity of CD86 level has a higher sensitivity and specificity together with mean fluorescence ratio in predicting the mortality of post inflammatory; in immunodeficiency patients. It can be used on day one and two in order to determine the trend of expression.

Corresponding author
M. Khaled
Critical Care Department, National Cancer Institute, Faculty of Medicine, Cairo University, Egypt
mkhicu@yahoo.com

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The Potential Pharmacological and Histological Benefits of Carvedilol on the Hippocampal Post- Stroke Seizures in Rats

Omnyah Ali El-Kharashi[*1] and Abeer A. Abd El Samad[2]

Departments of Pharmacology[1] and Histology[2], Faculty of Medicine, Ain Shams University, Cairo, Egypt
omnyah2011@gmail.com

Abstract: Stroke is the most common cause of seizures in the elderly, and seizures are among the most common neurologic sequel of stroke. About 10% of all stroke patients experience seizures, from stroke onset until several years later. We have investigated in the current study the possible protective effects of carvedilol versus the use of carvedilol immediately post stroke in a global cerebral ischemic model in rats. Twenty six male Wistar albino rats were divided into normal control (n=6), ischemic vehicle treated group n= (8) with neck tourniquet for 7.5 minutes and preinjected with phentolamine (0.5 mg/rat), group received daily injections with carvedilol (3mg/kg) for four days before induction of ischemia and group treated with carvedilol single injection (3mg/kg) immediately after induction of ischemia. Our results demonstrated that carvedilol either pre or post treatment significantly decreased the duration and the severity of seizures and consequently the mortality of the rat. Histological examination showed that pyramidal cells had features of cell degeneration and there was significant increase in the immunohistochemical reactions for GFAP, caspase-3 and TNF- α in ischemic group. Whereas, the pre-treated group showed protection of the pyramidal cells with significant decrease in the immunoreactions, the post-treated group showed less improvement in signs and immunoreactions than that of the pre-treated group. Therefore, it is regarded that the use of carvedilol has a neuroprotective beneficial effect over the use of carvedilol just after ischemia. Weather the repeated injections with carvedilol after stroke for a given duration will give a more neurotherapeutic effect or not, this is for further evaluation.

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Keywords: carvedilol, cerebral ischemia, seizures, TNF-α, GFAP, caspase

1. Introduction

Post-stroke seizure and post-stroke epilepsy are common causes of hospital admissions, either as a presenting feature or as a complication after a stroke. Cerebrovascular disease is the commonest cause of epilepsy in the elderly population **(Kramer, 2001).** Around 45% of early onset post-stroke seizures occur within the first 24 hours. It is described as a late onset seizure, when it occurs after two weeks of stroke onset. Late onset seizure has a peak within 6 to 12 months after the stroke and has a higher recurrence rate of up to 90% in both ischemic and haemorrhagic stroke **(Myint *et al.*, 2006).**

There are several causes for early onset seizures after ischemic strokes. An increase in intracellular Ca^{2+} and Na^{+} with a resultant lower threshold for depolarization, glutamate excitotoxicity, hypoxia, metabolic dysfunction, global hypoperfusion, and hyperperfusion injury (particularly after carotid end arterectomy) have all been postulated as putative neurofunctional aetiologies. Late onset seizures are associated with the persistent changes in neuronal excitability and gliotic scarring is most probably the underlying cause **(Silverman *et al.*, 2002).**

In the hippocampus, which is one of the regions most sensitive to ischemic challenge, global ischemia induces a complete loss of Cornu Ammonis area (CA)1 pyramidal neurons, whereas the resistant CA3 pyramidal neurons display a long-term hyperexcitability several months after the insult **(Epsztein *et al.*, 2006).** As regarding, pro-inflammatory cytokines play key roles in the epileptogenic cascade including seizure-related pathological changes in hippocampus, such as neuronal death, reactive gliosis and aberrant mossy fiber sprouting **(Fabene *et al.*, 2010)**.

Astrocytes also play a critical role in epileptogenesis **(Tian *et al.*, 2005).** Reactive astrocytosis may exacerbate inflammation by inducing the migration of other leukocytes into the injured site, interrupting blood-brain-barrier function **(Vezzani *et al.*, 2010)**, producing reactive oxygen species **(Hamby *et al.*, 2006)** and causing cytotoxic edema **(Zador *et al.*, 2009).**

Glial fibrillary acidic protein (GFAP) is expressed in the central nervous system in astrocytes. It is involved in many cellular functioning processes, such as cell structure, movement, cell communication, and the functioning of the blood brain barrier. GFAP has been shown to play a role in mitosis by adjusting the filament network present in the cell and maintenance of CNS myelin integrity.

GFAP is also proposed to play a role in astrocyte-neuron interactions, Purkinje cell communication and possibly many other neural cells. Moreover, GFAP levels are already used as a marker of neurologic damage in adults who suffer strokes and traumatic brain **(Vos *et al.*, 2010)**

Caspases are cysteine proteases that mediate apoptotic death in a variety of cellular systems, including neurons. Caspases are activated through extrinsic or intrinsic pathways. The latter is used by most neurons in most situations. In response to harmful stresses, cells induce programmed cell death (PCD); apoptosis. Seizures can induce neural damage and activate biochemical pathways associated with PCD. Since seizures trigger intraneural calcium overload, it has been presumed that the intrinsic cell death pathway mediated by mitochondrial dysfunction would modulate cell death following seizures **(Meller *et al.*, 2006).**

Kwan and Wood (2010) assessed the antiepileptic drugs (AED) for the primary and secondary prevention of seizures after stroke. They found three randomised controlled trials that have assessed the effects of several different AED for the secondary prevention of post-stroke seizures. Then, they concluded that, there is insufficient evidence to support the effectiveness of AED in the primary or secondary prevention of seizures after stroke.

Myint *et al.* (2006) limited the use of AED around an associated sedation. Other special considerations of AED use in older population are the possibility of drug interaction because of hepatic enzyme induction by commonly used AED such as carbamazepine and phenytoin, the higher chance of toxic effects because of the pharmacokinetic and pharmacodynamic changes associated with ageing. Drug compliance can also be an issue in older patients. They all recommended further well-conducted new strategy concerning this important clinical problem and targeting either the mechanisms or the mediators leading to development of ischemic seizures.

Carvedilol is a non-selective beta blocker/alpha-1 blocker indicated in the treatment of mild to moderate congestive heart failure (CHF). The neuroprotective efficacy of carvedilol might be related to its properties such as endothelial protection, antioxidant, anti-platelet effects and anti-inflammatory effects (**Watanabe *et al.*, 2011**).

In this work, we try to find the possible effect of carvedilol on hippocamal post-stroke seizures that enabled us to model a clinically relevant scenario with behavioural and histological studies.

2. Material and Methods

2.1. Drugs and Chemicals

Carvedilol powder ***(GlaxoSmithKline Egypt, GSK Egypt)*** was dissolved in 5% **DMSO** (Sigma, St. Louis, MO, U.S.A.), then diluted in a 0.9% saline solution. DMSO/saline vehicle was administered as a control *(Savitz et al., 2000).* All the materials used in histological study will be mentioned in their condition.

2.2. Animals

Twenty six adult male Wistar rats (200–250 g) were housed in plastic Perspex cages under controlled conditions (ambient temperature of 22 ± 1°C, natural light-dark cycle) for acclimatization and classified into 4 groups. Standard laboratory chow pellets and tap water were freely available. All experiments were done at the same time of day (between 9.00 a.m. and 12.00 a.m.) to minimize circadian influences on seizure susceptibility at the department of pharmacology in Ain Shams University.

Groups

Group I: (Control group) n= (6)

Group II: (Ischemic group) n= (8) exposed to ischemic injury (Cizkova ***et al.,*** 2000), vehicle-(DMSO and saline 9%) treated.

Group III: (Carvedilol pre-treated group) n= (6): Carvedilol (3mg/kg b. wt. subcutaneously) was injected daily, 4 days before the induction of ischemia *(Savitz et al., 2000).*

Group IV: (Carvedilol post ischemic treatment group) n= (6): Rats were injected with a single dose of carvedilol (3mg/Kg b. wt., subcutaneously) just after induction of cerebral ischemia.

2.3. Induction of global cerebral ischemia *(Cizkova et al., 2000)*

A 7.5-min period of cerebral ischemia was produced (ischemia group). This was done by inflating the neck tourniquet till cyanosis was observed, while inducing systemic hypotension for a 5 min period by giving phentolamine (0.5 mg/Rat, IV). Starting 4 hours post-ischemia convulsive behaviour responding to hot air was assessed.

2.4.The seizure severity Score *(Raedt et al.,2011)*

Seizure severity was assessed based on the observation of behavioral manifestations. Preconvulsive behavior in the form of initial akinesia, tremor of the whole body and/or incomplete limbic gustatory automatisms, salivation and head scratching was measured. The latency of the first seizure and the longest seizure (min.) were determined. The seizure severity score (SSS) was adapted from Racine's scale *(Racine, 1972)* to take into account the typical behavioral changes associated with hot air. This scale

consists of six stages that correspond to the successive developmental stages of motor seizures: 0: normal non-epileptic activity; 1: snout and facial movements, hyperactivity, grooming, sniffing, scratching, and wet dog shakes; 2: head nodding, staring, and tremor; 3: forelimb clonus and forelimb extension; 4: rearing and salivating; and 5: falling and status epilepticus. Seizure duration was the duration of limbic seizures (stage 1–2) and motor seizures (stage 3–5). Number of rats with clonic and tonic seizures was counted. Additionally animals exhibited **Status Epilepticus** (SE) > 120 min and animals exhibited no seizures were determined. Finally the mortality rate (%) was measured.

2.5. Histological study:

After 6 hours of induction of cerebral ischemia, the rats were sacrificed by intra-cardiac injection of 10% neutral buffered formalin under ether anesthesia. The brain was removed immediately and cut in coronal section. The specimen was fixed in 10% neutral buffered formalin and processed for light microscopic study to get paraffin sections of 5 µm thickness. They were stained with (Bancroft & Gamble, 2008):

A- Haematoxylin and Eosin (H&E)

B- Immunohistochemical technique for:

Glial fibrillary acidic protein (GFAP) of astrocytes and Caspase-3 to detect apoptosis (mouse monoclonal antibody purchased from Lab Vision, USA).

Tumour necrosis factor-alpha (TNF-α) (AAR33, polyclonal antibody) as pro-inflammatory marker (purchased from AbD Serotec, UK).

Serial paraffin sections were deparaffinized and dehydrated, including the positive control sections. The endogenous peroxidase activity was blocked with 0.05% hydrogen peroxide in absolute alcohol for 30 minutes. The slides were washed 5 min in phosphate buffered saline (PBS) at PH=7.4. To unmask the antigenic sites, sections were put into 0.01M citrate buffer (PH=6) in the microwave for 5 min. The slides were incubated in 1% bovin serum albumin dissolved in PBS for 30 min at 37°c in order to prevent the non specific background staining. The slides were divided to apply the three markers GFAP, caspase-3 and TNF-α. Two drops of ready to use primary antibody of GFAP and caspase-3, while dilution of 1:1000 of TNF-α were applied to sections, except for negative control. Then, they were incubated for one hour and half at room temperature. The slides were rinsed with PBS, then incubated for one hour with anti-mouse immunoglobulins (secondary antibody) conjugated to peroxidase labeled dextran polymer (DAKO, Denmark). In order to detect the reaction, the slides were incubated in 3,3-diaminobenzidene (DAB) for 15 min. The slides were counterstained by Haematoxylin, then dehydrated, cleared and mounted by DPX.

2.7. Morphometric study:

The number of the immuno-reactive star-shaped astrocytes stained by GFAP and pyramidal cells with positive brownish caspase-3 immuno-reaction were counted per high power field (HPF) in CA1 area of the hippocampus. Five fields from three serial sections of six rats per group were examined using x40 objective (final magnification x640) by Zeiss microscope in Histology Department, Faculty of Medicine, Ain Shams University. Color intensity of TNF-α was evaluated in CA1 area of the hippocampus using Olympus $BX40F^{-3}$ microscope and analyzed by image analysis software at regional centre for mycology & biotechnology in Al-Azhar University.

2.8. Statistical Analysis:

Mean and standard deviation (±SD) of the values were statistically analyzed. Then, one way ANOVA with post-hoc test of SPSS 17 was used to find significance between groups. The calculations were considered significant if $P < 0.05$.

3. Results

3.1. Effect of pre and post treatment of carvedilol on the seizure severity Score:

Table (1) and figure (1) show that, treatment with carvedilol either pre (repeated injections for 4 days) or post (single injection just after the ischemic injury) significantly decreased the duration and the severity of seizures and consequently the mortality of the rats.

In3.2. Effect of pre and post treatment of carvedilol on histological study

H&E examined sections of group I, CA1 area of the hippocampus showed its three layers (polymorphic, pyramidal and molecular). Pyramidal cells appeared with large vesicular nuclei and basophilic cytoplasm (Fig. 2-A). Immunohistochemichal stained sections for GFAP of the same group showed few brownish star-shaped astrocytes (Fig. 3-A). While the sections stained by caspase-3 showed no brownish immuno-reaction in the pyramidal cells (Fig. 4-A). The TNF-α stained sections showed minimal brownish immune-reaction in the different layers of CA1 area (Fig. 5-A).

In group II, the H&E stained sections showed that some pyramidal cells were degenerated with pyknotic nuclei and surrounded by halos, while others appeared having karyolitic nuclei (Fig. 2-B). Figures 3-B, 4-B and 5-B showed increase in the

immuno-reaction of GFAP, caspase-3 and TNF-α respectively. By statistical measures, table (2) showed a significant increase in mean astrocyte number (P<0.05) of GFAP immune-stain, a significant increase (P<0.05) in mean number of pyramidal cells with brownish immuno-reaction of the caspase-3 stained sections and a significant increase (P<0.05) of colour intensity of the TNF-α stained sections, all compared to group I.

In H&E stained sections of group III, the three layers of CA1 area appear nearly similar to group I (Fig. 2-C). Figures 3-C, 4-C and 5-C showed decrease in the immuno-reaction of GFAP, caspase-3 and TNF-α respectively compared to group II. By statistical measures, table (2) showed a significant decrease in mean astrocyte number (P<0.05) of GFAP immune-stain, a significant decrease (P<0.05) in mean number of pyramidal cells with brownish immuno-reaction of the caspase-3 stained sections and a significant decrease (P<0.05) of colour intensity of the TNF-α stained sections, all compared to group II.

Group IV showed in H&E stained sections few degenerated and shrunken cells of pyramidal layer with more condensed nuclei (Fig. 2-D). The GFAP stained sections appeared with immuno-reactive star-shaped cells nearly similar to group I (Fig. 3-D). The caspase-3 stained sections showed some pyramidal cells with brownish immuno-reaction (Fig. 4-D). Whereas, the brownish immuno-reaction of TNF-α was apparently more than group III and nearly similar to group II (Fig. 5-D). Statistical measures, (Table 2) showed a non-significant increase (P>0.05) in mean astrocyte number of GFAP immuno-stain compared to group I, a significant increase (P<0.05) in mean number of pyramidal cells with brownish immuno-reaction of the caspase-3 stained sections compared to group I, but a non-significant decrease (P>0.05) compared to group II and a significant increase (P<0.05) of colour intensity of the TNF-α stained sections compared to group III, but a non-significant decrease (P>0.05) compared to group II.

Table 1: Effect of carvedilol on poststroke seziures and mortality

Parameters \ Groups	Ischemic (Vehicle treated)	Carvedilol	
		Pre-treatment	Post- treatment
Longest Seizure (min)	57 ± 5.18 (6)	27±2.08# (4)	33.8 ± 3.03#(5)
Average Racine's score	2.4 ± 0.22 (6)	1.75 ± 0.96	2.4 ± 1.5 (5)
Rats with tonic seizures (%)	66	0	0
Rats with clonic seizures (%)	100	25	50
Rats exhibiting SE >120 min (%)	83.3	0	0
Rats exhibiting no Seizures (%)	0	30	17
Mortality rate (%)	25	0	0

N.B Preconvulsive behavior onset, latency to first seizure, longest seizure and average Racine's are mean ± SD with the number of animals shown in parentheses.

-Rats with tonic and clonic seizures; animals exhibiting SE >120 min, exhibiting no seizures and mortality rate are percentage (%) of rats.

-# significantly different from ischemic group at p< 0.05 - $ significantly different from carvedilol pre-treatment group at p< 0.05

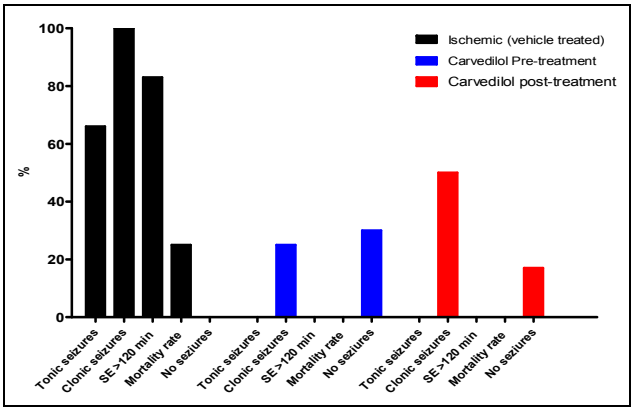
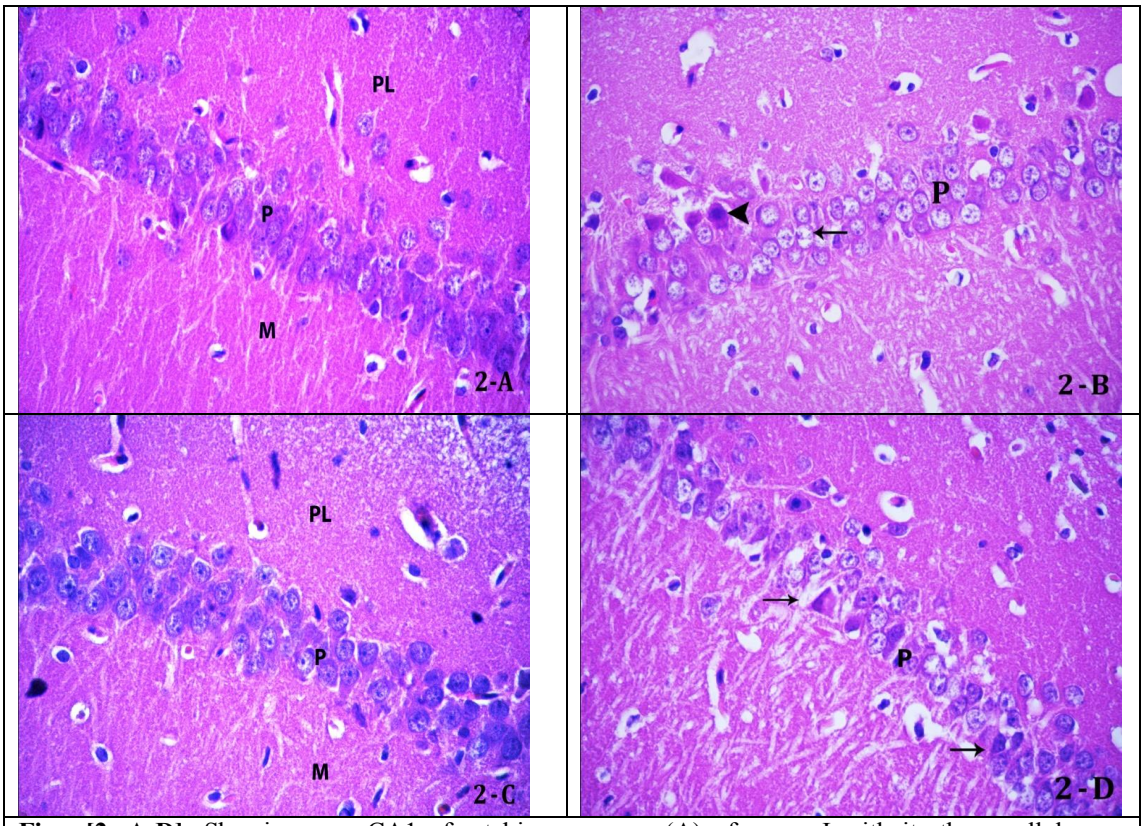


Figure (1) Effect of carvedilol on post-stroke seizures and mortality

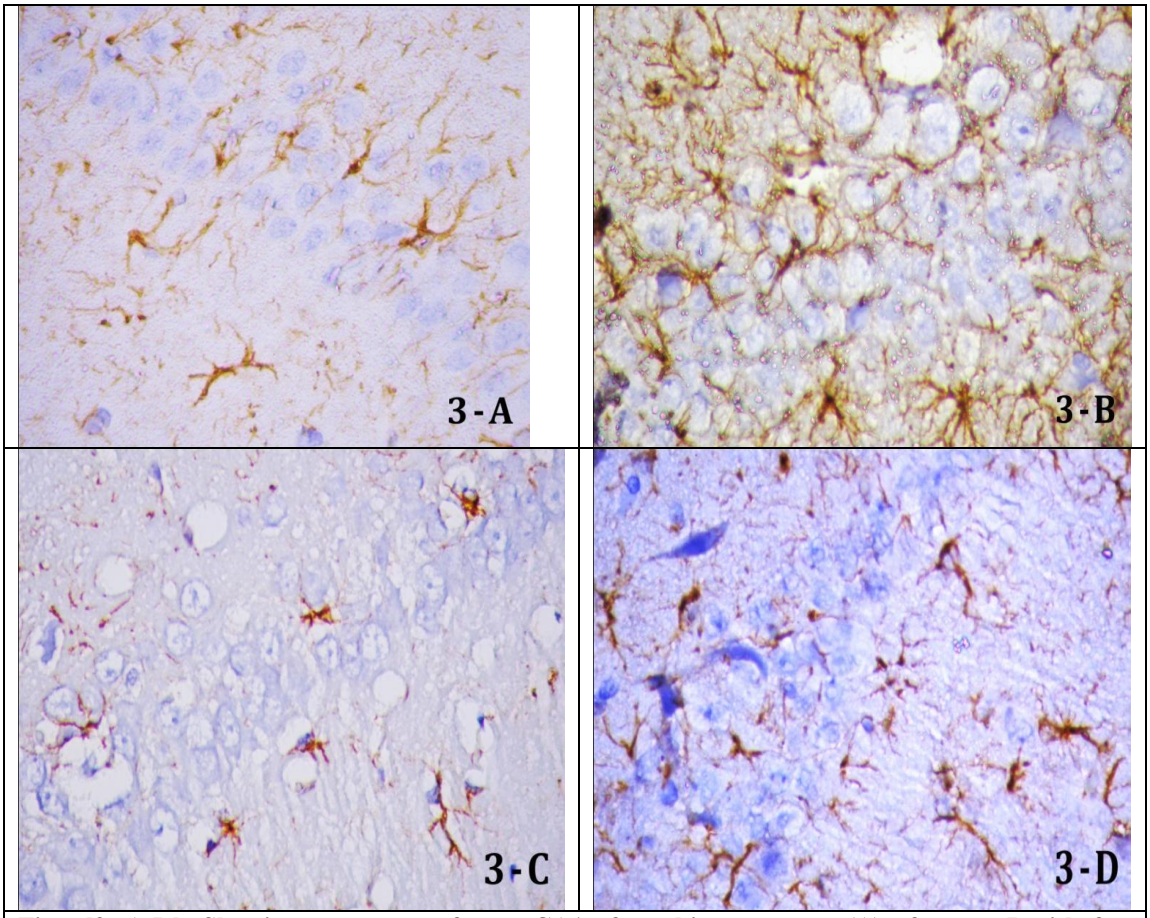
Table (2): Showing the mean ± SD of different immunohistochemical techniques per high power field (HPF) (GFAP for astrocytes detection, caspase-3 for apoptotic cell detection and TNF-α color intensity):

Groups / Parameters	Group I (control;normal)	Group II Ischemic (Vehicle treated)	Group III Carvedilol (pre-treated)	Group IV Carvedilol (post-treated)
Number of GFAP immune-reactive cells	4 ± 1.25	7.06 ± 2.15*	3.53 ± 1.84$^{\#}$	4.46 ± 1.12$^{\#}$
Number of caspase-3 immuno-reactive cells	0.8 ± 0.78	3.50 ± 1.71*	1.40 ± 0.96$^{\#\$}$	3 ± 0.81*
Color intensity of TNF-α	232.13 ± 17.49	271.16 ± 6.96*	224.52 ± 5.84$^{\#\$}$	259.97 ± 22.85

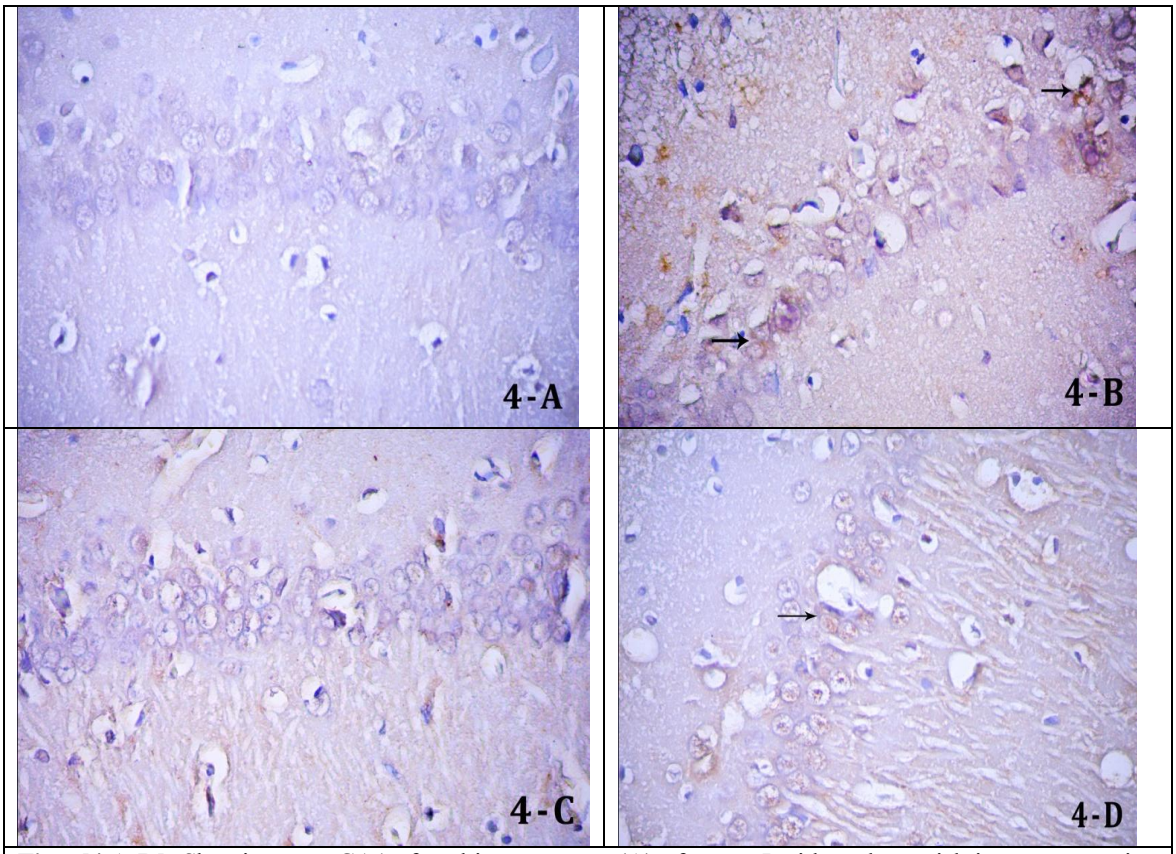
-Values are expressed as mean ± SD (standard deviation). - Number of rats = 6 per group
-Significant difference at $p<0.05$ - * $p<0.05$significance of difference by LSD vs group I
- $^{\#}$ $p<0.05$significance of difference by LSD vs group II - $^{\$}$ $p<0.05$significance of difference by LSD vs group IV



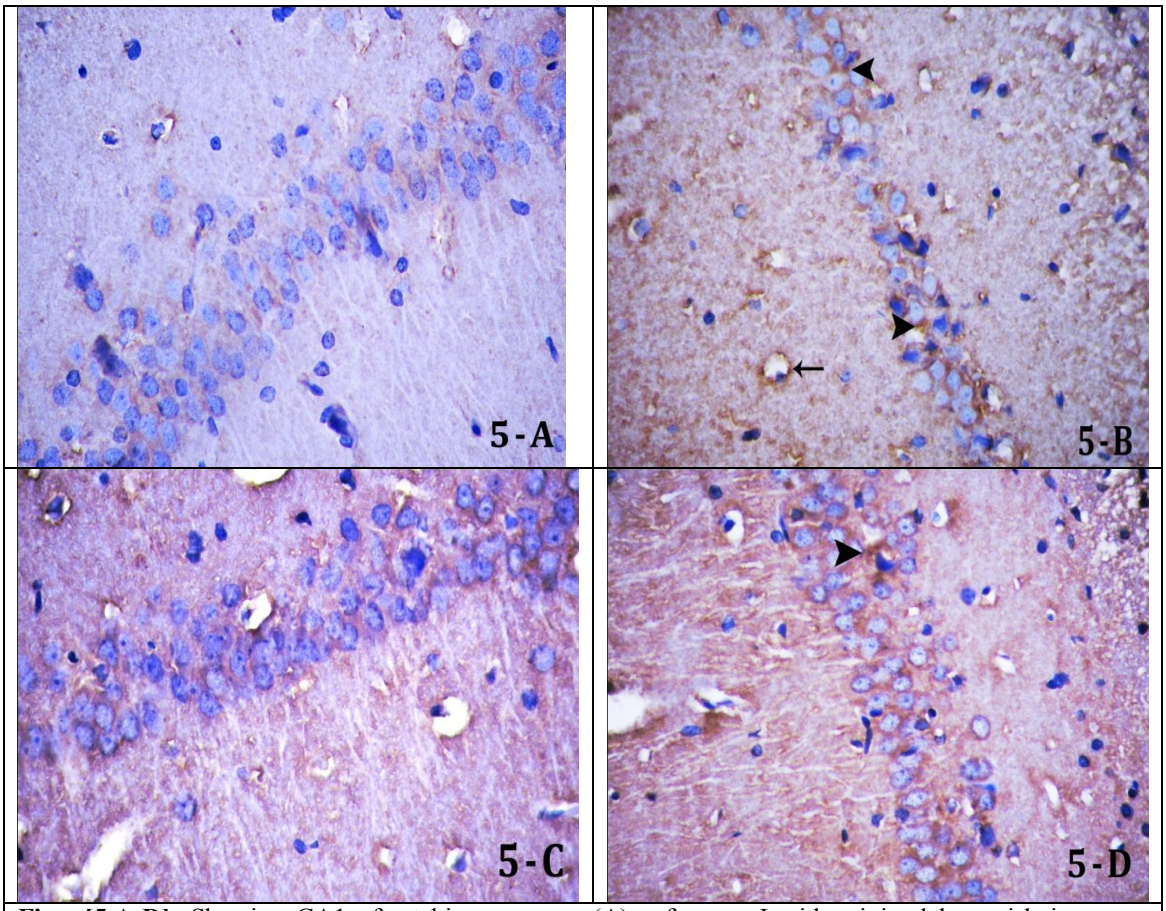
Figs. [2, A-D]: Showing area CA1 of rat hippocampus: (**A**) of group I with its three cell layers: polymorphic (PL), pyramidal (P) and molecular (M). Pyramidal cells of the middle layer appear with large vesicular nuclei. (**B**) of group II with some cells of pyramidal layer (P) appearing degenerated (▲) having pyknotic nuclei, while others having karyolitic nuclei (↑). (**C**) of group III with the three layers polymorphic (PL), pyramidal (P) and molecular (M) appearing nearly similar to group I. **(D)** of group IV having few degenerated and shrunken cells (↑) of pyramidal layer (P). (**H&E X400**)



Figs. [3, A-D]: Showing astrocytes of area CA1 of rat hippocampus. (A) of group I with few immuno-reactive star-shaped cells appearing with brownish granules. (B) of group II with apparent increase in their number, size and immuno-reaction. (C) of group III with apparently less in number, size and immune-reactivity than cells of group II. (D) of group IV with immuno-reactive star-shaped cells nearly similar to group I. (GFAP X640)



Figs. [4,A-D]: Showing area CA1 of rat hippocampus. (**A**) of group I with no brownish immuno-reaction appearing in pyramidal cells. (**B**) of group II with brownish immuno-reaction in many pyramidal cells (↑). (**C**) of group III with minimal brownish immuno-reaction in some pyramidal cells less than those of group II. (**D**) of group IV with brownish immuno-reaction in some pyramidal cells (↑). (**Caspase-3 X400**)



Figs. [5,A-D]: Showing CA1 of rat hippocampus. (**A**) of group I with minimal brownish immuno-reaction in pyramidal cell layer. (**B**) of group II with brownish immuno-reaction (▲) in pyramidal cell layer and in endothelial cells (↑). (**C**) of group III with brownish immuno-reaction apparently less than group II. (**D**) of group IV with brownish immuno-reaction (▲) in pyramidal cell layer. (**TNF-α X400**)

4. Discussion

Over the years, however, β-blockers have been associated with an incidence, albeit low, of central nervous system (CNS) side effects. In addition, noradrenergic receptors play a modulatory role in many nerve functions, including vigilance, attention, reward, learning and memory (Kumar *et al.,* 2011). Therefore, the present study has been designed to explore the possible role of carvedilol, an adrenergic antagonist on an ischemic injury induced seizures in rats.

In this work, treatment with carvedilol (a non-selective beta blocker/alpha-1 blocker) either pre (repeated dose injections for 4 days) or post (single dose injection just after the ischemic injury) significantly decreased the duration and the severity of seizures and consequently the mortality of the rats. Several authors; (Kumar and Dogra, 2009, Arrieta-Cruz *et al.,* 2010 and Kumar *et al.,* 2011) proved experimentally that carvedilol had neuroprotective potential, improved cognitive dysfunction and oxidative damage induced by aluminium chloride, colchicine and in a genetic model of Alzheimer, respectively.

The current study showed degenerated and shrunken pyramidal cells with pyknotic nuclei in H&E stained sections, as well as significant increase in caspase-3 immuno-reactive cells in CA1 area of hippocampus in group II (ischemic group). These findings could be explained by Li *et al.* (1997), who suggested that the cerebral ischemia triggered the release of free radicals that interact with many biological molecules such as nucleic acids, proteins and lipids to destroy them. The stressed mitochondria of the affected neurons released cytochrome c which activated caspases. Activated caspase-9 acted on caspase-3 to initiate apoptosis. Créple *et al.* (2003), also, reported that oxidative stress is one of many pathways that can activate neuronal apoptosis. Furthermore, Hattiangady *et al.*(2011) demonstrated that post-ischemic changes affected the CA1 area of hippocampus leading to neuronal degeneration.

The free radicals, also, generated after cerebral ischaemia increased the expression of

proinflammatory cytokines such as TNF-α and IL-1β (Lambersten *et al.,* 2009). Endothelial cells, leucocytes, astrocytes and even neurons released cytokines in cerebral ischemia (Clausen *et al.,* 2008). This explained the significant increase in the TNF-α color intensity of group II compared to group I in this study. The resident microglial cells are the main source of TNF-α (Lambersten *et al.,* 2009). In the present study, there was a significant increase in mean number of GFAP immune-reative astrocytes in group II. This might be a response to ischemic injury as ischemia triggered secretion of TNF-α, which activated astrocytes to produce other cytokines as interleukin-6. Whereas, the activated microglia in the affected middle pyramidal layer might initiate phagocytosis with TNF-α production (Uno *et al.,* 1997). On the other hand, some authors suggested that TNF-α might have a neuroprotective role and that the microglia acted as key regulator of neuronal survival after ischemic tissue injury (Lambersten *et al.,* 2009).

The present study showed improvement of the histological results in H&E stained sections with significant decrease in mean number of GFAP positive astrocytes, caspase-3 positive cells and TNF-α color intensity in group III, which was pretreated with carvedilol for 4 days, compared to group II (ischemic vehicle treated group). These findings could be explained by Abreu *et al.* (2000) who mentioned that carvedilol, in isolated rat liver mitochondria, is an antioxidant. Several authors (Savitz *et al.,* 2000 and Goyagi *et al.,* 2006) reported that carvedilol versus propranalol protects the neurons after transient focal cerebral ischemia in rats, through the preservation of mitochondrial function. It has an antiapoptotic role and can downregulate the inflammatory cytokine gene expression of TNF-α and IL-1β.

Savitz *et al.*(2000) proved that carvedilol inhibited a number of inflammatory processes during brain damage and suppress the release of cytokines in neurological animal disease models and lastly, Kumar and Dogra (2009) stated that carvedilol had a neuroprotective effect against colchicine-induced cognitive impairment and could attenuate the oxidative damages.

In the present study, the improvement of ischemic changes in pre-treated group was more evident than that of the post ischemic carvedilol treated group. There was a non-significant decrease (P> 0.05) of mean number of caspase-3 positive cells and a non-significant decrease (P> 0.05) of TNF-α color intensity in post ischemic carvedilol treated group compared to ischemic vehicle treated group . This could be explained by the need of multiple dosing regimens to accumulate the drug for efficacy (Lysko *et al.,* 1992).

Strosznajder *et al.* (**2005**) revealed that carvedilol (7-70mg/kg) administered subcutaneously directly after transient (5 min) forebrain ischemia protected significant population of neurons in the hippocampal CA1 area in gerbils, thus carvedilol raises high expectations also in the therapy of ischemia.

Given our findings that carvedilol (3 mg/kg/day subcutaneous injections for 4 days) showed a significant neuroprotective effect than single carvedilol (3mg/kg) post ischemic subcutaneous injection which might find a beneficial use in the prevention of neuronal impairment, such as brain ischemia and post stroke seizures. Weather the repeated injections after stroke with carvedilol for a while will give a more neurotherapeutic effects or not, this is for further evaluation.

Corresponding Author:
Omnyah Ali El-Kharashi
Departments of Pharmacology, Faculty of Medicine,
Ain Shams University, Cairo, Egypt
omnyah2011@gmail.com

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Three dimensional assessment of a newly designed distalizer (Bidirectional Distalizer)

Wael M Refai* & Ahmed H El Sherbini**

* Orthodontic Department, Faculty of Dentistry, Minia University, Egypt
** Orthodontic Department, Dentistry Division, National Research Centre, Cairo, Egypt
w_refai_67@hotmail.com

Abstract: Aim: The study was designed to three dimensionally assess the effects of a newly designed distalizer: bidirectional distalizer. **Method:** Ten patients suffering from Class II molars were included. Force was applied from the buccal and palatal sides. Cone beam CTs were taken before and after distalization. The cone beam images were then transferred to Mimics Dicom 10.01. The pre and post distalization images were superimposed and the difference was calculated. Depending on three reference planes, each cusp and root of the distalized molar was three dimensionally assessed. In addition, the loss of anchorage in the anterior region was inspected. Moreover, the plane in which most movement took place was detected by taking the centre of the crown and centre of the triangle connecting the three roots as references. **Results:** The crowns moved distally, laterally and occlusally while the roots moved distally, medially and occlusally. Anteroposterior movement was almost triple rotation and four times intrusion. Mesio-buccal cusp moved laterally. Disto-buccal cusp showed maximum antero-posterior movement. Maximum extrusion and intrusion were presented in Mesio-buccal and Mesio-palatal cusps respectively. Lateral movement was equal among Mesio-buccal & Palatal roots. Palatal root extruded. Maximum antero-posterior movement was found in disto-buccal root. Mean distal molar tipping was 1.68°. Protrusion of anterior incisors in relation to SN plane indicated loss of anterior anchorage. **Conclusion:** Bidirectional distalizer proved to be effective in molar distalization

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Keyword: Distalization, three dimensional analysis, Cone beam.

1. Introduction

Molar distalization is an important strategy for correction of molar relation in Class II malocclusion. Various modalities have been used including trans-palatal arch, head-gear, removable distalizing plate, inter-arch appliances and intra-arch appliances.

Trans-palatal arch (Goshgarian arch) is a very useful and simple method for unilateral molar distalization. If bilateral distalization is required, then one side is distalized followed by the other side[1].

The removable distalization plate (Cetlins plate) is also simple. The major drawbacks of these appliances are the need for patient compliance that could not be always ensured. In addition, the resultant movement is usually a tipping movement that requires further molar uprightning[2].

Similarly, the head-gear is a very efficient method that can be used with fixed or removable orthodontic appliances. However, it also requires patient compliance[3].

Inter-arch appliances such as Jasper jumper, Herbst appliance, Twin force and many others are mainly used as fixed functional appliances for correction of skeletal Class II cases. Distalization is a side effect that is favourable.

Intra-arch appliances are very helpful in molar distalization as they eliminate the need for patient compliance. The type of tooth movement is one of the most important factors that affect the operator's choice. Since force application is mostly on coronal portion of the molar, thus the resulting movement is *tipping movement* rather than bodily movement.

Byloff and Darendeliter[4] proved that Pendulum appliance tipped the molars distally. The results were in accordance with those conducted by Bussick and McNamara.[5] In their study of assessing changes consequent to maxillary molar distalization with the pendulum appliance, Fuziy et al [6] and Polat-Ozsoy et al[7] revealed same results. Many modifications were performed by osteo-integrated implant combination to improve the type of molar movement.[8,9,10]

Mini screws were used as additional anchorage[11,12,13] Franzulum appliance was a new appliance that was based on the idea of Pendulum[14] Viewing Wilson distalizer, distal tipping movement of the molars took place.[15] Same results were also obtained when using Jones-Jig appliance.[16,17,18,19] Consequently, the NiTi springs and repelling magnets were used for molar distalization. Similarly, both produced molar tipping.[20]

Distal-jet is a commonly used distalizer. It also resulted in tipping movement. [21] Various modifications such as having additional support from mini screws to prevent loss of anchorage had been done.[22]

Keles distalizer resulted in bodily molar movement as the forces were applied at the level of centre of resistance.[23] However, the results were controversial. Mini screws were again used to provide additional anchorage. [24]

From all the previously mentioned, no perfect distalizer design was attained in terms of molar tipping, extrusion, rotation, bite-opening beside the anchorage loss. A new design for a distalizer was to be achieved.

Accordingly, Saad * and Sherbini ** invented a new distalizer: Bidirectional distalizer *** in order to minimize or eliminate these problems. The study conducted at Minia University proved the efficacy of this distalizer.[25] For further assurance, the effect of this distalizer had to be assessed three-dimensionally. This study was conducted to highlight this aim.

2. Materials and Methods

The study was conducted over 10 adult cases with ages between 15-25 years. Patients had Skeletal Class I or mild Class II. Class II molar relationship was presented bilaterally. Horizontal growth pattern had to be present. Also, the selected cases were not indicated for extraction.

Ethics regulations:

- The whole treatment procedure was explained to the patients. Any unexpected outcomes were clarified. The patients were also informed that the new appliance will be inserted in their mouth. (Fig 1 &2)
- Treatment consent was signed by the patient or parents.

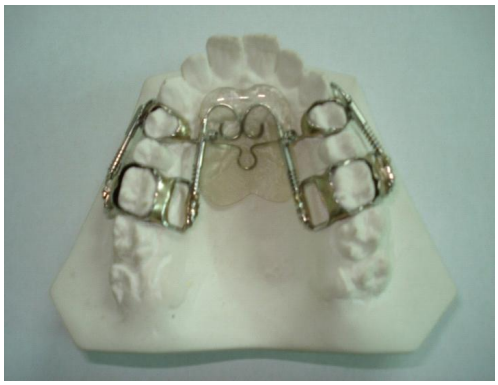

Fig 1: Bidirectional distalizer (Intra-oral view)

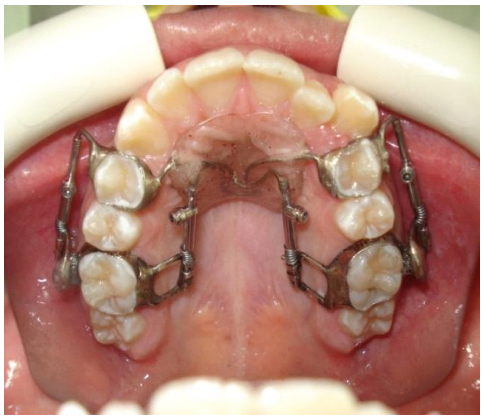

Fig 2: Bidirectional distalizer (Intra-oral view)

Construction of the appliance:

Proper sized bands were selected and fit to the right and left first premolars as well as for the right and left first permanent molars. This was done after separation of the first premolars and first molars.

An alginate impression was then taken with bands in patient's mouth. Proper seating of the bands in place was carried out. The impression was then poured with dental stone into a working cast.

Lab work:

Assuming the the idea of applying force through the center of resistance of molars (Trifurcation). The appliance consisted of the following: (Fig 3).

- ***Connectors:***

The metal connectors were fabricated of 1.1mm.stainless steel wires. They had a trapezoid shape. They were soldered to the molar bands both buccally and palatally. 2mm of clearance was present between the connectors and palatal tissues.

- ***Metal sleeves***

These were soldered to the metal connectors.

- ***Trans-palatal arch***

A trans-palatal arch connecting the two premolar bands was constructed from 0.8 mm. stainless steel wire and soldered to the premolar bands.

- ***Buccal Force applying component***

The buccal force applying component was constructed of metal tubes of Distal-jet soldered to the buccal surface of the premolar bands.

- ***Palatal force applying component***

The palatal force applying component was constructed of wire extension of Distal-jet tube imbedded in large acrylic button together with the transpalatal arch.

During wire bending, the height of the mesial end of buccal and palatal force applying components

were designed to be ***one millimeter*** less than the connectors soldered to the molar bands.

The acrylic button was extended to the incisive papilla anteriorly, till about 2 mm. from gingival margin laterally and posteriorly till region between second premolar and first molar. Solders and acrylic were finished and polished.

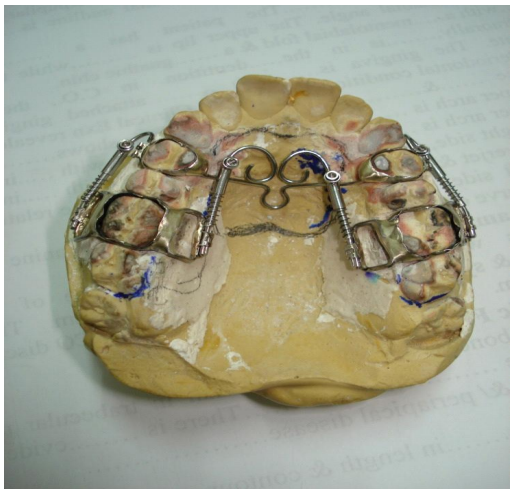

Fig 3: Distalizer with all metal components and soldres complete before curing of acrylic button.

Fitting of appliance:

After trying to ensure proper seating and absence of complains, teeth to be banded were isolated and dried. The distalizer was then cemented using glass ionomer cement. The excess cement was removed.

Concerning activation, the Alan Key was unlocked. NiTi coil springs were compressed. Alan keys were then closed. The total force level was measured using force gauge to be 200gm for both sides buccal and palatal. Patients were then scheduled for four weeks interval visit for follow up and reactivation.

The target of the distalization was considered to be achieved when Class I molar relation was obtained. Once Class I was achieved,, the distalizer was removed. .The post treatment records were then taken immediately.

Three-dimensional assessment:

Preoperative and postoperative Cone beam CTs were taken for each patient. The cone beam images were then transferred to Mimics Dicom 10.01 to be measured and extract the readings of the movement. Cross sectional view (Fig 4 A & B), coronal (Fig 5 A & B), sagittal (Fig 6 A & B) and panoramic ones (Fig 7 A & B) were depended upon to assess the movement of each cusp and root of the upper first molars.

Fixed planes were added to the 3D image to be used as reference for measurements. This enabled to accurately detect the movement of both crowns and roots in the three planes (Figs 8, 9, 10& 11). The facial axis plane Na-Pog was selected to measure the distalization movement antero-posteriorly. The plane SN was selected to measure intrusion or extrusion. SN plane also helped in inspecting incisor position after distalization. Concerning rotation, the distance between the mesio-buccal cusp tips and the apicies of the mesio-buccal roots was linearly measured. Superimposition of pre & post 3D reconstructions was performed to exactly show the molars movements and the loss in anterior anchorage. (Fig 12).

For comparison between the movements in each plane, another calculation was carried out. A rectangle representing the outline of the crown and a triangle connecting the roots apicies were drawn. The centre of the square and the triangle were identified. Superimpositon of the pre and post distalization images revealed the difference. The centre of the rectangle was extended to form a line dividing the rectangular to two equal halves. Superimposition allowed the calculation of the distal tipping. (Fig 13).

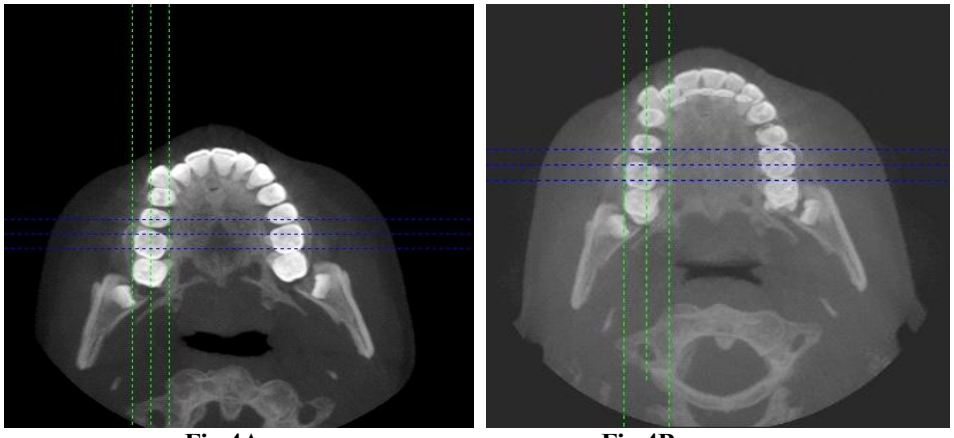

Fig 4A **Fig 4B**

Fig 4: Cross sectional view A: preoperative B: postoperative

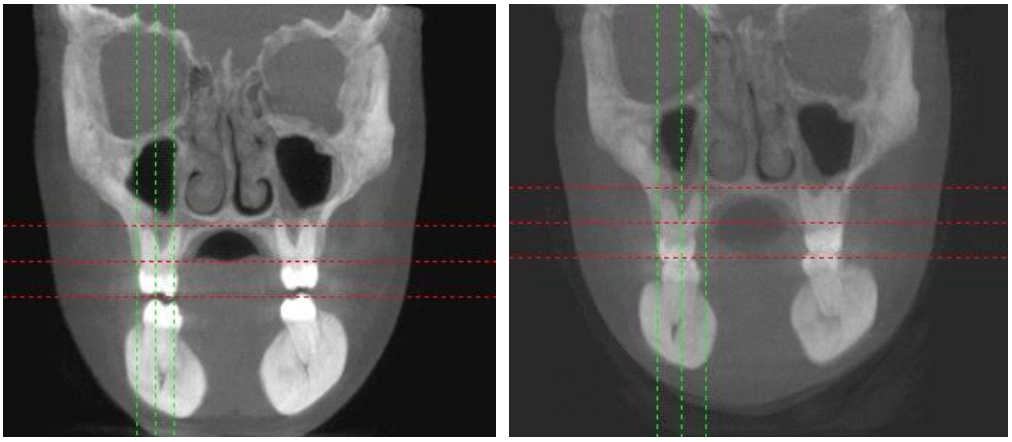

Fig 5A Fig 5B

Fig 5: Coronal view A: preoperative B: postoperative

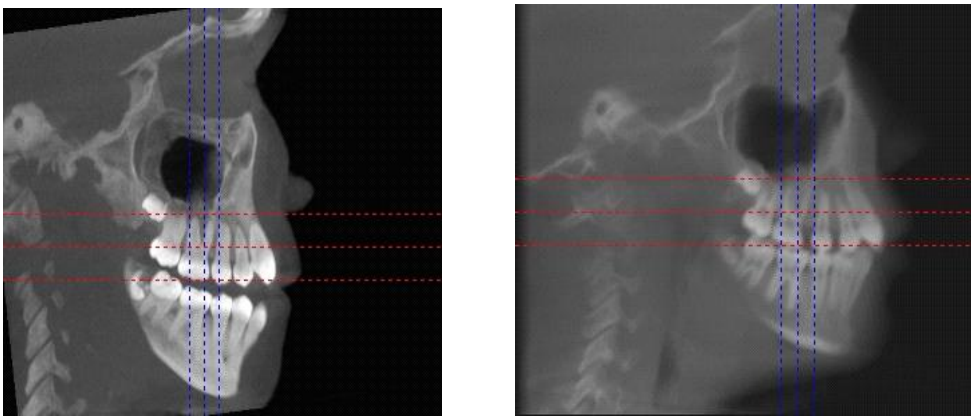

Fig 6A Fig 6B

Fig 6: Sagittal view A: preoperative B: postoperative

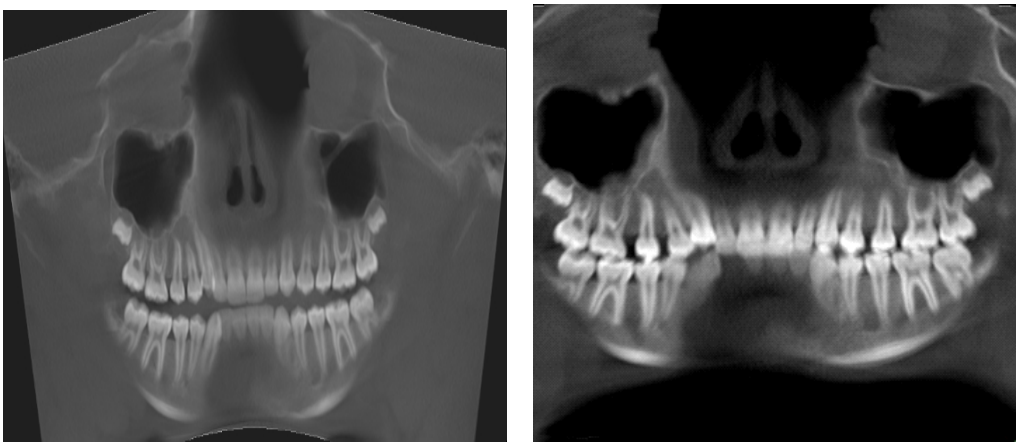

Fig 7A Fig 7B

Fig 7: Panoramic view A: preoperative B: postoperative

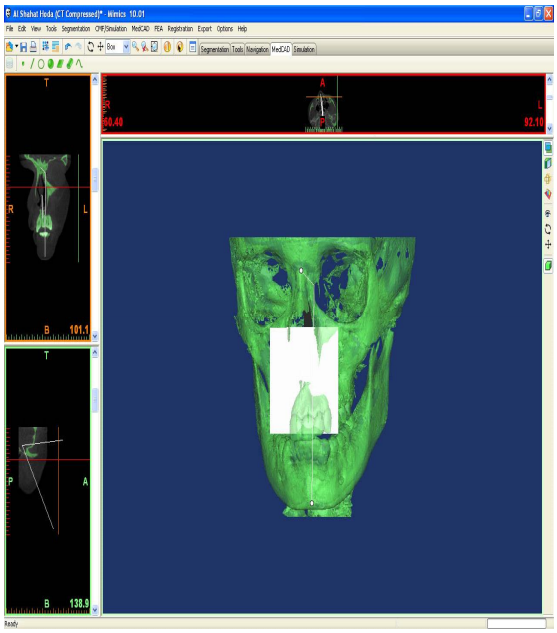

Fig 8: Antero-posterior facial axis plane N-Pog

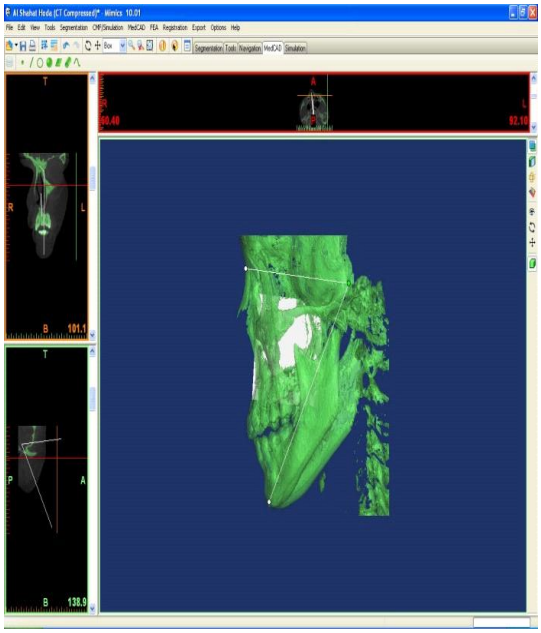

Fig 9: Lateral view of SN plane

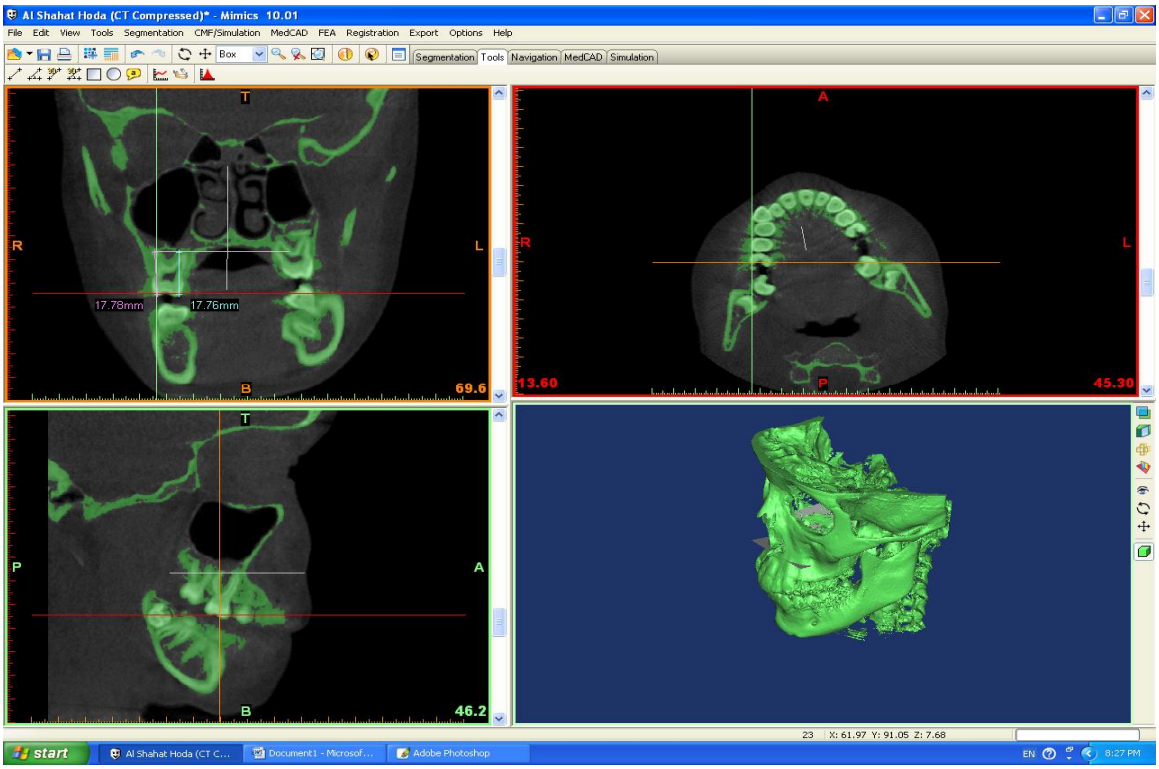

Fig 10: Rotation calculation by defining MB cusp tip and MBR apex.

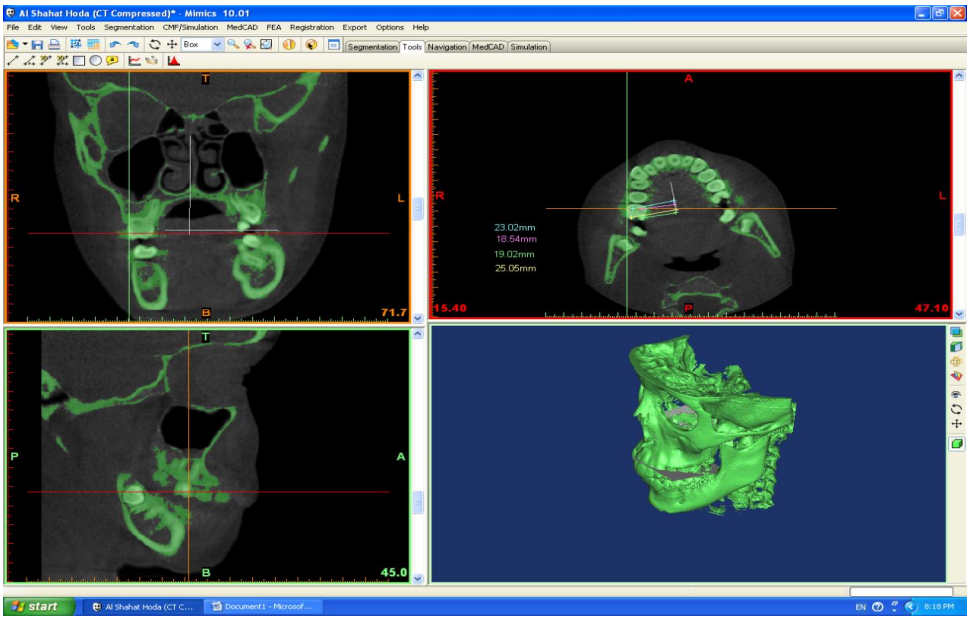

Fig 11: Postoperative rotation calculation.

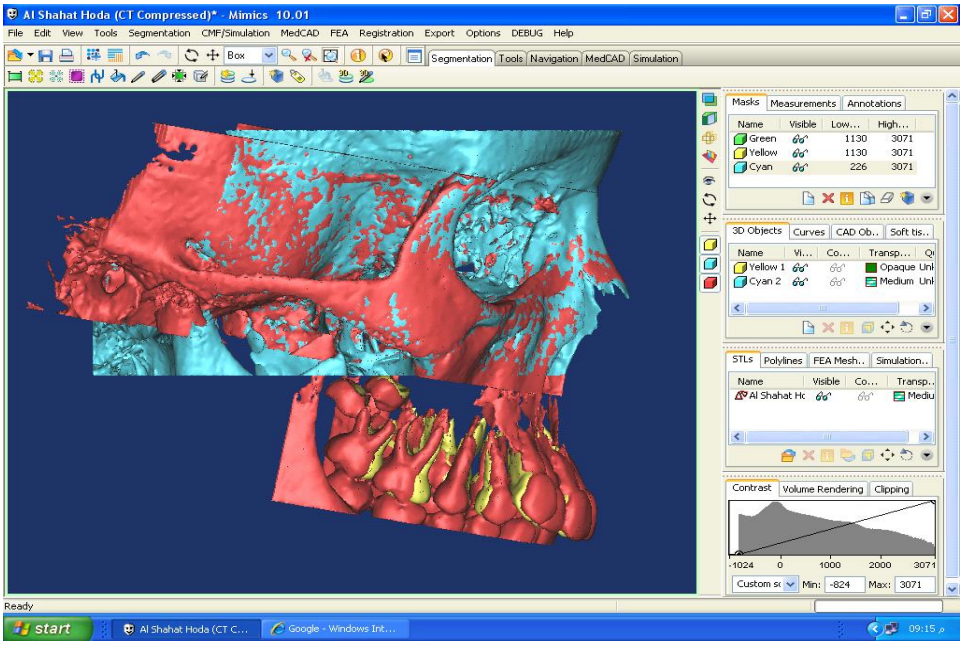

Fig 12: Superimposition of pre & post 3D reconstructions (Yellow color indicates preoperative position of the molars and the red one indicates the postoperative position).

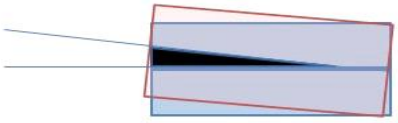

Fig 13: Schematic presentation of the calculation of the distal tipping.

3. Results

Concerning crown movement (Fig 14), each cusp was inspected separately. Maximum transverse movement occurred in Mesio-buccal cusp. Disto-buccal cusp showed maximum antero-posterior movement. Maximum extrusion and intrusion were presented in Mesio-buccal and Mesio-palatal cusps respectively. Analyzing the whole crown movement, it

can be denoted that the crowns moved laterally, distally and occlusally.

Examining root movement (Fig 15) it was observed that transverse movement was equal in both Mesio-buccal & Palatal roots with minimal movement in Disto-buccal root. Vertical movement was found to be extrusive in palatal root. Maximum antero-posterior movement was found in disto-buccal root. Accordingly, roots moved medially, occlusally and distally. The superimposition of the pre and post distalization photos revealed protrusion of anterior incisors in relation to SN plane indicating loss of anterior anchorage. (Fig 12 & 18). The results are illustrated in Table 2. The mean distal molar tipping was 1.68° with a maximum value of 3.56° .and a minimum value of 0.0°.

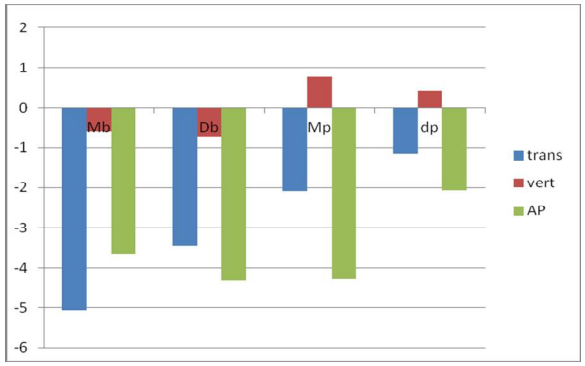

Fig14: Crown movement in the 3 planes of space. Mb: mesio-buccal cusp, Db: disto-buccal cusp, Mp: mesio-palatal cusp, DP: disto-palatal cusp.

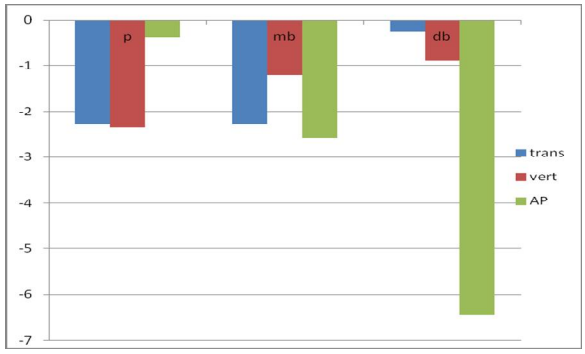

Fig 15: Root movement in the 3 planes of space. P: palatal root, mb: mesio-buccal root, db: disto-buccal root.

Comparison of crown movements: distalization, rotation and intrusion revealed that anteroposterior movement was almost triple the vertical one. It was also greater by four folds than the transverse movement (Fig 16). Same results were observed when comparing roots movement (Fig 17). The mean and standard deviation of both the crowns and roots movements in the 3 planes are illustrated in Table 1.

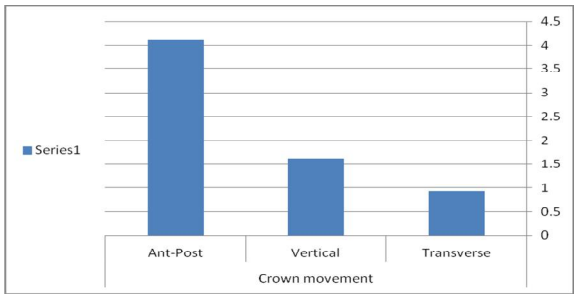

Fig 16: Crown movement (mm) in the three planes.

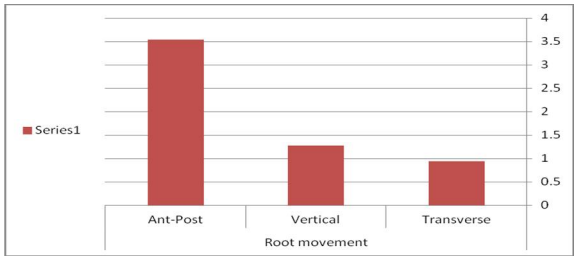

Fig 17: Root movement (mm) in the three planes

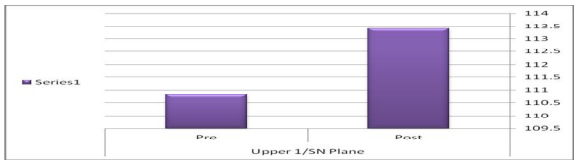

Fig 18: The pre and post distalization of the angulation of upper incisor in relation to SN plane

Table 1: Crown and root movements (mm) in the three planes.

Root			Crown			
Transverse	Vertical	Ant-Post	Transverse	Vertical	Ant-Post	
0.94	1.26	3.54	0.925	1.62	4.11	Mean
0.60	0.81	2.02	0.53	0.85	2.18	St Dev

Table 2: Change in incisor angulation

Maximum	Minimum	ST Dev	Mean	
119.4	104.2	5.99	110.8	Pre
121.7	105.8	6.44	113.4	Post

4. Discussion:

Three dimensional computed tomography (the Cone Beam System) has been used for diagnostic purposes in orthodontic treatment.[26] Cevidanes *et al*[27] stated that cone- beam computed tomography (CBCT) provides simulation tools that can help bridge the gap between imaging types

CBCT is used to determine the optimal locations and angulations for miniscrews used as skeletal anchorage in orthodontic treatment.[28,29,30] It is also useful in the Assessment of orthognathic surgeries,[31] rapid palatal expansion [32,33] and temporary anchorage devices. [34, 35]

In the present study CBCT was depended upon to assess upper first molar distalization. The distalizer: bidirectional distalizer is a newly designed one.

Forces were applied buccally and palatally in order to obtain a resultant force through the centre of resistance of the first maxillary molar. Thus, bodily movement can be obtained which eliminates the need for further molar uprightning.

Although, the appliance construction requires multiple steps, it is less complicated than the bulky and hardly controlled magnets.[36] The construction steps are similar to those of the Distal-jet.[37]

In the contrary to Jones-jig Appliance[38] no bite opening was observed. Also, no molar uprightning was required. However, anchorage loss was obvious from the protrusion of the upper incisors in relation to SN plane. This was the issue with nearly all the distalizers: Pendulum,[39] Jones-Jig,[16] NiTi coil-springs, Rare earth magnets,[20] Distal-Jet [21] and Keles distalizer,[23,24]

The presence or absence of the third molar was also put into consideration. Previous research revealed that extraction of upper third molar is recommended to provide enough space for the distalization. [25]

Concerning results, not only, the whole tooth movement was assesed but also the movement of each cusp and each root. The loss of anchorage in the anterior region was also inspected.

Results revealed movement of the molar in the three planes. Anteroposterior movement was greater than the vertical and transverse movements. Intrusion of molar helped in preventing bite opening. In addition, molar uprightening was not needed not only due to the dominance of anteroposterior movement but also due to the minor distal tipping. Another positive result is the buccal movement of the crown. This denotes that the design help the molar to follow the arch perimeter during distalization.

5. Conclusions:

Bidirectional distalizer proved to be effective in molar distalization.

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Histopathological and Immunohistochemical Studies on the Adrenal Cortical Tumors of Egyptian Patients

[1]Samia, M. Sanad, [2]Mahmoud, A. El-Baz, [3]Omar, I. Ghonemy and Hassan, [4]F. Abo El-Nazar

[1]Zoology Department, Faculty of Science, Zagazig University, Egypt
[2]Pathology Department, Faculty of Medicine, Mansoura University, Egypt
[3]Zoology Department, Faculty of Science, Benha University, Egypt
[4]Urology and Nephrology Center, Mansoura University, Egypt
egypt_sbes@hotmail.com

Abstract: The presnet study provides guidelines for the diagnosis of adrenal cortical tumors in Egyptian patients. This retrospective study included 40 patients of adrenal cortical tumors (28 adenoma and 12 carcinoma). They were admitted to Mansoura Urology and Nephrology Center between 1985-2002. All patients were surgically treated by adrenalectomy. Patients with adenoma were followed for a period ranging from 24-67 months. Clinical and laboratory improvement of adenoma was observed. Nine patients of carcinoma died of distant metastasis after 8 months and the other 3 patients were still alive 24 months after surgery. Hyaline globules which are PAS positive were detected in adrenal cortical adenoma and carcinoma and both types were positive for reticulin stain. Immunohistochemically; cytokeratin was expressed in 22/28 cases of adenoma and all cases 12/12 of carcinoma. Vimentin was expressed in 20/23 cases of adenoma and 8/12 cases of carcinoma. The present study concluded that: (1)- Reticulin staining is useful for the diagnostic differentiation of adrenal cortical carcinoma from adrenal cortical adenoma. (2)- The expression of cytokeratin and vimentin is helpful in diagnosis, but the histopathological examination of paraffin sections remains the basic method. (3)- No significant correlation between immunohistochemical pattern of adrenocortical tumors and survival was observed.
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Key words: Histopathology, Immunohistochemistry, Adrenal cortical tumors, Cytokeratin, Vimentin

Introduction

Tumors in the adrenals originate from the adrenal cortex and medulla or as metastases from-extra adrenal primaries (Tatic *et al.,* 2002). Differentiation between these three groups is the first task a pathologist to tackle when dealing with specimens from the adrenal region. The second great problem is the dignity of adrenal tumors, which cannot be determined in many adrenomedullary and some adrenocortical tumors. Immunostaining is helpful but the histopathological examination of paraffin sections remains the basic method (Saeger, 2000).

Adrenocortical cancer is a rare cancer with a very poor prognosis (Tissier *et al.,* 2005)**.** According to Barzon *et al*. (2005), adrenocrotical tumors may originate from the zona glomerulosa, zona fasiculata or zona retecularis and be associated with syndromes due to overproduction of mineralocorticoids, glucocorticoids or androgens respectivley. During the past decade, many monoclonal antibodies have been developed, some of which have been assessed in adrenocortical neoplasmas using various methods. Antibodies directed against cytokeratin and vimentin can help in diagnosis (Saeger *et al.,* 2003).

The present study has been carried out on some of the adrenal cortical tumors obtained from patients in the Urology and Nephrology Center, Mansoura University, Egypt. The study was conducted to:

1- Threw light on the origin of the different tumors of the adrenal cortex.
2- Study the clinical approachs of the patients with these tumors.
3- Differentiate between the different types of adrenal tumors using the histological and immunohistochemical methods.
4- Examine the diagnostic usefulness of the co expression of cytokeratin and vimentin in adrenal cortical tumors.

2. Patients and Methods

This retrospective study was carried out on 40 cases of adrenal cortical tumors (28 Adenoma and 12 carcinoma). These cases were obtained from the Urology and Nephrology Center belonging to Mansoura University during the period from 1985-2002. All patients were routinely investigated after hospitalization to evaluate the extent of the tumor. The study included clinical examination, conventional laboratory investigation, intravenous urography, abdominal ultrasonography, chest x-ray to rule out pulmonary metastases and radio isotopic bone scan to exclude skeletal metastases. Also, computerized axial and tomography and radical

adrenalectomy in all patients were performed. Clinical data which are recorded in the present study were obtained from surgical requests. These data included patients' age, sex and size of each tumor.

Histopathological examination:

After adrenalectomy, the resected tumor was fixed in 10% buffered formalin for 24 hours and processed as usual for the preparation of paraffin wax blocks. These blocks were sectioned at 4-6 µm and were stained by the following methods:

1. Routine hematoxylin and eosin stain for general histological examination (Harris, 1900).
2. Periodic acid-Schiff's (PAS) reaction for detection of intracytoplasmic hyaline globules (Hotchkiss, 1948).
3. Reticulin stain by Gordon method for revealing the distribution of reticular fibers in the tumor tissues (Gordon and Sweets, 1936).
4. Moreover, immunohistochemical stainings were performed using different monoclonal and polyclonal primary antibodies.

Immunohistochemical examination:

Immunohistochemical staining of 4-6 µm paraffin sections was performed on the Dako Autostainer (Dako corporation, Carpentaria, Calif) using streptavidin-biotin peroxidase method of Hsu *et al.,* (1981). Histostain plus Kits (Zymed USA) which contain 10% non-immune serum, biotinylated secondary antibody and streptavidin-perxidase for antibodies against pan-cytokeratin (NCL-PAN-CK, 1:25; Novo castra Vector, Burlingame, Calif) and vimentin (Dako Carpentaria Calif, USA) for adrenal cortical tumors were used.

1- Pan cytokeratin (NCL-PAN-CK):

Pan Cytokeratin (NCL) is a mouse monoclonal antibody that reacts with human cytokeratins 5,6,8 and 18 (58 Kd, 56 Kds, 52 Kd & 45Kd), respectively. It is designed to recognize almost epithelial tissues and their tumors. NCL-PAN-CK labels all carcinomas of simple and squamous epithelial origin. The positive control for cytokeratin is the skin **(Angus *et al.*, 1988).**

2- Vimentin:

This vimentin **(57 Kd)** is a mouse monoclonal antibody that reacts with human vimentin intermediate filament subunit. In normal tissues, cell types which vimentin includes are endothelial cells, fibroblasts, smooth muscle cells and lymphoid cells. A number of tumors co-express vimentin and cytokeratin e.g. thyroid carcinomas, pleomorphic adenomas of the salivary glands and renal carcinoma.

Immunohistochemical procedure (Elias *et al.*, 1989):

Serial sections were cut at 4-6 µm and deparaffinized with xylene, rehydrated with descending grades of alcohols and immersed in deionized water.

Prior to immunohistochemistry, selected tissue sections were pretreated twice in an 1100- W microwave oven at 70% power for 4 minutes in 1% citrate buffer at PH 6.0 (Lab. vision corporation, Fremont, Calif) for vimentin. Sections for pan-cytokeratin immunostaining were pre-treated with 0.01% pepsin solution for 30 minutes at 37◦C.

Deparaffinized sections were incubated with 0.3% hydrogen peroxide in methyl alcohol to block the endogenous peroxidase activity for 30 minutes. Then, washed in phosphate-buffered saline (PBS) at pH 7.4 and incubated with non-immune serum. After application of the primary antibody, a biotinylated secondary antibody was added followed by the avidin-biotin-peroxidase complex. After each immunostaining procedure, sections were incubated with 3, 3′ diaminobenzidine-hydrogen peroxide substrate and counterstained with hematoxylin. Appropriate positive and negative control sections were also used.

Result of stain:

Immunohistochemical reactions revealed cytoplasmic brown stain in positive cases with the used antibodies. Accumulation of cytokeratin and vimentin was observed in tumor cells which take a brown color.

Follow up of patients:

The patients were followed postoperatively in the outpatient clinic for available period (mean = 47.46 month). This included, clinical examination, body weight and hypertensive state. Moreover, radiological follow up for local recurrence and distant metastasis in malignant cases was carried out.

3. Results

In the presnet study 40 patients represented the cortical tumors. These tumors were subdivided into two subdivisions, adenoma and carcinoma with a percentage of 24.7% and 10.6%, respectively.

I-Clinical findings:

1- Adrenal cortical adenoma (ACA):

In this study, 28 patients had cortical adenoma with a percentage of (24.7%). The clinical data of these patients is outlined in Table (1).

Age distribution:

Age distributions in patients with ACA are shown in table (1a). Most of the cases were in the 6th decades of life, while, in the first and second decades, no patients were recorded. The age of patients ranged from 22-60 years (the average was 46.8 years).

Sex distribution:

Sex distribution in patients with ACA is shown in table (1b). 28 patients had cortical adenoma with a

percentage of 24.7%. They were 12 males and 16 females. The male to female ratio was 1:1.3.

Tumor Size:

The tumor size in patients with ACA is recorded in table (1c). The size ranged from 2 to 10 cm. with an average 6.65 cm. More than 60 % of the tumors have sizes ranging from 6-10 cm, while less than 40 % of them possessed sizes ranged from 0-5 cm.

2-Adrenal cortical carcinoma:

12 patients had cortical carcinoma with a percentage of 10.6%. The clinical data of patients with cortical carcinoma is outlined in table (2).

Age distribution

The age of the patients ranged from 10 up to 53 years with an average age of 42 years. Nine cases were equal to or above 42 years and three cases were below the age of 42 year. Most cases were in the 5th decade of life.

Sex distribution:

Sex distributon in patients with ACC is shown in table (2). 12 patients had cortical carcinoma with a percentage of 10.6%. They were 5 males and 7 females. The male to female ratio was 1:1.4.

Tumor size:

Tumor sizes which were recorded in all cases of ACC are shown in table (2). The tumor size ranged from 10 to 18 cm. The average diameter was 12.8 cm. A male patient with an age of 24 year represented the largest ACC tumor size, while three patients represented the smallest one. Their age ranged from 42-53 years. One patient was female and the other two patients were males.

II- Histopathological Observations:

1- Adrenal cortical adenoma (ACA):

The most common architectural patterns of ACA are cells grouped in a nesting or alveolar pattern. These cells have compact eosinophilic cytoplasm. Their nuclei are deeply stained and vesicular with central to eccentric nucleoli (Fig. 1). In addition, an organoid architectural was observed in most of the studied ACA (Fig. 2). In all cases of ACA, neither mitotic figures nor tumor necrosis were observed.

In the present study, the tumor tissues of all cases were stained by PAS reaction and by Gordon technique to demonstrate the total hyaline globules and Reticulin fibers, respectively. The results of staining are recorded in table (3). This table shows that, the PAS Positive intracytoplasmic hyaline globules (Fig. 3) were detected in 13 cases only from the studied ACA 28 cases. The other 15 cases revealed negative reactivity to the PAS reaction.

Moreover, most of the ACA, (23/28) stained by Gordon technique were highly rich in the reticular fibers (Fig. 4). Moderately staining reactivity for reticular fibers was observed in 5 cases only. The reticular fibers surround the individual cells.

2- Adrenal cortical carcinoma (ACC):

The tumor has a diffuse or solid pattern of irregular-pleomorphic cells. Most tumor cells have moderate to abundant eosinophillic compact cytoplasm. Mitotic figures were numerous with many atypical forms (Fig. 5). Sections of tumors were stained with PAS and Gordon technique for reticulin stain. The result of staining is recorded in table (3). From this table, it can be observed that, the PAS positive intracytoplasmic hyaline globules were detected in eight cases of ACC (Fig. 6), while the other four cases showed negative staining. Most of the ACC tumors studied (10/12) showed a relative deficiency of the reticular fibers (Fig. 7). Two cases only of ACC gave negative results with reticulin stain.

III- Immunohistochemical observations:

The immunohistochemical reactions appeared as granular brown deposits precipitated in the cytoplasm of tumor cells. Meanwhile, the expression of cytokeratin and vimentin in the ACA and ACC are summarized in Table (4).

Expression of cytokeratin in the adrenal cortical tumors (ACT):

1- Adrenal cortical adenoma (ACA):

From table (4), it can be noticed that, most of the studied ACA cases (21/28) showed relative expression of cytokeratin with different grades of intensity. However, a wide range of expression was noticed. The staining was observed to be focal and the nuclei were stained blue with prominent nucleoli (Fig. 8). Seven cases showed a strong staining pattern and five cases showed a weak staining pattern. Moreover, the last seven cases of ACA gave negative immunoreactive staining pattern for cytokeratin.

2- Adrenal corticar carcinoma (ACC):

In the adrenal cortical carcinoma (ACC), cytokeratin expression was seen in all cases of tumors. However, a wide range of expression was noticed, 3 cases demonstrated a strong staining pattern and 9 cases showed a moderate staining pattern (Fig. 9).

Expression of vimentin in the adrenal cortical tumors (ACT):

1- Adrenal cortical adenoma (ACA):

ACA exhibited a wide range of expression with vimentin. Some tumors showed immunostaining reactivity mainly in stromal elements, while others showed strong expression of vimentin in the neoplastic cells (Fig. 10). Vimentin expression was observed in 20 cases of adrenal cortical adenomas. The staining was generally strong in eight cases, moderately also in eight cases and four cases gave weakly staining, while the other 8 cases of the tumor

showed negative results for the staining reactivity (Table 4).

2- Adrenal cortical carcinoma ACC:

In ACC, vimentin was seen in the cytoplasm of the tumor cells and its expression was observed in (10/12) ACC cases (Fig. 11). The staining was generally strong in three cases, moderately in five cases and weak immunoreactive in two cases. The last two cases were negative for immunoreactivity staining.

Table (1): Clinical data of the patients with adrenal cortical adenoma.

Case No.	Sex	Age (Year)	Size (cm.)	Follow up (month)	Status
1	F	30	7	168	S
2	M	22	7	120	S
3	F	53	8	124	S
4	F	56	5	84	S
5	F	30	8	120	S
6	M	53	8	82	S
7	F	35	5	78	S
8	M	46	4	72	S
9	F	58	8	68	S
10	M	59	7	60	S
11	F	54	7	62	S
12	M	56	5	48	S
13	F	51	8	36	S
14	M	43	3	35	S
15	M	53	6	32	S
16	F	27	4	28	S
17	F	60	7	26	S
18	F	53	5	24	S
19	M	58	8	20	S
20	F	57	6	12	S
21	F	35	10	26	S
22	F	54	9	32	S
23	F	37	8	48	S
24	M	34	4	32	S
25	M	50	6	24	S
26	M	52	3	30	S
27	F	37	5	27	S
28	M	60	2	18	S

S = Survival M = Male F = Female

Table (1a): Age distribution in patients with adrenal cortical adenoma.

Age group	No. of patients	Percentages %
First decade (1-10)	0	0%
Second decade (11-20)	0	0%
Third decade (21-30)	4	14.2%
Fourth decade (31-40)	5	17.8%
Fifth decade (41-50)	3	10.7%
Sixth decade (51-60)	16	57.1%
Total	28	100.0 %

Table (1b): Sex distribution in patients with adrenal cortical adenoma.

Sex	No. of patients	Percentages %
Male	12	42.8%
female	16	57.2%
Total	28	100.0%

Table (1c): Tumor size in patients with adrenal cortical adenoma.

Size group (cm)	No. of patients	%
0-5	11	39.28%
6-10	17	60.72%
Total	28	100.0

Table (2): The clinical presentation of Adrenal Cortical Carcinoma.

Case	sex	Age (years)	Size (cm)	Followup (months)	Status
1	M	45	12	36	D
2	M	42	10	11	D
3	F	46	10	16	D
4	M	10	13	24	D
5	F	46	15	24	D
6	M	43	11	37	D
7	F	32	16	38	D
8	F	24	18	27	D
9	F	36	13	24	S
10	M	53	10	39	D
11	F	44	11	36	S
12	F	42	14	23	S

D = Died, S = Survival, M = Male, F = Female.

Table (3): The results of PAS and Reticulin stain for adrenal cortical tumors (ACA & ACC).

Stain / Tumor	PAS		Reticulin stain		
	+ve	-ve	High +2	Low +1	-ve
ACA	13/28	15/28	23/28	5/28	-
ACC	8/12	4/12	-	10/12	2/12

+ve Positive -ve Negative

Table (4): Immunohistochemical expression of cytokeratin and vimentin in adrenal cortical tumors (ACA & ACC).

Immunostaining / Tumor	Expression of cytokeratin				Expression of vimentin			
	+3	+2	+1	0	+3	+2	+1	0
ACA	7/28	9/28	5/28	7/28	8/28	8/28	4/28	8/28
ACC	3/12	9/12	-	-	3/12	5/12	2/12	2/12

+3 intense immunoreactivity. +2 moderate immunoreactivity +1 weak immunoreactivity. -ve negative immunoreactivity.

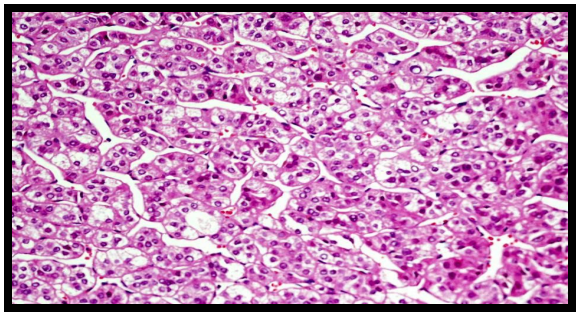

Fig. (1): Photomicrograph of adrenal cortical adenoma showing alveolar or nesting pattern. Cells have compact eosinophilic cytoplasm. (H & E x 200)

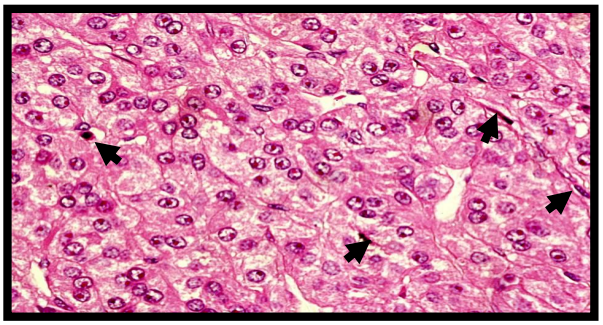

Fig. (2): Photomicrograph of adrenal cortical adenoma showing organoid arcitecture (arrows). No mitotic figures are observed, nuclei are vesicular with central to eccentric nucleoli. (H & E x 400)

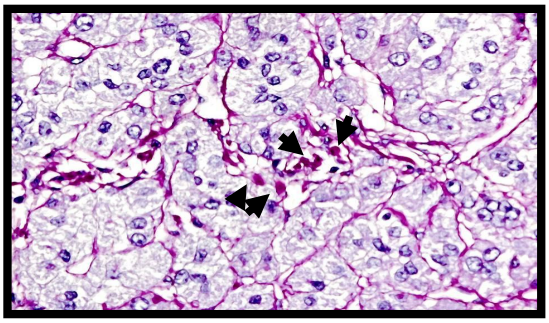

Fig.(3): Photomicrograph of adrenal cortical adenoma showing intra-cytoplasmic hyaline PAS positive globules, (arrows). (PAS X 400)

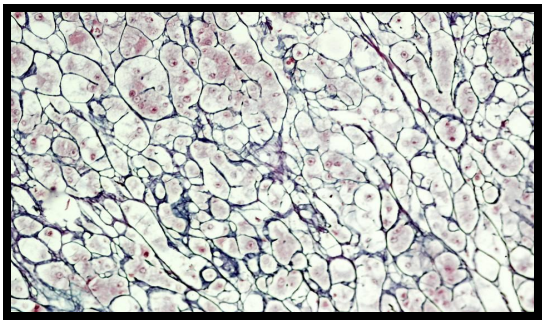

Fig. (4): Photomicrograph of adrenal cortical adenoma showing abundant reticular fibers. (Gordon stain X 200)

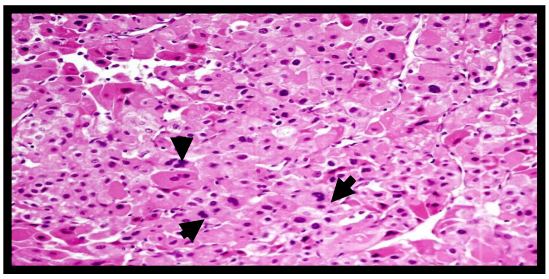

Fig. (5): Photomicrograph of adrenal cortical carcinoma showing diffuse pattern of irregular pleomorphic tumour cells. Most tumour cells have moderate to abundant eosinophilic compact cytoplasm. Mitotic figures are numerous with many atypical forms (arrows). (H & E X 200)

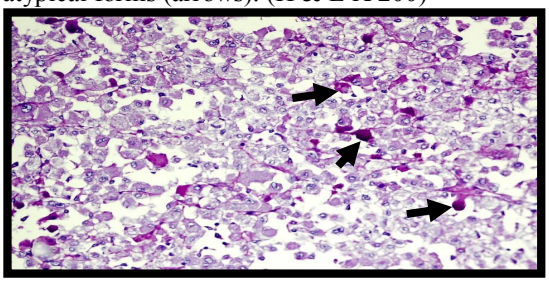

Fig. (6): Photomicrograph of adrenal cortical carcinoma showing intracytoplamic hyaline globules (arrows). (PAS X100)

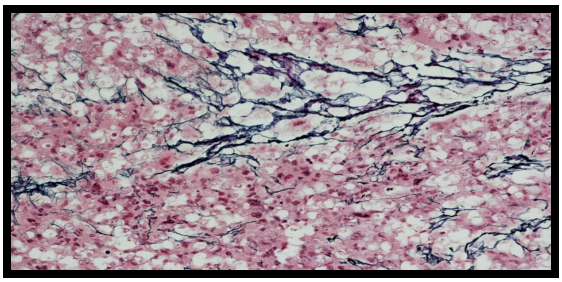

Fig. (7): Photomicrograph of adrenal cortical carcinoma showing deficient reticular fibers. (Gordon stain X 200)

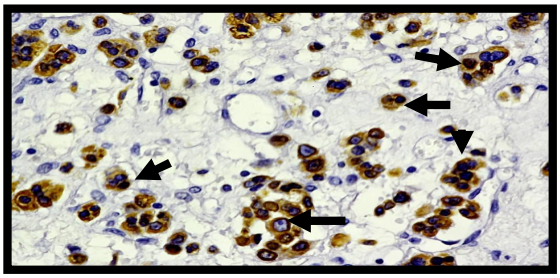

Fig. (8): Photomicrograph of adrenal cortical adenoma showing intense cytoplasmic immunoreactivity for Cytokeratin. (Immunoperoxidase X 400)

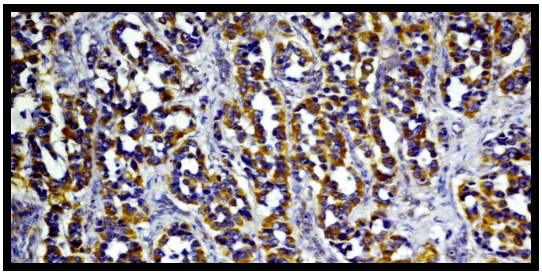

Fig. (9): Photomicrograph of adrenal cortical carcinoma showing intense cytoplasmic immunoreactivity for Cytokeratin in a brown colour. (Immunoperoxidase X 200)

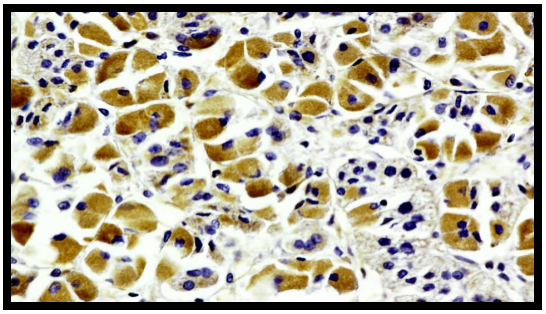

Fig. (10): Photomicrograph of adrenal cortical adenoma showing intense cytoplasmic immunoreactivity for Vimentin in a brown colour. (Immunoperoxidase X 400)

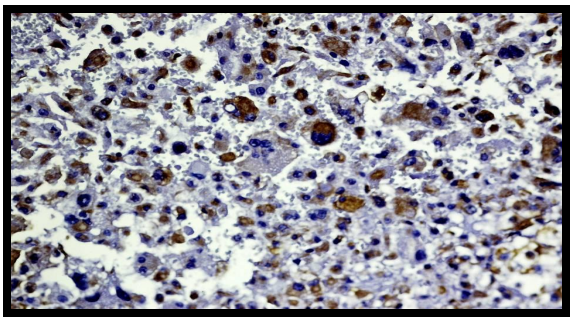

Fig. (11): Photomicrograph of adrenal cortical carcinoma showing intense cytoplasmic immunoreactivity for Vimentin in a brown colour. (Immunoperoxidase X 200)

4. Discussion

In the present study, forty cases of adrenal cortical tumors with a nearly mean of 5 years follow-up were investigated. They were 28 adenoma (ACA) and 12 carcinoma (ACC).

The studied patients were between 22-60 years of age, average age 46.8 years. Most patients were in 6th decade of life and they were 16 female and 12 male. The size of the tumor ranged from 2 to 6 cm with mean diameter of 3.8 cm. No patients with adenoma had recurrence of tumor after excision. Lack *et al*. (1990) reported that, there is a distinct predilection for female patients and most tumors are diagnosed in the third to fifth decades of life. The tumor measures 2-3 cm in diameter. Neville *et al.* (1985) showed that, 92% of tumors were less than 2 cm in diameter, while Conn *et al.* (1964) reported that 72% of tumors were less than 3 cm.

In the present study, the most common architectural patterns are cells in a nesting or alveolar arrangement. Adenomas typically had a compact eosinophilic cytoplasm. ACA tumor cell nuclei are usually single and their shapes are round to oval. Margination of chromatin along the nuclear membrane produces a vesicular appearance. Most nuclei contain a single dot-like nucleolus which is central or eccentric in location. Organoid architecture is seen, no mitotic figures are present, predominantly clear or foamy cytoplasm and no tumor necrosis in all cases is present. In addition, the intracytoplasmic hyaline globules were detected in 13/28 adrenal cortical adenoma. These globules are round to oval and are identified positive by periodic-acid Schiff's reaction (PAS). Lack *et al.* (1997) observed also the same result. They reported the presence of intracytoplasmic hyaline globules in a few number of adrenal cortical tumors both benign and malignant. Moreover, Handler (2003) showed that, intracytoplasmic hyaline globules have also been observed in about 10% of adrenal cortical tumors; both benign and malignant. The present study revealed that most cases of adrenal cortical adenoma were rich in reticular fibers by staining tissues with reticulin stain.

In the present investigation, twelve cases of adrenal cortical carcinoma were studied. The patients were between 24-53 years of age; average age was 42 years. Most patients were in the 4th and 5th decade of life and they were seven females and five males. Size of the tumor ranged from 10-18 cm with mean 12.8cm of diameter. Four patients were survival and the other eight patients of carcinomas had died with disease. Wooten and King, (1993) reported that, individuals in the 4th and 5th decades of life are most frequently affected, although adrenal cortical carcinoma can occur at any age. Weiss *et al.* (1989) showed that, most large clinical series indicate a predilection for female patients, with a ratio of 1.5 to 1 (over 900 patients studied). However, there was a predilection for male patients including one that reviewed all non-hormonal tumors (Lack *et al.,* 1999). The average size of tumors recorded in several documented studies was 12.0cm (Card *et al.,* 1992), 12.4cm (Henley *et al.,* 1983), 14.0 cm (*Weiss et al.,* 1989) and 16.6 cm (Cohn *et al.,* 1986), but the range wide was from 3 to 40 cm (Lewinsky *et al.,* 1974). Scheingar *et al*., (2005) reported that ACC, were highly malignant tumors that accounts only 0.2% of death due to cancer, and were usually resistant to chemotheraby.

Carcinomas were characterized by mitotic figures in the most active area. All cases were lack of significant nest growth pattern components. Nine cases were solid or trabecullar growth was more common and a considerable proportion of cells showed eosinophillic cytoplasm. Tumor necrosis was found in 10 cases. In the present study, the intracytoplasmic hyaline globules were detected in eight cases of adrenal cortical carcinomas. Most of the cases of adrenal cortical carcinomas were deficient in reticular fibers.

It can be concluded that, carcinomas were usually larger than adenomas. The average size of adrenal cortical adenoma and adrenal cortical carcinoma are about 3.8cm and 12.8cm, respectively. The patients with adenomas were older in the average than those with carcinoma (average 46.8 years versus average 42 years). No patients with adenoma had recurrence of tumor after excision, whereas 8 carcinomas patients were dead of tumor. These data are in accordance with Evans *et al.* (1996) who reported in their study of 56 cases of adrenal tumors that, carcinomas were usually larger than adenomas and the patients of adenomas were older in the average than those with carcinoma. In addition, these results are in agreement with Wieneke *et al.* (2003) who revealed that, all patients with tumor classified

adenomas were alive, without evidence of disease, whereas 21 patients of carcinomas had died with diseases.

Concerning the immunohistochemical results, 21 of 28 cases of adrenal cortical adenoma (ACA) showed cytokeratin positive cells while all 12 cases of adrenal carcinoma (ACC) showed immunoreactivity for cytokeratin. Lack *et al.* (1999) reported that, at present there is no pathogenomonic immunohistochemical profile for adrenal cortical carcinoma, but even negative results can sometimes aid in a difficult differential diagnosis. Cote *et al.* (1990) reported positive results in cortical cells and adrenal cortical adenoma, but none of the adrenal cortical carcinoma cells expressed cytokeratin. Our findings are in agreement with those observed by Nakano (1988) who reported that, 24 of 62 adrenal cortical carcinomas were cytokeratin positive, while all of the 42 adrenal cortical adenomas were negative. In a study reported by Haak *et al.* (1995), nine of 18 adrenal cortical carcinomas were positive for cytokeratin and in most cases, less than 25% of cells were relatively immunoreactive. None of the adrenal cortical adenomas was immunoreactive for cytokeratin. Our results are in accordance with Hoang *et al.* (2002) who reported that, all tumors studied (four adrenal cortical carcinomas) were immunoreactive for cytokeratin.

In the present study, 20 of 28 cases of adrenal cortical adenoma showed scattered vimentin-positive cells considering the fact that, the adrenal cortex is of mesodermal origin. Vimentin positive tumor cells were detected in 8 out of 12 cases of adrenal cortical carcinoma. This finding may suggest differentiation of the tumor to fetal tissue. Vimentin has been reported to be positive in tumor cells of adrenal cortical carcinoma. Nevertheless, the frequency reported in the literature varies considerably; 65% of the adrenal cortical carcinoma (Gaffey *et al.,* 1992), 73% of adrenal carcinoma versus only 14% of adrenal adenomas (Nakano, 1988) and 100% of adrenal cortical carcinomas (Diman *et al.,* 1992) were positively immunoreactive for vimentin. Meanwhile, Song *et al.,* (2004) observed also that, all the studied adrenocortical carcinomas were immunoreactive for vimentin. Generally, despite the variable results reported in the literature, a tumor that has the typical morphology of adrenal cortical carcinomas would statistically be expected to be vimentin positive and cytokeratin negative. Raikhlin *et al.,* (2002) revealed in their study that, adrenocortical tumors were associated with positive reaction to vimentin and a negative one to cytokeratin. These characteristics were used to differentiate adrenocortical tumors from adenomas that were reactive for cytokeratin and hardly to vimentin. On the contrary, our results revealed that adrenal cortical tumors were positive immunoreactive for cytokeratin and vimentin in most studied cases.

Corresponding author
Samia, M. Sanad
Zoology Department, Faculty of Science, Zagazig University, Egypt
Email: egypt_sbes@hotmail.com

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Expression of p27kip and XIAP in patients with Hepatocellular carcinoma

Maaly M Mabrouk[1],Enaam Salah[1], Medhat A Ghazy[2], Dina H. Ziada[3], Hassan El Batae [3], Ghannam Amr and [4] Abd Elfattah Omnia[*4]

Departments of Clinical Pathology[1], Internal Medicine[2], Tropical Medicine and Infectious Disease[3] and Clinical Oncology Department[4], Faculty of Medicine -Tanta University, Tanta, Egypt
[*]omniaabdelfattah@yahoo.com

Abstract: Introduction: The increasing incidence of hepatocellular carcinoma (HCC) being a major health problem. Functional alterations of cell cycle regulators (e.g. p27kip) can be observed in HCC as it considered being potent tumor suppressors. Dysregulation of the balance between proliferation and cell death represents a pro-tumorigenic principle in human hepatocarcinogenesis. X-linked of apoptotic inhibitors (XIAP) is a regulator of apoptosis, cytokinesis and signal transduction. The aim was to evaluate the expression of p27kip and XIAP in HCC and their clinico-pathological significance. Subjects & Methods: The study was carried on forty patients with newly diagnosed HCC and 10 controls matched for age. Liver function tests, Serum alpha-feto protein, serologic markers for viral hepatitis, abdominal ultrasonography, triphasic computer tomography abdomen, liver biopsy and real-time PCR expression of p27kip and XIAP were done for all cases of the study. Result: There was a significant change in expression of p27kip or XIAP in HCC patients either by decrease (p27kip) or increase (XIAP) as compared to the controls ($p<0.05$). The decreased expression of p27kip or increased XIAP expression were associated with decrease of overall survival, increased incidence of recurrence and associated with more unfavorable prognosis. Conclusion: p27Overexpression may expect good prognosis while overexpression of XIAP suggests poor prognosis for HCC patients and they can be used as an independent prognostic factors for predicting disease-free and overall survival rates of these patients.

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Keywords: Carcinoma; Hepatocellular; p27kip; XIAP; Prognosis

1. Introduction

Hepatocellular carcinoma (HCC) is a major health problem, represents the fifth most common cancer in men and the seventh in women. Most of the burden of disease (85%) is born in developing countries. HCC is associated with a poor prognosis being the third leading cause of oncology-associated death [(1)] making this disease a major challenge that is highly resistant to conventional chemotherapy and radiation [(2)].

HCC pathogenesis has been related to exposure to environment toxins, alcohol and drug abuse, autoimmune disorders, genetic factors, elevated hepatic iron levels, obesity, and infections with hepatotropic viruses especially hepatitis B (HBV) & hepatitis C (HCV) viruses as it has been estimated that chronic infections with HBV and HCV account for up to 80% of HCC [(3)]. Many of liver insults, chronic inflammation, liver regeneration, fibrosis, and cirrhosis are thought to contribute to the development of HCC in these patients [(4)].

Cell division is controlled by a highly conserved group of proteins, the changes in their expression or activity levels are usually observed in human cancer cells and in genetic alterations which lead to cancer formation [(5)].The cyclin kinase inhibitor p27kip1 acts as a tumor suppressor in a variety of human cancers [(6)]. P27kip1 function as an inhibitor of cyclin E or cdk2 (cyclin dependent kinase 2) complexes activities leading to regulating cell progression from a quiescent state into the G1 phase and from the G1 phase into S-phase [(7)]. Several studies have demonstrated that decreased expression levels of p27 in primary cancer tissue correlates with reduced overall and progression free survivalas well as poor response to chemotherapies or targeted treatments. The inverse relations were observed in tumors of breast, prostate, bladder, lung, liver, larynx, ovary, and stomach [(8-10)].

Apoptosis is a physiological mechanism to remove excess cells during liver development and regeneration [(11)]. However, insufficient apoptosis has been involved in development and progression of liver tumors [(12-13)]. Several studies have approved that the increased expression of anti-apoptotic factors like the inhibitors of apoptosis proteins (IAPs) in a variety of solid tumors and cancer cell lines [(14)].

The inhibitor of apoptosis proteins (IAP) include a large family of endogenous caspases inhibitors, which include X-linked IAP (XIAP), c-IAP1, c-IAP2 and Survivin [(15)]. XIAP is the most important and efficient caspases inhibitor as it inhibit caspase initiator activation, caspase-3,-7 and -9 via its interaction through the BIR3 and BIR2 domains [(16)]. XIAP could

inhibit efficiently extrinsic death receptor and the intrinsic mitochondria pathways to generate the effector caspases 2p3. Increased XIAP has been reported in a several human tumors including esophageal carcinoma [17], clear cell renal carcinoma [18], ovarian carcinoma [19], lymphoma [20] and HCC [21]. In some cases, this expression was found to be associated with survival reduction [18].

The aim of this study is to evaluate p27kip and XIAP genes expression in patient with HCC and their clinical importance.

2. Subjects and Methods:

Patients

The study was carried out on forty patients suffering from HCC diagnosed from inpatients of Internal Medicine, Tropical and Oncology departments, Tanta University Hospital. They were 31 males and 9 females. Their ages ranged from 40-62 years.

Methods

All subjects in this study underwent the following

1. Complete history taking including disease duration.
2. Full clinical examination.
3. Doppler abdominal Ultrasonography.
4. Ultrasound and\or CT-guided liver biopsies .
5. Triphasic computer tomography (CT) abdomen.
6. Chest X-ray.
7. Bone scan if there is bone pain or elevated alkaline phosphatase.

Laboratory Investigation

- Complete blood count.
- Fasting and post prandial blood sugar
- Kidney function tests
- Liver function tests were done, including serum bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (Alb) and prothrombin time and activity.
- Hepatitis B surface antigen was assayed using enzyme immunoassay kits {SURASE B-96 (TMB) from General Biologicals Corporation, Innovation First Road, Science-Park, Hsin Chu,Taiwan,R.O.C.}
- Circulating anti-HCV antibodies were detected using the Murex enzyme immunoassay kit (Innotest® HCV Ab. From Innogenetics N.V. Belgium) (18).
- Serum alpha-feto protein by immunoassay method.
- Routinely Hematoxylin-Eosin-stained sections of HCCs and surrounding tissue were reviewed by the same histopathologists.
- The tumors were staged according to according TNM staging system [22].

None of the patients received chemotherapy or radiation before enrollment in the study or suffered from any other malignancies.

The Different treatment modalities of the patients with HCC are shown below (table 4).

The follow-up period ranged from 1 to 30 months.

Normal control tissues of the liver were taken from ten HCV patients who underwent liver biopsy before start HCV treatment and have Ishak fibrosis stages 0–1(F=0-1) [23]

Statistical analysis

Data were analyzed using statistical package for social sciences (SPSS) version I7 (SPSS Inc., Chicago, IL, USA) and the Graph Pad Prism software (GraphPad Prism Software Inc. San Diego, California, USA).Descriptive statistics were done. Categorical data were presented as number and percent. Statistical significance was defined as a P value of <0.05.Chi-square test was used to compare between the significant differences and match between the studied groups and some other qualitative variables. Comparison of continuous data between two groups was made by using Mann-Whitney test. Overall survival was measured from the date of random assignment until death. Patients who had not died or who were lost to follow-up were censored for overall survival when they were last known to be alive. The survival charts were depicted on Kaplan Meier plots. Analysis of the disease prognostic factors was done using the log rank test. To investigate whether expression levels of each gene and protein were associated to patient survival, HCC samples were categorized in high or low expressor groups if the target level wasabove or below the median expression value, respectively.

Expression of XIAP and p27kip mRNA was assessed by real-time PCR in liver tumor specimens.

Isolation of total RNA from liver tissues specimen using **PAXgene** Tissue RNA Kit (Qiagen, Germany). Purification of total RNA from tissues fixed and stabilized in PAXgene Tissue Containers according to the manufacturer's instructions and quantified by spectrophotometry (Pharmacia Biotech) at 260 nm. Total RNA was stored at -80°C until molecular investigation was performed.

Synthesis of cDNA from total RNA samples is the first step in using TaqMan Gene Expression Assays. The process involves the following procedures:1. Preparing the RT master mix. 2. Preparing the cDNA archive reaction plate.3. Performing reverse transcription.

Preparing RT Master Mix:

To prepare RT master mix:

The Total volume 50 µL: 10 µL Reverse Transcription Buffer, 4µL dNTP's, 10µL random primer,5 µL Multiscribe Reverse Transcriptase ,50 U/µL and 21µL Nuclease – free H_2O. Mix 50µL of the RT Mix with 50µL of isolated RNA (High-Capacity cDNA Archive Kit; Applied Biosystem, Foster City, CA, USA) under standard conditions (Table 2).

Preparing the PCR Master Mix for Each sample:

The cDNA of tumor tissue was used as a template to amplify the studied genes and cDNA of normal tissue was used to amplify GAPDH using the primers listed in Table 1. The assay identification numbers of target and housekeeping genes are as follow: p27kip gene Hs00153277_m1,XIAP gene Hs00236913_m1, and cDNA of normal tissue was used to amplify GAPDH gene Hs99999905_m1using the primers listed in Table 1. The reaction mixture was performed in a final volume of 25 µL: containing 5 µL cDNA, 1.25 µL Forward primer, Reverse primer, TaqMan Probe (FAM Dye) Target Gene (p27kip,XIAP genes), 1.25 µL Forward primer, Reverse primer, TaqMan Probe of reference Gene (GAPDH) , 12.5 µL TaqMan® Universal PCR Master Mix with UNG Supplied AS a 2 x concentration (Applied Biosystems,USA)which consist of: AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with dUTP, Passive Reference ROX dye was used for the PCR analysis and optimized buffer, and 5 µL nuclease-free water.

Table 1: Nucleotide sequence of the primers and probes used in the study were generated by using Primer 3 software (http://fokker.wi.mit.edu/cgi-bin/primer3/ primer3_www_slow.cgi)

Oligonucleotides	Nucleotide Sequence
P27kip-forward	5`- AGCACACGCATTTGGTGGA-3`
P27-reversed	5`- TAGAAGAATCGTCGGTTGCAGGT-3`
P27kip-TaqMan probe	FAM- 5`-AAAGACTGATCCGCGGACAGCCAGA-3`TAMRA
	5`-AGTGGTAGTCCTGTTTCAGCA-TCA-3`
XIAP- forward	5`- CCGCACGGTATCTCCT-TCA-3`
XIAP-reversed	FAM 5`- CACTGGCACGAGCAGGGTTTCTTTATACTG-3`TAMRA
XIAP- TaqMan probe	
	5`- GAAGGTGAAGGTCGGAGT-3`
	5`- GAAGATGGTGATGGGATTTC-3`
	FAM 5`-CAAGCTTCCCGTTCTCAGCC-3`TAMRA
GAPDH-forward	
GAPDH-reversed	
GAPDH- TaqMan probe	

Table 2: Times and Temperatures (two-step RT-PCR)

Times and Temperatures (Two-steps RT-PCR)				
1.RT step	Hold	Hold	Hold	
	10 min@25°C	120 min@37°C	5 sec@85°C	
	Initial Steps		PCR(Each of 40 cycles)	
2.PCR Step	AmpErase® UNG Activation	AmpliTaq Gold® DNA polymerase,	Melt	Anneal/Extend
	Hold	Hold	°Cycle	
	2 min@50°C	10 min@95°C	15sec@95°C	1 min@60°C

By using real time PCR Step One instrument and software (Applied Biosystems, Foster City, CA). Calculation of the results were done by application of comparative CT method for relative quantitation , $2^{-\Delta\Delta CT}$ $\Delta CT_{unknown} = CT_{target} - CT_{reference}$

The difference in threshold cycles for target and reference gene.

$\Delta\Delta CT = \Delta CT\ CT_{unknown} - \Delta CT_{Calibrator}$.

(RQ) Relative Quantitation = $2^{-\Delta\Delta CT}$

The Fold Change (FC) was calculated, defined as the ratio between averaged normalized expression level of targets in neoplastic and corresponding non-neoplastic samples. Normalized RQ were log 2-transformed for statistical analysis.

There is a significant increase in expression of both p27 kip and XIAP genes in HCC patients as compared to the control (p =0.02, 0.04 respectively) as fold change of p27expression in the patients ranged from 0.02 to 5.0 while in the control ranged from0.01 to 0.9. Fold change of XIAP expression in the patients ranged from 0.32 to 5.99 while in the control ranged from 0.21 to 1.3.

(A)

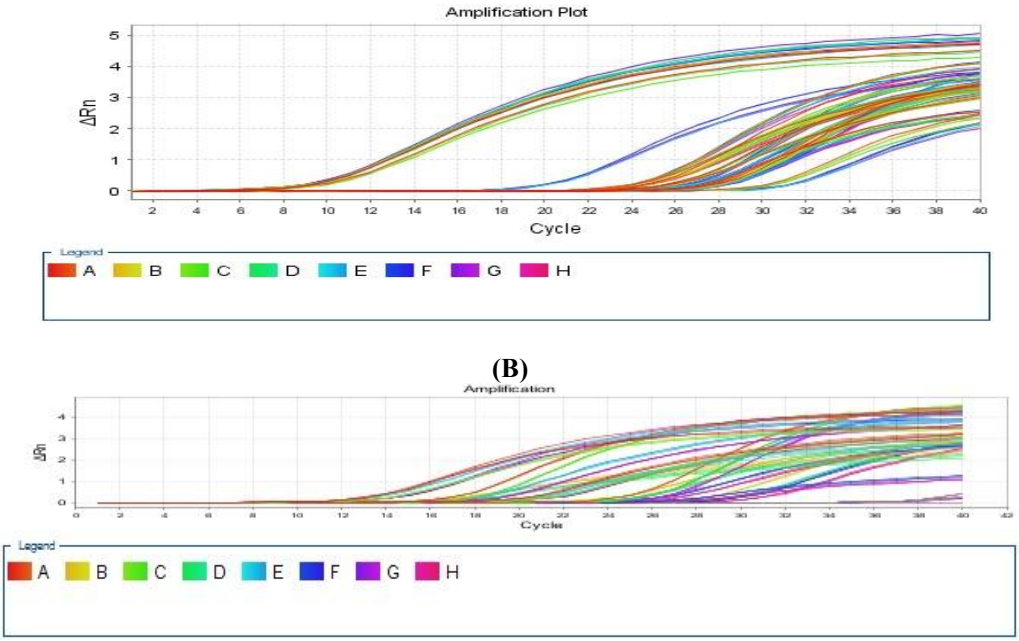


Figure 1. Amplification plots of p27kip and XIAP using real time PCR. A represents the amplification plot of p27kip expression and B represents that of XIAP. 3. Results

p27Kip expression was significantly increased with cirrhosis (p=0.02), portal venous invasion (p=0.02), histologic grade (p=0.00), TNM staging(p=0.00), tumour recurrence (p = 0.00), and metastasis (p = 0.00),whereas no significant change was found between p27Kip1 expression and other clinicopathologic variables. Table3. On the other hand, XIAP expression was significantly increased with portal venous invasion (p=0.02), TNM staging(p=0.00), tumour recurrence (p = 0.00), and metastasis (p = 0.00), whereas no significant change was found between XIAP expression and other clinicopathologic variables. Table 3.

Correlation of the expression of p27kip and XIAP with the patients' survival

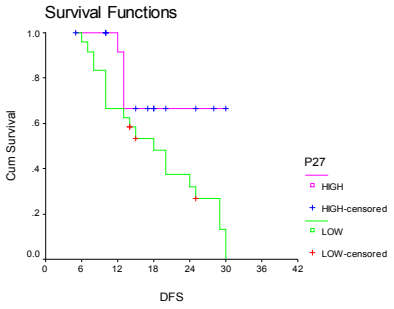
By using the Kaplan-Meier analysis, the patients with statistical analysis also indicated that both the disease free survival and overall survival rate of the p27kip overexpression group were significantly higher than that of the low expression group (P<0.05) (Fig. 1A,B).While their data of the XIAP overexpression group were significantly lower than that of the low expression group (P<0.05) (Fig. 1C,D).

Discussion

The prognosis of HCC has been significantly improved in the past few years due to earlier diagnosis and more effective treatments. However, tumor recurrence and metastasis are still the major problems for long term survival [(24-25)].

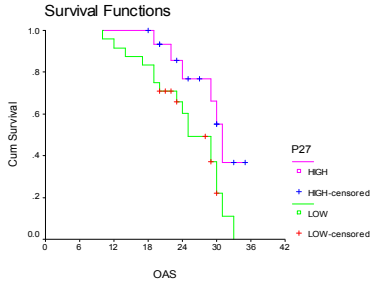
There is a wide agreement that liver cirrhosis caused by HBV or HCV infection is an important risk factor for the development of HCC as they develop a microenvironment of chronic liver injury, inflammation, and regeneration [(1,26)].

Although surgical resection and liver transplantation are the only treatment modalities that enable prolonged survival in patients with hepatocellular carcinoma (HCC), the majority of HCC patients presents with advanced disease and do not undergo resective or ablative therapy. Transarterial chemoembolization (TACE) is indicated in intermediate/advanced stage unresectable HCC even in the setting of portal vein involvement (excluding main portal vein). Sorafenib has been shown to improve survival of patients with advanced HCC in two controlled randomized trials. Yttrium 90 is a safe microembolization treatment that can be used as an alternative to TACE in patients with advanced liver disease or in case of portal vein thrombosis. External beam radiation can be helpful to provide local control in selected unresectable HCC. These different treatment modalities may be combined in the treatment strategy of HCC and also used as a bridge to resection or liver transplantation. Patients should undergo formal multidisciplinary evaluation prior to initiating any such treatment in order to individualize the best available options [(27)].



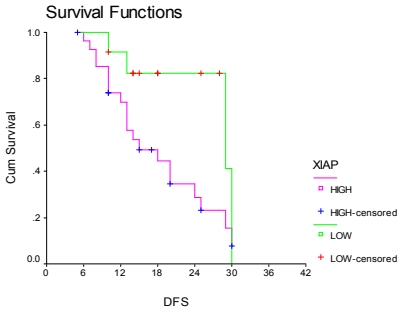
A

P=0.03



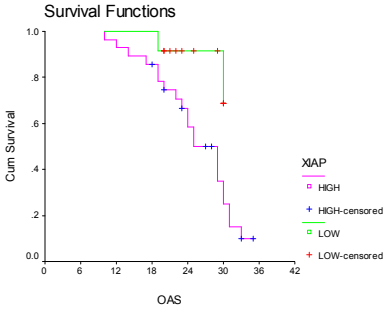
B

P=0.02



C

P=0.04



D

P=0.02

Fig 2. Kapalan-Meier survival curves of patients with HCC. (A,B) Disease and Overall survival rate are significantly higher in patients with p27kip overexpression. (C,D) Disease and Overall survival rate are significantly lower in patients with XIAP overexpression.

Deregulated apoptosis has a major role in pathogenesis of cancer, abnormality of cell differentiation, and chemotherapy resistance.

Caspase inactivation is contributed to enhanced survival and reduced apoptosis in tumor cells. X-linked inhibitor of apoptosis proteins (XIAP) is a principal inhibitor of apoptosis through its ability to inhibit caspase-3 and caspase-7 [(28)].

In this study, there was an increase in XIAP expression in patients with HCC and this was significantly associated with TNM stage, recurrence and metastasis. While, there was no relation with gender, age, presence of cirrhosis or histological grade. There was a decrease in overall and disease free survival in patients with high expression of XIAP.

These results in agreement with the results of Shiraki et al., [(29)]who found that XIAP is mainly expressed in all HCC cell lines and in approximately 70% of HCC tissue, whereas little or no expression is seen in chronic hepatitis or cirrhotic tissue and XIAP expression was inversely correlated with apoptosis.

And with Shi et al.,[(30)] who found that the level of XIAP expression in the three established HCC lines correlated with their metastatic capability by providing survival advantage to the metastatic cells via its anti-apoptosis effect. They suggested that XIAP could be an important factor in determining clinical outcomes of HCC patients. As majority of HCC samples were found to be highly positive for XIAP. In contrast, less than half of the adjacent liver tissues expressed XIAP, suggesting that high XIAP expression was characteristic of the HCC cells. They also noticed that increased XIAP expression had significantly shorter disease-free survival indicating that XIAP expression could provide additional prognostic factor. They also noticed that suppression of XIAP could be beneficial in cancer therapy and anti-sense oligonucleotides against XIAP have been developed and found to possess therapeutic effects in both in vivo and vitro studies.

Additionally, Notarbartolo et al.,[(31)] observed that XIAP high expression in HCC samples was associated with a more unfavorable prognosis and inhibitors of apoptotic proteins might play in the adverse biology of hepatocellular carcinoma. Augello et al.,[(32)] also found that XIAP high expression correlated with HCC recurrence and shorter overall survival while there was no association with other clinicopathological parameters.

Cyclin/cyclin-dependent kinase (CDK) complexes are integrators of multiple signals from both the extracellular and intracellular environment for efficient regulation of cell cycle progression [(33)].

Table 3. Expression of p27Kip1& XIAP genes and clinicopathological parameters in HCC patients

	No of patients	P27kip Low expression	P27 high expression	X^2	XIAP Low expression	XIAP high expression	X^2
Gender							
Males	31	19	12	0.75	11	20	0.16
Females	9	5	4		1	8	
Age							
<45years	13	8	5	0.89	3	10	0.50
≥45years	27	16	11		9	18	
Viral status							
HB	7	3	4	0.49	3	4	0.06
HC	29	19	10		6	23	
HB&HC	4	2	2		3	1	
Cirrhosis							
Presence	32	22	10	**0.02***	11	21	0.22
Absence	8	2	6		1	7	
No. of liver Tumors							
1-5	31	18	13	0.66	12	19	0.19
>5	9	4	5		3	6	
Tumor size							
<5 cm	26	14	12	0.27	7	19	0.56
≥5 cm	14	10	4		5	9	
Bilobar disease							
Yes	17	8	9		10	7	
No	23	10	13	0.33	12	11	0.44
Portal vein invasion							
Yes							
No	19	15	4	**0.02***	9	10	**0.023***
	21	9	12		3	18	
Histological grade							
Well							
Moderate							
Poor	8	4	4	**0.00***	2	6	0.66
	19	16	3		7	12	
Serum AFP (ng/ml)	13	4	9		3	10	
<100							
≥ 100							
TNM staging	24	15	9	0.69	5	19	0.12
I-II	16	9	7		7	9	
III-IV							
Recurrence							
Yes	29	21	8	**0.00***	6	23	**0.03***
No	11	3	8		6	5	
Metastasis							
Presence	24	10	14	**0.00***	10	14	
Absence	16	14	2		2	14	**0.04***
	6	4	9	**0.00***	7	6	
	34	20	7		5	22	**0.02***

Table 4. Different treatment modalities of forty patients with HCC

Radiofrequency ablation (RF)	**11**
Transarterial chemoembolization (TACE)	**16**
Cheomtheraputic &targted agents	**4**
Supportive therapy	**9**

There is an obvious relation of CDKs inhibitors and disease pathogenesis. p27kip1 is a CDK inhibitor that controls CDK activity throughout the cell cycle. As a CDK inhibitor, p27KIP1 has tumor suppressor activity [34].

In this study, there was a decrease in p27kip1 expression in patients with HCC and this was

significantly associated with presence of cirrhosis, histological grade, TNM stage, metastasis and recurrence. While, there was no relation with gender or age. There was a decrease in overall and disease free survival in patients with low expression of p27kip1.

These results with the results of Huang et al., [35] who reported that p27Kip1 expression correlated significantly with histological grade, venous invasion and cirrhosis, whereas no significant correlation was found between p27Kip1 expression and gender or age. The low expression of p27Kip1 was associated with short survival rate.

Chen et al.,[36] also found in their study on patients with HCC that the low expression of p27 Kip1 was inversely significantly related to cancer differentiation and tumor metastasis. There was no significantly difference between low expression and over expression in terms of age and gender. The survival rate of the p27 overexpression group was significantly higher than that of the low expression group. Multivariate analysis using the Cox's proportional hazards model showed that p27 Kip1 protein are independent prognostic indicators for patients' overall survival. They explained that as p27 Kip1 regulate cell proliferation as CDK inhibitor by inhibiting cell cycle progression from G1 to S phase in a dose dependent fashion.

El Bassiouny et al., [37] noticed in their study that the expression of p27Kip1 significantly decreased in HCC cases. Matsuda et al., [38] found that p27 is abundantly expressed in quiescent cells and is downregulated in many aggressive cancers.

It has been found [39], that p27 is frequently inactivated in HCC, and is considered to be a potent tumor suppressor as it is a negative regulator of G1-S phase transition through inhibition of the kinase activities of Cdk2/Cyclin E. in addition to other studies that reported the decreased expression of p27Kip1 is related to tumor progression and could be used as a potential predictor for HCC [40-41].

In conclusion, the decreased p27kip and increased expression of XIAP in HCC is involved in cancer cell behavior including proliferation, differentiation, and metastasis. Abnormal expression of them either separate or in conjunction may expect prognosis for patients with HCC. Moreover, effective therapy directed against them can be used in treatment of HCC, so, further studies about their used as a tool of treatment should be evaluated on wide scale of patients.

Corresponding author
Abd elfattah omnia
Clinical Oncology Department[4], Faculty of Medicine -Tanta University, Tanta, Egypt
omniaabdelfattah@yahoo.com

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DNA Image Cytometry of the Liver of Chronic Hepatitis C Virus Infected Patients and Hepatocellular Carcinoma in Sharkia Governorate in Egypt

Samia, M. Sanad[1], Amal, M. Mangoud[2], Amr A. Shalaby[1] and Mahmoud S. Abd El-Wahed[2]

[1]Zoology Department, Faculty of Science, Zagazig University, Egypt
[2]Pathology Department, Faculty of Medicine, Zagazig University, Egypt
egypt_sbes@hotmail.com

Abstract: In the present investigation forty three (11 HCC, 12 cirrhosis and 20 chronic hepatitis) cases were chosen from the paraffin blocks and unstained slides of fine needle aspirates biopsies which were preserved in the archive of the Early Cancer Detection Unit (ECDU) belonging to the Faculty of medicine, Zagazig University, Egypt. All the cases were previously diagnosed and proved by PCR to have HCV. 50 µm thick paraffin sections and fine needle aspirates smears were prepared for evaluating DNA ploidy and S- phase fraction (SPF) by DNA image analysis. The results indicated that, the hepatocyts of chronic hepatitis C cases have a regular multiplicity of normal diploid DNA (Euploid polyploidization). All of the studied HCCs cases and only one cirrhotic case revealed aneuploidy. It was concluded that, patients infected with chronic HCV are predominantly affected by HCC and the DNA image analysis techniques can be considered as early predictors of cellular abnormality and different malignant criteria, which can lead to early and well diagnosis as well as rapid manipulation of the patients.
[Samia, M. Sanad, Amal, M. Mangoud, Amr A. Shalaby and Mahmoud S. Abd El-Wahed zar. **DNA Image Cytometry of the Liver of Chronic Hepatitis C Virus Infected Patients and Hepatocellular Carcinoma in Sharkia Governorate in Egypt.** Life Science Journal 2011;8(4):987-995]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 126.

Keywords: DNA image cytometry, Liver, Hepatitis C virus, Hepatocellular carcinoma. Sharkia Governorate, Egypt.

1. Introduction

Objective analysis of microscopic images of cells and tissues for purposes of classification and measurements of cell components has been a goal of human pathology and cytology since the 19th century (Koss, 1987). During the last decade, image cytometry has become an established technique in the field of analytical cellular pathology. The new development of image analysis technology and the availability of commercial instruments capable of making reliable and so measurements, provide an advantage for quantitative immunohistochemistry (Wells *et al.,* 1992). According to Wells *et al.* (1996), all specimens of exfoliative-and aspiration cytology are suitable for static DNA measurements after staining according to Feulgen. Formalin fixed and paraffin embedded tissues are suitable after combined mechanic and enzymatic cell separation.

According to Cohen (1996), image cytometry enables use of internal controls and use of small amount of tissue. On the other hand, technical problems include nuclear overlap, which may give false optical density and a time consuming, quantitation required many nuclei to be cut apart or rejected. For minimal nuclear overlap and sharp focusing 50µm tissue sections are required.

Cell DNA content measurements, including determination of tumor ploidy and S-phase fraction; have been performed on a wide variety of human tumors for more than 20 years. There are two techniques to perform DNA quantitation: flow cytometry and static cytometry. The analysis in both techniques, is based upon the principal that DNA-specific dyes should stain the cellular DNA in a stoichiometric manner (i.e. the amount of stain is directly proportional to the amount of DNA within the cell (Batsakis *et al.,* 1993).

The prognostic value of DNA ploidy in HCC however has been questioned because of contradictory results (Ezaki *et al.,* 1988 and Ng IOL *et al.*, 1994). This discrepancy may be attributed to the limitations of sample size, patient pool, methodological differences, potential differences in the natural history of disease in various geographic locations, and last but not least potential features of HCC, such as multicentric origin and intratumor heterogeneity, which may lead to disparate sampling (Lapis *et al*., 1995).

The diagnosis of liver cell dysplasia and HCC is often difficult to establish in both cytologic material and needle-core biopsy specimens. The potential for misdiagnosis is greatest in attempting to discriminate well-differentiated HCC from dysplastic hepatocytes in cirrhosis (Crawford, 1990). Previous monophotometric IA studies have shown statistically significant differences between benign and malignant hepatocytes. The mean values of nuclear area, perimeter, and maximum diameter measured in fine needle aspirates (FNA) were significantly different between cases of HCC and benign hepatocytes in

cirrhotic nodules (Kondo *et al.,* 1999). Statistically significant differences in histologic nuclear density expressed as nucleocytoplasmic ratio have been demonstrated in histologic sections of HCC and benign hepatocytes in normal, atrophic and cirrhotic liver (Kondo *et al.,* 1999). However reliable classification criteria based on IA measurements have not been developed because there is considerable data overlap between benign and malignant hepatocytes. The accurate diagnosis of HCC may be difficult, because specimens frequently consist only of cytological materials or needle core biopsies. Erler *et al*. (1993 & 1994) suggested that, image analysis methods offer a potentially useful adjunct in discriminating between benign and malignant hepatocytes, because the require on a small amount of tissue and do not rely on architectural pattern. The present investigation is aimed to study DNA image cytometry of chronic hepatitis c virus infected patients and hepatocellular carcinoma in Sharkia Governorate, Eegypt

2. Patients and Methods

The present study has been carried out in the Early Cancer Detection Unit (ECDU) belonging to the Faculty of Medicine, Zagazig University, Egypt. Forty three (11 HCC, 12 cirrhosis and 20 chronic hepatitis) cases were chosen from the paraffin blocks and unstained slides of fine needle aspirates biopsies which were preserved in the archive of ECDU. All the cases were previously diagnosed during the period from January 1998 to December 2002 and proved by PCR to have HCV.

Preparation of smears:

Unstained slides of fine needle aspirates biopsies which were preserved in the archive of ECDU were stained with Feulgen method for DNA image cytometry.

Preparation of nuclear suspension for DNA image analysis:

Five 50 μm sections in 15 ml 16x 100 mm centrifuge tubes were deparaffinized by adding 10 ml of xylene for 20 minutes and this step was repeated twice. The sections were next rehydrated using a sequence of 10 ml of 100 %, 95 %, 70 % and 50 % ethanol incubation for 20 minutes each at room temperature (RT) and each step was repeated twice. The tissue was then washed in 2 changes of distilled water (d.w.) for 20 minutes and left in 10 ml d.w. for 24 hours.

Nuclear suspension preparation was done according to the method described by Hedley *et al*.(1983), with slight modifications. The tissue was resuspended in 1.5 ml of 0.5 % pepsin solution (Sigma Chemicals Co., St. Louis, MO, USA) in normal saline (0.9 % NaCl in d.w. w/v), adjusted to pH 1.5 with concentrated HCl. This mixture was incubated in a water bath for 30 minutes at 37 C°, then vortexing at 5 minutes intervals for 30 minutes. The proteolytic reaction was terminated by adding 2 ml of 10 % fetal bovine serum (FBS) in phosphate buffered saline (PBS) (10% FBS/PBS). The samples were immediately filtered through 40 μm nylon mesh. After centrifugation for 10 minutes at 1000 rpm (round per minute), the nuclear pellet was resuspended in 2 ml 5% FBS/PBS, then centrifugation for 10 min. at 1000 rpm. The supernatant was decantated and the pellet was flattened on a cleaned glass slides. These slides were air dried at room temperature overnight, then post-fixed in 10% neutral buffered formalin for 30 minutes and washed in d.w. for 10 min. and left to dry overnight at RT.

Cytometric Measurements:

1- Reagents:

Feulgen method using CAS DNA staining commercial Kit (Qualitative DNA staining Kit, Cell analysis, Inc., Elmhurst, IL, USA) was used. The Feulgen staining solution was prepared by mixing one stain reagent vial and 100 ml of 0.1 N HCl. The stain solution was stirred on a magnetic stirrer for 60 min. and it was tightly blocked (closed) to prevent SO2 loss. Solution was filtered immediately before using. The rinse solution was prepared by addition of the entire contents of one rinse reagent vial to 300 ml of 0.05 N HCl in a 500 ml flask and mixed well until completely dissolved. The Feulgen reaction produced specific blue staining of nuclear DNA.

2- DNA Staining and Analysis according to Schulte and Wittekind (1990):

Air-dried prepared slides and the unstained fine needle aspiration (FNA) smears were treated for 60 min. in 5N HCl to hydrolyze nuclear DNA. The slides were directly transferred to freshly prepared Feulgen stain in tightly capped coplin jar for 60 minutes, and then rinsed in 3 changes of rinse solution for 30 seconds, 5 minutes and 10 minutes, subsequently. After that, the slides were washed in distilled water and placed in 1% acid alcohol for 5 minutes. They were then dehydrated in 2 changes of absolute alcohol for 2 minutes each. The slides were then cleared in 2 changes of xylene (2 minutes, each) and mounted with DPX and coverslips.

The stained slides were analyzed with Hund CML image analyzer and software *(Hund H500, Wetzlar, FRG)*. The instrument calculates the DNA index (DI) and coefficient of variation (CV) of measured peaks. For each slide, 20 lymphocytes were

used as an internal diploid DNA content standard for the slide. Four hundred (400) non-overlapping hepatocyte nuclei were then measured. Peak statistics are based on user demarcation of the histogram. The CV of each peak was calculated in the standard fashion in the standard deviation of demarcated divided by the mean.

3- DNA Histogram Interpretation:

1-Histograms were regarded as uninterpretable for ploidy if the CV for the DNA diploid Go/G1 peak was < 8

2-The histogram was considered diploid when the single peak occupied the diploid position (DI range: 0.9 – 1.1), and fewer than 15% of cells were present at the tetraploid position.

3-If an additional distinct peak was present, the tumor will be classified into one of the 5 non-diploid categories depending on DI. Thus tumors will be considered DNA hypodiploid for DI<1.0, hyperdiploid for DI in the range 1.1- 2.1 and hypertetraploid for a DI greater than 2.1. If more than one non-diploid peak was observed, the tumor will be classified as multiploid. For tetraploid tumors, the additional peak should be in the tetraploid region and should contain ≥ 15% of cells in the presence of recognizable G2/M peak. The term aneuploid is used to describe hypodiploid, hyperdiploid and hypertetraploid subgroups of non-diploid tumors as one group.

3. Results

In the present study, observations of Image cytometry are illustrated in table (1). According to the criteria of Bocking *et al*. (1992), a single G1 peak was regarded as diploid DNA and two or more G1 peaks as aneuploid. The CV values were relatively broad (3.0 – 7.0), but were still within an acceptable range. All the cases of chronic HCV were diploid. The mean of SPF value for the studied chronic HCV cases was (7.12 ± 3.67), and for the cirrhotic cases was (12.23 ± 5.18), while for the HCC cases was (26.46± 7.14); the difference is a highly significant (F= 90.55 and P = 0.0001) (Table 1). Also, statistical analysis showed a highly significant correlation (P<0.0001) between the DNA ploidy and the average SPF values of the HCCs (table 1 and Fig. 1).

Table (1): Cell Cycle fractions and DNA ploidy according to different pathological changes of the studied cases.

Histopathol.changes / Cell Cycle	Chronic Hepatitis N	Cirrhosis	HCC	Test of sig.	P
S-phase					
x ± SD	7.12 ± 3.67	12. 23 ±5.18	26.46 ± 7.14	F= 90.55	0.0001
Range	2. 20 - 0.75	5.85 – 17.90	21.00 – 38.30		S.
DI1					
x ± SD	0.99 ± 0.08	1.03 ± 0.03	1.035 ± 0.4	F=	0.093
Range	0.91 – 1.08	0.95 –1.07	0.99 – 1.08	252	N. S.
DI 2					
x ± SD	1.98 ± 0.07	2.01± 0.11	1.59 ± 0. 29	F=	0.0001
Range	1.91 – 2.08	1.67 - 2.09	1.23 – 2.3 2	12.88	S.
Prolif. F.					
x ± SD	24.37 ± 4.36	36.14 ±6.28	64. 23 ± 7.08	F=	0.0001
Range	17.90 –33.80	27.5 – 46.60	53.50 – 74.9	61.93	S.
DNA	No %	No %	No %		
Diploidy	20 100.0	11 91.7	0.0 0.0	X 2	0.004
Aneuploidy	0 0.0	1 8.3	11 00.0	8.45	S.

By correlating the DNA index (DI1) and the DNA ploidy, average of (DI1) was 0.99±0.08 for chronic HCV cases, while it was 1.03±0.03 and 1.035±0.4, for cirrhotic and HCC cases respectively, revealing no significant association between histopathological lesions and DI1 value in this study (F=252, P=0.093) as illustrated in table 1 and Fig. 2.

By correlating the DNA index (DI2) and the DNA ploidy, there was a high significant correlation between the average of (DI2), which was 1.98±0.07 for chronic HCV cases, and 2.01±0.11 and 1.59±0.29 for cirrhotic and HCC cases respectively and for different histopathological leions, where (F=12.88, P <0.0001) as illustrated in table (1) and (Fig. 3).

Concerning the mean of proliferation fraction values, it has been found that, it showed a high significant correlation with the type of histopathological lesions. The mean values of proliferation fraction of the studied cases as recorded in table (1) were significantly high (64. 23 ± 7.08) for

HCC comparing to (24.37 ± 4.36 and 36.14 ± 6.28) for chronic HCV and cirrhotic cases, (F=161.93 and P< 0.0001).) as illustrated in table (1) and (Fig. 4).

The DNA ploidy progressively changed with the increased hitopathological changes particularly with the presence of malignancy. By correlating DNA ploidy with the histopathological changes using Chi square , a high significant correlation was recorded, where, (*p* value = 0.004) as illustrated in table (1) and Fig. (5).

According to DNA image analysis data, aneuploid cases were 12 representing (28%) of the studied cases. One of them was cirrhotic (Fig. 10) and 11 cases were HCCs as shown in (Figs. 11, 12, 13, & 14). The remaining cases (31, 72%) were diploid as shown in Figs. (6, 7, 8, & 9). Among the 7 cases of well differentiated HCCs, 6 cases were aneuploid and one case was tetraploid, resulting in (100%) of foci being either aneuploid or tetraploid (Fig. 13). Also, the two (100%) moderately differentiated HCC cases were abnormal, each of them was obviously aneuploid. The two cases of poorly differentiated HCC revealed also abnormal histograms, one of them was aneuploid and the other one was multiploid (Fig. 14).

The DNA index (DI) of the aneuploid peaks ranged from 1.1 to 1.9 or below 0.9 and above 2.1. Among the HCC foci, tetraploidy as defined by a large G2/M peak and a small S-phase population was presented in one sample. Whether a high G2/M includes near-tetraploid aneuploid populations or is merely a manifestation of polyploidy is particularly difficult to resolve in the liver, which has a sizable polyploid population both normally and in HCC. It has been also noticed that, human hepatocellular tumor growth was associated with a decreased tendency toward polyploidy. Observations of the present study indicated that, the poorly differentiated HCCs has a higher frequency of DNA abnormal populations.

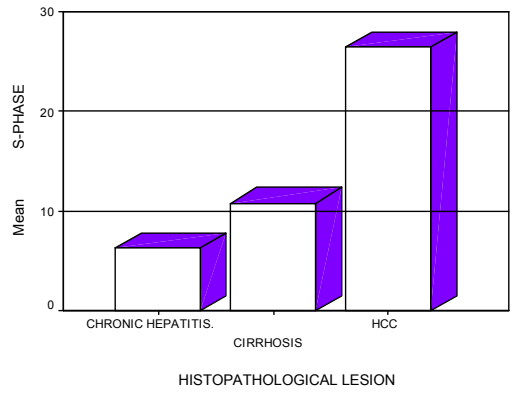

Fig. 1: Comparison of the mean value of S-Phase fraction of different pathological changes.

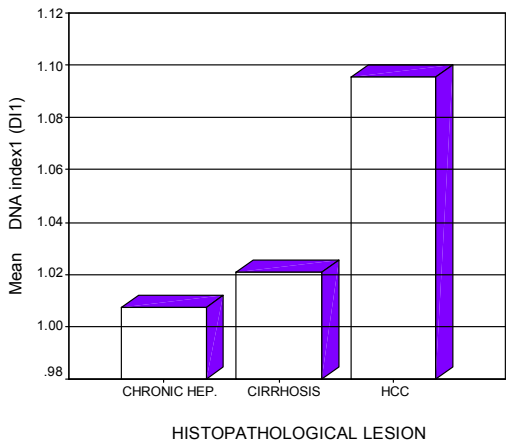

Fig. 2: Comparison of the mean value of DNA index 1 (DI1)of the different pathological changes.

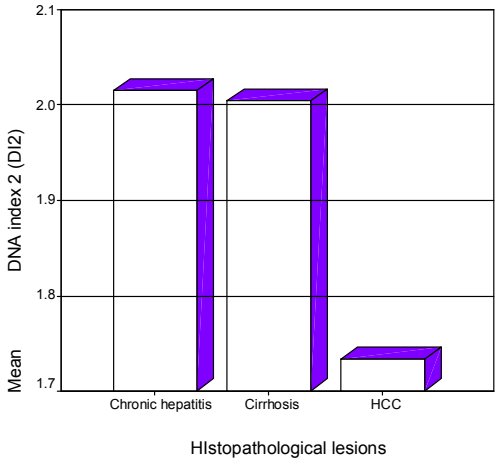

Fig. 3: Comparison of the mean value of DNA index (DI2) of the different pathological changes

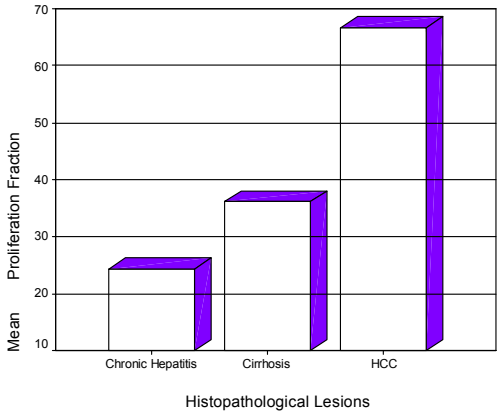

Fig. 4: Comparison of the mean value of proliferation fraction the different pathological changes

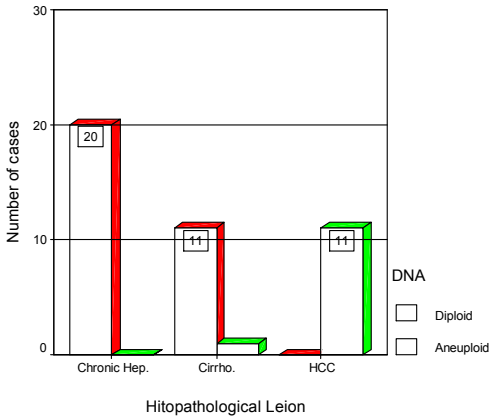

Fig. 5: Comparison of the DNA ploidy and the different pathological changes.

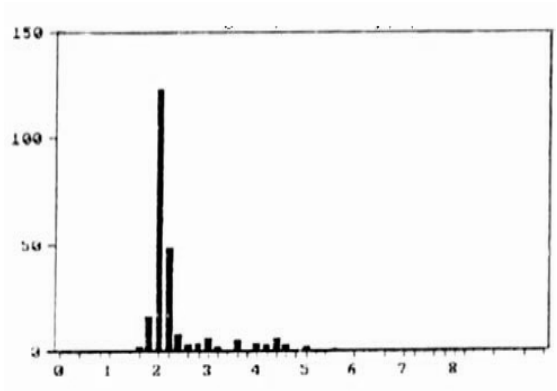

Fig. 6: DNA histogram of a female patient aged 29 years and infected with HCV showing normal diploid peak (non-aneuploid) using (Helmut Hund GmbH D6330 Wetzlar 21, Germany), (SPF=2.98) and (DI1 = 1.00).

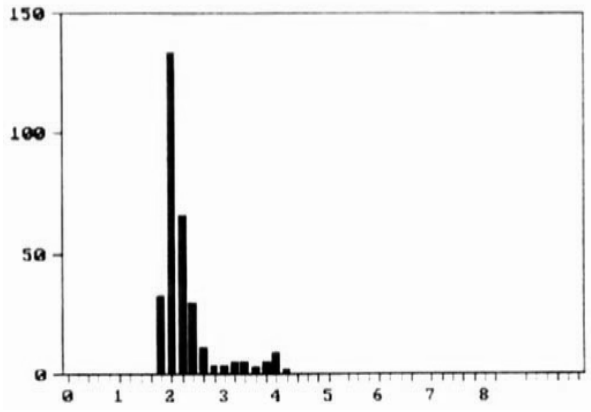

Fig. 7: DNA histogram of a male patient aged 32 years and infected with HCV showing normal diploid peak (non-aneuploid) using (Helmut Hund GmbH D6330 Wetzlar 21, Germany), (SPF=4.23) and (DI1 = 1.00).

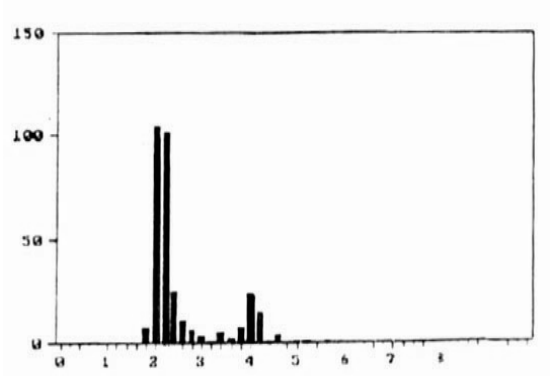

Fig. 8: DNA histogram of a male patient aged 41 years and infected with HCV showing normal diploid peak (non-aneuploid) using (Helmut Hund GmbH D6330 Wetzlar 21, Germany), (SPF=7.11, DI1 = 0.99, DI2 = 1.97).

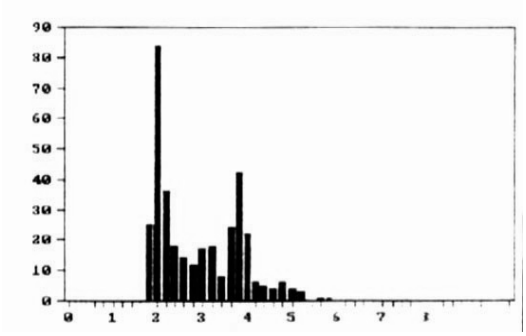

Fig. 9: DNA histogram of a female patient aged 57 years and infected with HCV and marked cirrhosis showing normal diploid peak (non-aneuploid) using (Helmut Hund GmbH D6330 Wetzlar 21, Germany), (SPF=10.28) and (DI1 = 1.03, DI2 = 2.07).

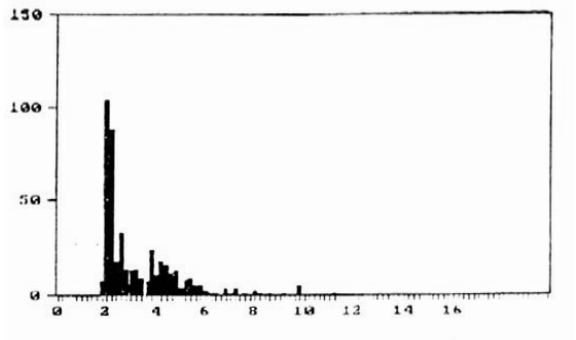

Fig. 10: DNA histogram of a male patient aged 49 years and infected with HCV and marked cirrhosis showing aneuploid peak using (Helmut Hund GmbH D6330 Wetzlar 21, Germany), (SPF=13.47) and (DI1 = 1.06, DI2 = 1.34).

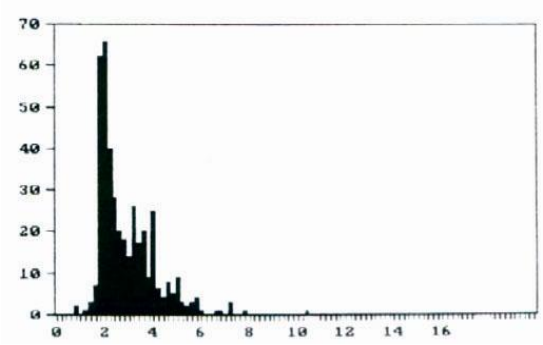

Fig. 11: DNA histogram of a female patient aged 67 years and infected with HCV and well differentiated HCC showing aneuploid peak using (Helmut Hund GmbH D6330 Wetzlar 21, Germany), (SPF=24.68) and (DI1 = 1.04, DI2 = 1.61).

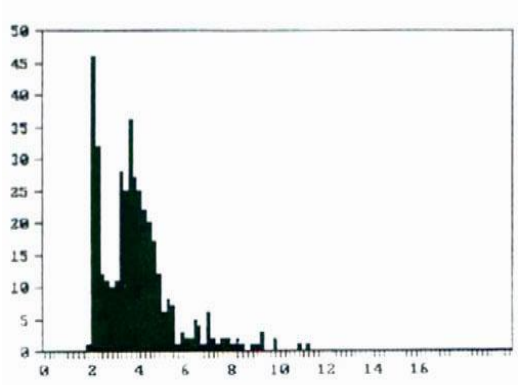

Fig. 12: DNA histogram of a male patient aged 67 years infected with HCV and well differentiated HCC showing aneuploid peak using (Helmut Hund GmbH D6330 Wetzlar 21, Germany), (SPF=26.17) and (DI1 = 1.02, DI2 = 1.98).

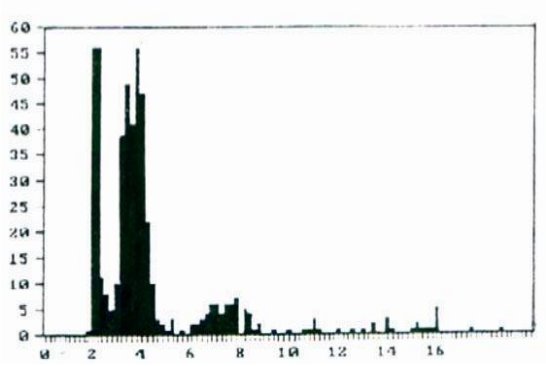

Fig. 13: DNA histogram of a male patient aged 61 years infected with HCV and moderately differentiated HCC showing tetraploid peak using (Helmut Hund GmbH D6330 Wetzlar 21, Germany), (SPF=33.65) and (DI1 = 1.09, DI2 = 1.66).

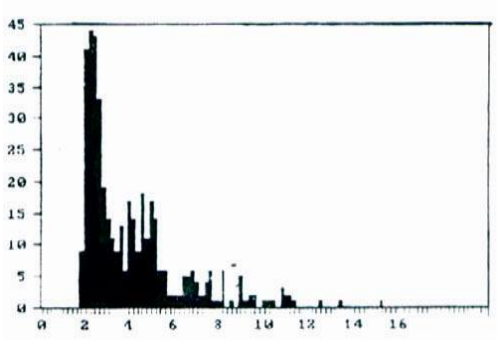

Fig. 14: DNA histogram of a male patient aged 61 years infected with HCV and poorly differentiated HCC (trabeculr pattern) showing multiploid peak using (Helmut Hund GmbH D6330 Wetzlar 21, Germany), (SPF=31.73) and (DI1=1.03, DI2=1.23).

4. Discussion

The development of techniques for measuring the quantity of DNA in a population of tumor cells has resulted in the refinement of diagnostic criteria and development of new treatment protocols based on prognosis and the likelihood of response to therapy (Look *et al*., 1995, Hyman *et al.,* 2002, Clingoz *et al.,* 2003, Keller *et al.,* 2003 and Lorenzato *et al.,* 2004**).** The presence of normal hepatocytes and stroma as well as inflammatory cells served as internal controls for each specimen. As the staining intensity of fixed nuclei varied from one sample to another, the lymphocyte external diploid control was excluded from DNA analysis. The first peak to the left was regarded as representing the diploid cells and the DNA index (DI) values of other peaks were calculated with this peak as a reference. Consequently, tumors were considered to be diploid when a single peak was observed; tumors with more than one peak were non-diploid and the first peak was always regarded as the diploid peak (Bocking *et al*., 1992). Evaluating of cell ploidy of the smears of (FNA) which have been also stained with blue Feulgen are somewhat better than those prepared from paraffin sections because the nuclear chromatin is better spread on the slide. The findings of Rubin *et al*. (1994) in that direction are, in accordance with our observations. The same observations were also noticed by Coon et al. (1987), who showed that, formalin-fixed tissue has additional disadvantages of producing histograms with a higher CV and more subnuclear debris than fresh specimens. A high CV may be due either to a true-near diploid aneuploid population or to deterioration of DNA as a result of poor fixation or embedding (Thomas *et al*., 1992).

DNA index (DI) is a crude measure of true DNA content. The range of diploid value (0.9 to 1.1) would include cells that had gained or lost up to 10%

of their DNA. Thus, it is possible that, diploid clones harbor cytogenetic abnormalities not detectable by ploidy analysis and aneuploid clones with the same DNA index could contain very different karyotypic abnormalities (McEntee *et al*., 1993). Thus cytogenetic differences could exist between primary and recurrent tumor cell populations with the same DNA index (DI). Also, of the diploid clones may actually be near diploid.

In the present study, DNA image cytometry of the chosen cases of chronic HCV showed non aneuploid (diploid) histograms for each of them (100%). Also, the mean of coefficient of variation (CV) of G0/G1 peaks of the samples extracted from paraffin-embedded tissue was (5.5%) and (3.8%) of the FNA cases and the DNA index (DI) was (1.0 ± 0.7) and these values may be entirely consistent with the values obtained with normal diploid populations, as was also observed by Bocking et al. (1992).

The occurrence of aneuploidy in cirrhotic livers without regard to the presence of dysplasia has been evaluated in the present study. Image cytometry of the DNA inclusions of the cirrhotic liver tissues revealed that, two cases representing (8.33%) of them were aneuploid. This finding is in agreement with Lin *et al*. (1990), who noticed that, two of (17) cases were aneuploidy, suggesting that, the occurrence of dysplastic cells is sometimes seen in cirrhotic livers. In addition, when aneuploidy was assessed in cirrhotic livers harboring HCC, the nonneoplastic tissue contained DNA aneuploid cells, while the frequency of DNA aneuploidy was much less in cirrhotic livers of patients without HCC (Ballardini *et al*., 1999**).**

In the present study, one case (9.1%) of the HCCs was diploid, 9 cases (81.8%) were aneuploid while, one case (9.1%) was multiploid. The two diploid and multiploid histograms represented well differentiated and poorly differentiated HCC, respectively. These findings are approximately in accordance with An *et al*. (1997) who observed that, 14 HCC were diploid, 24 0f 40 aneuploid, and 2 cases of them were multiploid. On the other hand, few studies, however, have compared the ploidy status large cell dysplasia with normal, cirrhotic and HCC cases (Rubin *et al*., 1994**).** An *et al*. (1997)**,** showed that, 92% of the dysplastic cases were aneuploid compared with 60% of the HCC cases and 43% of the benign cases exhibited aneuploidy. Zeppa *et al*. (1999) studied 84 HCCs diagnosed by FNA; eight of them were well differentiated HCC. They found that, 68 cases were aneuploid and 16 were euploid (9 diploid and 7 polyploid). Four of the eight cytologically suspect cases were aneuploid. Statistical analysis showed an association between size and cytologic grading, and between aneuploidy and multiple tumors. They concluded that, DNA ploidy evaluation by static cytometry of hepatic tumors may be useful in the diagnosis of cytologic samples and could represent independent prognostic parameter in predicting the survival outcome of patients with HCC.

The role of liver cell dysplasia in the evolution of HCC is a matter of scientific debate. A progression over time from cirrhosis to dysplasia and then to HCC has been hypothesized (Anthony, 1976)**.** However, Cohen and De Rose (1994) argued that, the mean age of cirrhotic patients with dysplasia is less than the mean age of cirrhotic patients without dysplasia, implying that dysplasia is not chronologically related to cirrhosis. Other studies suggest that, dysplasia lacks features usually associated with HCC, in particular, the reticulum framework in dysplasia is preserved, the serum alpha-fetoprotein is normal, and the nuclear to cytoplasmic ratio is unchanged (Watanabe *et al.,* 1983)**.** A normal nuclear to cytoplasmic ratio suggests that "dysplastic cells" are simply polyploid. The presence of polyploid cells in normal liver is well-established (Saeter *et al*., 1988).

Many studies examined the DNA ploidy SPF as potential prognostic factors in human liver diseases and HCC (McEntee *et al*., 1993; Rim *et al*., 1993 and Ng IOL *et al*., 1994). The conclusions reached by investigators range from attributing no prognostic significance to these parameters to considering them significant and independent prognostic factors (McEntee *et al*., 1993). In the current study, DNA ploidy and the fraction of cells undergoing DNA synthesis, expressed as S-phase fraction (SPF) were evaluated in each of chronic hepatitis, cirrhotic and HCC cases. The DNA ploidy was similar in chronic HCV and most of the cirrhotic cases, which was non-aneuploid (diploid), except two cirrhotic cases were aneuploid. The SPF value for the aneuploid HCCs was significantly higher than for the diploid cirrhotic cases, which also in turn have a significantly higher SPF than chronic HCV examined cases.

The proportion of aneuploid HCC cases in this study was considerably higher than that which was previously reported by other investigators (Chen *et al*., 1991; Kopper *et al*., 1991 and Nagasue *et al*., 1992). In this context, it is worth mentioning that, a DNA image cytometric and flow cytometric studies, which were carried out on paraffin embedded human HCC, using the same technique showed that 70% of the tumors were aneuplod (Rim *et al*., 1993).

Different tumors and host factors have been related to prognostic significance of HCC. An association between DNA ploidy and tumor size was made by Japanese scientists (Fujimoto *et al*., (1992) and higher incidence of aneuploid tumors has been

found in poorly differentiated HCC. In the present findings, significant correlation was found between DNA ploidy and cytological grade. In reviewing the literature on DNA ploidy versus age in human HCC, the reports are contradictory (Nagasue *et al*., 1992). Morphometric parameters are useful in discriminating benign from malignant hepatocytes. Studies have also compared the nuclear morphometric characteristics of dysplastic hepatocytes with those of benign and malignant hepatocytes (Marchevsky *et al.,* 1994& 1997)**.** On the other hand, few experimental studies were carried out in this field and took in consideration the role of some nuclear parameters such as *p53* in the liver DNA ploidy. Yin *et al.* (1998) showed that, the hepatocytes of the heterozygous or homozygous *p53*-mice as well as mice expressing one allele of *p53* ser246 do not undergo normal polyploidization with aging and show an increase in the number of cycling (G1-, S-, M- phase) cells. Also, they concluded that, loss of *p53* removes blocks in the cell cycle, leading to increase proliferation, whereas expression of the *p53* ser246 mutation stimulates G0 to G1 and/or M to G1 transition of hepatocytes.

In the present work, it has been noticed that, image analysis is potentially useful as an adjunctive tool for the evolution of liver biopsy material that is potentially difficult to interpret and can be used to distinguish objectively normal hepatic tissue from hepatic dysplasia, HCC and non-neoplastic liver. These findings agree with An *et al*. (1997)**,** who concluded that, foci of morphological atypical hepatocytes found in liver with cirrhosis may contain cells with a distinct DNA aneuploid peak detectable by image cytometry. Foci with high grade dysplasia had a higher frequency of aneuploidy compared with foci of low-grade dysplasia, although this tendency was not statistically significant. These findings support the recognition of liver cell dysplasia as a morphologic entity containing an aneuploid subpopulation. As such, this lesion may be a precursor for the development of HCC. On the other hand, high AFP levels have been found in cases with poorly differentiated HCC, but the relationship between DNA ploidy and AFP production is questionable. According to Chen *et al.* (1991), hyperploid tumors have been associated with high AFP levels and diploid tumors with low AFP wheras Nagasue *et al.,* (1992) found no correlation between DNA ploidy and AFP production.

Corresponding author
Samia, M. Sanad
Zoology Department, Faculty of Science, Zagazig University, Egypt
egypt_sbes@hotmail.com

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Contributing Factors of Iron Deficiency Anemia among Children under Two Years Attending Family Health Centers in Alexandria.

Amina Ahmed Mohamed and Ahmed samir abo-donia

Community Health Nursing Department, Faculty of nursing, Alexandria University
Department of Home Economics, Faculty of Agriculture- El-Shatby, Alexandria University
dr.amiina@hotmail.com

Abstract: Iron deficiency is one of the biggest contributing factors to the global burden of anemia. It is an indicator of both poor nutrition and poor health. Iron deficiency is considered as one of the ten leading global risk factors in terms of its attributable disease burden. Hence, the aim of the present study was to investigate the contributing factors of iron deficiency anemia among children under 2 years attending family health centers in Alex. Exploratory descriptive study design was adopted to carry out this study. The study was conducted at four family health centers in Alexandria chosen randomly from seven zones .Total sample was 400 healthy children aged 12 to 24 months and their mothers. Two tools were developed and used by the researcher in order to collect the necessary data. The findings of the present study revealed that, the prevalence of anemia was 77% among children under 2 years. Minimum to maximum age was 12-21months for not anemic infants with a mean age of 15.3±3.1 and 12-24 months for anemic infants with a mean of 17±2.6. male infants constitute more than half (54.3%) not anemic and 50.3% anemic infants, while, female infants constitute 45.7% of not anemic infants, and 49.7% of anemic infants. Statistically significant differences was found between two groups regarding level of father & mother education ,residence, weight ,height ,hemoglobin% of children and intake of iron. About 50% of not anemic mothers' practices regarding weaning practices were scored as good (correct) practices (which means correct time &types of food) compared to only11.7% of anemic mothers' practices. The study concluded that the socio economic factors, faulty weaning and feeding practices are the main contributing factors of anemia among children under 2 years. It is recommended that all efforts should be directed to promote nutrition well-being of young children. As the nutritional well of young children reflects household, community, and national investments in family health and contributes in both direct and indirect ways to the country's development.

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Key Words: contributing, factors, Iron, deficiency, anemia / children

1. Introduction:

Anemia is a medical condition characterized by abnormal decrease of blood capability for gas and nutrient exchange .It is a worldwide health problem for all ages and both sexes [1]. Anemia, defined as hemoglobin concentration below established cut-off levels [2], it is a widespread public health problem with major consequences for human health as well as social and economic development [3,4]. Although estimates of the prevalence of anemia vary widely and accurate data are often lacking, it can be assumed that in resource-poor areas significant proportions of young children and women of childbearing age are anemic. Iron deficiency anemia is the most frequently hematological disease of infancy and childhood [5]. WHO estimates the number of anemic people worldwide to be a staggering two billion and that approximately 50% of all anemia can be attributed to iron deficiency [6]. While, more than 75% of Indian toddlers are anemic [7,8] . Moreover, more than 100 million African children are thought to be anemic [8]. In Egypt the Demographic Health Survey 2008 reported that about 48.5% of children under 2 years had iron deficiency anemia (IDA) [9]. where the study done in Côte d'Ivoire demonstrated that 40–50% of children and adult women were anemic and that IDA accounted for about 50% of the anemia in schoolchildren and women, and 80% in preschool children (2–5 years old) [10].

The body of the newborn contains about 0.5 gram iron, where as the adult content is 5gram. In order to make up this 4.5g difference, 0.8-1.5mg of iron must be absorbed each day to fill the stores and to fulfill the growth requirements [11]. This is essential during the first stages of life. Since less than 10% of iron in diet is absorbed, a diet containing 8-15 mg iron is necessary each day. This is difficult to obtain in the first year if the infant depends mainly on breast milk. For this reason, the diet should include infant cereals starting from 6 months [12]. There are two forms of dietary iron: heme and non heme. Heme iron is derived from hemoglobin, the protein in red blood cells that delivers oxygen to cells. Heme iron is found in animal foods that originally contain hemoglobin,

such as red meats, fish, and poultry. Iron in plant foods such as lentils and beans is arranged in a chemical structure called nonheme iron [13]. This is the form of iron added to iron-enriched and iron-fortified foods. Heme iron is absorbed better than nonheme iron, but most dietary iron is nonheme iron [13]. The highest risk of ID occurs during times of rapid growth and nutritional demand, during infancy (ages 6-24 months), early childhood, adolescence, and pregnancy [14]. Moreover, the second half of an infant's first year is an especially vulnerable time because infants are learning to eat and if nutritional intake is inadequate, the consequences persist throughout life [15, 16].

Iron deficiency leads to reduced oxygen-carrying capacity and can impact immunity, growth, and development [17]. Iron has multiple roles in neurotransmitter systems and may affect behavior through its effects on dopamine metabolism [18]. The association between ID and dopamine metabolism is highly relevant to children's cognitive development as dopamine clearance has strong effects on attention, perception, memory, motivation, and motor control .The etiology of iron deficiency anemia among children is one of multiple and interacting causes [19].

Among the numerous factors that contribute to the onset of anemia especially, iron deficiency anemia are, both nutritional such as vitamins and mineral deficiencies, suboptimal breastfeeding practices, poor quality complementary foods and non-nutritional such as infection, contamination of complementary food and feeding utensils [20].

Basically there are three types of interventions to increase the iron status of populations; improvement of diet in terms of bioavailable iron, fortification of foods, and medicinal iron supplements [21]. Although anemia has been recognized as a public health problem for many years, little progress has been reported and the global prevalence of anemia remains unacceptably high [21]. WHO, UNICEF and Ministry of Health and Population therefore reemphasize the urgent to combat anemia and stress the importance of recognizing its multi factorial etiology for developing effective control program [22]. As a result of that, The ministry of health is conducting and implementing a national preventive program that aims at child's health welfare such as program for prevention of anemia among children under 2 years (This was done through estimation of blood hemoglobin level of infants at 12 &18 months during immunization and follow up of infants or children and giving the recommended iron supplementation for anemic children according to the schedule of WHO of iron supplementation) [23]. But the prevalence of iron deficiency anemia is still high.

So, a complete study of maternal knowledge and practices in our community is essential to identify malpractices toward feeding, weaning of infants which in turns, will reflect the causes of iron deficiency anemia and malnutrition among infants and young children.

Aim of the study was to investigate the contributing factors of iron deficiency anemia among children under 2 years attending at family health centers in Alexandria.

Research questions:

- What are the contributing factors of anemia among children under 2 years?
- Dose the feeding & weaning practices affect the level of hemoglobin %?
- Dose the socio-economic factors affect the level of hemoglobin %?

2. Materials and Methods

I - Materials

Design:

Exploratory descriptive study design was adopted to carry out this study.

Setting

The study was conducted at four family health centers in Alexandria chosen randomly from seven zones, namely; El montaza from El montaza zone, Bacau's from east zone, El Hadara from middle zone and EL Laban from EL Gomrok zone .

Subjects

On hundred of mothers and their infants or young children were selected randomly from the well baby clinic affiliated to the previously mentioned settings s were included in the study. Total sample was 400 healthy children aged 12 to 24 months and their mothers.

The criteria of selection: Children aged 12 to 24 months - Healthy children (free from any diseases- Attended clinic for immunization and \or follow up.

Tools

Two tools were developed and used by the researcher in order to collect the necessary data.

Tool I Structured interview questionnaire:

It included the following parts.

First part – was concerned with socio-demographic data such as child's age, sex, birth order, mothers and father's age, level of education, occupation and income.

Second part was concerned with information related to feeding patterns such as time& pattern of breast feeding, and age of introducing food, age of stop breast feeding.

-Mothers practices of weaning which includes correct time and types of weaning food, food that increases /or decreases absorption of iron.

-Feeding during sickness and the nutritional problems facing the mothers during feeding her infant.
- 24 recall method (sheet to recall last 24 hours received food).

Third part– Health care data: This part was developed based on child health record in order to obtain information which could influence the infants nutritional state, such as use of vitamin supplementations, iron, calcium, or any prescribed medication -Result of blood investigation especially level of blood hemoglobin. (HB% was done as a routine during follow up of infant aged 12 months and 18 months according to the protocol of ministry of health & population for early detection and treatment of iron deficiency anemia during childhood).

Tool II

Anthropometric measurements:

Weight and length were measured for all studied children

II- Methods

- Permission was adopted to collect the data from the selected family health centers. The researcher explained to the director of selected centers the purpose of the research to insure their cooperation and proper communication.
- The tools were submitted to 5 juries who were expert in the field of community health nursing for its content validity; the validity for the various questions varied between 80-100% .The tools were also tested for its reliability where alpha reliability was 0.74.
- A pilot study was conducted for 30 children and their mothers from another setting (they were not including in the study). The revised form was used in this study.
- Every mother was interviewed individually in previously mentioned settings when she came for follow up or immunization of their infants.
- The children's weight and length were measured and recorded :

(a) Weight – was measured for each child using a baby beam scale. The weight was recorded based on the formula of normal weight = age in years×2+8 [(24)]

(b) Length – The recumbent (crown-heel) length was measured, using a wooden length board. The infant was laid on the examination table with this head positioned firmly against the fixed head board. The knees were stretched. Length =age in years ×5+80[(24)]

Hemoglobin estimation was done by lab technicians {a sample of blood from finger tip was collected from children by using a sterilized blood lancet).

The data were collected during the period of three months (January 2011 to march 2011)

The collected data were coded, analyzed, tabulated and presented in percentage, mean, standard deviation and chi-square test & mann-whitnenty Z test at 5% level of significance by using the SPSS version 10 for windows.

A scoring system for mother's practices regarding weaning & feeding practices was adopted from the assessment sheet. The correct answers were predetermined according to the literature and based on Egyptian food table practices for children feeding. The dietary practices were answered either by "Good practice for correct time & types of food consumption, Fair practices for incorrect time or types of food consumption and Poor or dangerous for incorrect both time &types of food consumption". If (Good) answer it scored "two"; if (Fair) answer, it scored "one" and if (Poor) it scored "zero". All items were summed together to form a score as follows: The mothers feeding practices (by questioning them) total score equaled "26" -Good practices had a score "20-26".- Fair practices had a score "13- 19".- Poor practices had a score "< 13".

Calculation of percent predicted weight and length:

Percent predicted weight and length of the studied infants were calculated by dividing actual weight or length by their predicted weight or length multiplied by 100.

3. Results

The prevalence of iron deficiency anemia is 77% among children under2 years.

Table (1) presents parent's socio-demographic characteristics among normal and anemic infants &/young children. The vast majority 63% of not anemic and 67.2% of anemic infants had mother's aged between 20 and 30 years old. No Statistically significant difference was found between two groups as; (X^2= 4.47, P= 0.107). The table also shows that the vast majority of mothers of not anemic (31.5%) and anemic infants (28.6%) had secondary level of education. Statistically significant difference was found between two groups as (X^2= 21.8, P< 0.0001). About 17.4% of not anemic and 15.6% of anemic infants had working mothers, and the remaining 82.6% of not anemic and 84.4% of anemic infants had not working mothers (X^2=0.17, P=0.68). On the other hand, the vast majority, 38% of not anemic and 29.9% of anemic infants fathers' level of education was the preparatory one. Statistically significant difference was found between two groups (X^2= 20.054, P< 0.0001). All fathers (100%) of not anemic and 98.1% of anemic infants were working fathers.

No Statistically significant difference was found between two groups (X^2= 1.82, P=0.18). Table (1) also presents birth order of infants which was ranged from 1 to 5 of not anemic with a mean 2.3 ± 1.1SD, and 1-5 of anemic with a mean 2.1±1.1SD (Z=1.81, P=0.07). Monthly income of most parents (73.7%) of anemic infants was not enough compared to only 19.6% of not anemic parents. No Statistically significant difference was found between two groups as (X^2= 1.751, P=0.417). More than three quarters (77.2%) and (61%) of not anemic and anemic infants reside urban areas.

Table (2) presents personal and medical characteristics among normal and anemic infants. The table shows that male infants constituted more than half (54.3%) of not anemic and 50.3% of anemic infants, while, female infants constituted 45.7% of not anemic infants, and 49.7% of anemic infants. Minimum to maximum age was 12-21months for not anemic infants with a mean age of 15.3±3.1 and 12-24 months for anemic infants with a mean of 17±2.6. (Z=4.631, P<0.0001) Minimum to maximum hemoglobin level of infants was 11.0-12.6 gm/dl with mean±SD 11.4±0.4 for not anemic infants, and 7.5-10.9 gm/dl with mean±SD 9.9±0.6 for anemic children. Statistically significant difference was found between two groups (Z=14.667, P<0.0001). Presence of previous health problems were found in 22.8% of not anemic infants and in17.9% for anemic infants (X^2=1.14, P=0.286). Anemia was the most common health problem 14.1% for not anemic, and 9.4% for anemic infants followed by GIT, congenital anomalies & respiratory problems (respectively 5.4%, 2.2% & 1.1%) for not anemic, and (respectively 4.5%, 0.3% & 3.6%) for anemic infants. The table also shows that only 37% of not anemic, and 26.3% of anemic infants took medication (appetizers, vitamins, calcium). Statistically significant difference was found between two groups (X^2=3.928, P=0.047). Regarding, iron intake, it was observed that, 67.4% % of not anemic and only 20.5% anemic infants were not taking iron. Statistically significant difference was found between two groups (X^2=5.86, P=0.015).

Table (3) shows anthropometric measures among normal and anemic infants &/ young children. The table shows minimum to maximum weight was 7 to 15 kg with a mean±SD 10.6±1.7 for not anemic infants and 6.5 to 15 kg with mean±SD 10.2±1 for anemic infants (Z=2.076, P=0.038). Height was 60 to 87 cm with a mean±SD 76.8±6.1 for not anemic, and 59 to 88 cm with a mean±SD 75.9±5.7 for anemic infants (Z=1.416, P=0.157). The percentage of predicted weight was 55.6 to 133.3 with a mean±SD 88.4±15.6 for not anemic, and 48.2 to 128.6 with a mean±SD 79.3±14.4. Statistically significant difference was found between two groups (Z=4.883, P=0.001). Finally, the percentage of predicted height was 68.6 to 99.4 with a mean±SD 88.9±6.5for not anemic and 68.3 to 100.6 with a mean±SD 87.1±6.3 for anemic infants. Statistically significant difference was found between two groups (Z=2.38, P=0.018).

Table (4) shows feeding practices among normal and anemic infants. The table shows that the vast majority (75%) of not anemic, and (62.3%) of anemic infants were breastfed. statistically significant difference was found between two groups (X^2=5.01, P=0.025). Most (60.9 %) of not anemic, and (60.4%) of anemic infants started breast feeding within 12 hours, followed by immediately after delivery and after 24 hours [respectively, 21.7%& 17.4% for not anemic & 19.3% & 20.3% for anemic infants]. No statically significant differences was found between two groups (X^2=0.38, P=0.828). Means of duration of breast feeding [Age of stop breast feeding (months)] were 19.0±3.7 for not anemic and 17.7±4.2 for anemic infants. Statistically significant difference was found between two groups (P=0.007) .On the other hand, minimum to maximum age of introducing food was 1 to 10 with mean±SD of 5.7±1.8 for both anemic and not anemic infants (Z=0.377, P=0.706). Regarding problems occurred as a result of weaning& feeding; this table portrays that the majority of anemic (90.9%) and not anemic (95.7%) children had no problems. Concerning feeding of children during illness it appears from this tables that the majority (87.0%) of not anemic children was breast feeding and soft diet compared to only 26.3% of anemic children . No significance was noticed [X2=2.819, P=0.093].

The 24 hour food recall is a simple tool for dietary assessment. Interviewees were asked to recall the exact contents of their child's diet over the previous 24 hours, using standardised cups, bowls and spoons for estimation of food volume. The nutritional content of each dish was then calculated using standardized tables[3,25]. Table (5) shows the distribution of the mothers of anemic and not anemic children by their practices of complementary feeding during the last 24 hours. (24hours recall of food quality). Animal milk consumption was reported by 71.4% of anemic children and 70.4% of not anemic, yogurt and cheese by 66.2% of the anemic children and 66.3%. of not anemic. Animal source of proteins was consumed by 71.7% of not anemic sample compared to 30.2% of anemic children. Regarding eggs, they were consumed nearly by the same percent among anemic and not anemic sample (78.6% and 79.3% respectively). Among the plant source of proteins (beans- legumes), they contributed to the diets of 79.9% of the anemic ample and 80.4% of not anemic. The table also, reveals that the majority of

mothers of anemic children (82.5%) and of not anemic (83.7%) reported giving carbohydrates (any food prepared from grains, maize, and flour). Dark green leafy vegetables were used only by 6.5% of the anemic sample, and 38.3% of them (anemic children) consumed any raw fruits juice.

Table (6) presents weaning practices among normal and anemic infants. The good weaning practices which means that correct time of introducing foods and correct types of food according to the age of infants was observed in half (50%) of the not anemic and for 11.7% of anemic infants. Significant difference was observed between two groups (X^2=6.69, P=0.035).

Table (7) shows feeding practices which affect iron absorption among normal and anemic infants &/young children. Regarding, receiving iron rich foods (Recommended Daily Allowance of iron) (as red meat, liver, apple) it was reported by 78.3% of mothers of not anemic infants and by 6.2% of anemic infants. Statistically significant difference was found between two groups (X^2=19.41, P=0.0001). The table also, shows food increases absorption of iron such as food rich in vitamin C as orange, lemon, guava juices was reported by the majority (92.4%) of not anemic and only 6.5% in anemic infants. No statistically significant difference was found between two groups (P=0.708). And finally, food decreases absorption of iron (as tea, phytate, calcium) was received by the majority (94.2%) of anemic infants. Compared to only 13.1% of not anemic, there was a significant differences between two groups (X^2=5.29, P=0.021).

Table (1): Parents socio-demographic characteristics among normal and anemic infants and young children.

Parents socio-demographic characteristics	Studied infants (n=400)				Significance
	Not anemic (n=92)		Anemic (n=308)		
	No.	%	No.	%	
Mother age (years)					
Less than 20	5	5.4	32	10.4	**X^2=4.47**
20-	58	63.0	207	67.2	**P=0.107**
30-	29	31.6	69	22.4	
Level of mother education					
Illiterate	8	8.7	99	32.1	
Read and write	15	16.3	39	12.7	**X^2=21.775**
Preparatory education	22	23.9	46	14.9	**P<0.0001***
Secondary education	29	31.5	88	28.6	
University education	18	19.6	36	11.7	
Mother occupation					
Working	16	17.4	48	15.6	**X^2=0.172**
Not working	76	82.6	260	84.4	**P=0.678**
Level of father education					
Illiterate	8	8.7	81	26.3	
Read and write	6	6.5	42	13.6	**X^2=20.054**
Preparatory education	35	38.0	92	29.9	**P<0.0001***
Secondary education	27	29.4	59	19.2	
University education	16	17.4	34	11.0	
Father occupation					
Working	92	100.0	302	98.1	**X^2=1.82**
Not working	0	0.0	6	1.9	**P=0.177**
Birth order					
Min-Max	1-5		1-5		**Z=1.807**
Mean±SD	2.3±1.1		2.1±1.1		**P=0.071**
Monthly income					
Not enough	18	19.6	227	73.7	**X^2=1.751**
Enough	69	75.0	72	23.4	**P=0.417**
More than enough	5	5.4	9	2.9	
Residence					
Rural	21	22.8	120	39.0	**X^2=8.08**
Urban	71	77.2	188	61.0	**P=0.004***

X^2: Chi-Square test Z: Mann Whitney test *significant at P≤0.05

Table (2): Personal and medical characteristics among normal and anemic infants and young children.

Personal and medical characteristics	Studied children (n=400)	Significance

of infants	Not anemic (n=92)		Anemic (n=308)		
	No.	%	No.	%	
Gender					
Male	50	54.3	155	50.3	**X^2=0.459**
Female	42	45.7	153	49.7	**P=0.498**
Age (months)					
12-	47	51.1	68	22.1	
16-	41	44.6	229	74.3	
20-	4	4.3	11	3.6	
Min-Max	12-21		12-24		**Z=4.631**
Mean±SD	15.3±3.1		17.0±2.6		**P<0.0001***
Hemoglobin level (gm/dl)					
Min-Max	11.0-12.6		7.5-10.9		**Z=14.667**
Mean±SD	11.4±0.4		9.9±0.6		**P<0.0001***
Previous health problems					
None	71	77.2	253	82.1	**X^2=1.14**
Present	21	22.8	55	17.9	**P=0.286**
Respiratory problems	1	1.1	11	3.6	
GIT(diarrheal	5	5.4	14	4.5	
Anemia	13	14.1	29	9.4	
Infectious disease	2	2.2	1	0.3	
Medication intake					
Yes (appetizer, Vit.,& calcium)	34	37.0	81	26.3	**X^2=3.928**
No	58	63.0	227	73.7	**P=0.047***
Iron intake					
Yes	30	32.6	245	79.5	**X^2=5.86**
No	62	67.4	63	20.5	**P=0.015***

X^2: Chi-Square test Z: Mann Whitney test *significant at P≤0.05

Table (3): Anthropometric measures among normal and anemic infants &/ young children.

Anthropometric measures of infants	Studied children (n=400)				Significance
	Not anemic (n=92)		Anemic (n=308)		
	No.	%	No.	%	
Weight (Kg)					
Min-Max	7-15		6.5-15		**Z=2.076**
Mean±SD	10.6±1.7		10.2±1.7		**P=0.038***
Height (cm)					
Min-Max	60-87		59-88		**Z=1.416**
Mean±SD	76.8±6.1		75.9±5.7		**P=0.157**
Percentage of predicted weight					
Min-Max	55.6-133.3		48.2-128.6		**Z=4.883**
Mean±SD	88.4±15.6		79.3±14.4		**P<0.0001***
Percentage of predicted height					
Min-Max	68.6-99.4		68.3-100.6		**t=2.38**
Mean±SD	88.9±6.5		87.1±6.3		**P=0.018***

Z: Mann Whitney test t: T-test *significant at P≤0.05

Table (4): Feeding practices among normal and anemic infants &/ young children.

Feeding practices	Studied children (n=400)	Chi-Square test

	Not anemic (n=92)		Anemic (n=308)		
	No.	%	No.	%	
Breast feeding					
Yes	69	75.0	192	62.3	**X^2=5.01**
No	23	25.0	116	37.7	**P=0.025***
Start of breast feeding	(n=69)		(n=192)		
Immediately after delivery	15	21.7	37	19.3	X^2=0.38
Within 12 hours	42	60.9	116	60.4	P=0.828
After 24 hours	12	17.4	39	20.3	
Duration of breast feeding [(Age of stop breast feeding (months)]					
Min-Max	**10-24**		**6-24**		**X2 Z=2.693**
Mean±SD	**19.0±3.7**		**17.7±4.2**		**P=0.007***
Age of introducing food (months)					
Less than 2	2	2.2	12	3.9	
2-	9	9.8	23	7.5	
4-	26	28.3	77	25.0	
6-	43	46.7	153	49.7	
8-	12	13.0	43	13.9	
Min-Max	1-10		1-10		Z=0.377
Mean±SD	5.7±1.7		5.7±1.8		P=0.706
Problems occurred during feeding					
Yes diarrhea	4	4.3	28	9.1	**X^2=2.17**
No	88	95.7	280	90.9	**P=0.141**
Feeding of children during illness					
- Breast feeding +fluid, vegetables soup, soft food.	80	87.0	81	26.3	**X^2=2.819**
-Breast feeding only.	2	2.2	200	64.9	**P=0.093**
-Usual feeding.	10	10.8	27	8.8	

*significant at P≤0.05

Table (5): Feeding practices among normal and anemic infants &/young children.

Food consumed in the last 24 hours *	**Studied children (n=400)**							
	Anemic (n=308)				**Not anemic (n=92)**			
	No		**Yes**		**No**		**Yes**	
	No.	%	No.	%	No.	%	No.	%
Animal milk	88	28.6	220	71.4	27	29.3	65	70.7
Cheese-yogurt	104	33.8	204	66.2	31	33.7	61	66.3
Fruit juice	260	84.4	48	15.6	15	16.3	77	83.7
Animal source of proteins (meat, poultry, fish, liver)	215	69.8	93	30.2	26	28.3	66	71.7
Eggs	66	21.4	242	78.6	19	20.7	73	79.3
Plant source of proteins (beans, lentils, legumes)	62	20.1	246	79.9	18	19.6	74	80.4
Carbohydrates (bread, rice, macaroni, cakes, biscuits, cerelac,….)	54	17.5	254	82.5	15	16.3	77	83.7
Red-orange or yellow vegetables	251	81.5	57	18.5	12	13.0	80	87.0
Dark green vegetables (spinach, kale, amaranth leaves)	288	93.5	20	6.5	7	7.6	85	92.4
Any raw fruits	190	61.7	118	38.3	32	34.8	60	65.2

***according to standardized servings & Recommended Daily Allowance (RDA) of food groups according to children's age** [3,25].

Table (6): Weaning practices among normal and anemic infants and/ young children.

Weaning practices	**Studied infants (n=400)**	**Significance**

	Not anemic (n=92)		Anemic (n=308)		
	No.	%	No.	%	
Good(Correct time and type)	**46**	**50.0**	**36**	**11.7**	
Fair (Correct type only)	**20**	**21.7**	**158**	**51.3**	
Poor (Incorrect time and type)	**26**	**28.3**	**114**	**37.0**	

- X^2: Chi-Square test *significant at P≤0.05
- According to standardized servings & Recommended Daily Allowance (RDA) of food groups according to children's age [3,25].

Table (7): Feeding practices affect iron absorption among normal and anemic children.

Feeding practices affect iron absorption	Studied children (n=400)				Chi-Square test
	Not anemic (n=92)		Anemic (n=308)		
	No.	%	No.	%	
Food increase absorption of iron					
Yes	85	92.4	20	6.5	**X^2=0.14**
No	7	7.6	288	93.5	**P=0.708**
Receive (RDA of iron)iron rich food					
Yes	72	78.3	19	6.2	**X^2=19.41**
No	20	21.7	289	93.8	**P<0.0001***
Food decrease absorption of iron					
Yes	12	13.1	290	94.2	**X^2=5.29**
No	80	86.9	18	5.8	**P=0.021***

RDA of iron = Recommended Daily Allowance of iron according to the report of National Nutritional Institute 2004[25]. *significant at P≤0.05

4. Discussion

The development of optimal nutrition among children during the first two years of life is continuum that results from a series of breast feeding and complementary feeding practices and behaviors as well as access to the appropriate mix of food [26]. From the age of six months onwards when breast milk alone is no longer sufficient to meet all nutritional requirements, infants enter a particularly vulnerable period of complementary feeding during which they make a gradual transition to eat ordinary family food [27].The incidence of malnutrition rises sharply during the period from 6 to 18 months of age in most countries and the deficits acquired at this age are difficult to compensate for later in childhood [28,29].

Since nutritional anemia, especially iron deficiency anemia, is by far the most prevalent and since it constitutes a real public health challenge amenable to preventive interventions, more emphasis should be given to this type of anemia at the national or international level [30,31]. So, the aim of this research is to investigate the contributing factors of iron deficiency anemia among children under two years attending at family health centers in Alexandria.

The present study found that the prevalence of anemia is 77% among children under two years. This is in agreement with a study conducted in Brasilia at 2002 which found a high prevalence (63%) of anemia associated with age, weight and height deficit [32]. The etiology of iron deficiency anemia is multi factorial. The dietary inadequacy of absorbable iron and other relevant nutrition, is the main immediate cause of iron deficiency anemia [33].

The results illustrated in table (1) indicated that the incidence of anemia among infant of mothers less than 20 years was 10.4%. On the other hand, the Egypt Demographic Health Survey 2005, found that the incidence of anemia among infants of mothers less than 20 years was 65.9% [34].

The present study indicated that about one third of the illiterate mothers had anemic infants more than the highly educated one (11.7%). Also the finding of Egypt Demographic Health Survey 2005, found that the anemia among infant of illiterate mother was 55.5% [34] .This results is in accordance with the study done by Hussein, 2006 who found that with lower levels of education of the family, there is lowered quantity and quality of the diet [26]. Anemia among infant living in urban area was greater than to those living at rural area (61%), these results are opposing to the study done by Pasricha et al. (2010) who found that, anemia among rural Indian infants was (72%)[35]. This may be returned to that the majority of urban mothers in our country are working mothers and they leave their infants after only three months of age at nursery centers for long time of the day which in turn affect on their feeding practices. Whereas, children living in rural areas are exclusively

breast fed for a longer time and completely weaned at a later age, compared to urban children. These differences suggest that among rural and urban mothers, traditional patterns remain influential beside the availability of weaning foods, either actual or in terms of cost is less [36]. Studies have found maternal educational level to be positively related to better nutritional status of children. Maternal education is usually connected with greater use of health services, lower fertility and more centered care giving behaviors [37].

Regarding income, it denotes (from table 1) that, there was no significant difference between anemic and not anemic children as more than enough income constituted only 2.9% and 5.4% respectively. This finding is supported by the report of world bank atlas method of Country classification in which Egypt is classified as lower middle income country (gross national income per capita ranged from $ 976 to $ 3.855) based on 2008 gross national income per Capita [38,39] .

In Egypt iron supplementation is a routine activity of family health centers and MCH. It follows the WHO schedule for daily use of iron "60-120 mg given to pregnant mothers, as one tablet for mild anemia, two tablets for moderate to severe anemia, in addition to 2mg/kg body weight given to infant and Preschool for 6 months [31].The current study revealed that, there was a significant association between iron intake among anemic and not anemic children (table 2

It is apparent from the findings of this study regarding the anthropometrics measurement that there was a significant difference in weight and height among anemic and not anemic child table (3). This is in agreement with a research done, in Brasilia by Brunken et al. (2002) who found that the prevalence of malnutrition (Z score < -2) was 0.8% according to weight-for-height, 5.0% according to weight-for-age, and 10.3% according to height-for-age [32].

Throughout infancy, the weaning period is particularly critical. At the same time as breast milk with its nutritional and immunological properties gradually diminishes with age of the child, the weaning food is generally inadequate in poor societies. The main concerns with weaning food lie not only on its nutritional quality, but also on the risk of contamination and its effect on the household budget [40,41].

Regarding the practice of complementary feeding, finding of this study showed that only 2.2 % of not anemic & 3.9% of anemic children of the present sample started in the first month, while 9.8% and 7.5% of not anemic and anemic children respectively started in second & third months (table 4), which is lower than corresponding figures in Ismailia, Egypt (2004) where they were 19.2 and 41% respectively. The reported figures for Riyadh, Kingdom Saudi Arabia (2004) were 11.2% and 22.4% respectively[42].In a study done in Latin American countries it was found that early introduction of complementary feeding is associated with lower maternal educational level [43].

WHO recommended that during children' illness fluid intake should be increased, including more frequent breastfeeding, and encouraging the child to eat soft, varied, appetizing, favorite foods. Moreover, the IMCI mother's care, recommended the same principles of child feeding during illness [23,44] . These principles are in agreement with the results of the present study, as the majority of not anemic children were following the recommendation of WHO regarding feeding of infant during illness (table 4) and with another study done by Hussein, 2006 as the study found that more than three quarter of the mothers sample reported that they didn't encourage their children to eat if they didn't want to but give them more fluids [26].

Although the incidence of intestinal diseases is on the decline, the intestinal infection exist allover Egypt. Diarrheal diseases also present one of the most important health problems among children as, insufficient refrigeration, presence of flies and improper disposal of wastes and refuse keep the incidence of this disease high [45].In this study, anemic child suffering from problem during feeding mainly diarrhea, more than the not anemic one (table IV) Child nutritional problems due to faulty feeding practice is widely observed in Egypt [46].

Animal protein is a sensitive indicator of quality of diet on which depends the bioavailability of iron[24].The finding drawn from the present study indicated that animal source of protein eaten only by one third of the anemic study sample, while plant source eaten by more than two third of the study sample (table 5). That also indicated a high prevalence of low intakes of Vitamin B12 & iron. Since vitamin B12 is present only in animal products, so vitamin deficiencies lead to a lot of risks [3]. In all cases the ever increasing food prices in relation to incomes are resulting in reduction in quality and quantity of diets which may explain why the nutritional status is not improving in spite of the government intervention in this regard. The results of the present study is in agreement with the study done by Utomo et al. (2000) which found that eggs were the most common sources of protein, while meat and fish were rarely consumed by young children [47].

Since weaning is a potential source of physical and psychological stress to the infant, it should be done gradually owing to the reduction in food

choices, it requires considerable knowledge and experience to ensure that the transition from nutritionally adequate mother's milk to solid food is achieved successfully .This has often proved to be difficult [18]. Finding drawn from the present study showed that only 11.7% of the mother's of anemic children had good practice regarding principles of weaning (correct time of introducing different types of foods (table 6). This means that the majority of anemic children didn't receive recommended daily allowance of different types of food groups. This result is in accordance with study done by Arimond and Ruel (2004), at Ethiopia which showed that in all age groups under nourished children received less nutrient than non under nourished children [48]. This may be attributed to the majority of parents of anemic children were illiterate with low income. While with increasing education, women have more power within the family to allocate resources on food and other expenditure for their children's health and welfare [24]. These results are opposing to the study done by Mohammed (2002) who found that most mothers either breast feeding or artificially feeds, had very high awareness regarding feeding & weaning practices [49].

It is surprising to find that only 6.5% of anemic children received iron rich food according to their RDA compared to 92.4% of not anemic children (Table 7). This result is opposite to the result reported by a research done by Rizkalla N.1999, which revealed that about one third of preschoolers get less than 90% of their RDA of iron [50]. This may be attributed to, in Egypt "baladi" or unrefined bread (85% extraction) is the most popular [51]. On one hand the presence of phytates and bran in bread inhibits the absorption of dietary iron from plant origin. On the other hand tea with biscuit is a wide spread habit among mothers of anemic children. Tea contains tannic acid which inhabits the absorption of dietary iron from plant origin [52]. As most of the dietary iron consumed by children is of plant origin which may particularly explain the wide spread prevalence of iron deficiency anemia.

Conclusion and Recommendations

Based on the findings of the present study, it could be concluded that, the prevalence of anemia was 77% among children under 2 years. About half of not anemic mothers' practices regarding weaning practices were scored as good (correct) practices (which means correct time &types of food) compared to only one tense of anemic mothers' practices. Moreover, the study concluded that the socio economic factors (as level of father & mother education, residence), faulty weaning and feeding practices are the main contributing factors of anemia among children under 2 years . It is a serious concern that, iron inadequately is severe among children's diet.

It is recommended that all efforts should be directed to ensure effectiveness and efficiency of intervention programs that target to pregnant, lactating women and preschool children to ensure their health and wellbeing. Also, encourage the private sector and media to have an active role especially in information, education and communication activities such as; increasing health awareness among population regarding exclusive breast feeding for 4-6 months for maintenance of the infant's iron status and all families should be empowered to increase the intake of bioavailability of iron-source foods and change their cooking and eating habits.

Establish dietary guidelines for healthy eating taking into consideration consumption of foods that are rich in iron, and reducing intake of that containing constituents that inhibit or interfere with iron absorption

In-service training programs to midwives and nurses regarding save delivery and stressed on the importance of delaying ligation of the umbilical cord until it has stopped pulsating (30-60 seconds),which is important in improving iron stores in infants born to anemic mothers.

Corresponding author
Amina Ahmed Mohamed
Community Health Nursing Department, Faculty of nursing, Alexandria University
dr.amiina@hotmail.com

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Study of Serum Tumor Necrosis Factor Alpha and Interleukin 6 in Type 2 Diabetic Patients with Albuminuria

Ahmed Zahran[1], Enas S. Essa[2] Waleed F. Abd Elazeem [2]

[1]Internal Medicine Department, Nephrology Unite and [2]Clinical Pathology Department Faculty of Medicine, Menoufia University, Egypt
ahmed173@hotmail.com

Abstract:Background: Chronic kidney disease (CKD) is one of the major complications of type 2 diabetes and is the leading cause of end stage renal disease (ESRD). There are Growing evidences indicating that chronic low-grade inflammatory response is a recognized factor in the pathogenesis of development and progression of diabetic renal injury. The aim of this study was to analyze the relationship between inflammatory markers tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) with urinary albumin excretion (UAE) as a marker of renal injury. **Methods:** A total of 73 type 2 diabetic patients were divided into three groups according to urinary albumin excretion, normoalbuminuria, microalbuminuria and macroalbuminuria In addition, 10 apparently healthy subjects were included as control group. TNF-α, IL-6, Glycated hemoglobin (HbA1c) and creatinine were measured in all population. Urinary albumin excretion was measured by morning spot sample albumin / creatinine ratio (ACR). **Results:** Levels of TNF-α and IL-6 were found to differ significantly among studied groups. Both markers showed significant positive correlation with duration of diabetes, Albumin creatinine ratio and glycated hemoglobin and showed significant negative correlation with estimated glomerular filtration rate (eGFR), however TNF-α showed a better correlation with these variables when compared with IL-6. Stepwise regression analysis demonstrated that TNF-α is the independent predictors for ACR in total population with adjusted R^2 0.512 and P value less than 0.01. **Conclusion:** TNF-α and IL-6 are higher in type 2 diabetic patients with albuminuria and correlate well with the severity of albuminuria, however TNF-α was found to be a predictor of ACR suggesting the possible role of TNF-α in pathogenesis and progression of renal affection in type 2 diabetic patients.

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Keywords: Diabetic nephropathy; Albuminuria; tumor necrosis factor alpha and interleukin 6.

1. Introduction

The global diabetes burden is predicted to rise to 366 million by 2030 and would present itself as a major health challenge (1). Chronic kidney disease (CKD) is one of the major complications of type 2 diabetes and is the leading cause of end stage renal disease (ESRD) (2).The literatures are replete with studies about the involvement of innate immune system and low grade inflammation in pathogenesis of DM. and there is now clear evidence that inflammatory markers, acute-phase reactants and proinflammatory cytokines are strongly associated with the risk of developing type 2 diabetes (3-10). Based on this perspective several studies were conducted to address the role of this inflammatory cytokines in the development of diabetic complications. The exact mechanisms leading to the development and progression of renal damage in diabetes are not yet completely known. Growing evidence indicates that activation of innate immunity with the development of a chronic low-grade inflammatory response is a recognized factor in the pathogenesis of this disease (11). Cytokines are a group of pharmacologically active, low molecular weight polypeptides that possess autocrine, paracrine, and juxtacrine effects. These molecules cluster into several classes (i.e. interleukins, tumor necrosis factors, interferons, colony-stimulating factors, transforming growth factors and chemokines) (12). The cytokine TNF-α is a well-known member of the TNF superfamily consisting of 157 amino-acid peptide produced mainly by monocytes, macrophages, B and T lymphocytes. TNF signals through two distinct receptors TNFR1 and TNFR2 thereby controlling expression of cytokines, immune receptors, proteases, growth factors and cell cycle genes which in turn regulate inflammation, survival, apoptosis, cell migration, proliferation and differentiation (13). Human IL-6 composed of 184 amino acid and produced by various types of lymphoid and non-lymphoid cells, such as T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangium cells, and several tumor cells (14). Different inflammatory molecules, including pro-inflammatory cytokines such as TNF-α and IL-6 play a critical role in the development of micro vascular diabetic complications, including nephropathy (15-16). The objective of the present study is to examine the relation between inflammatory cytokines TNF-α and IL-6 with urinary albumin excretion (UAE) in type 2 diabetic patients in early stages of diabetic kidney disease.

2. Patients and Methods

The protocol for this study followed the ethical standards and approved by the ethical committee of our institution and all subjects gave informed consent to participate in this study. This study was conducted on 73 type 2 diabetic patients, 29 males and 44 females. In addition, 10 apparently healthy, age and gender matched, subjects were involved in this study as a control group (5 males, 5 females).

Patients with the following criteria were excluded: age over 70 years, body mass index (BMI) above 30, current acute infection or any acute illness, systemic hypertension defined as blood pressure more than 140/90, history of ischemic heart disease, cerebrovascular stroke, malignancy, dyslipidemia, hepatic disorders, smokers, hematological diseases and patients with serum creatinine more than 1.5 mg/dl.

Patients were divided according to UAE which was measured by early morning spot urine sample for albumin creatinine ratio (ACR) into 3 groups, diabetic without microalbuminuria (ACR less than 30 mg/gm), diabetic with microalbuminuria (ACR between 30 – 300 mg/gm) and diabetic with macroalbuminuria (ACR more than 300 mg/gm).

All subjects underwent full history taking and clinical examination including measuring blood pressure weight and height. Mean arterial pressure (MAP) was calculated as {(2 x diastolic blood pressure (mmHg) + systolic blood pressure (mmHg)}/3. BMI Was calculated as weight (Kg)/ {Height (m)} ^{2} GFR was estimated using Modification of Diet in Renal Disease Abbreviated Equation (MDRD):

[GFR = 186 × (serum Cr)$^{-1.154}$× (age)$^{-.203}$× (0.742 if female) × (1.210 if African American)] (17).

Laboratory assessment

Blood samples were collected by sterile venipuncture and divided into 2 parts; the first part was collected on dipotassiumethelenediamine tetra-acetic acid (EDTA) tube for glycated hemoglobin (HbA1c). The second part was transferred into another plain vacutainer tube, left to clot, then centrifuged for 10 minutes at 4000 r.p.m. and the serum obtained for determination of TNF α and IL-6 was kept frozen at -20 ºC till analysis. Early morning 10- 20 ml of midstream urine was collected for measurement of urinary albumin and creatinine for measurement of albumin creatinine ration (ACR). ACR was calculated using the following equation: ACR = Albumin mg/ d l ÷ Creatinine g/ dl

HbA1c was measured using quantitative colorimetric measurement of glycohemoglobin as percent of total hemoglobin using kits supplied by STANBIO LABORATORY, USA.

Albumin in urine was estimated by Beckman's microalbumin test kit on Synchron CX9 autoanalyser.Beckman.Urine creatinine is measured by a modified rate Jaffe method.

Serum TNF-α was determined by enzyme linked immunosorbent assay method, using IDELISA^{TM} Human TNF-α ELISA kit. It utilizes a monoclonal antibody (capture antibody) specific for human TNF α coated on a 96-well plate. Standards and samples are added to the wells, and any human TNF α present binds to the immobilized antibody. The wells are washed and biotinylated polyclonal anti-human TNF-α antibody (detection antibody) is added. After a second wash, avidin-horseradish peroxidase (avidin-HRP) is added, producing an antibody-antigen-antibody sandwich. The wells are again washed and a substrate solution is added, which produces a blue color in direct proportion to the amount of human TNF-α present in the initial sample. The stop buffer is then added to terminate the reaction. This results in a color change from blue to yellow. The wells are then read at 450 nm

Serum IL-6 was determined by enzyme linked immunosorbent assay method using AviBion Human IL-6 ELISA kit, Ani Biotech Oy, Orgenium Laboratories Business Unit, FINLAND. The assay employs an antibody specific for human IL6 coated on a 96-well plate. Standards, samples and biotinylated anti-human IL6 are pipetted into the wells and the IL6 present in a sample is captured by the antibody. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed. Following this second wash step, TMB substrate solution is added to the wells resulting in color development proportional to the amount of IL6 bound. The stop solution changes the color from blue to yellow and the intensity of the color is measured at 450 nm.

Statistical Evaluation

We used the statistical package of social signs (SPSS, version 16) to perform the analysis. Correlation between inflammatory markers (TNF-α and IL-6) with duration of diabetes, HbA1c, albuminuria and eGFR was performed by pearson correlation, one way ANOVA test or Kruskalwalis test was used as appropriate for comparison of quantitative variables among more than two independent groups. Multiple stepwise regression analysis was performed to determine the possible predictor for ACR among potential risk factors including inflammatory markers. P value ≤ 0.05 was considered significant.

3. Results

The cohort was divided according toACR into 3 groups. Group I; diabetics with normoalbuminuria, group II; diabetics with microalbuminuria and group III; diabetics with macroalbuminuria in addition to group IV; control group. Baseline characteristics and comparison of the studied groups are shown in table 1.

The groups are matched regarding age, sex, BMI and mean arterial blood pressure. Serum TNF-α and IL-6 differed significantly among the studied groups (Figure 1). Pearson's correlation coefficients (r) between serum TNF-α and Duration of diabetes mellitus, HbA1c & ACR showed a significant positive correlations with *P* value less than 0.001, while it showed a significant negative correlation with eGFR with *P* value less than 0.001.(Figure 2). IL-6 also showed significant correlations with the same parameters but with less r value (*P*< 0.01) (Figure 3). Interestingly both TNF-α and IL-6 correlated significantly positive with r value 0.521 and P value less than 0.001. Stepwise regression analysis demonstrated that TNF-α is an independent predictor for ACR in total population with adjusted R^2 0.512 and *P* value less than 0.01.

Table 1: Baseline characteristics and comparison of the studied groups.

	(Group I) Diabetics with normoalbuminuria ACR (mg/gm) 17.74 + 9.32 (n= 23)	(Group II) Diabetics with microalbuminuria ACR (mg/gm) 77.74 + 65.02 (n= 34)	(Group III) Diabetics with macroalbuminuria ACR (mg/gm) 468.69 + 57.40 (n=16)	(Group IV) Control group ACR (mg/gm) 4.40 + 4.01 (n= 10) Mean + SD	*P* value
*Age (years)	55.96 ± 6.20	57.12 ± 7.65	61.31 ± 6.42	55.10 ± 6.72	> 0.05
**Sex (M/F)	7/16 (30.4/69.6%)	16/18 (47.1/52.9%)	6/10 (37.5/62.5%)	5/5 (50/50%)	> 0.05
*Duration of D M (years)	7.17 ± 2.95	14.85 ± 5.59	21.25 ± 6.01	-	< 0.001
*MAP (mmHg)	94.78 ± 4.09	94.36 ± 4.14	93.33 ± 3.16	91.50 ± 5.58	> 0.05
*BMI	27.35 ±1.76	28.29 ± 1.50	28.42 ± 1.36	27.63 ± 1.40	> 0.05
*HbA1c	7.33 ± 1.56	9.25 ± 1.52	11.80 ± 1.55	5.57 ± 0.42	< 0.001
*eGFR (ml/min/1.73m^2)	81.96 ± 26.41	68.32 ± 18.15	56.14 ± 12.92	83.56 ± 25.40	< 0.01
*TNF-α (pg/ml)	65.56 ± 69.39	173.24 ± 84.95	312.69 ± 75.56	13.50 ± 3.31	< 0.001
*IL-6 (pg/ml)	96.52 ± 151.29	214.91 ± 165.40	346.75 ± 191.70	5.20 ± 3.36	< 0.001

*: Mean ± Standard Deviation, **: Number and percentage, M/F: Male/Female
D M: Diabetes Mellitus, MAP: Mean arterial pressure, BMI: Body mass index, HbA1C: glycated hemoglobin, ACR: Albumin creatinine ratio, eGFR: Estimated glomerular filtration rate, TNF-α: Serum tumor necrosis factor alpha, IL-6: Interleukin 6.

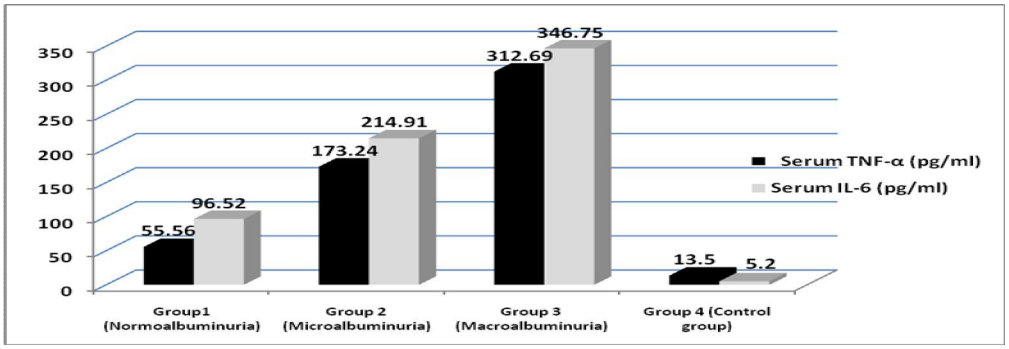
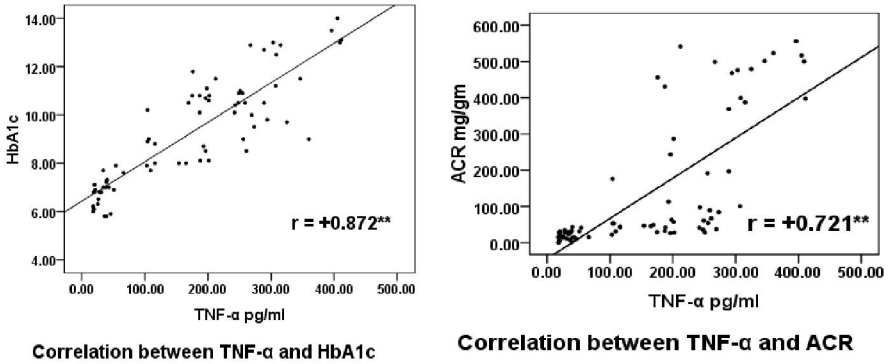
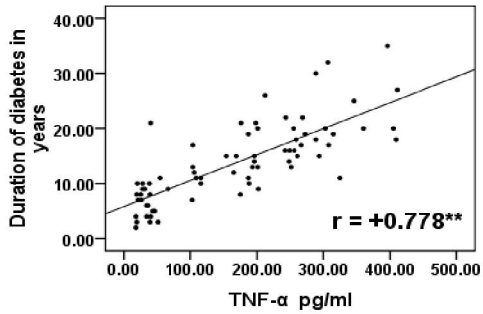


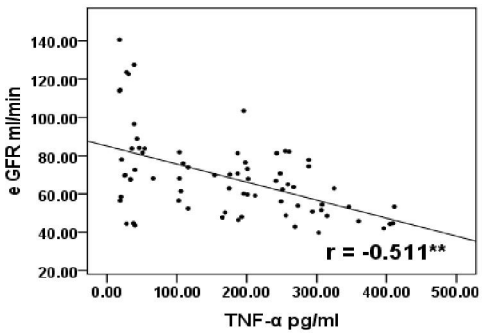
Figure 1: Comparison of TNF-α and IL-6 among the studied groups



Correlation between TNF-α and HbA1c **Correlation between TNF-α and ACR**



correlation between TNF-α and duration of diabetes in years

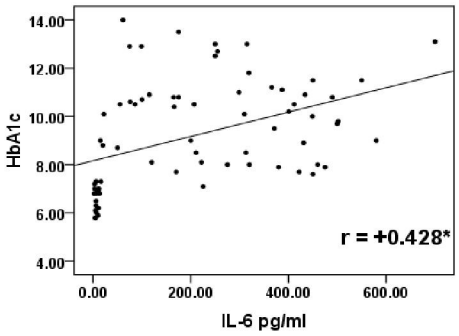


Correlation between TNF-α and e GFR

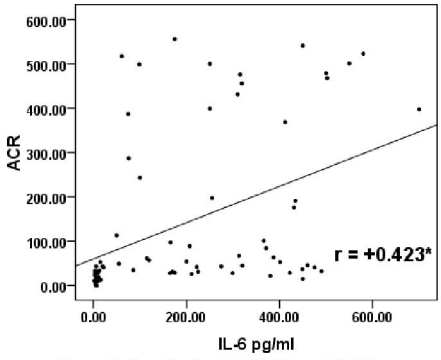
**: P value < 0.001

TNF-α: Serum tumor necrosis factor alpha,HbA1C: glycated hemoglobin, ACR: Albumin creatinine ratio e GFR: Estimated glomerular filtration rate

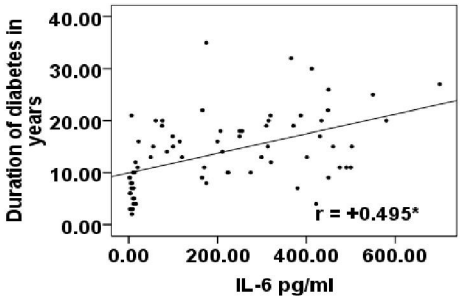
Figure 2: correlation between TNF-α with HbA1c, ACR, duration of diabetes in years and e GFR



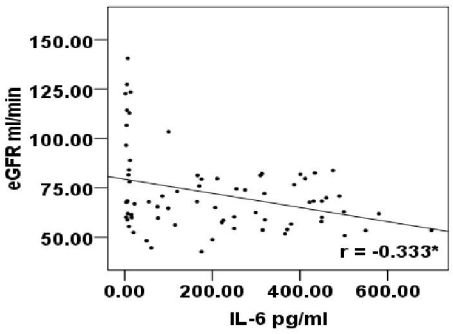
Correlation between IL-6 and HbA1c



Correlation between IL-6 and ACR



Correlation between IL-6 and duration of diabetes



Correlation between IL-6 and eGFR

*: P value < 0.01

IL-6:Serum Interleukin 6, HbA1C: glycated hemoglobin, ACR: Albumin creatinine ratio, eGFR: Estimated glomerular filtration rate,

Figure 3: correlation between IL-6 with HbA1c, ACR, duration of diabetes in years and eGFR

4. Discussion:

Diabetic nephropathy is one of the major microvascular complication of type 1 and type 2 diabetes mellitus and the leading cause of end stage renal disease. It was thought to be a result from interactions between hemodynamic and metabolic factors, however research during the past 10 years has provided insight into the etiology of diabetic nephropathy at the cellular and molecular level, and inflammation has emerged as being a key pathophysiological mechanism (18). Clinical investigations have implicated the roles of TNF-α and IL-6 in the development of diabetic nephropathy, suggesting that inhibition of those cytokines is promising remedy to ameliorate diabetic nephropathy.

In this present study we investigated the role of inflammatory cytokines TNF-α and IL-6 in relation to albuminuria in patients with type 2 diabetes. In our study we found that TNF-α and IL-6 differed significantly among studied groups. Furthermore there

were a significant correlation between each marker and HbA1c, albuminuria, eGFR & duration of diabetes. These results are consistent with other investigators who found higher levels of inflammatory cytokines among diabetic patients with albuminuria. Moriwaki et al. reported higher levels of TNF-α and IL-6 among diabetic patients without albuminuria compared to those with albuminuria and concluded that TNF-α and IL-6 may have some etiopathogenic roles in diabetic nephropathy (19). In a study included 95 diabetic patients conducted by Refaat H. et al. they found higher levels of serum and urine TNF-α in diabetic group with proteinuria and similar to our results found a good correlation between serum TNF-α and protein / creatinine ratio, HbA1c & duration of diabetes mellitus (20). Consistent with our results Maulana Azad studied IL-6 in 60 diabetic patients divided into three groups (normo, micro and macroalbuminuria) and found that IL-6 was higher in diabetic patients with macroalbuminuria compared to patient with microalbuminuria and normoalbuminuria and they also found a good correlation between IL-6 with HbA1c and urinary albumin excretion which was similar to our results but with a better r values (21). In our study stepwise regression analysis demonstrated that TNF-α was an independent predictor of urinary albumin excretion while IL-6 was not. In accordance with us Ng Dp et al. demonstrated that TNF-α system is likely to exert independent effects on albuminuria and renal function in type 2 diabetes while C reactive protein and IL-6 did not show that (22). Serum and urine TNF-α were found to be independently and significantly associated with ACR in diabetic patients (20, 23). Many investigators demonstrated a structural damage associated with inflammatory cytokines. Dalla Versa et al studied 74 type 2 diabetic patients regarding acute phase markers of inflammation including IL-6 in relation to structural kidney damage determined by mesangial fractional volume and glomerular basement membrane (GMB) width in a kidney biopsy and they found that IL-6 is significantly higher in group with increased GBM thickness and linear regression analysis demonstrated that IL-6 is one of the predictors of GBM thickness (24). Renal IL-6 expression has been related to mesangial proliferation, tubular atrophy and the intensity of interstitial infiltrates in diverse models of renal disease, suggesting a contributing role in the progression of renal disease (25). Experimental studies have demonstrated that urinary albumin excretion significantly correlates with renal cortical mRNA levels and urinary TNF-α excretion in animal models of diabetic nephropathy (26–27). Of more interest TNF-α inhibition by Infleximab reduced urinary albumin excretion in diabetic rat (28).

Conclusion

Serum TNF-α and IL-6 are elevated in diabetic patients with albuminuria and their levels are significantly higher with increasing levels of albuminuria. Both markers correlated well with ACR however TNF-α showed a better correlation than IL-6, moreover TNF-α found to be a predictor of ACR. This can suggest a possible role of TNF-α in pathogenesis and progression of renal injury in diabetic patients. Further studies are needed to confirm our finding and to study the possible role of TNF-α inhibitor in the prevention and treatment of diabetic nephropathy.

Corresponding author
Ahmed Zahran
Nephrology Unit, Internal Medicine Department Faculty of Medicine, Menoufia University, Egypt.
ahmed173@hotmail.com

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Sequential Ultrastructural Investigation of Pulp Tissue Responses to Rabbit`S Teeth Bleaching

Heba Ahmed Adawy[1], Mohamed Mohamed Fekry Khedr[2] and Mohamed Gomaa Attia-Zouair[3]

[1]Department of Oral Biology, Faculty of Dental Medicine (Girls), Al-Azhar University, Cairo, Egypt
[2]Department of Oral Medicine, Periodontology, X-Ray and Diagnosis, Faculty of Dental Medicine (Boys), Al-Azhar University, Cairo, Egypt
[3]Department of Oral and Dental Pathology, Faculty of Dental Medicine (Boys), Al-Azhar University, Cairo, Egypt
hebaadawy@yahoo.com; hebaadawy@hotmail.com

Abstract: Teeth bleaching have been documented for teeth whitening by oxidizing agent are associated with morphological alterations of the enamel surface. To investigate the sequential changes of the pulp tissue structures, using transmission electron microscopy (TEM), after enamel bleaching utilizing light enhanced bleaching, this study was performed. Rabbit's teeth were randomly divided into six groups (Gs) as follows: control group (G1) was received no treatment, while test groups included (G2: 24 hours following treatment, G3: one week following treatment, G4: two weeks following treatment, G5: one month following treatment, and G6: two months following treatment). After termination of each period, the rabbits were sacrificed, the teeth were carefully dissected and removed. Each tooth was split open with chisel and hammer and the pulp was removed with a sharp excavator, and prepared for TEM investigation. The results revealed that bleaching effects on pulp tissue elements were of various degrees cellular alterations. The observed pattern of tissue changes fell into four phases: a) mild to moderate pulp tissue injuries may be produced as an early response of teeth bleaching, b) development of localized regions of severe cellular injuries and/or necrosis, c) regression in the signs of degeneration accompanied initial recovery of cellular elements of pulp tissue, and d) complete recovery and reassuming of various tissue elements of the pulp.
[Heba Ahmed Adawy, Mohamed Mohamed Fekry Khedr and Mohamed Gomaa Attia-Zouair **Sequential ultrastructural investigation of pulp tissue responses to rabbit`s teeth bleaching.** Life Science Journal 2011; 8(4):10261033]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 129

Key Words: bleaching, pulp, TEM, ultrastructural

1. Introduction:

There are a number of methods and approaches that have been described in the literature for the bleaching of vital teeth with the common active ingredient being an oxidizing agent that acts on the organic matrix within the teeth [1-10]. Accelerating peroxide bleaching with simultaneous illumination with various sources having arrange of wave lengths and spectral power has such as halogen curing lights, plasma arc lamps, lasers and light emitting diodes [2,3,11,12]. A number of approaches of evaluating pulp tissue changes following tooth whitening exist, each with their evidence about a penetration of bleaching materials into the pulp tissue has been found [11-17]. While the central region of both the coronal and the radicular pulp contains large nerve trunks and blood vessels, the peripheral zone is circumscribed by specialized odontogenic region composed of odontoblasts, cell free zone and cell rich zone.[18] pulp inflammation could be seen in various features[19]. It has been reported that adversely affect pulp tissue structures could be found [20-22]. Weather the pulp tissues could be regenerated after exposing to harmful effects was a matter of discussion [23,24]. Ultrastructural study about cellular structure of pulp as a response of enamel bleaching, have only been previously investigated in a very limited study.[25] In this regard, the ultimate goal of the current study was to obtain more information about cellular structure of pulp as a response of rabbit`s enamel bleaching by transmission electron microscopy (TEM).

Material and Methods:

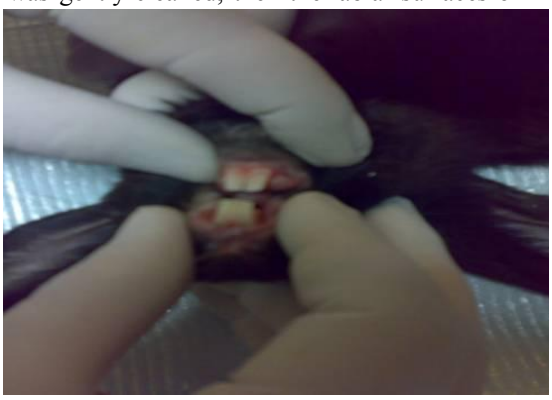
A total of forty two male New Zealand rabbits were selected from a reputable supply, the rabbit unit at the Faculty of Agriculture, Cairo University. The animals were divided into six groups, while a control group receiving no treatment (group 1: normal), the experimental groups were of five intervals (24 hours: (group 2) and one week: (group 3), two weeks: (group 4), one month: (group 5) and two months: (group 6). All animals were previously vaccinated and treated against scabies, coccidiosis and enteritis (viral hemorrhagic diseases). The animal`s weight ranged between 2.5 to 3 Kg with an age of 4-4.5 months. Two rabbits were kept in a separate cage, fed and maintained during the time of the study at a private animal housing unit. The rabbits were fed on a specific diet *ad labium* (about 150 g per day) and water. Rabbits were left for a few days before the procedure to settle down.

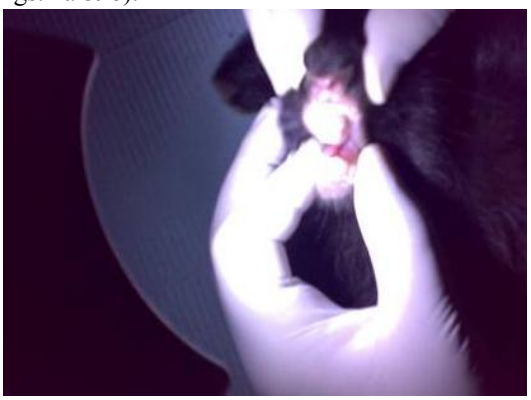
Anesthesia:

Rabbits were given an intramuscularly injection of tranquilizer Xylazine HCl (Xylaject®, The Egyptian Co. of Chemicals & Pharmaceuticalas {ADWIA}, 10th of Ramadan City, Cairo, Egypt) and anesthetic solution Ketamine HCl (Ketamar, Amoun Pharmaceutical Company, El-Obour City, Cairo, Egypt). 10%. Xylazine HCl was injected at a dose of 0.5 ml (2-3 mg / Kg) animal body weight. After ten minutes the second injection of Ketamine HCl was given at a dose of 50 mg per Kg animal body weight. This combination allowed an anesthetic duration of 20-30 minutes. The same doses were repeated when needed.

Procedures:

Prior to starting bleaching, the surface of each tooth was gently cleaned, then the labial surfaces of the rabbits` upper and lower anterior teeth were painted with a layer approximately 1 mm thick of a mixture of a 35% hydrogen peroxide solution and a bleaching agent according to the manufacture instruction. Then, exposed to a bleaching light source (Wave light, Schein, Melville, NY, USA) that contains a plasma arc lamp. The distance between the emitting tip of the light source and the tooth surface was at 15 mm. All the upper and lower incisors were exposed to the light source 3 s, then left standing for 5 min, and then exposed to the light source again for another 3 s. After they were left standing for another 2 min, each tooth was then exposed to the light source for another 3 s of final exposure. Then, the mixture that had been applied to the teeth was removed. This procedure was repeated three times (Figs. 1a & b).





Figs (1 A & B). Photomicrographs of rabbit`s teeth before and during the exposure to a bleaching light source that contains a plasma arc lamp.

Methods of Evaluation:

I. Clinical Inspection:

Animals were observed during the experimental periods; the teeth surfaces were inspected regularly before and after application of bleaching procedures.

II. Preparation of the specimens for transmission electron microscopy (TEM) examination:

Preparation and fixation:

After each period of investigation the rabbits were sacrificed, the teeth were carefully dissected and removed. The teeth were then placed in jars labeled by animal number and investigation duration. Specimens for TEM examination were prepared according to Bancroft and Stevens [26]. Each tooth was split open with chisel and hammer. The pulp was removed with a sharp excavator and rapidly immersed in a mixture of 2.5% glutaraldehyde and 10% formaldehyde (F/G solution) for 24-48 hours (Dard *et al.*) [27] . Each fixed pulp was placed on a glass slab and cut into three parts (superficial, middle and deep) using a sharp razor blade. From each part, cross sections (1 mm thick) were cut and washed several times in phosphate buffer solution with pH 7.2-7.4. The specimens were post-fixed in 1% osmium tetroxide for one hour, and washed again in phosphate buffer. The specimens were loaded in ascending concentrations of ethyl alcohol. After complete dehydration, the specimens were embedded in (EPON 812). Flat rubber moulds were used to obtain the specimen blocks. Semithin sections were cut with a diamond knife, mounted on glass slides and stained with 1.0% toluidine blue for light microscopic examination. The area of interest was selected for ultra-thin sectioning. The cut sections were stained with uranyl acetate and lead citrate to be examined with transmission electron microscope (Japan Electron Microscope 1010).

3. Results

Group 1 (control):

TEM revealed that odontoblastic layers were peripherally arranged in palisading pattern outlined the central part of the pulp. They appeared ovoid or columnar in shape with well-defined cell membrane. Odontoblastic nuclei are oval and most of them were

basally situated, while others occupied the whole cell. The nuclei showed normal chromatin distribution and dominance of euchromatin. There was distinct boundary between condensed and loose chromatin. The nuclei were surrounded by clear regular double nuclear membrane. Well developed strands of rough endoplasmic reticulum were arranged around the nuclei in the cytoplasm of odontoblasts. The electron dense strands of RER became are parallel to the long axis of the cell in the areas further from the nucleus (the supranuclear zone). Abundant mitochondria were detected in the odontoblastic cytoplasm especially in the apical region of the cells. Although they had a variety of shapes, each of them preserved its typical structure, which is a double membrane with internal cristae. Numerous junctions were seen subjacent to odontoblasts including tight junctions (Zonula occludens), belt desmosome (Zonula adherens) and gap junctions (nexus or hemidesmosome type) (Figs. 2&3). The central part of the pulp showed great numbers of fibroblasts that were immersed in a loose fibrillar connective tissue matrix. The nucleus of a fibroblast had elongated or fusiform apperance with normal chromatin. Moreover, the cytoplasmic extensions of fibroblasts were clearly apparent (Fig. 4). Vascular components of pulp tissue showed normal endotheliocytes lining the vascular lumen of blood vessel (Fig. 5).

Group 2 (24 hours-period):

Comparing this group to the previous one (control group), electron micrographs of pulpal tissue revealed minimal loss of orientation of odontoblastic cell layers associated with wide extracellular compartments. The cell membranes remained intact but the junctions between cell membranes were almost lost. Most of the odontoblasts appeared with regular nuclear membrane accompanied by slight peripheral or central chromatin clumping. Cell organelles within the cytoplasm were normally found and most of the RER and mitochondria were preserved (Fig. 6). Fibroblasts appeared normal in most of the specimens. The Blood vessels showed obvious hyperemia and widening of the lumen. Slight flattening of vascular endothelial cells was observed (Fig. 7). Some mylinated nerve endings showed hyalinization and loss of the stratification structure of myelin sheath (Fig 8).

Group 3 (one week-period):

Electron micrograph of pulpal tissue showed serious ultrastructural findings. The odontoblasts revealed an irregular nuclear membrane associated with severe peripheral and central chromatin clumping. The odontoblastic cytoplasm showed extensive vacuolization, swollen mitochondria with loss of their internal cristae and disrupted RER accompanied by much loss of ribosomes. The cell membrane was ill-defined in some samples. Complete loss of cell junctions between odontoblastic layer and wide extracellular matrix was noticed (Fig. 9). The fibroblasts showed intracytoplasmic vacuoles and irregularities in their nuclear membranes in a form of moderate peripheral and central chromatin condensation (Fig. 10). Severe hyperemia had showed up in the blood vessels of pulp tissue, increasing the width of the lumen of blood vessels. The endothelial cells were obviously affected; they showed vacuolization, irregular nuclear membranes and swollen mitochondria. Collagen degradation and hyalinization was observed throughout the ground substance (Fig. 11). Most of the mylinated nerves had lost their striations, expressed extensive hyalinization and vocalization (Fig. 12).

Group 4 (two weeks-period):

TEM revealed that the odontoblastic layers have minimal alterations. Most of the odontoblasts were shown to be preserved their ovoid regular outline of their nuclei with regular nuclear membrane and slight peripheral chromatin condensation. Swollen mitochondria were detected in the apical and paranuclear parts of the cell. RER showed moderate dilatation and loss of ribosomes. The cell membranes were preserved intact. The intercellular junctions were still lost and wide extracellular compartments were detected (Fig. 13). Few fibroblasts showed intracellular vacuolization and irregular nuclear membrane, while most of them showed normal ultrastructural findings, compared to the control group (Fig. 14). Hyperemia within blood vessels was seen to be decreased. However, the endothelial cells showed some vacuolization and irregularities in their nuclear membranes. Ultrastructural changes of mylinated nerves were less severe compared to that shown in the previous group (group 3; one week-period). Hyalinization and collagen fibrils degradation in the ground substance was still existed.

Group 5 (one month-period):

TEM, revealed that the odontoblastic layers have normal pattern on a whole although the intercellular wide compartments were still present (Fig. 15). Fibroblasts expressed nearly regular morphological features as in group 1 (Fig. 16). Most of the mylinated nerves were recovered on the whole, preserving the stratification of the myelin sheath. Hypremia within blood vessels was shown to be decreased and hyalinization of collagen fibrils was limited.

Group 6 (two months-period)

TEM examination of this group revealed nearly the same ultrastructural findings as those of group 1 , regarding the odontoblastic cell layer and the loose fibrillar connective tissue.

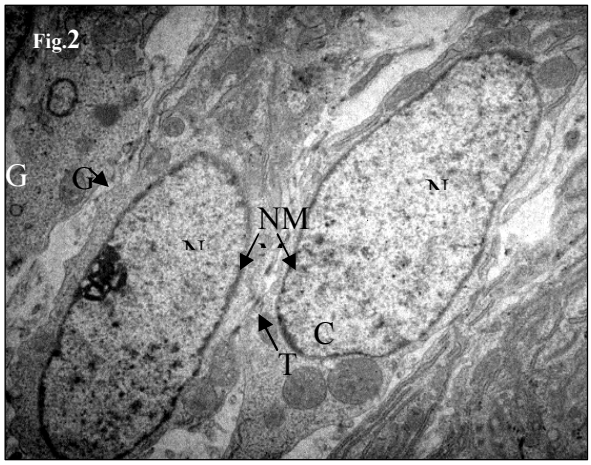


Fig. 2. Electron micrograph of group 1 showing; normal palisading odontoblasts with open faced nucleus (N), regular nuclear membrane (NM) and normal chromatin distribution (C). Notice tight (T) and gap junctions (G). X5000

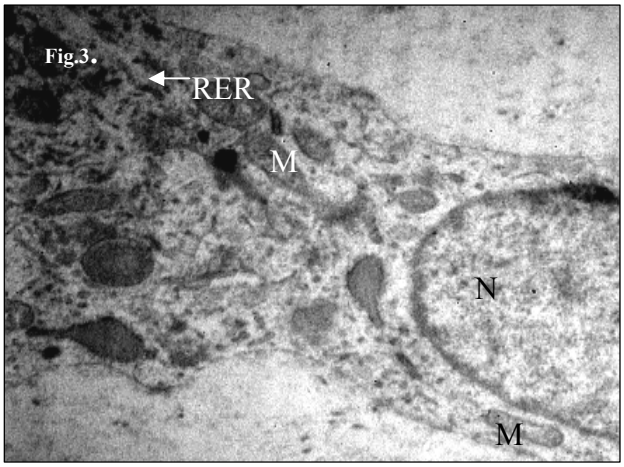


Fig. 3. Electron micrograph of mature odontoblast with abundant apical mitocondria and normal rough endoplasmic reticulum. X15000

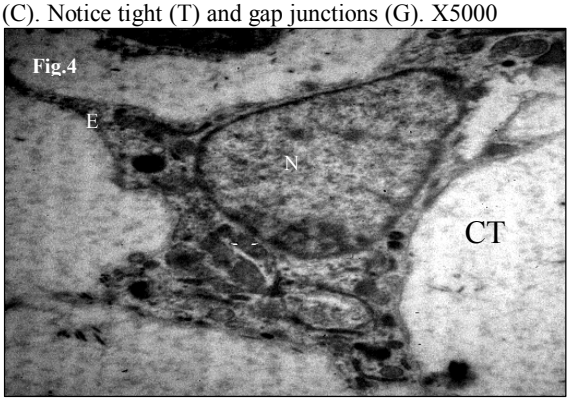


Fig. 4. Electron micrograph of normal fibroblasts immersed in a loose fibril connective tissue matrix (CT). Notice the cytoplasmic extensions of the cells (E) (X12000).

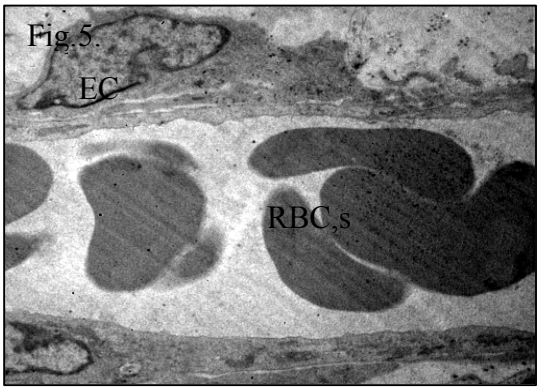


Fig. 5. Electron micrograph of normal blood vessel lined with endothelial cells (EC), and RBC,s in its lumen. (X8000).

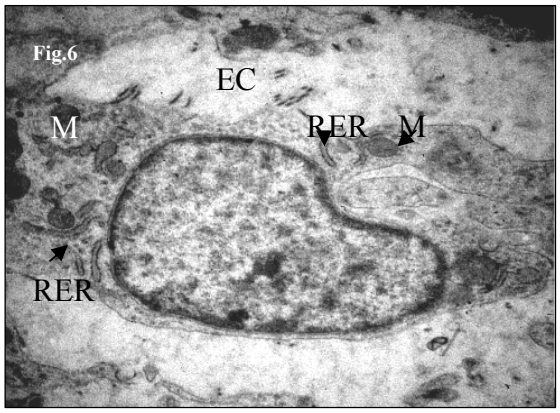


Fig 6. Electron micrograph of group 2 showing minimal loss of odontoblastic cell layer orientation with wide extracellular compartments (EC). Most of the RER and mitochondria (M) were preserved but the cell Junctions were nearly lost (X15000).

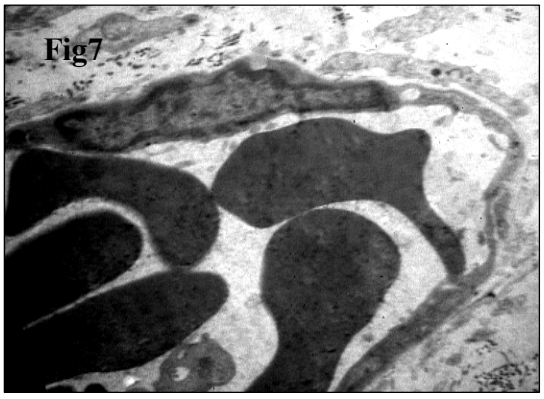


Fig. 7. Electron micrograph of group ll showing hyperemia and widening of the lumen of the blood vessels. Notice slight flattening of vascular endothelial cells (EC) (X10000,X4000).

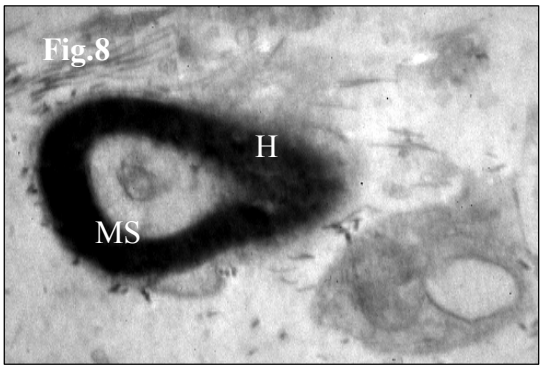


Fig. 8. Electron micrograph of group 2 showing endodontic nerve endings with moderate degree of hyalinization (H) and loss of the stratification structure of myelin sheath (MS) (X15000).

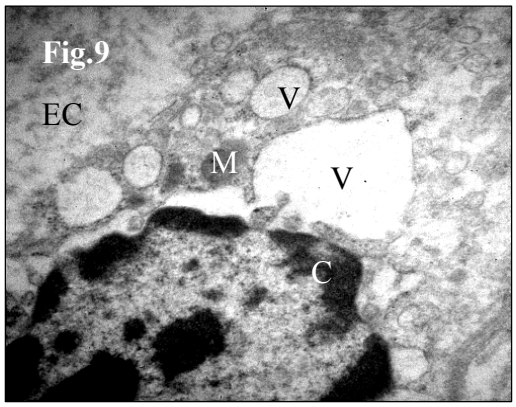


Fig. 9. Electron micrograph of group 3 showing serious ultra structural changes. The odontoblasts showed an irregular nuclear membrane (NM) with severe peripheral and central chromatin clumping (C). The cytoplasm showed severe vacuolization (V), swollen mitochondria (M). Notice the complete loss of cell junctions and wide extracellular compartments (EC), (X30000).

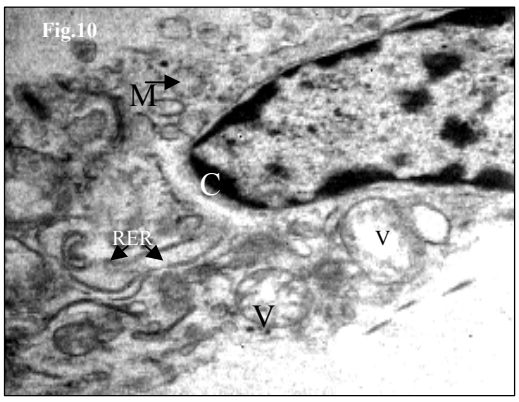


Fig. 10. Electron micrograph of group 3 showing some intracytoplasmic vacuoles (V), irregularity in the nuclear membranes(NM) , nuclear chromatin clumping (C)and swollen mitochondria (M) of fibroblasts. Notice areas of hyalinization in ground substance (H) and strands of RER (X6000, X15000).

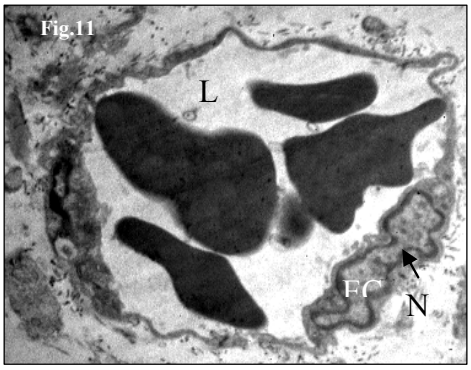


Fig. 11. Electron micrograph of group 3 showing severe hyperemia and increased width of the lumen (L) of blood vessels. The endothelial cells (EC) showed corrugated nuclear membranes (NM) (X8000).

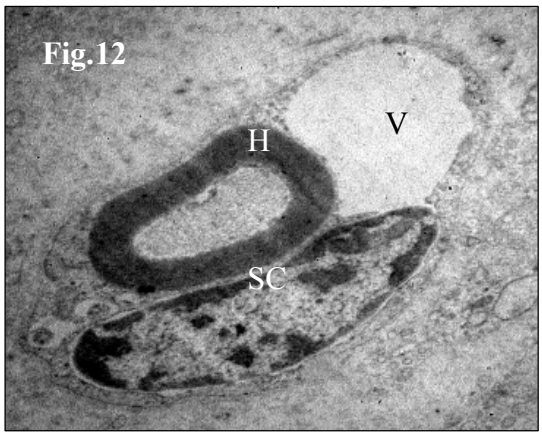


Fig. 12. Electron micrograph of group 3 showing loss of striations, hyalinization (H) and vocalization (V) of endodontic nerve endings. Notice Schwann cell (X15000).

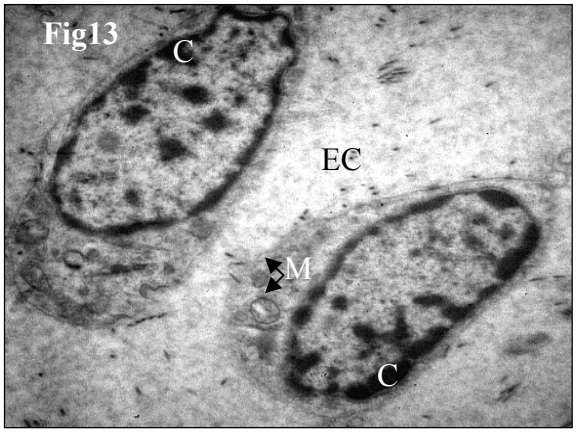


Fig. 13. Electron micrograph of group 4 showing odontoblastic cells with slight peripheral nuclear chromatin condensation (C) and swollen mitochondria (M). Notice loss of intercellular Junctions and wide extracellular compartments (EC) (X15000).

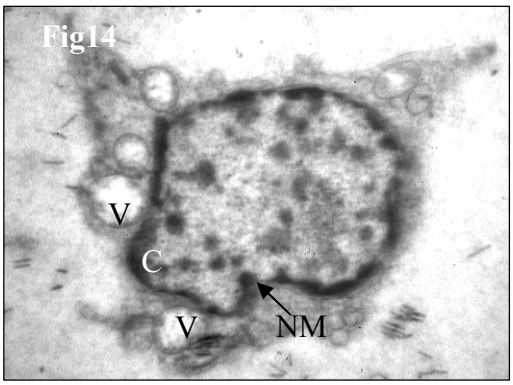


Fig. 14. Electron micrograph of group 4 of fibroblasts showing slight nuclear chromatin condensation (C) and irregularity in nuclear membrane (NM) and cytoplasmic vacuolization (V) (X26000).

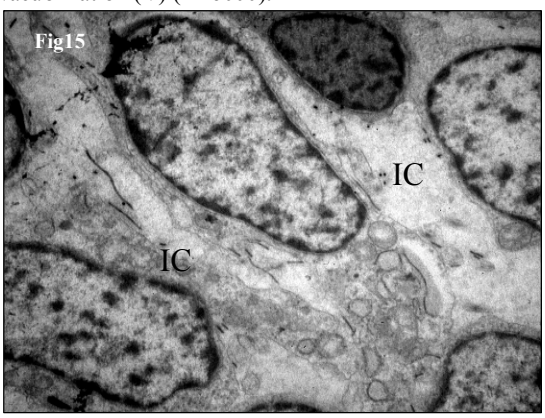


Fig. 15. Electron micrograph of group 5 showing normal odontoblastic layer on a whole except the wide intercellular compartments (IC) (X12000)

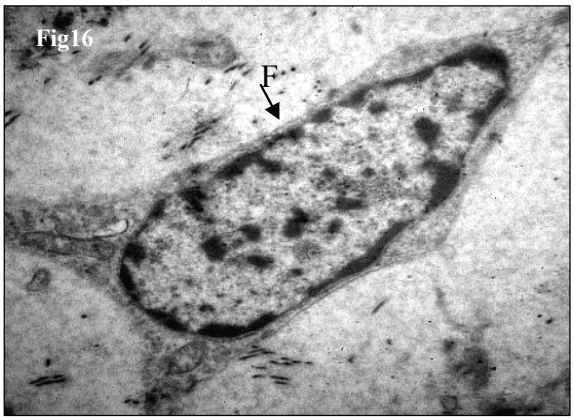


Fig. 16. Electron micrograph of group 5 normal fibroblast (F) in ground substance (X20000)

4. Discussion

To allow the observation of details not visible by the light microscope, magnified image of a specimen was performed, by transmission electron microscopy (TEM), to obtain more information about cellular structure of pulp as a response of enamel bleaching. The present study showed that male Germany rabbits were a successful animal model for this experiment. The plasma cell of odontoblasts seen in the low power electron micrograph displayed many of the main cytoplasmic components which are commonly encountered in the human odontoblasts. Moreover, at a high magnification micrograph, details of membranous and various cellular components and structures of stromal pulp tissues revealed similar results as in the human [18]. The present TEM results revealed that hydrogen peroxide in a combination with heat generation caused early alterations in pulp tissue elements at both the 24 hours and one week (groups 2&3) following the enamel bleaching application indicated that the pulp tissue did not remain healthy. Evidence of affection of the odontoblasts could be manifested as a loss of their orientation, in addition to chromatin clumping and RER affection. The primary degenerative changes of cellular organelles of odontoblasts, were in consistence with that reported by other researchers as a sequel of acute pulpitis[23], suggesting that the immediate and/or early effect of enamel bleaching may participate in the death of the severely damaged odontoblasts. This may be due to the penetration of the bleaching agent into the odontoblasts through the dentinal tubules as a second step to enamel and dentin demineralization [8,9]. At two weeks (group 3), in contrast to that shown by Essawy and Korany[25], pulpal blood vessels and nerve endings showed obvious alteration with loss of the stratification structure of myelin sheath. The severity of the changes was extended to include complete loss of cell junctions between odontoblastic layer Moreover, deeper pulp tissue structures showed pathological alterations. These included irregularities of the nuclear membranes of fibroblasts, severe vascular hyperemia, loss of nerve striations, swollen mitochondria and collaginolysis of ground substance accompanied hyalinization. This indicates that the light energy made the harsh peroxides penetrating deeper towards pulp tissue elements including odontoblasts, blood vessels and nerve tissues. The contradictory results compared to the aforementioned results can be attributed as, in their study, the bleach of preference for at-home teeth whitening is slower acting carbamide peroxide, which breaks down into hydrogen peroxide. Carbamide peroxide has about a third of the strength of hydrogen peroxide. This was in consistence with that reported by other researchers [20,21,23]. To answer how peroxide products can cause adverse effect on teeth enamel and pulp tissue elements: When peroxide-based oxidizing agents are used, a chemical reaction takes place which allows fluids to move through enamel defects into the

dentinal tubules directly towards the pulp tissue elements. Hydrogen peroxide (H_2O_2) can easily be broken down to hydroxyl (OH-) and per hydroxyl radicals (highly reactive molecules) [4]. Interestingly, at one month (group 4), electron micrograph revealed that the odontoblastic layer showed minimal alterations compared to the aforementioned groups. Most of the odontoblastic cells have preserved their ovoid regular outline of their nuclei with regular nuclear membrane, although, slight peripheral chromatin condensation was noticed. Moreover, RER showed moderate dilatation and the cell membranes were seen to be intact. While most fibroblasts showed normal ultrastructural finding, hyperemia within blood vessels was shown to be decreased and changes of endodontic nerves were less severe than that seen in the previous groups. Comparing the enamel bleaching effect of immediate response (24 hours & one week) of pulp tissue structures versus that of delay (one week), suggested that bleaching has only a modest positive impact on overall efficacy. Thus, pulp tissue affection appears to be over all limited and/or reversible. This can be attributed as: in vital pulp, there are sufficient mechanisms that protect the tissue from radicals generated from the reaction of hydrogen peroxide, and defense mechanism of the pulp would significantly reduce available levels of hydrogen peroxide [23] . It has been reported that the pulp may protect itself from damage by hydrogen peroxide to oxidize some other substrate, while catalase breaks down hydrogen peroxide to water and oxygen by enzymatic breakdown of the molecule. Hydrogen peroxide may be degraded by two classes of enzymes: peroxidase and catalase. The present results revealed a positive relationship between the intervals following bleaching and the magnitude of pulp response following enamel bleaching. This indicated that pulpal cells appeared to be resuming their functional activity.

At two months (group 5) the results revealed that almost all pulpal cellular elements seemed to be resuming their functional activity represented by the the appearance of pulp tissue normal ultrastructrual findings. odontoblastic layer almost appeared normal on a whole although the intercellular compartments were still wide. However, hypremia within blood vessels was shown to be decreased. Also, hyalinization and collagenolysis of the ground substance were limited. Moreover, most of the endodontic nerves were shown to be recovered, preserving the stratification of the myelin sheath. This indicated that the pulpal tissue structures appeared to be reversed. Similar results were reported by others [16,23]. Regeneration of pulp tissue elements can be explained as follow: stimuli that induce degeneration and/or necrosis in some cells can trigger the activation of replication pathways in others; recruited inflammatory cells not only clean up the necrotic debris but also elaborate mediators that drive the synthesis of new extracellular matrix (ECM). A collagen matrix acts as a molecular grid with channels and pores, which changes the original dense collagen structure into a sponge-like structure allowing cell migration. The remaining healthy pulp tissue structure, is a promising, allows cellular migration. Moreover, cell proliferation can be stimulated by intrinsic growth factors, injury, cell death or even mechanical deformation of tissues. Proliferating cells progress through a series of checkpoints (cell cycle). At three months (group 6) revealed that appearance of pulp tissue almost mimic normal ultrastructural findings. This can be explained as the ECM is much more than a space filler around cells; its various roles include: mechanical support for cell anchorage, determination of cell orientation, control of cell grpwth, maintainance of cell differentiation, scaffolding for tissue renewall, establishment of tissue microenvioenmentals and storage and presentation of regulatory molecules.[24]

Corresponding author
Heba Ahmed Adawy
Department of Oral Biology, Faculty of Dental Medicine (Girls), Al-Azhar University, Cairo, Egypt
hebaadawy@yahoo.com
hebaadawy@hotmail.com

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1/12/2012

Genetic Diversity among Five Egyptian Non-Poisonous Snakes Using Protein and Isoenzymes Electrophoresis

Nadia H. M. Sayed

Zoology Dept., College for Women for Science, Arts and Education, Ain Shams University, Heliopolis, Cairo, Egypt
dr1nadiah@gmail.com

Abstract: The present work is an attempt to discover the genetic diversity among five Egyptian non-poisonous snakes; *Psammophis sibilans sibilans, Psammophis schokari aegyptius, Spalerosophis diadema, Lytorhynchus diadema and Coluber rhodorachis* by using SDS-PAGE electrophoresis for two water soluble isoenzymes as well as protein of liver samples. Obtained results revealed that, protein samples showed a total of 21 bands with molecular weight ranged from 250-18 kDa. 12 common bands were recorded in all species. Also, the genetic similarity is 83.8% among all species. The *Spalerosophis diadema* is closer to the *Coluber rhodorachis* (90%) than to *Lytorhynchus diadema* (89%) while the highest similarity is present between *Lytorhynchus diadema* and *Coluber rhodorachis* (92%). Moreover, there is a high similarity between *Psammophis sibilans sibilans* and *Psammophis schokari aegyptius* (91%). The two isoenzymes; *α*-esterase (*Est*) and peroxidase (*Px*) yielded 9 heterogeneous alleles arranged in six loci. The genetic similarity is 27.2% between all species. The high similarity observed between *Spalerosophis diadema* and *Coluber rhodorachis* (67%) than between *Spalerosophis diadema* and *Lytorhynchus diadema* (50%) and the similarity between *Lytorhynchus diadema* and *Coluber rhodorachis* is 50%. It is concluded that, the Species in the same subfamily have high similarity coefficient. The phylogenetic tree showed that, the *Psammophis* species are grouped in one cluster and the other colubrid species are grouped in other cluster.
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Keywords: Egyptian snakes; Serpents; SDS-protein; Isoenzymes; SDS-Page; Electrophoresis.

1. Introduction:

Extensive molecular genetic diversity has been discovered within and among populations and species whoever its first discovery in proteins (**Zuckerkandl and Pauling, 1965**), isozymes/allozymes (**Lewontin, 1974**) and DNA (**Kimura, 1983**). The technique of protein electrophoresis has contributed greatly to resolving systematic problems in many groups of organisms (**Avise, 1994; Mishra *et al*., 2010**). The usefulness of allozyme data to identify phylogenetic relationships has long been recognized as genetic markers (**Murphy *et al*., 1990 and 1996**). The suborder Serpents is distributed in the entire world (**McDowell, 1987; Zug *et al*., 2001**).The family Colubridae is the most diverse, widespread, and contains greater than 1800 species within all of Serpents (**Pough*et al*., 2004**). **Goodman and Hobbs (1994**) recorded the distribution of colubrid species of the family Colubridae in the northern portion of the Egyptian Eastern Desert which include: *Coluber florulentus,* C. *rhodorachis,* C. *rogersi, Lytorhynchus diadema, Malpolon moilensis, Psammophis schokari, P. aegyptius,* and *Spalerosophis diadema.* There are as within Egypt where *P. aegyptius* and *P. schokari* are sympatric and both have been collected in the Egyptian Eastern Desert (**Goodman *et al*., 1985**). Previous descriptions of the external and taxonomical features of some snakes have been ambiguous and unreliable. Therefore, several authors used the karyological studies (**Pinou and Dowling, 1994**), biochemical electrophoresis (**Dowling *et al.*, 1983 and 1996; Murphy and Crabtree, 1985; Dessauer*et al*., 1987; Cadle, 1988; Highton, *et al*., 2002**) and molecular sequence analysis (**Zaher *et al*., 2009; Pyron *et al*., 2011**) to resolve the cladistic relationships among snakes and to clarify their phylogeny. Snakes classification into subfamilies remains dissenting subjects (**McDowell, 1987; Kelly *et al*., 2003**). The monophyly of the subfamilies Colubrinae, Natricinae, Psammophinae, and Xenodontinae appears to be common to several molecular studies (**Dowling *et al*., 1996; Kelly *et al*., 2003**). Family Colubridae is now represented by twelve genera (*Dolichophis*, *Eirenis*, *Hemorrhois*, *Lytorhynchus*, *Malpolon, Natrix*, *Platyceps*, *Psammophis*, *Rhagerhis, Rhynchocalamus*, *Spalerosophis* and *Telescopus*) including 24 species **(Amr and Disi, 2011)**. The generic diagnosis for *Psammophis sp* carried out by **Kelly *et al.* (2008)**. *Psammophis aegyptius* (**Marx, 1958**) was formerly considered as a subspecies of *Psammophis schokari* but later on it is currently recognized as a distinct species **(Schleich *et al.* 1996).**

This research aimed to illustrate the genetic diversity between and within some common Egyptian colubrid snakes of the family Colubridae by using the electrophoresis analysis of two isoenzymes and SDS-protein. Moreover, an attempt was carried out to

verify the traditional morphological classification of the species of this study.

2. Materials and Methods:

2.1. Species:

Five Egyptian colubrid species were collected from different localities of Egypt (Table 1). Morphological identification and classification of the animals as well as scientific and common names of these species was carried out according to previous studies **(Anderson, 1898; Marx, 1968; Goodman and Hobbs, 1994)**. The work is carried out on two samples of *Psammophis sibilans sibilans, Psammophis Schokari aegyptius* and *Spalerosophis diadema* and one sample of Coluber *rhodorachis* and *Lytorhynchus diadema*. The five species are belonging to four genera and two subfamilies.

2.2. Tissue preparation:

Liver sample of each animal was taken and homogenized in phosphate buffer and centrifuged with high speed centrifuge (10,000 rpm for 10 min). Supernatants (water soluble proteins and isoenzymes) were stored at -20^{o}C for further electrophoretic analysis.

2.3. SDS-Protein:

SDS-polyacrylamide gel electrophoresis was performed in 11 % acrylamide slab gels following the system of **Laemmli (1970)**. A volume of 20 µL of the mixture was loaded in the gel. After electrophoresis, the gel was stained by Coomassie brilliant blue. The gel was distained and after the appearance of the bands is photographed.

2.4. Isoenzymeselectrophoresis:

Polyacrylamide gel electrophoresis (PAGE) was performed in a vertical system, thermostated at 4–6°C by a circulator cooling bath. Enzyme samples were loaded on to 21 x 22.8 cm plates with 4 mm thickness. Reservoir buffer was cold tris–glycine (pH 8.3) while bromophenol blue was used as marker front–dye. Gels were electrophoresed in 10% native-polyacrylamide gel as described by **Stegemann *et al.* (1985)** and at a constant current of 10 mA and 20-30 volts. For the two enzymes, 50 µl extract per well was loaded. Each isoenzyme was run in a separate gel.

2.5. Isoenzymes staining:

The gel was stained for α-esterase (α-*Est*) according to **Desborough *et al.* (1967)** with some modifications. The gel was stained in a freshly prepared mixture composed of 100 mg fast blue stain; dissolved in 100 ml phosphate buffer (1.3 g NaH_2PO_4 and 0.3 g Na_2HPO_4) and 125 mg α-naphthyl acetate; dissolved in 1 ml acetone and diluted with 10 ml phosphate buffer, filtered). The gel is kept in staining mixture at 37 °C overnight in dark place.

The gel was stained for peroxidase isoenzyme (*Px*) according to **Van Loon (1971)** with some modifications. The gel was stained in a freshly prepared mixture composed of (200 mg benzidine dissolved in 100 ml dis. water, 1 ml glacial acetic acid and few drops of H_2O_2) The gel is kept in staining mixture at 37°C overnight in dark place.

Gel fixation was carried as follow; the gel was washed two or three times with tap water; fixed in equal volumes of glycerol and water, after 24 h the gel is washed with tap water and photographed.

2.6. Statistics:

All gels of protein and isoenzyme electrophoresis were documented using a digital camera (SONY®, 5 MP) and on the basis of the band mobility. The clear bands were scored using Totallab® 120 Gel analysis program (Nonlinear Inc., Durham NC, USA). As "1" for presence while "0" for absence in a binary data form, while the unclear unidentified bands were excluded automatically by the program. For isoenzymes, the bands of enzyme activity were designated using the known system of nomenclature (**Allendorf and Utter, 1978**). An abbreviation which corresponds to the name of the enzyme designated each locus. When multiple loci were involved, the fastest anodal protein band was designated as locus one, the next as locus two and so on. The allozyme and the specific locus was designated by numerical numbers superscripting the enzyme and the locus number e.g., *EST*–2^{a} means allele no. (a) at *EST* locus–2 and *PX*–1^{b} means allele no. (b) at *PX* locus–1. Genetic similarity and genetic distance were estimated within and among species according to **Nei and Li (1979)**. The similarity coefficients were used to construct dendrogram using the unweighted pair group Methods with Arithmetic averages (UPGMA) method from NTSYS-pc package (**Rohlf, 2000**).

3. Results

1-Comparison SDS-PAGE analysis of liver soluble proteins for the five serpent species:

Liver soluble proteins of five Egyptian snake species were separated by SDS-PAGE technique. Table (2) shows the gel analysis within and among five species. The soluble liver proteins are separated into 21 bands that ranged from 250 to 18 kDa (Fig.1 and Table 2) and band frequency ranged from 0.25-1.00 with mean value equal 74%. Both species share 12 common bands. Table (3) shows that the similarity coefficient between the individual within the same

species is higher than the similarity coefficient between the different species. The similarity matrix was ranged from 73% to 92% with average 83.8% and the genetic distance was ranged from 8% to 27%with average 16.2% between all species. Table (4) demonstrates the similarity coefficient between *Psammophis sibilans sibilans and Psammophis schokari aegyptius* is high (91%). Moreover, similarity coefficient between *Spalerosophis diadema* and Coluber *rhodorachis and* is higher (90%) than between *Spalerosophis diadema* and *Lytorhynchus diadema* (89%). The highest similarity is present between *Lytorhynchus diadema* and *Coluber rhodorachis* (92%).As shown in figure (2), the dendrogram is divided into two main clusters; one for Psammophis species while, the other cluster is formed by the rest species. The *Coluber rhodorachis* is clustered to *Lytorhynchus diadema* and the two species are sister clade to *Spalerosophis diadema*.

2-Comparaison isoenzymes analysis for the five serpent species:

Two enzymes were examined by native-polyacrylamide gel electrophoresis; α -esterase (*α-Est*) and peroxidase (*Px*). Figures 3 and 4 show gel profiles and zymograms of isoenzyme α-esterase and peroxidase, respectively. Table (5) shows 9 heterogeneous alleles (bands) with 6 loci. *α-Est* shows four genotypes with seven alleles while *Px* isoenzyme demonstrates two genotypes and two alleles. All of these alleles did not express simultaneously in one sample and they showed different isoenzyme forms. All genotype loci are monomers with one allele except Est-1 in the sample 2 (*Psammophis sibilans sibilans*), *Est-2* in the sample 3 (*Psammophis schokari aegyptius*) and *Est*-4 in samples 1 and 2 (*Psammophis sibilans sibilans*) are dimers with two alleles. *Est*-1 is established in all species except *Lytorhynchus diadema* and *Coluber rhodorachis*. *Est*-2 and *Px*-1 were limited to *Psammophis* species and were not recorded in other species. *Est*-4 is recorded only in *Psammophis sibilans* and *Coluber rhodorachis* samples. Moreover, *Est*-3 is documented in *Spalerosophis diadema* and *Coluber rhodorachis* while *Px-2* is limited to Colubrinae species. Moreover, *Lytorhynchus diadema* showed no *Est* activity. The similarity matrix was calculated according to the total number of alleles and the number of sharing bands, within and among species. Table (6) shows the similarity coefficient between the members within the same species is higher than the similarity coefficient between the different species. As shown in table (7), the similarity coefficient was ranged from 0% to 67% with average 27.2% and genetic distance was ranged from 33% to 100% with an average 72.8% between all the species. The high similarity coefficient is presented between *Spalerosophis diadema* and *Coluber rhodorachis* (67%) than between *Spalerosophis diadema* and *Lytorhynchus diadema* (50%) and the similarity coefficient is 50% between *Lytorhynchus diadema* and *Coluber rhodorachis*. In addition to that, the similarity coefficient is 36% between *Psammophis sibilans sibilans* and *Psammophis schokari aegyptius*. In figure (5), the similarity matrix has been calculated according to the number of sharing bands which constructed the clustering of *Psammophis* species in same cluster and the other colubrid species in the other cluster. Moreover, the constructed tree displays that the *Coluber rhodorachis* is sister to *Spalerosophis diadema* and not to *Lytorhynchus diadema*.

Table 1.Scientific name, Common name, Arabic name and locality of five Egyptian snakes

No.	Scientific name	Common name	locality
1	*Psammophis sibilans sibilans* **(Linnaeus, 1758)**	African Beauty snake, Abu Essuyur	Abu Rawash-Giza
2	*Psammophis schokari aegyptius* **(Marx, 1858)**	Egyptian Sand snake, Saharan Sand snake, Harseen	Egyptian Sahara, Faiyum
3	*Spalerosophis diadema* **(Schlegel,1837)**	Clifford's Royal snake, ArqamAhmar	Abu Rawash-Giza
4	*Lytorhynchus diadema* **(Dumeril, Bibron and Dumeril, 1854)**	Diademed Sand Snake, Bisbas	Abu Rawash-Giza
5	*Coluber rhodorachis* **(Jan,1865)**	Azrude Gabaly, **Jan' s** Desert Racer	Sinai

Table (2): The binary data obtained from protein gel electrophoresis based on SDS-PAGE of liver protein data among and within five Egyptian snake species.

B.N	RF	MW	1	2	3	4	5	6	7	8	B.F
1	0.03	250	0	0	0	0	1	1	0	1	0.38
2	0.06	234	1	1	1	1	1	1	1	1	1.00
3	0.11	202	0	0	0	1	1	0	0	0	0.25
4	0.16	174	1	1	1	1	1	1	1	1	1.00
5	0.23	142	1	1	1	1	1	1	1	1	1.00
6	0.27	126	0	0	0	0	1	1	0	0	0.25
7	0.31	112	1	1	1	1	1	1	1	1	1.00
8	0.47	70	1	1	1	1	1	1	1	1	1.00
9	0.52	62	1	1	1	1	1	1	1	1	1.00
10	0.47	57	1	1	1	1	1	1	1	1	1.00
11	0.54	54	1	1	1	1	1	1	1	1	1.00
12	0.58	50	1	1	1	1	1	1	1	1	1.00
13	0.62	45	1	1	1	1	1	1	1	1	1.00
14	0.65	41	1	1	1	1	0	0	0	0	0.50
15	0.69	36	1	1	1	1	0	0	0	0	0.50
16	0.71	34	1	1	1	1	1	1	1	1	1.00
17	0.82	25	1	1	1	1	1	1	1	1	1.00
18	0.83	24	1	1	1	1	0	0	0	1	0.63
19	0.86	22	1	1	0	0	0	0	0	0	0.25
20	0.89	20	0	0	1	1	0	0	0	0	0.25
21	0.93	18	1	1	1	1	0	0	0	0	0.50

BN= band number RF=relative front MW= molecular weight in kilo Dalton BF= band frequency

- columns 1-2 *Psammophis sibilans sibilans*; columns 3-4 *Psammophis schokari aegyptius*; columes5-6, *Spalerosophis diadema*; column 7, *Lytorhynchus diadema* and column 8, *Coluber rhodorachis*

Table 3: The similarity matrix and genetic distances based on SDS-PAGE of liver protein dataamong and within five Egyptian snake species.

G.D G.S	1	2	3	4	5	6	7	8
1	100	0	6	9	25	23	17	23
2	1	100	6	9	25	23	17	23
3	94	94	100	3	25	23	17	16
4	91	91	97	100	21	25	20	19
5	75	75	75	79	100	3	11	10
6	77	77	77	75	97	100	8	7
7	83	83	83	80	89	92	100	8
8	77	77	84	81	90	93	92	100

Table 4: The genetic similarity (upper) and genetic distance (lower) based on SDS-PAGE of liver protein data among the five Egyptian snake species.

G.S G.D	P. si. si.	P. sc. ae.	S. di.	L. di.
P. sc. ae	91 09			
S. di.	75 25	73 27		
L. di.	83 17	80 20	89 11	
C. rh.	84 16	81 19	90 10	92 08

P. si. si=*Psammophis sibilans sibilans*; ***P. sc. ae***=*Psammophis schokari aegyptius;* ***S. di***=*Spalerosophis diadema*; ***L. di***=*Lytorhynchus diadema* and ***C. rh***=*Coluber rhodorachis*

Table (5): The alleles; presence (1) and absence (0) data obtained from electrophoretic pattern of α-*Est* and *Px* isoenzyme within and among five Egyptian snakes.

B.N	RF	Alleles	1	2	3	4	5	6	7	8	B.F
1	0.336	*Est*-4^{b}	1	1	0	0	0	0	0	0	0.25
2	0.386	*Est*-4^{a}	1	1	0	0	0	0	0	1	0.38
3	0.619	*Est*-3	0	0	0	0	1	1	0	1	0.38
4	0.696	*Est*-2 ^{b}	1	0	1	0	0	0	0	0	0.25
5	0.736	*Est*-2 ^{a}	0	1	1	1	0	0	0	0	0.38
6	0.817	*Est*-1 ^{b}	1	1	1	1	1	1	0	0	0.75
7	0.850	*Est*-1^{a}	0	1	0	0	0	0	0	0	0.13
8	0.800	*Px*-2	0	0	0	0	1	1	1	1	0.5
9	0.848	*Px*-1	1	1	1	1	0	0	0	0	0.5

BN= band number RF=relative front BF= band frequency

α-*Est* = α-esterase *Px* =peroxidase

Columns 1-2, *Psammophis sibilans sibilans*; columns 3-4 *Psammophis schokari aegyptius*; columns 5-6, *Spalerosophis diadema*; column 7, *Lytorhynchus diadema* and lane column 8, *Coluber rhodorachis*.

Table (6): The similarity matrix and genetic distances based on isoenzyme electrophoresis data among and within five Egyptian snake species.

G.D G.S	1	2	3	4	5	6	7	8
1	100	27	33	50	75	75	100	75
2	73	100	40	33	78	78	100	78
3	67	60	100	71	71	71	100	100
4	50	67	86	100	67	67	100	100
5	25	22	29	33	100	0	50	33
6	25	22	29	33	100	100	50	33
7	0	0	0	0	50	50	100	50
8	25	22	0	0	67	67	50	100

Table (7): The genetic similarity (upper) and genetic distance (lower) based on isoenzyme electrophoresis data among five Egyptian snake species.

G.S G.D	**P. si. si.**	**P. sc. ae**	**S. di**	**L. di**
P.sc.ae	**36 64**			
S.di	**20 80**	**29 71**		
L.di	**0.00 100**	**0.00 100**	**50 50**	
C.rh	**20 80**	**0.00 100**	**67 33**	**50 50**

P. si. si=*Psammophis sibilans sibilans*; ***P. sc. ae***=*Psammophis schokari aegyptius;* ***S. di***=*Spalerosophis diadema*; ***L. di***= *Lytorhynchus diadema* and ***C. rh***= *Coluber rhodorachis*

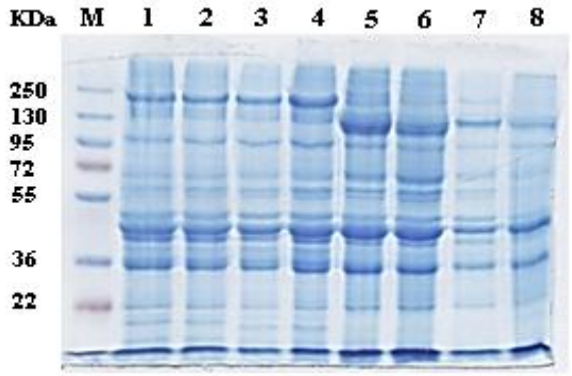

Figure (1): Liver protein profile bands of five Egyptian snakes separated on a SDS-PAGE. M represents protein marker. Lanes 1-2, *Psammophis sibilans sibilans*; lanes 3-4 *Psammophis schokari aegyptius*; lanes5-6, *Spalerosophis diadema*; lane 7, *Lytorhynchus diadema* and lane 8, *Coluber rhodorachis*.

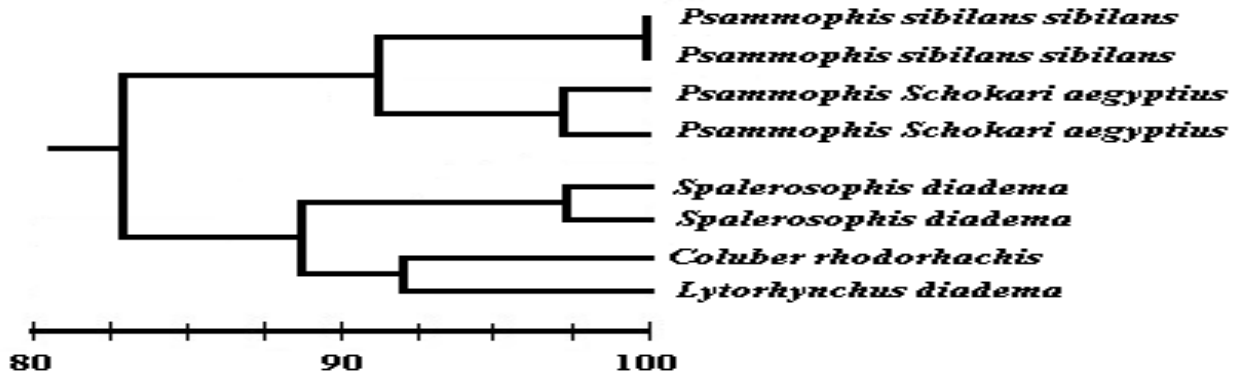

Figure (2): Dendrogram on genetic relationship within and among the five Egyptian snakes based on protein data.

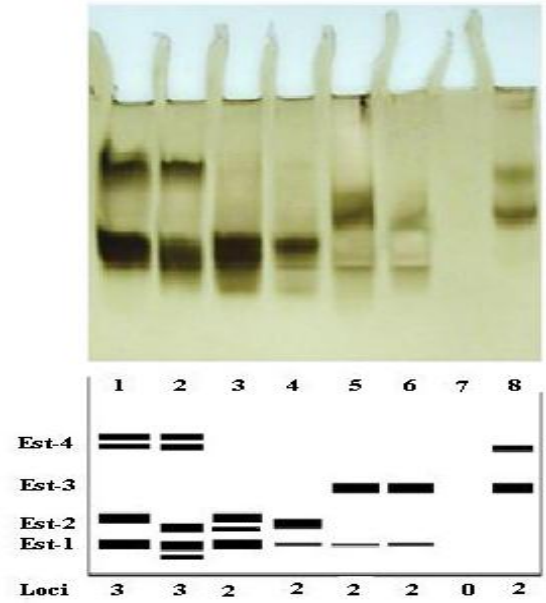

Figure (3): Profile and zymogram of α–esterase isoenzyme bands of five Egyptian snakes.

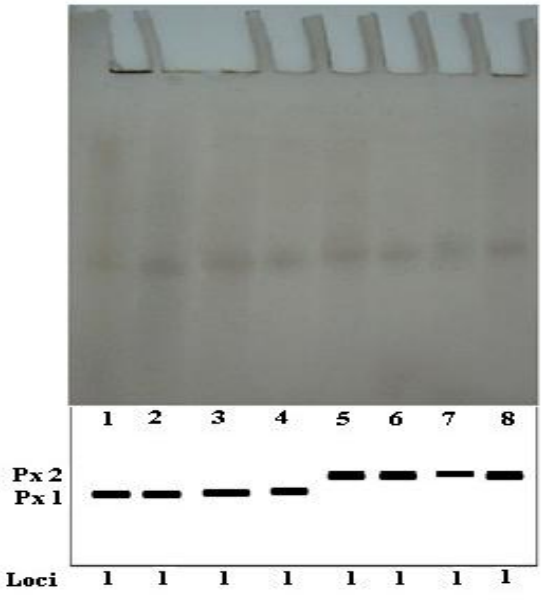

Figure (4): Profile and zymogram of peroxidase isoenzyme bands of five Egyptian snakes.

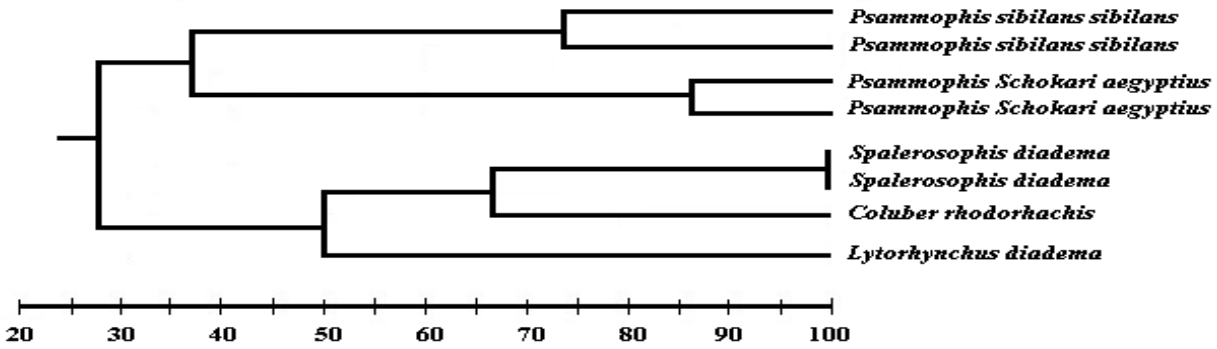

Figure (5): Dendrogram on genetic relationship within and among the five Egyptian snakes based on isoenzymes data.

4. Discussion:

Polymorphic proteins are useful markers for identifying different snakes and for studying their breeding forms, population genetics, and problems concerned with species development (**Dessauer *et al*., 1987**). Although many questions remain unsolved about the evolution of snakes, their genetic diversity, population structure, speciation, historical biogeography and phylogeny are evidently resolved through revising the protein structure. In the present study, the Colubridae snakes were clearly distinguished by using two electrophoretic parameters; the enzymes loci and the SDS-PAGE of liver protein into two clusters and several clades at different levels which are similar with previous studies suggesting paraphyly or polyphyly of this family (**Lawson and Dessauer, 1981**; **Dowling *et al.,* 1983**). However, the phylogenetic relationships among major clades of the Colubridae remain unresolved (**Dessauer *et al*., 1987**). In the present study, the electrophoretic banding of proteins indicated that the species of the family Colubridae have high sharing bands within the molecular weight of the range between 234;174-142;112 -45 kDa and in the range from 34-35 kDa. In the present work the high sharing bands between the colubrid species suggest that the subfamilies, Psammophinae and Colubrinae are belong to the family Colubridae. These results are similar to the previous studies (**Kelly *et al.,* 2003**; **Lawson *et al*., 2005**) by using DNA sequences.

In the present work, unexpected great close relationship between *Spalerosophis diadema*, *Coluber rhodorachis* and *Lytorhynchus diadema* was detected. This result supports the previous studies by **Lawson *et al.* (2005)** and **Pyron *et al*. (2011)** which presented that the genus *Lytorhynchus* is sister to a clade composed of the genera *Spalerosophis* and *Coluber*. In addition, the present work showed that, there is high similarity between *Spalerosophis diadema* and *Coluber rhodorachis* (90%) and the genetic distance between them is smaller (10%) by using SDS-PAGE of protein. This result is similar to that present by **Schatti and Utiger (2001)** which found 7% genetic diversity between *Spalerosophis diadema* and *Platyceps (Coluber) rhodorachis* by using molecular DNA sequences. Therefore these two species are considered to have the same monophyletic ancestor (**Schatti and Utiger, 2001**).

Spalerosophis diadema is more closer to *Coluber rhodorachis* (90%) than to *Lytorhynchus diadema* (89%) but *Coluber rhodorachis* is more related to *Lytorhynchus diadema* (92%) by using SDS-PAGE of protein. Moreover, *Spalerosophis diadema* is closer to *Coluber rhodorachis* (67%) than to *Lytorhynchus diadema* (50%) and *Coluber rhodorachis* has less similar to *Lytorhynchus diadema* (50%) by using isoenzymes of protein. Therefore, the evolutionary history of snakes still remains controversial and ambiguous. In addition to this, *Lytorhynchus diadema* has not any fragment with the other snakes in this work at *Est* loci. This result is similar to blind snakes (Typhlopidae-Colubrinae) which share no alleles with any other snake (**Dowling *et al*., 1996**). Then this study suggests that allozyme evolution among colubrid snakes has been conservative for some loci. The species in the subfamily Colubrinae shows more similarity to each other. These results were similar to **Dowling *et al.* (1983)** and **Lawson (1987)** which indicated that the members of Colubrinae are relatively closely related worldwide by using Albumin and transferrin immunological comparisons and electrophoretic studies.

However, phylogenetic studies using small allozymes and small sample sizes provide capable to distinguish relationships at the species level or at subfamilies level. This has shown similarity with other data (**Hillis, 1987**; **Crother *et al*., 1992**; **Crother, 1999**) which indicated that, the reduced

samples did not change inferred relationships found from larger samples and suggest that small sample sizes often are enough to positively estimate phylogeny. In spite of that, the small number of loci (two) and the sampling of only one and two illustrative of each species. Esterase enzyme profile for the eight snakes showed a variation between snakes of *Psammophis sibilans sibilans* and those of *Psammophis schokari aegyptius* that expect to be produced an identical band pattern. This variation in relative mobility and band numbers may be related to their physiological, biochemical and immunological processes to tolerate seasonal variation and environmental conditions (**Silva *et al*., 2011**; **Tosunoglu *et al*., 2011**).

In conclusion, the species in the same subfamily are more related to each other than between the species in the different subfamilies and displayed significant supports with some molecular and taxonomical studies.

Corresponding author
Nadia H. M. Sayed
Zoology Dept., College for Women for Science, Arts and Education, Ain Shams University, Heliopolis, Cairo, Egypt.
dr1nadiah@gmail.com

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Histopathological and Immunohistochemical Studies on the Adrenal Medullary Tumors in Egyptian Patients

Samia, M. Sanad[1], Mahmoud, A. El-Baz[2], Omar, I. Ghonemy[3] and Hassan, F. Abo El-Nazar[4]

[1]Zoology Department, Faculty of Science, Zagazig University, Egypt
[2]Pathology Department, Faculty of Medicine, Mansoura University, Egypt
[3]Zoology Department, Faculty of Science, Benha University, Egypt
[4]Urology and Nephrology Center, Mansoura University, Egypt
egypt_sbes@hotmail.com

Abstract: The present study provides guide lines for the diagnosis of adrenal medullary tumors in Egyptian patients. This retrospective study included, 73 cases of adrenal medullary tumors (39 pheochromocytoma, 13 neuroblastoma, 12 ganglioneuroblastoma and 9 ganglioneuroma) admitted to Mansoura Urology and Nephrology Center, Egypt.. All tumors were studied histologically and immunohistochemically. In pheochromocytomas, 33 patients became normal after 24 hours, the other 6 died from distant metastases. 6 patients with neuroblastoma and ganglioneuroblastoma were still living after adrenalectomy, while the other 19 patients received chemotherapy and were non-living after 24 months. Nine patients with ganglioneuroma were still living after adrenalectomy. All prepared slides were stained with periodic-acid Schiff' reaction (PAS) and reticulin stains. Hyaline globules which were (PAS) positive were pheochromocytomas, while, they were not detected in neuroblastoma groups. All tumors were positive for reticulin stain. All cases of adrenal medulalry tumors were examined immunohistochemically using antibodies against chromogranin A, S-100 protein and neuron-specific enolase . Chromogranin A was expressed in all cases (39/39) pheochromocytoma, 5/13 neuroblastoma, 7/12 ganglioneuroblastoma and 7/9 ganglioneuroma. S-100 protein was expressed in 32/39 pheochromocytoma, 9/13 neuroblastomas, and all cases of ganglioneuroblastoma and ganglioneuroma. Neuron-specific enolase was expressed in all cases of pheochromocytoma, neuroblastoma, ganglioneuroblastoma and ganglioneuroma. The neuroendocrine tumors were stained with high specificity and sensetivity for the neuroendocrine markers; chromogranin A and neuron-specific enolase. Histomorphological features of benign and malignant pheochromocytomas may be similar. Neuroendocrine markers (chromogranin A, neuron-specific enolase) are useful in diagnosis of pheochromocytoma. Frequency of S-100 protein positive sustentacular cells is high in benign pheochromocytomas and low in malignant pheochromocytoma (our results suggest that, S-100 immunostaining is a useful marker to predict malignant behavior in pheochromocytoma. Intensity of neuron-specific enolase may be similar in both benign and malignant pheochromocytoma). No significant correlation was observed between expression of chromogranin A and neuron-specific enolase in pheochromocytoma and survival. The features of histopathological changes are the most important basis to make diagnosis for neuroblastomas group. Immunohistochemical staining can verify it further and play an important role in its differential diagnosis.

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1. Introduction:

Tumors in the adrenals originate from the adrenal cortex and medulla or as metastases from-extra adrenal primaries (Tatic *et al.* 2002). Differentiation between these three groups is the first task a pathologist to tackle when dealing with specimens from the adrenal region. The second great problem is the dignity of adrenal tumors, which cannot be determined in many adrenomedullary and some adrenocortical tumors. Immunostaining is helpful but the basic methods remain the histopathological examination of paraffin sections (Saeger, 2000). The adrenal medullary tumors include pheochromocytoma and neuroblastoma groups. They arise from chromaffin cells and neuroblast, respectively (Tatic *et al.*, 2002).

With the development of immunohistochemical techniques a greater understanding of abnormal neural and neuroectodermal differentiation was achieved (Achilles *et al.*, 1991). The developments of monoclonal antibodies have permitted the localization of single peptides and proteins that are produced by the cells of the tumor. However, it is a difficult to predict clinical outcome for patients with paraganglioma on morphologic backgrounds only. So, the use of monoclonal antibodies directed against S-100, NSE and chromogranin A can help in their localization and consequently their expression is

noticed in benign and malignant lesions (Feng *et al.*, 2005). As regard neuroblastoma group that are derived from primitive neuroblasts,these tumors can be conceptualized as three different maturational manifestation of a common neoplasm (Shimada *et al.*, 2004). The expression of S-100 protein is associated with good prognosis.

The present study has been carried out on the adrenal medullary tumors obtained from patients in Urology and Nephrology centre, Mansoura University, Egypt. The morphologic distiniction of adrenal medullary tumors is still difficult. Therefore, this study was conducted to:

1-Threw light on the origin of the different adrenal medulary tumors.

2-Study the clinical approachs of the patients infected by these tumors.

3-Differentiate between the different types of adrenal tumors using thehistological and immunohistochemical methods.

4- Investigate the expression of chief cell markers and sustentacular cell markers in the adrenal medullary tumors as pheochromocytoma and ganglioneuroblastoma groups.

5-Examine the diagnostic usefulness of immunohistochemical techniques in determining the presence of differentiation and maturation in case of neuroblastoma.

2. Patients and Methods

This retrospective study was carried out on 73 cases of adrenal medullary tumors (39 pheochromocytoma, 13 neuroblastoma, 12 ganglioneuroblastoma and 9 ganglioneuroma). These cases were obtained from Urology and Nephrology Center, Mansoura University, Egypt during the period from 1985-2002.

All patients were routinely investigated after hospitalization to evaluate the extent of tumor. The study included clinical examination, conventional laboratory investigation, intravenous urography, abdominal ultrasonography, chest x-ray to rule out pulmonary metastases and radio isotopic bone scan to exclude skeletal metastases. Also, computerized axial and tomography and radical adrenalectomy in all patients were performed. Clinical data included in this study were obtained from surgical requests including patients' age, sex and the size of each tumor.

Histopathological examination:

After radical of the adrenal medullary tumor, the resected tumor was fixed in 10% buffered formalin for 24 hours and processed for the preparation of paraffin wax blocks. These blocks were sectioned at 4-5 µm and were stained by the following methods:

1.Routine hematoxylin and eosin stain for general histological examination (Harris, 1900).

2.Periodic acid-Schiff (PAS) reaction for the detection of intracytoplasmic hyaline globules (Hotchkiss, 1948).

3.Reticulin stain by Gordon method for demonstrating the distribution of reticular fibers in the tumor tissues (Gordon and Sweets, 1936).

4.Moreover, immunohistochemical stainings were carried out using different monoclonal and polyclonal primary antibodies.

Immunohistochemical examination:

Immunohistochemical staining of 4-6 µm paraffin sections was performed on the Dako Autostainer (Dako corporation, Carpentaria, Calif) using streptavidin-biotin peroxidase method of Hsu et al. (1981). Histostain plus Kits (Zymed USA) which contain 10% non-immune serum biotinylated secondary antibody used in this study. These kits contain 10% non-immune serum, biotinylated secondary antibody and streptavidin-perxidase for antibodies against chromogranin A and Neuron specific enolase (monoclonal, 1:50, Zymed) and S-100 protein (polyclonal, 1:50; Zymed) for adrenal medullary tumor .

Immunohistochemical reagents:

Immunohistochemistry was performed by using a panel of three monoclonal primary antibodies and 2 polyclonal primary antibodies. A panel of 5 reagents (3 monoclonal and 2 polyclonal) was also used in this study. All reagents were mouse antibodies.

1- Chromogranin A:

This is a second generation of polyclonal mouse antibody. This antibody is especially designed for sensitive detection of chromogranin A. It is purchased from Zymed laboratories INC., So. San Francisco. It is a cytoplasmic marker as it presents in cytoplasm.

2- Neuron -specific Enolase:

This is a monoclonal mouse anti-NSE protein. It reacts predominantly with the gamma subunits of NSE of neuronal origin but not with alpha subunits of glial origin. It may cross react with beta subunits from muscle. It is purchased from zymed lab., INC., So. San Francisco. It is a cytoplasmic marker.

3- S-100 protein:

This S-100 protein (21-24Kda) is a mouse polyclonal antibody. S-100 stains almost all benign and malignant melanomas and their metastases. It is purchased from Zymed laboratories, INC, South San Francisco, Calif. It is a nuclear and cytoplasmic marker as it is present in both cytoplasm and nuclei.

Immunohistochemical procedure (Elias et al., 1989):

Semi serial sections were cut at 4-6 µm and deparaffinized with xylene, rehydrated with

descending grades of alcohols and immersed in deionized water.

Deparaffinized sections were incubated with 0.3% hydrogen peroxide in methyl alcohol to block the endogenous peroxidase activity for 30 minutes. Then, washed in phosphate-buffered saline (PBS) at PH 7.4 and incubated with non-immune serum. After application of the primary antibody, a biotinylated secondary antibody was added followed by the avidin-biotin-peroxidase complex. After each immunostaining procedure, sections were incubated with 3, 3´ diaminobenzidine-hydrogen peroxide substrate and counterstained with hematoxylin. Appropriate positive and negative control sections were also used.

Result of stain:

Immunohistochemical reactions revealed cytoplasmic brown stain in positive cases with the used antibodies. Accumulation of Cg A and NSE in tumor cells which take a brown color. Whereas these reactions revealed cytoplasmic and nuclear accumulation of staining with a brown color in case of S-100 protein especially in the sustentacular cells.

Follow up of patients:

The patients were followed postoperatively in the outpatient clinic for available period (mean = 47.46 month). This included, clinical examination, body weight and hypertensive state. Moreover, radiological follow up for local recurrence and distant metastasis in malignant cases was carried out.

3. Results

Histopathological examination of the excised adrenal medullary tumors revealed that these tumors could be divided into two main types, pheochromocytoma and neuroblastoma groups. The last group was subdivided, on some histopathological criteria, into neuroblastoma, ganglioneuroblastoma and ganglioneuroma.

Meanwhile, the pheochromocytoma was represented by 39 patients (32 benign and 7 malignant). Of the 39 patients, 21 were males and 18 females. A total number of 34 patients represented the neuroblastoma group. The number of patients was 13, 12, and 9 for NB, GNB and GN, respectively .

It could be observed that, the pheochromocytoma represents the high frequency, while ganglioneuroma represent the lowest one. In view of the fact that these tumors represent different clinical entities, their results will be individually displayed.

I- Clinical findings:

1- Pheochromocytoma:

The clinical data of the patients with pheochromocytoma is outlined in table (1). Thirty-nine cases of pheochromocytoma were examined clinically to illustrate the sex and age of patients. In addition, the size of the tumors was recorded. The patients were followed for a definite period. Most of them were died after a follow up period reaching 58 months.

Age distribution:

The age distribution among the patients with pheochromocytoma is outlined in table (1a). The age of patients ranged from 19 up to 61 years with an average age of 36.4 years. Most of the cases are in the 4^{th} and 5^{th} decade of life.

Sex distribution:

Sex distribution in patients is represented in table (1b). 39 cases of pheochromocytoma were 15 males with a percentage 38.46% and 24 were females with a percentage 61.54%. The male to female ratio was 1:1.6.

Tumor size

The tumor size in patients with pheochromocytoma is outlined in table (1c). The sizes ranged from 4 -14 cm. with an average size 8.543 cm.

2- Neuroblastoma and ganglioneuroblastoma:

The clinical data in patients with neuroblastoma represented by 13 patients and ganglioneuroblastoma represented by 12 patients is outlined table (2).

Age distribution:

The age distribution among the patients with neuroblastoma and ganglioneuroblastoma is outlined in Table (2a). The age of patients ranged from 4 months up to 44 years (average age 6 years). Most cases are in the first year of life.

Sex distribution:

Sex distribution among the patients with neuroblastoma and ganglioneuroblastoma are recorded in table (2b). There were 13 male with percentage 52% and 12 females with a percentage 48%. The male to female ratio is 1.08:1.

Tumor Size:

The size of tumor in 25 patients with neuroblastoma and ganglioneuroblastoma is recorded in table (2c). The size of tumors ranged from 9.5 to 20 cm. with the average size of 14.56 cm.

3- Ganglioneuroma:

Nine patients had ganglioneuroma; there were five males and four females. The male to female ratio was 1.25:1. The clinical data are shown in table (3).

Age distribution:

Age distribution in patients with ganglioneuroma is recorded in table (3). The age of the patients ranged from 15 up to 35 years with average value (23.2) years. Most of the cases are in the 2^{nd} decade of life.

Tumor size:

Tumor size was recorded in nine patients with ganglioneuroma with an average diameter of 11.7

cm. The size of tumors ranged from eight to 14cm (Table 3).

The outcome of patients:

All patients were followed postoperatively in the out patient clinic for available periods ranged from 11 to 192 months in case of pheochromocytoma (Table 1), from 16 to 52 months for NB and from 10 to 45 months for GNB patients (Table 2). The GN patients were followed in the outpatient clinic for a period ranged from 23 to 121 months (Table 3).

All patients with adenoma and GN are alive but in case of ACC 3 patients from 12 were alive while nine patients with a percentage of 66.7 % were died from distant metastases. In case of pheochromocytoma, 32/39 patients were alive during the follow up period while 7 cases were died. Moreover, 2/13 patients with NB and 4/12 cases with GNB are still alive after adrenalectomy. The patients of this study were examined clinically to determine the body weight, hypertension, monitoring blood pressure. Moreover, radiological investigations were carried out for local or distant metastases in malignant cases.

II- Histological Observations:

1- Pheochromocytoma:

The pheochromocytoma showed the typical cell nests (Zellballen pattern) of the chief parenchymal cells in 35 cases of the studied 39 cases (Fig. 1). Whereas, four cases only of the studied tumors showed spindling of the chief parenchyma cells (Fig. 2). Generally, the cytoplasm of the tumor cells is often lightly eosinophillic and finely granular. The nuclei were also lightly basophilic and possessed spherical shape with prominent nucleoli. Two cases of composite pheochromocytoma were studied (Fig. 3), spindle cell schwannian stroma admixed with pheochromocytoma. The sections of the tumor were stained with PAS and reticulin stains. The results of staining are recorded in table (4). This table showed that, the PAS positive intracytoplasmic hyaline globules were detected in most cases (28/39) of pheochromocytoma (Fig. 4). No hyaline globules were demonstrated in 11 cases of pheochromocytoma. Most cases of tumor were rich in reticular fibers (Fig. 5). Moreover, the results of reticulin staining showed highly rich stain ability (33/39), moderately rich (4/39) and weak stain ability (2/39) for the reticular fibers.

2- Neuroblastoma and ganglioneuroblastoma:

Neuroblastoma showed accentuation of fibro vascular stroma and tumor nodules formed of monotonous primitive cells (Fig. 6). Some areas suggest the formation of homer Wight pseudo rosettes. These rosettes appear as pale zones with fibrillar matrix corresponding to neuritic cell processes (Fig. 7). Sometimes, there are areas with a more diffuse or solid pattern with patches of calcification (Fig. 8).

Ganglioneuroblastoma showed patchy nodules of immature neuroblasts set within a full-grown Ganglioneuromatous stroma (Fig. 9).

From table (4), it can be observed that, no intracytoplasmic hyaline globules were detected in all cases of neuroblastoma and ganglioneuroblastoma (Figs. 10 and 11). Moreover, both NB and GNB were rich in reticular fibers (Figs. 12 and 13). These figures showed that, the reticular fibers are more abundant in case of Neuroblastoma.

3-Ganglioneuroma:

Microscopically, it can be observed that, two distinct cell groups were identified. The first is ganglionic cells and the second is Schwannian cells placed in an eosinophillic matrix (Fig. 14). From table (4), it can be observed that, no intracytoplasmic hyaline globules were detected in all cases of tumor (Fig. 15). Positive PAS reaction is observed in the stroma of ganglioneuroma. All cases of tumors are rich in reticular fibers (Fig. 16). The abundant reticular fibers are observed in between the tumor cells of GN.

III- Immunohistochemical observations:

Table (5) shows the immunoreactivity of the adrenal medullary tumors with Cg A, NSE and S-100 protein.

1- Expression of Cg A :

a- Pheochromocytoma:

Chromogranin A showed diffuse cytoplasmic staining in all chromaffine cells (Fig. 17). Cg A staining was consistently intense in most tumors, 34 cases of tumor showing intense staining pattern and 5 cases of tumor show moderate staining pattern. The two cases of composite pheochromocytoma (pheochromocytoma and ganglioneuroma) showed intense immunoreactivity for Cg A in pheochromocyte components (Fig. 18), whereas the other components for ganglioneuroma were negative for Cg A immunoreactivity (Table 5).

b- Neuroblastoma (NB):

In five cases of 13 Neuroblastoma Cg A expression was observed. It revealed intense immunoreactivity in one case of 13 neuroblastoma and the other four cases being moderately immunoreactive for Cg A. The other eight cases were completely negative for Cg A. The immunoreactivity of Cg A is detected as brown deposits in the fibrillar areas with overlapping inter twisting neuritic processes (Fig. 19).

c- Ganglioneuroblastoma (GNB):

The immunoreactivity of Cg A revealed intense cytoplasm staining in the large neuroblasts and

immunostaining in neuritic processes in five cases of tumors (Fig. 20). Two cases only gave weak immunoreactivity. The other five cases were completely negative for Cg A (Table 5).

d- Ganglioneuroma (GN):

The immunoreactivity of Cg A is noticed in the cytoplasm of the ganglionic cells that gave an intense reaction in five cases of tumors (Fig. 21). The other four cases were not expressed for Cg A giving negative results.

2- Expression of NSE:

a- Pheochromocytoma:

All pheochromocytoma were immunoreactive for NSE with different degree of staining as follow; 37 cases of tumors being intense, 2 cases showed moderate staining. The other two cases were negative for NSE immunoreactivity. The reactions appear as brown color and diffuse in the cytoplasm of the chief cells of tumor (Fig. 22).

b- Neuroblastoma (NB):

Expression of NSE has been observed in almost all the cell of NB. An intense reaction is seen in the cytoplasm of theses cells, whereas the stroma is unstained. It also can be observed in abundance in the neuritic process (Fig. 23). The staining was intense in 5 cases, moderate in also five cases and the other three cases being slight immunoreactivite for NSE.

c- Ganglioneuroblastoma (GNB):

Expression of NSE immunostaining was observed in abundant stromal septa. In addition, the cytoplasm of cell bodies is intensely positive. All cases of ganglioneuroblastoma (GNB) were intensely reactive for NSE (Fig. 24).

d- Ganglioneuroma (GN):

The immunoreactivity of NSE is observed in the ganglionic cells and Schwann cells of GN. These cells showed a wide range of immuno- reactivity. In addition, the stroma of GN was stained in a blue colour by haematoxylene as a counter stain (Fig. 25). Six cases of tumor showed intense immunostaining reactivity and the other three cases gave negative immunoreactions (Table 5).

3- Expression of S-100 protein:

a- Pheochromocytoma:

S-100 protein was absent in chromaffin cells but was present in the cytoplasm and nuclei of sustentacular cells surrounding chromaffin cells (Fig. 26). S-100 protein, was stained moderately to intensely in all cases. 27 cases were intensely stained and five cases showed moderate staining. The other seven malignant pheochromocytoma were negative for the immunoreactivity of S-100 protein.

b- Neuroblastoma:

The immunoreactions of S-100 protein are seen adjacent to the vascular septa. The cells of NB are consistent with Schwann or sustentacular cells that gave intense reaction. However, S-100 protein expression was observed in all cases of Neuroblastoma (Fig. 27). The staining was generally strong in 7 cases and the other 6 cases of tumor showed moderate immunoreactivity cells.

c- Ganglioneuroblastoma (GNB):

In GNB, the cells are in contact with ganglionic cells. These cells showed intense immunoreactive S-100 protein. They are in the typical location of satellite cells. All 12 GNB were positively stained for S-100 protein (Fig. 28) and the other three cases of tumor showed moderate immunoreactivity for S-100 protein.

d- Ganglioneuroma (GN):

S-100 protein expression revealed a brown staining in the cytoplasm of the ganglionic cells. In addition, an intense S-100 protein immuno reactivity was observed in the Schwann cells as well as in the matrix of GN. In the GN stained positively with S-100 protein, immunoreactivity was consistently present in nerve fibrils. All cases of tumors demonstrated a strong staining pattern for S-100 protein (Fig. 29).

Table (1): The clinical data of patients with pheochromocytoma

Case	Sex	Age (year)	Size (cm)	Follow up (months)	Status
1	F	36	5	192	S
2	F	43	7	120	S
3	M	35	8	96	S
4	F	58	8	82	S
5	F	21	10	94	S
6	M	19	8	86	S
7	F	30	10	81	S
8	F	36	11	78	S
9	F	43	13	72	S
10	F	40	14	66	S
11	M	28	7	58	S
12	F	60	5	58	D
13	M	53	4	55	D
14	F	45	4	71	S
15	F	52	7	51	S
16	M	41	6	49	S
17	F	35	6	47	S
18	M	46	7	44	S
19	M	46	5	42	S
20	F	54	13	38	D
21	M	36	10	37	S
22	F	24	11	32	S
23	F	34	8	30	S
24	M	61	7	28	D
25	M	20	11	26	S
26	F	25	13	24	S
27	F	35	7	24	S
28	F	24	6	21	S
29	F	19	9	20	S
30	F	42	11	19	S
31	M	44	4	17	D
32	F	19	7	15	S
33	M	44	6	14	S
34	F	20	5	13	S
35	M	42	12	11	S
36	F	34	14	24	S
37	F	54	12	58	D
38	F	30	11	36	S
39	F	37	12	48	S

Table (1a): Age distribution in patients with pheochromocytoma.

Age group	No. of patients	Percentage %
1-10	0	0%
11-20	5	12.8%
21-30	8	20.51%
31-40	10	25.64%
41-50	9	23.07%
51-60	6	15.38
Over 60	1	2.5%
Total	39	100.0

Table (1b): Sex distribution in the patients with Pheochromocytoma.

Sex	No. of patients	%
Male	15	38.46%
Female	24	61.54%
Total	39	100.0

Table (1c): Tumor sizes in 39 patients with pheochromocytoma.

Tumor size (cm)	No. of patients	%
0-5	6	17.1%
6-10	17	48.6%
11-15	16	34.3%
Total	39	100.0

Table (2): Clinical data of patients with neuroblastoma and ganglioneuroblastoma (25 cases)

Tumors	Case	Sex	Age	Size	Follow up	Status
I-Neuroblastoma	1	F	4 m	10	30	D
	2	M	3 y	9.5	23	D
	3	M	4 y	17	19	D
	4	M	7 m	17	20	D
	5	M	10 m	10	18	D
	6	M	3 y	13	24	S
	7	M	7 m	17	26	D
	8	M	3 y	12	52	D
	9	F	6 m	17	16	S
	10	F	11 m	20	22	D
	11	F	1 y	13	60	D
	12	F	9 m	10	96	D
	13	F	5 y	12.5	20	D
Ganglioneuroblastoma	14	F	4 y	14	23	D
	15	M	44 y	16	20	D
	16	M	3 y	10	30	D
	17	M	10 m	11	16	S
	18	F	8 m	18	24	D
	19	M	43 y	12	45	D
	20	M	5 y	13	24	D
	21	F	2 y	15	12	S
	22	F	16 y	10	10	S
	23	F	3 y	17	17	D
	24	M	1 y	20	24	D
	25	F	4 y	18	17	S

y= Year m=Months, S= survival, D= died

Table (2a): Age distribution in patients with neuroblastoma and ganglioneuroblastoma.

Age group	No. of patients	%
1 month- 11 m	9	36%
1 y-10 y	13	52%
11 y-20 y	1	4%
21 y-30 y	0	0
31 y-40 y	0	0
41 y-50 y	2	8%
Total	25	100%

Table (2b): Sex distribution in (25) patients with neuroblastoma and ganglioneuroblastoma

Age sex	No. of patients	%
Male	13	52%
Female	12	48%
Total	25	100.0%

Table (2c): Tumor size in (25) patients with neuroblastoma and ganglioneuroblastoma.

Tumor size	No. of patients	%
0.5	0	0
6-10	6	20%
11-15	9	36%
16-20	10	40%
Total	25	100.0

Table (3): Clinical data of 10 patients with ganglioneuroma

Case	Sex	Age (years)	Size (Cm)	Follow up (Months)	Status
1	F	20	13	30	S
2	M	19	14	23	S
3	M	31	8	23	S
4	M	35	13	31	S
5	M	17	10	18	S
6	F	24	12	121	S
7	F	28	15	46	S
8	M	15	11	52	S
9	M	20	10	36	S

Table (4): Results of PAS and Reticulin stains for adrenal medullary tumors (AMT).

Stain / Tumor	PAS		Reticulin stain		
	+ve	-ve	high +2	low +1	-ve -
Adrenal medullary tumor					
- Pheo.	28/39	11/39	33/39	6/39	-
- NB	-	13/13	13/13	-	-
- GNB	-	12/12	12/12	-	-
- GN	-	9/9	9/9	-	-

+ve Positive **-ve Negative**

Table (5): The immunoreactivity of AMT with Chromogranin A (Cg A), Neuron-specific enolase (NSE) and S-100 protein.

Stain / AMT	Intensity for Cg A +3 +2 +1 -ve	Intensity for NSE +3 +2 +1 -ve	Intensity for S-100 +3 +2 +1 -v
- Pheo.	34/39 5/39 -	37/39 2/39 -	27/39 5/39 -
- NB	-	-	7/39
- GNB	1/13 4/13 -	5/13 5/13	7/13 6/13 -
- GN	8/13	3/13 -12/12	-
	5/12 - 2/12	- - -	9/12 3/12 -
	5/12	6/6 3/9 -	-
	5/9 - -	-	9/9 - -
	4/9		-

+ 3 intense immunoreactivity. + 2 moderate immunoreactivity. + 1 weak immunoreactivity. - ve negative immunoreactivity.

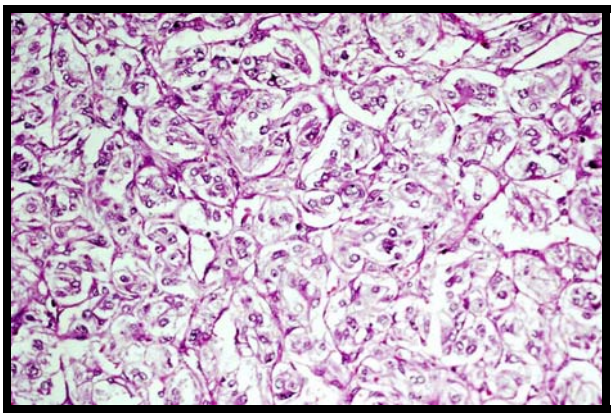

Fig. (1): Photomicrograph of adrenal pheochromocytoma showing the typical nesting pattern (Zellballen appearance). (Hx & E X 200).

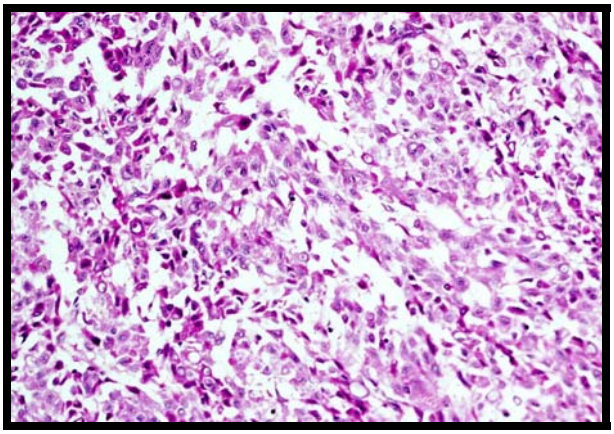

Fig. (2): Photomicrograph of adrenal pheochromocytoma, spindle cell pattern. (Hx & E X200).

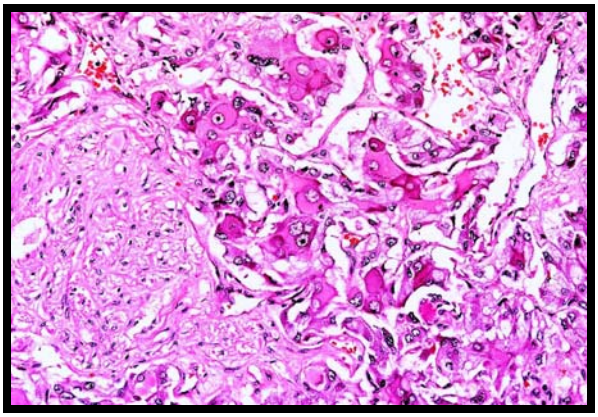

Fig. (3): Photomicrograph of composite pheochromocytoma showing spindle cell schwannian stroma admixed with pheochromocytoma. (Hx & E X 200).

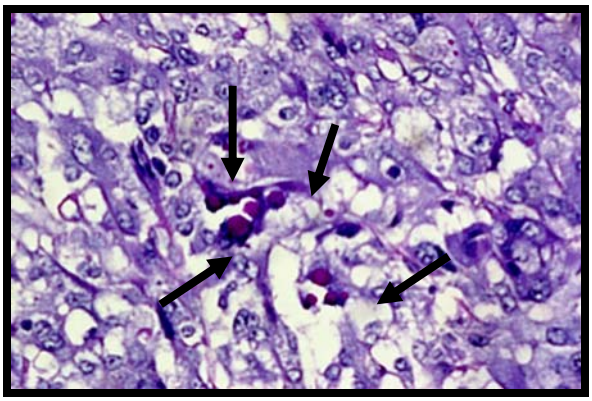

Fig. (4): Photomicrograph of pheochromocytoma showing intra-cytoplasmic hyaline globules (arrows). (PAS x400).

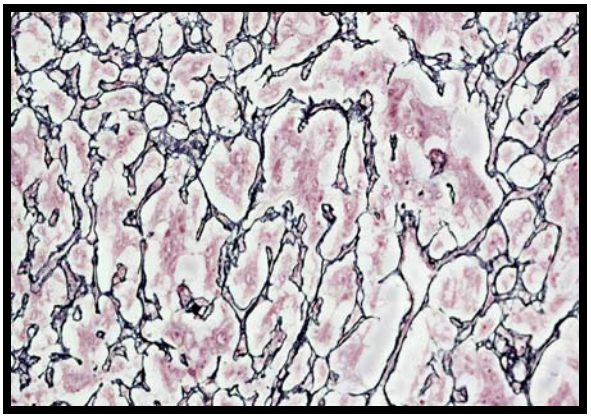

Fig. (5): Photomicrograph of pheochromocytoma showing accentuated alveolar pattern bounded by condensation of reticular fibers.(Gordon stain X 200).

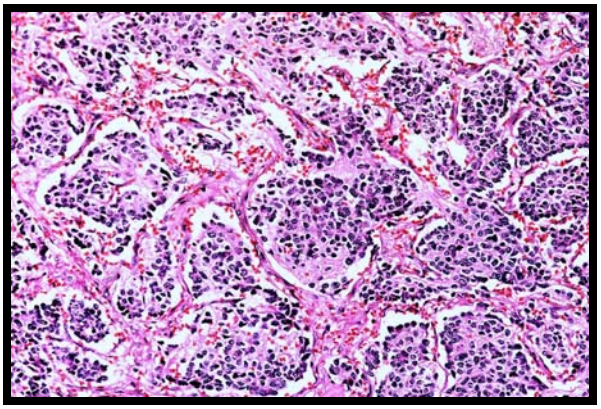

Fig. (6): Photomicrograph of neuroblastoma showing accentuation of fobrovascular stroma and tumour nodules formed of monotonous primitive cells. (H x & E X 200).

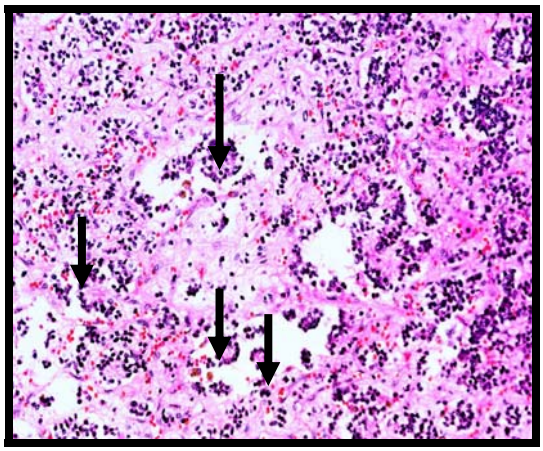

Fig. (7): Photomicrograph of neuroblastoma showing numerous Homer Wright rosettes. (H x & E X 200).

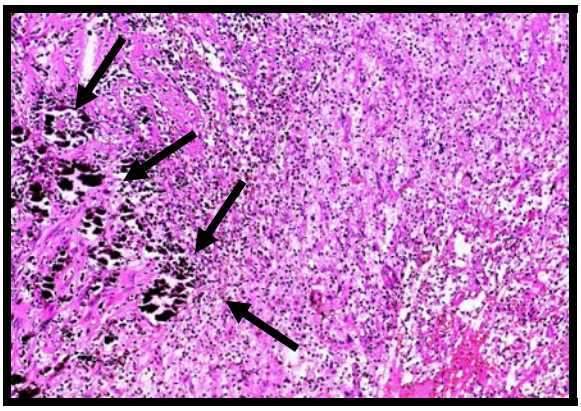

Fig. (8): Photomicrograph of neuroblastoma showing patches of calcification. (Hx & E X 100).

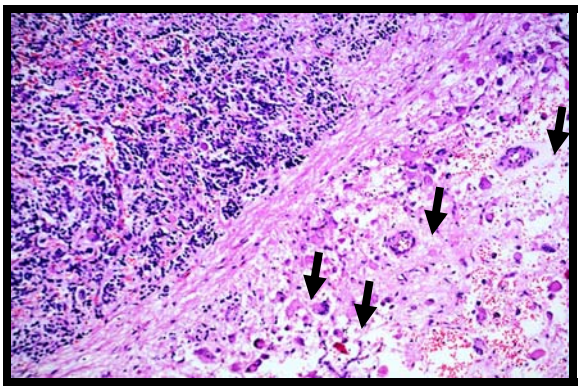

Fig. (9): Photomicrograph of ganglioneuroblastoma showing patchy nodules of immature neuroblasts set within a mature ganglioneuromatous stroma arrows.(Hx& E X100).

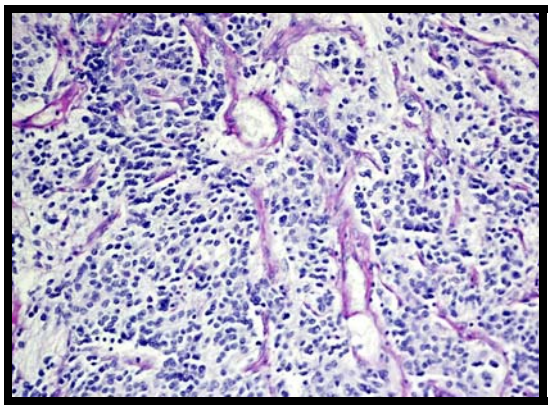

Fig. (10): Photomicrograph of neuroblastoma showing no intracytoplasmic hyaline globules. (PAS stain X 200).

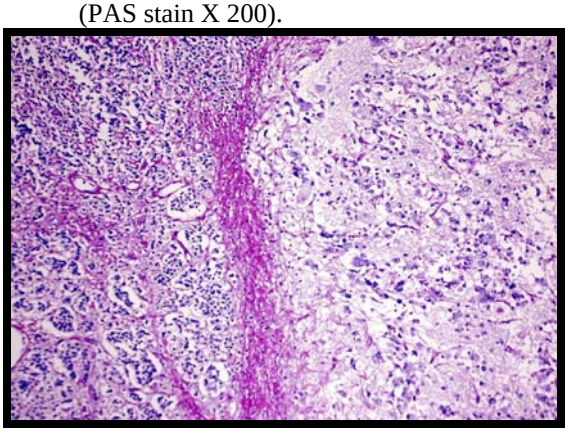

Fig. (11): Photomicrograph of ganglioneuroblastoma showing no intracytoplasmic hyaline globules. (PAS stain X 100).

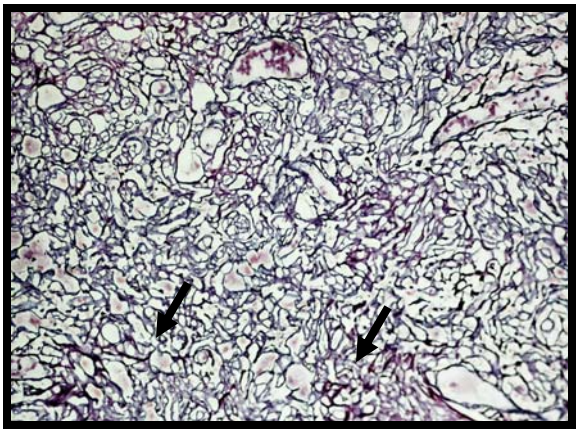

Fig. (12): Photomicrograph of neuroblastoma showing abundant reticular fibers in tumour cells. (Gordon stain X 200).

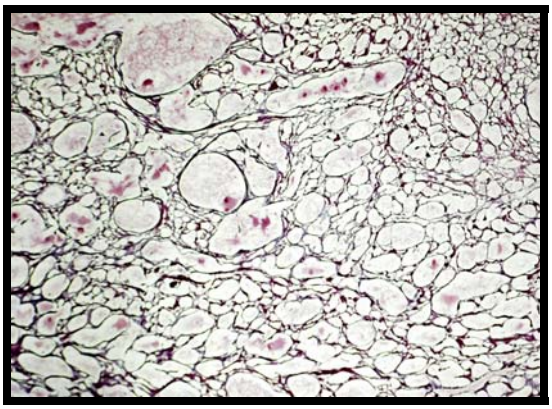

Fig. (13): Photomicrograph of ganglioneuroblastoma showing abundant reticular fibers in tumour cells. (Gordon stain X 200).

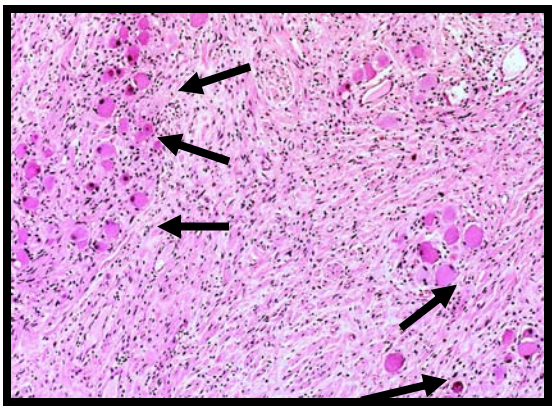

Fig. (14): Photomicrograph of ganglioneuroma showing mature ganglion cells (arrows) in Schwannian cell dominant stroma.(H & E X200).

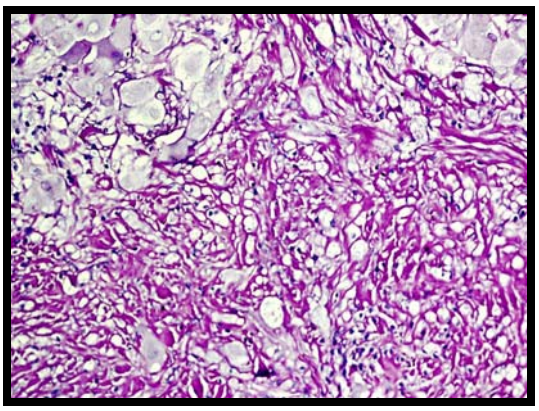

Fig. (15): Photomicrograph of ganglioneuroma. Note that the intracytoplasmic hyaline globules are not detected in tumour cells.(PAS stain X 200).

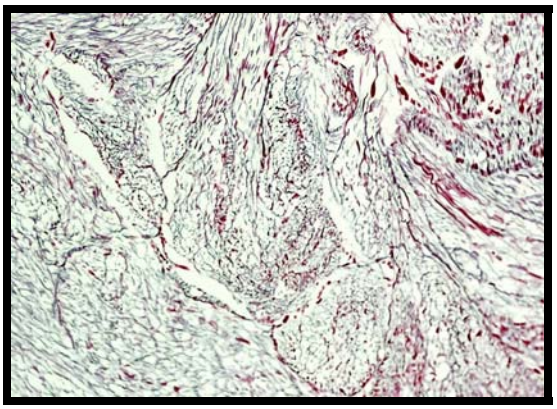

Fig. (16): Photomicrograph of ganglioneuroma showing abundant reticular fibers in tumour cells. (Gordon stain X 100).

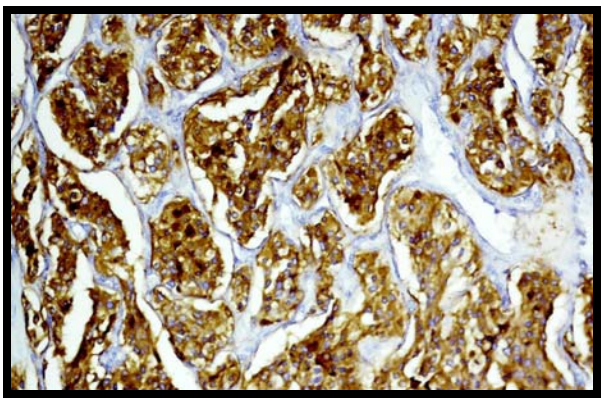

Fig. (17): Photomicrograph of pheochromocytoma showing intense cytoplasmic immunoreactivity of chromaffin cells for Chromogranine (The brown color) (Immunoperoxidase X 200).

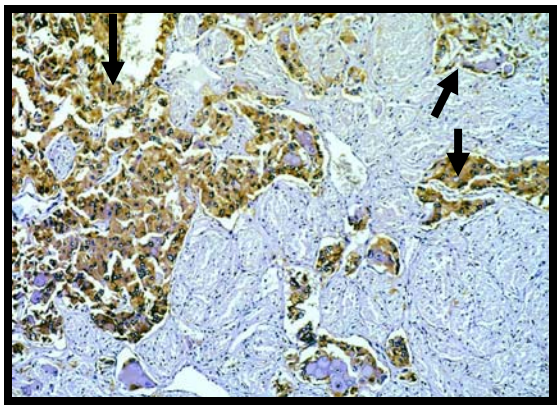

Fig.(18):Photomicrograph of composite pheochromocytoma showing intense cytoplasmic immunoreactivity for Chromogranine within the chromaffin cells (arrows). (Immunoperoxidase X 200).

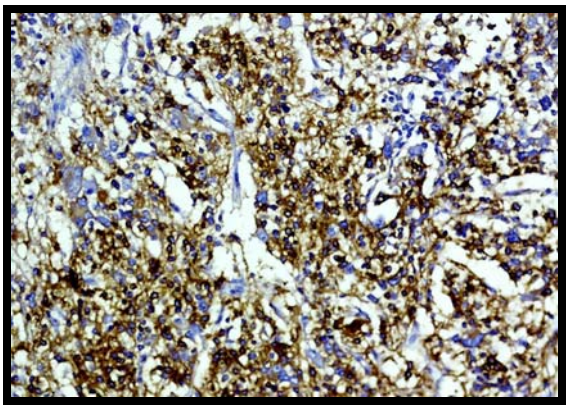

Fig.(19): Photomicrograph of neuroblastoma . immunoreactivity for chromogranin A was seen in brown fibrillar areas with overlapping interwining neuritic processes (brown color) (Immunoperoxidase X 200).

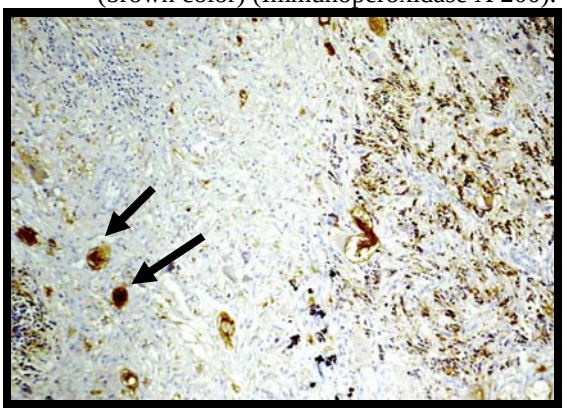

Fig. (20): Photomicrograph of ganglioneuroblastoma, Immunohistochemical analysis directed to chromogranin A. Note the large neuroblasts with intense cytoplasmic staining also staining in processes. (Immunoperoxidase X 100).

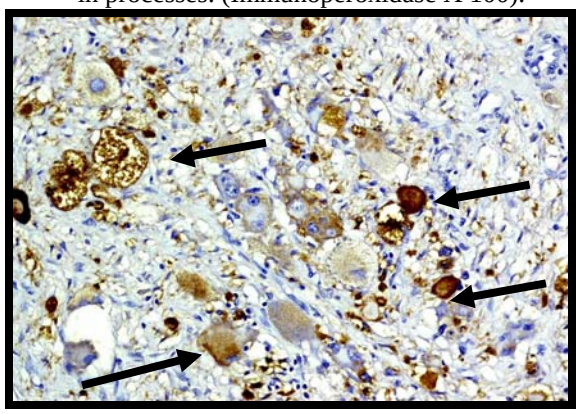

Fig. (21): Photomicrograph of ganglioneuroma showing immunoreactivity for chromogranin A. Note ganglion cells(arrows) are strongly stained. (Immunoperoxidase X 200).

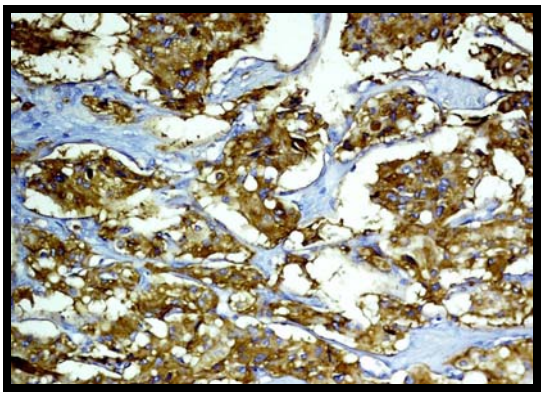

Fig. (22): Photomicrograph of pheochromocytoma showing immunoreactivity for neuron specific enulase, Note the diffusely strong staining within the cytoplasm of Chief cells. (Immunoperoxidase X 200).

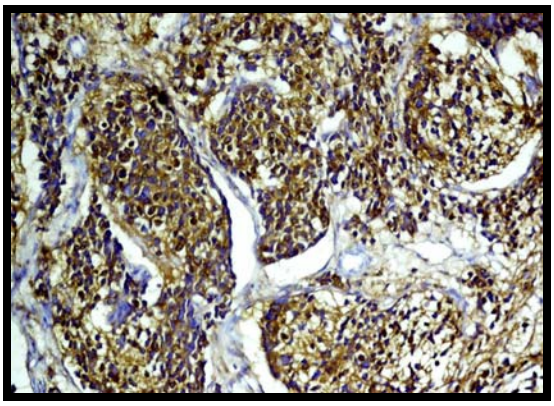

Fig. (23): Photomicrograph of neuroblastoma stained for neuron specific enulase. Almost all of the cells show a positive cytoplasmic reaction, whereas the stroma is unstained. (Immunoperoxidase X 200).

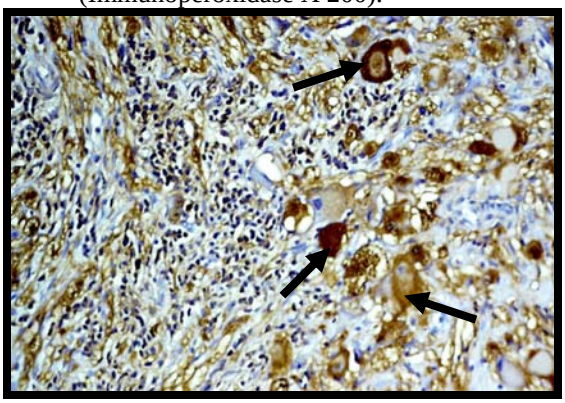

Fig. (24): Photomicrograph of ganglioneuroblastoma stained for neuron specific enolase, highlights abundant stromal septa. The cytoplasm of ganglion cells (arrows) is also intensely positive.(Immunoperoxidase X 200).

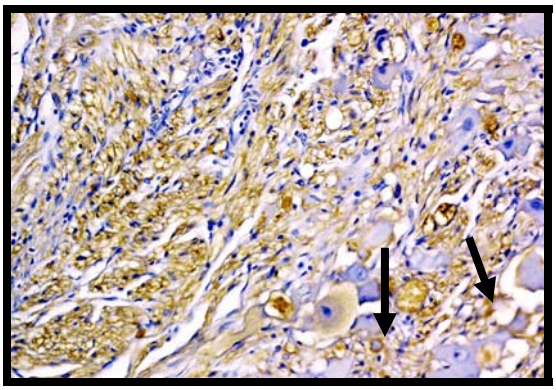

Fig. (25): Photomicrograph of ganglioneuroma showing immunoreactivity for NSE. Ganglion cells are strongly stained (arrows). Schwann cells are also stained positively. (Immunoperoxidase X 200).

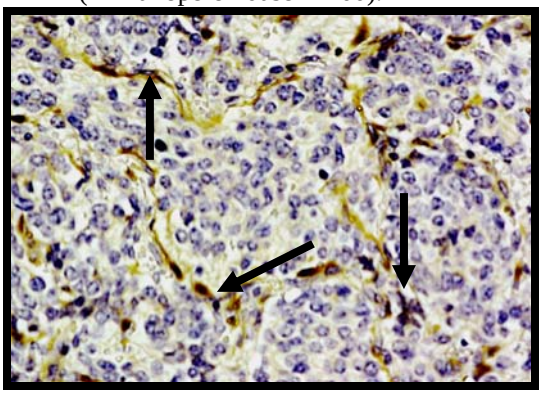

Fig. (26): Photomicrograph of pheochromocytoma showing immunoreactivity for S-100 protein in sustentacular cells (arrows) surrounding the chromaffin cells. (Immunoperoxidase X 200).

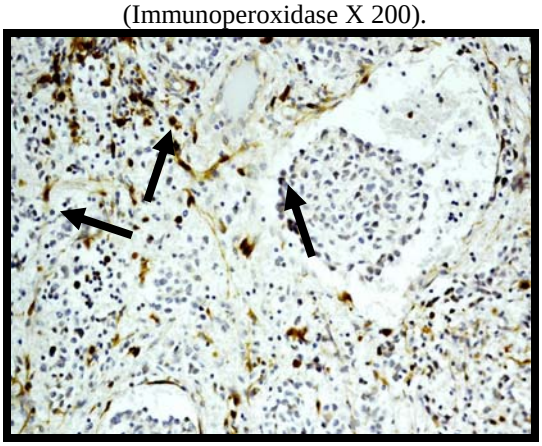

Fig. (27): Photomicrograph of neuroblastoma showing satellite to dendritic cells immunoreactive for S-100 protien are adjacent to vascular septa (arrows). Cells are consistent with Schwann or sustenticular cells. (Immunoperoxidase X 100).

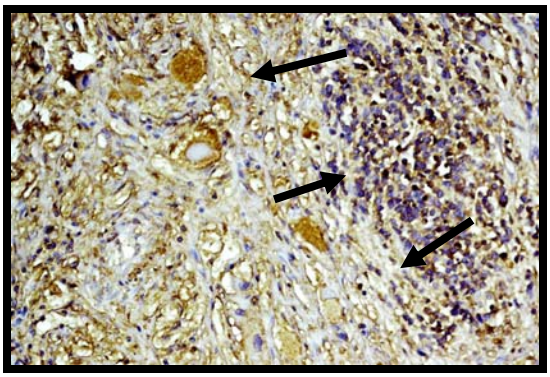

Fig. (28): Photomicrograph of ganglioneuroblastoma, immunostained for S-100 protein. Cells in intimate contact with ganglion cells show intense staining (arrows), and are in the typical location of satellite cells. (Immunoperoxidase X 100).

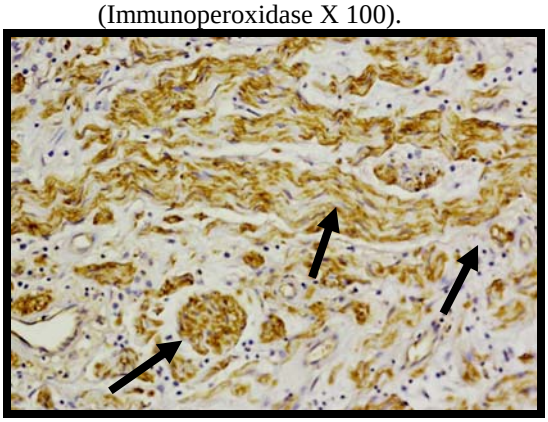

Fig. (29): Photomicrograph of ganglioneuroma showing immunoreactivity for S-100 protein, revealed cytoplasmic stain of the ganglion cells (arrows). Matrix and schwann cells were also strongly positive. (Immunoperoxidase X 100).

4. Discussion

a- Pheochromocytoma:

Pheochromocytomas are rare tumors of the adrenal medulla or the paraganglion system. There are no histological or immunohistochemical markers available that define the malignant behavior of these tumors, so for only the discovery of metastases reveals malignancy (Salmenkivi *et al.*, 2001 and David *et al.*, 2004). Thus, 39 pheochromocytomas (32 benign and 7 malignant) were analyzed histomorphologically and immunohistochemically. The patients were between 19 and 61 years of age (average age 36.4 years). Most of the cases are in 4th and 5th decade of life and there were 24 females (61.5%) and 15 males (38.4%). The largest pheochromocytoma had the diameter of 14cm. In this respect, Lack (1994) documented that, the peak age at diagnosis is in the 5th decade of life, but

pheochromocytomas can affect any age group. Most progressive information revealed a roughly equal sex incidence, but some show a slight predilection for both male and female (Tatic *et al.,* 2002). However, they reported that, the incidence of pheochromocytoma is similar in both sexes and most frequent between the ages of thirty and fifty. The largest pheochromocytoma had a diameter of 12 cm. In this study, histological examination of pheochromocytomas showed that the cells appeared with significant irregularities in shapes , dimensions and their patterns. Pheochromocytes cells were mostly of polygonal shape (35 cases of 39); whereas in the other four cases spindling cells were evident. This result is in agreement with other international published studies reported by Saeger *et al.* (2003). They reported that, histological criteria for aggressive biological behavior of pheochromocytoma include limited pattern of growth, 3-5 mitotic figures on 10 microscopic high power fields and invasion of a capsular lymphatic and blood vessels.

In the present study, the intracytoplasmic hyaline globules which are periodic acid-Schiff positive were detected in 26 of 32 clinically benign and only 2 of 7 malignant pheochromocytoma. Dekker and Oehrle (1971) in their study reported that there might be intracytoplasmic hyaline globules that are periodic acid-Schiff (PAS) positive and resistant to diastase pre- digestion. These globules are identical to those observed in the normal and hyperplastic adrenal medulla. In the meantime, hyaline globules were detected in 38 of 64 clinically benign and only eight of 34 malignant sympathoadrenal paragangliomas (Linnoila *et al.,* 1990). It has been found that these globules are related to secretory activity in some way, but their functional significance is not clear (Mendlesohn *et al.,* 1978). All cases of pheochromocytoma in the present study were positive for reticulin stain. This stain for reticulin clearly accentuates the alveolar anatomizing trabecullar patterns with distinct nests of cells (Zellballen appearance).

All the studied pheochromocytomas (39 benign and 7 malignant) showed chromogranin A and neuron specific enolase immuno-positive reaction in nearly all tumor cells. Similarly, Pace *et al.* (2002) and Feng *et al.* (2005) reported that all pheochromocytomas were strongly immunoreactive for chromogranin A and neuron-specific enolase. In our study, there is no differences between expressions of Cg A in malignant or in benign pheochromocytoma. Whereas, Feng *et al.,* (2005) concluded that, there was statistically significant difference of Cg A expression between ACT and AMT and also between benign and malignant pheochromocytoma.

The results of the present study revealed that, the intensity of staining for neuron specific-enolase is supreme in cases comprising benign and all malignant pheochromocytoma. This result is different with Kliewer *et al.* (1987). They showed that, the intensity of staining of neuron-specific enolase is furthermost (strong) in benign paragangliomas and weak (faint) in malignant tumors. Tatic *et al.* (2002) reported that, immunohistochemical analysis has confirmed the importance of pan-neuron endocrine markers (Cg A and NSE) in pheochromocytoma identification. Pheochromocytomas have a multiple synthetic activity as chief neuroendocrine feature.

Giovanella (2005) reported that, chromogranin A is a member of the granin family contained in secretory vesicles of chromaffin adrenal cells. Despite pheochromocytoma tumor cells heterogeneity chromogranin A and neuron-specific enolase are the most common neuoropeptides synthesized. They are associated with the presence of neuroendocrine storage granules. Report of Moreno *et al.* (1999) showing the presence of neuron-specific enolase in all pheochromocytomas extended our initial observation on the localization of neuron-specific enolase in pheochromocytomas and other neuroendocrine tumors.

The detection of neuron-specific enolase in pheochromocytomas can be helpful in cases in which the differential diagnosis between pheochromocytomas and other tumors, such as ACC, may be a diagnostic problem although the histological features of pheochromocytomas occurring in patients with malignant and benign tumors are variable. These neuroendocrine tumors all possess a distinguishing histochemical marker that is the neuron-specific enolase.

Giovanella (2005) and Feng et al. (2005) showed that, the expression of chromogranin A was strongly positive in chief cells that contained granins in their vesicles. These results correspond to our results in two cases of composite pheochromocytoma in which the component of pheochromocytes are strongly positive for chromogranin A and neuron-specific enolase. The other components of ganglioneuroma were positive for chromogranin A in some ganglion cells and were positive for neuron-specific enolase. Since, in this material, staining for S-100 protein was restricted to dendritic cells which are located at the periphery of the trabecullar or solid main cell clusters, all such S-100 immunoreactive elements were assumed to represent sustentacular cells. S-100 positively of sustentacular cells in paragangliomas has been reported in several studies covering adrenal and extra-adrenal sympathetic neoplasms (Guido Belleza *et al.,* 2004). The present work showed that, S-100 protein positive

sustentacular cells were found in 32 of 39 benign pheochromocytomas in immunohistochemical assessment. It can be observed that, complete loss of sustentacular cells was in malignant pheochromocytoma (7 of 39 cases). Achilles *et al.* (1991) discovered that, S-100 protein staining of sustentacular cells was seen in both adrenal and extra-adrenal lesions. The 10 malignant tumors are entirely devoid of S-100 protein positive cells. Therefore, the presence of S-100 protein positive cells may help to exclude malignancy in individual pheochromocytoma cases. Achilles *et al.* (1991) stated also that, the immunohistochemical staining for S- 100 protein has been designated on appropriate tool to further subclassify paragangliomas, at least in the case of adrenal pheochromocytoma.

b- Neuroblastoma and ganglioneuroblastoma:

The age of patients was between 4 months and 44 years of age (average age 6 years). Most of the patients occur in the first 4 years of life and they were 12 females (48%) and 13 males (52%). Size of tumors ranged from 9.5-20 cm with the average diameter of 14.5cm. In this respect, Young *et al.* (1986) reported that, 85% of all neuroblastoma and ganglioneuroblastoma cases occur in the first 4 years of life and there are no sex-related differences in incidence rate. Koike *et al.* (2003) reported that, adrenal ganglioneuroblastoma is extremely rare in adults. The present study revealed that 19 patients with neuroblastoma and ganglioneuroblastoma are still living after adrenalectomy, while six of the cases received chemotherapy and none was living after 24 months. Evans *et al.* (1996) reported that, neuroblastoma at different stages has an 80% of survival rate and no evidence indicates that chemotherapy improves survival.

Torrisi *et al.* (2003) reported that, there are obvious difficulties in determining the incidence of neuroblastoma giving its capacity for regression, maturation and early death. Treatment complications prohibit reproduction and prevent multigenerational pedigrees for evaluation. Bundy *et al.* (1982) indicated essentially no risk of offspring of surviving patients with neuroblastoma or ganglioneuroblastoma developing similar tumors. Koike *et al.* (2003) studied a case of adult type ganglioneuroblastoma and they observed that, there has was no evidence of recurrence for 2.5 years after the operation.

In the current results, histological appearance of neuroblastoma showed accentuation of fibrovascular stroma and tumor nodules formed of monotonous primitive cells. Sometimes, there are areas with a more diffuse or solid pattern, while ganglioneuroblastoma showed a patchy nodules of immature neuroblasts set within a mature ganglioneuromatous stroma. Shimada *et al.* (1984) and Lack *et al.* (1990), reported that, neuroblastoma and ganglioneuroblastoma often have a lobular growth pattern with delicate, often incomplete fibrovascular septa. Sometimes there are areas with a more diffuse or solid pattern. Hachitanda *et al. (1994)* showed that, a distinct organoid pattern has been recently described in which a thin fibrovascular meshwork isolates regular nest of neuroblastoma cells. This is reported to be an indicator of good prognosis in younger children.

c- Ganglioneuroma:

The patients were between 15-35 years of age (average age 23.2 years) and there were four female and five males. Size of tumors range from eight to 15cm with the average diameter of 11.7 cm. In general, survival in our population was excellent with no deaths seen at a mean follow up of 42.5 months (range 18-121 months*).*

Bove and Mcadams (1981) reported that, ganglioneuroma usually occur in an older age group than neuroblastoma and showed that many patients are 7 years. Nelims *et al.* (2004) showed in some cases, ganglioneuroma was the end stage of maturation of less differentiation tumors such as NB and GNB. It was based on the age at diagnosis over 10 years of ages and the anatomic location of these tumors appears to arise de novo.

The present work revealed that, two distinct cell groups were identified, ganglion cells and Schwann cells placed in a loose myxoid stroma. In this respect, Torrisi *et al.* (2003)found that, the tumor of ganglioneuroma consisted of proliferation of well differentiated Schwann cells and ganglion cells in the lamina properia.

In the present study, special stains such as periodic acid Schiff reaction (PAS) and reticulin stain were applied on all cases of neuroblastoma groups (Neuroblastoma, ganglioneuroblastoma, and ganglioneuroma). The results showed that, most of cases were rich in reticular fibers in tumor cells but there was no hyaline globules in neuroblastoma group. Triche *et al.* (1987) were be able to support this result. They reported that, neuroblastomas are usually glycogen-poor and PAS negative. This property is classically used to distinguish neuroblastoma from Ewing's tumor.

In this study, it can be concluded that, the tumor cells, in a large number, of neuroblastoma groups (NB, GNB and GN) were positively stained by several neuroendocrine antibodies as well as by antibodies specific for chromogranin A, neuron-specific enolase (NSE) and S-100 protein.

Examination of chromogranin A showed that, 19 of 30 neuroblastoma groups were chromogranin A-positive (5 cases NB, 5 cases GNB and out of 9 cases GN). This result can be compared to those of

Douglas *et al.* (1994) who reported that, five of six pure neuroblastoma were reacted positively with chromogranin A. In addition, Molenar *et al.* (1990) showed that, immunostaining for chromogranin A has been demonstrated in neuroblastomas, ganglioneuroblastomas and ganglioneuromas. Positive results are expected where the concentration of neurosecretory granules is greatest such as ganglionic cells with active synthesis or areas with overlapping or intertwining of numerous neuritic processes (e.g., Homer Wright rosettes) or matted neuropil. Eder *et al.* (1998) studied several cases for neuroblastomas and ganglioneuroblastomas and they reported that chromogranins are a class of acidic proteins found in large secretory granules of neuroendocrine tissues and tumors derived from them.

Tornoczky *et al.* (2004) reported that, both neuronal cells expressed immunoreactivity for chromogranin A and neuron-specific enolase. Small neuronal cells showed more intense chromogranin A immunoreactivity, indicating an earlier stage of neuronal differentiation. Liu *et al.* (2003) showed neuron specific enolase and chromogranin A were expressed in small cells in neuroblastomas. Other authors often showed positive immunoreactivity for multi-neural markers such as chromogranin A, synaptophysin and neuron-specific enolase but were negative for S-100 protein, CD34 Desmin and CD45 in 8 cases of neuroblastomas (Hasegawa *et al.*, 2001). The results of the present study revealed that, four cases of ganglioneuroma stained with chromogranin A were negative and 5 cases were positive in cortical tissues. Kazantesva *et al.* (2002) confirm our results when they observed chromogranin A in cells of cortical adenoma and in 20-75% of carcinoma cells. These ultramicroscopically findings were confirmed by the observation of typical neuroendocrine granule. In this study, it can be concluded that, the tumor cells of a large number of neuroblastomas are positively stained by antibodies specific for neuron- specific enolase. All neuroblastomas irrespective of their degree of differentiation stained positively with the neuron-specific enolase antibody. NSE stained both cytoplasm in precursor cells and nerve processes in neuroblastomas.

Thomas *et al.* (1987) studied the immunostaining of neuron-specific enolase in neuroblastomas. They found that, immunostaining for neuron- specific enolase is usually apparent in neuroblastoma and ganglioneuroblastomas, with highlighting of neuritic extensions in the form of rosettes, matted neuropil or sparse internuclear fibrillar matrix.

In the present study, the immunohistochemical analysis of the adrenal neuroblastomas directed to S-100 protein revealed cytoplasmic staining of the ganglion cells in ganglioneuroma. The matrix and Schwann cells were also positive for S-100 protein. While in ganglioneuroblastoma, the cytoplasm of some cell bodies was also intensely positive for S-100 protein. Immunostaining for S-100 protein may demonstrate small to elongate dendritic-shaped cells in NB and GNB, particularly in fibro- vascular septa separating the tumor cells. These have been regarded as Schwann cells or precursor cells, suggesting possibility of differentiation within the tumor. Pace *et al.* (2002) in his study found that, matrix and schwann cell were positive for S-100 and for glial fibrillary acidic protein. Aoyama *et al.* (1990) suggested that, immunoreactivity for S-100 protein has been used to evaluate differentiation of neuroblastoma cells into Schwann cells, and may provide some information regarding prognosis, even though it is not entirely specific for Schwann cells. Liu *et* al. (2003) reported that, S-100 protein was positive in the area of bunch of neurofibrils. The present results also keep in toutch with Wallace *et al.* (2003) who found the spindle cell component stained positively for S-100 protein in ganglioneuroma. The ganglion cells in the studied tumors were stained positively with S-100 protein. On the contrary, this result is dissimilar with the last authors who reported that, the ganglion cells were unstained positively with S-100 protein but were stained positively for glial fibrillar acidic protein by routine immunohistochemical staining.

Corresponding author
Samia, M. Sanad
Zoology Department, Faculty of Science, Zagazig University, Egypt. egypt_sbes@hotmail.com

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Genes Reprogramming During ATRA-induced Differentiation of Acute Promyelocytic Leukemia Cells

Yiwu Sun, Kafeel I Muhammad, Neal Hakimi

Department of Medicine, Brookdale University Hospital and Medical Center, Brooklyn, NY 11212, USA
yiwu_sun@hotmail.com

Abstract: The therapeutic and preventive activities of retinoids in cancer are due to their ability to modulate the growth, differentiation, and survival or apoptosis of cancer cell. An acute promyelocytic leukemia cell line AP-1060 presents an abrupt response to all-trans retinoic acid (ATRA) which depends on its mutant PML/RARα. Microarray analysis of 9265 sequences demonstrated a complex cascade of reprogramming of AP1060 upon treatment with ATRA characterized by the differential expression of gene sets between induced (100nM ATRA) and sub-induced (10nM ATRA) cells. Among 1550 modulated genes by ATRA, 47% shown differential expression. A number of the small G-protein family was extensively involved in this reprogramming. The early up-regulated IL1 and down-regulated its angonistor may initiate the apoptosis pathway mediated by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Seven in absentia (Drosphila) homologies (SIAH-1, 2, and 3) considered as the inducers of apoptosis were repressed. Thus, a balanced functional network seem emerge and contribute to ATRA inducing differentiation based on the uniquely biologic and molecular characters of AP1060.
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Keywords: Acute promyelocytic leukemia; Retinoic acid; Induced differentiation

1. Introduction

Acute promyelocytic leukemia (APL) is an interesting system in the study of human cancer because it is sole human malignancy that can be successfully treated with the induced differentiation therapy. The most of APL are characterized by the chromosome translocation t(15;17), and the fused PML/RARα resulted from this translocation is responsible for the leukemogenesis. It used to be believed that PML/RARα as a negative dominant factor abrupt PML and RXR by hterodimerization with PML/RARα (Raelson, 1996), which respectively interferes the function of RXR and PML for growth inhibition and apoptosis regulation and the differentiation pathway dependent on RXR (Kastner 1992; Grignani 1993; Rousselot 1994; Wang 1998). The early studies implies that the response of APL to ATRA base on the intrinsic molecular target PML/RARα to fusion gene which involved the gene transcriptional events that initiate the differentiation (Nervi and Nason-Burchenal 1998).

Both RARα and PML/RARα as interact with nuclear corepressor (N-CoR), mSin3 and a histone deacetylase (HAD) to form co-repressor complex: RARα-CoR or/and PML/RARα-CoR, the later is more stable (Dyck and Weis 1994; Lin and Minucci 2000). Deacetylation of histones by this complex may result in chromatin assembly and transcriptional repression. The exposure to ATRA orchestrates an ordered response of this apparatus involving initial dissociation of co-repressor from RARα or the RARα-portion of PML/RARα, but not from PLZF/RARα (an APL phenotype resistant to ATRA) (Dyck and Weis 1994), which permit association of co-activator (CoA) for forming a co-activator complex (CBP/P300, P/CAF, NcoA-1/SRC-1, P/CIP, and ACTR) to relieve the negative repression through post-transcriptional down-regulation of PML/RARα. The functional significance of histone deacetylase recruitment in APL pathogenesis was demonstrated by ability of histone deacetylase inhibitors in combination with ATRA to overcome the repressor activity of PML/RARα and PLZF/RARα in transient transfection assays, and to enhance the terminal differentiation of APL cells (Alland and Hong 1997; Lin , Grignani and Guidez 1998). In addition, recent evidence suggests that PML-RARα is also able to recruite the methylating enzymes (DnmT1 and Dnmt3a), leading to the hypermethylation of the RA target promoters resulting in transcriptional repression (Di Croce 2002; Villa 2006). These studies suggested that differentiation of APL by ATRA is triggered directly through a PML/RARα dependent signaling pathway.

We developed a new APL cell line AP1060 (Sun 2004), which harbored a missense mutation (Pro407Ser) in PML/RARα and presented an additional translocation t(3; 14) in addition to t(15;17). The very abrupt ATRA concentration response of AP-1060 may make this cell line of unusual value for analyzing subsets of response

genes. For example, evidence was presented that some genes, including CD18, CD38 and myeloblastin, can be turned on by ATRA (Shao 1997). This suggests that some genes are activated from normal RARα-responsive loci that are not susceptible to dominant negative repression by PML/RARα or possibly that some non-RARα mechanism is involved (Kang 1999). We undertook a study base on large-scale expression analysis, microarray to identify gene expression patterns in APL cells upon treatment with ATRA.

2. Material and Methods

Cell culture: AP-1060 cells were maintained in IMDM containing 10% FBS and 10% agar leukocyte conditioned medium (ALCM, StemCell Technologies, Vancouver, BC). For induced differentiation, cells at $1X10^6$ were suspended in IMDM containing 10% FBS supplemented with 5% ALCM, and treated by addition to the culture medium of either 10 nM, 100nM ATRA (Sigma, St. louis, MO). Control cells were treated stimultaneously carrier, and for each time point, a control flask was maitained for the same length of time and harvested in parallel.

Evaluation of the cellular maturation: A panel of monoclonal antibodies (CD11a, CD11b, CD15, CD15s, CD18, CD38, CD45, CD65s, CD117, MPO) as described (24) were used to identified the immunophenotyping markers before and after treating AP1060 cells with ATRA. The procedures used for treatment of cells in suspension culture with RA and for the measurement of terminal cell differentiation by the nitroblue tetrazolium dye reduction (NBT) test was performed as previously described (Paietta 1994). Cytologic evaluation of cell differentiation features was determined from Wright –stained cytospin preparations.

Retinoic Acid Binding Assay: Assay the levels of [3H]RA binding in cytoplasmic extracts of AP1060 measured by fast performance liquid chromatography (FPLC) (Jetten 1990). Briefly, 10^8 Cos-1 cells were suspended in 5ml phosphate buffered saline. PSG5 expression vector DNA (400ug), containing either wilde-type long form (P-R-L) or mutant PML-RARa (Pro407Ser), was mixed with the cell suspension for 10 minutes at room temperature. Electro-poration was done by using a Gene Pulser II at 250 uF and 350 V. After 3 days' culture in Dulbecco modification of Eagle medium (DMEM) with 10 % fetal bovine serum (FBS), the cells were harvested for nuclear protein extraction, as described. To test RA binding, 0.2 ml nuclear extract was incubated for 15 hrs at 4 C with 10 nmol/L [^{3}H] RA (30 Ci/mmol) (Dupont-NEN, Boston, MA) in the absence or presence of 200-fold excess of unlabeled RA. The unbound RA was removed by incubation with dextran-coated charcoal for 15 minutes and then centrifuged for 15 minutes at 10000g. The supernatant was analyzed at 4 C by fast performance liquid chromatography (FPLC) , using a Superose 6HR 10/30 size-exclusion column (Pharmacia Biotech, Piscataway, NJ).

RNA and probe preparation: Total RNA was prepared with Trizol (Life Technologies, Inc). For each hybridization, two separate probes were made by using 3DNA Submicro Expression Array Detection Kit (Genisphere, PA): one labeled with Cy3 (control) and another with Cy5 (treated). The 11ul reactions mix was set up by adding 10ug RNA, 3ul Cy3 or Cy5 RT primer (0.067pm0l/ul), and heated at 80°C for 10 minutes to denature the nucleic acid. The following were then added: 1ul Rnase inhibitor (Promaga, MN), 4ul 5X fist-strand buffer (250mM Tris, 375mM KCL, and 15mM MgCl, pH 8.3), 1ul dNTP Mix (10mM each dATP, dCTP, dGTP, and ATTP), 2ul DTT (0.1M), and 1ul Superscript II Reverse Transcriptase (200units/ul; Life Technologies, Inc). The reaction was carried out for 2 hour at 42°C, and stop by adding 3.5ul of 0.5M NaOH/50mM EDTA and heating at 65°C for 10 minutes. After neutralized with 5ul of 1M Tris-HCL(pH7.5), The probes were precipitated by addition of 15ug Linear Acrylaminde and 2.5 volumes of cold ethanol:0.25 volumes 10M Ammonium Acetate, harvested by centrifugation, and resuspended in 15ul Hybridization buffer (40% Formamide/4X SSC/1% SDS).

Hybridization of microarray: Microarray array was printed by Albert Einstein College of Medicine facility, and consisted of the PCR-amplified products of 9256 cloned sequences (Cheung 1999). The slides were prepared for hybridization by moistening over boiling water and then immediately cross-linked with 80 mJ of UV light (UV Stratalinker, Stratagene, La Jolla, CA). Slides were again moisten over boiling water, snap-dried on a hot plate, and soaked for 1 hour in 0.6 M succinic anhydride, 0.02 M sodium borate (pH8.0) in 1-methyl-2-pyrrolidinone. After briefly rinses in 0.1% SDS and distilled water, they were soaked in boiling water for 5 minutes, followed by an ethanol risne at -20°C. The chips were pre-hybridized for 3 hour at 55°C with 2X SSC/0.1%SDS/1%BSA, washed with 2XSSC and 0.2X SSC each for 5 minutes. The hybridization mixture (15ul above probe, 2ul oligo dT blocker and 500ng Cot 1 DNA) was heated at 80°C for 10 minutes. After cooling to room temperature, the mixture was incubated with 5ul of each Cy3 and Cy5 Capture reagent at 55°C for 30 minutes to pre-hybridize the cDNA to the dendrimer, and applied to the pre-hybridized slide,

covered with cover-slip, and hybridized at 55°C for 16 hours. The slide was washed in 2X SSC/0.2% SDS at 60°C for 15 minutes and then in 0.2X SSC/0.1% SDS and 0.1X SSC at 37°C each for 20 minutes.

Scanning and Analysis: Albert Einstein College of Medicine facility provided scanning service. The excitation for Cy3 and Cy5 was at 532 and 633 nm, respectively, and detection centered on 570-580nm and 670-680nm. The emission from the Cy3 and Cy 5 fluorochromes was recorded in two separate high-resolution scans. Two images were superimposed, and the emission at each wavelength was quantified by Genepix 4.0.

The above data were transferred to an Excel spreadsheet, where the signal:background ratio for each channel was calculated as well as the ratio between these ratios (i.e., red signal:background ratio was divided by the green signal:background ratio). The data were then normalized among dots by expressing this value as a ratio to the average of these values for all 9265 genes. The data were transferred to Microsoft Access, where a combination of Access, Microsoft Excel, GeneCluster and TreeView were used for analysis (Eisen 1998).

The time points and RA concentrations were considered as two given agents in a single experiment. For each agent, data for each gene was collected if the signal:background ratio was >1.25 for either the red or green channel.

3. Results

Differentiation response of AP1060 has typical cytological features of the M3 variant form of APL, including a high nuclear:cytoplasmic ratio and cytoplasmic hypogranularity. Following short-term culture in the presence of 100nM ATRA, there was obvious evidence of cellular differentiation compared to untreated cells (Fig. 1A, B). The impression that these nuclear changes reflected increased differentiation was confirmed by NBT test. However, no discernible differentiation was observed with 10nM ATRA (Fig. 1C). After for 5 days, The NBT positive cells increased to 98% in the percentage of 100nM ATRA, compared to increase from the baseline value of 2% in the absence or presence of 10nM ATRA (Fig. 2). At 10nM, expression of myeloid differentiation antigens (CD65s, CD15, CD11b) remained unchanged compared to cultures in the absence of ATRA. However, expression of the precursor antigen CD117 decreased by 50% and CD38 increased from 10 to 44%. At 100nM, all myeloid antigens were present at > 90% of cells while CD117 were lost (Fig. 2).

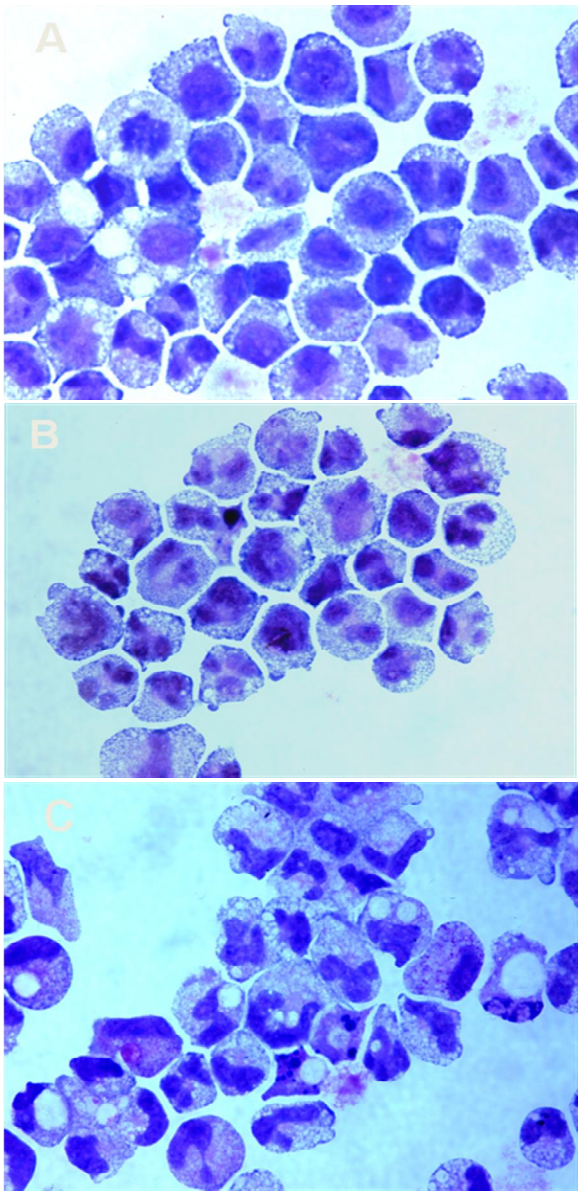

Figure 1. Cytologic evaluation of AP1060 in the absence or presence of ATRA. The cells were cultured for 5 days. Photograhps of cytospin slide were taken at 1000X magnification of modified-wright's stained. (A) without ATRA treatment; (B) 10nM ATRA; (C) 100nM ATRA.

Our published data, which the trans-criptional transaction was performed in COS-1 cells with a reporter vector containing a repeat retinoic acid response element (DR5-tk-luc) to determine whether the RA-dose-response of AP1060 cells dependent on the missense mutation (P407), revealed its abrupt transcriptional transactivation response to ATRA comparing with the L-form PML-RARα (P-R-L) (Sun 2004). This is consistent with the differentiation responsive to ATRA (Fig 2). With

size-exclusion FPLC analysis, RA binding activity was tested by incubating variable concentrations of [3H]RA with nuclear extracts from COS-1 cells which were transiently transfected with pSG5 vector expressing mutant PML-RARα. Nuclear extracts were incubated with the respective concentrations of [3H]RA in the absence or presence of a 200-fold excess of unlabeled ATRA for 15 hr at 4°C. This mutant PML/RARAα completely lack RA binding activity at 1 nM ATRA, but bound ligand in a monomeric and dimeric form respectively at 10 and 100 nM RA. This contrasts to the wild-type L-form (P-R-L) that presents more characteristic high molecular mass, multimeric complexes (Fig. 3). It indicated that the mutant PML-RARα (Pro407Ser) product may represent the mutant PML-RAR homodymers and/or low-molecular-weight complexes with other nuclear proteins at 10 nM ATRA.

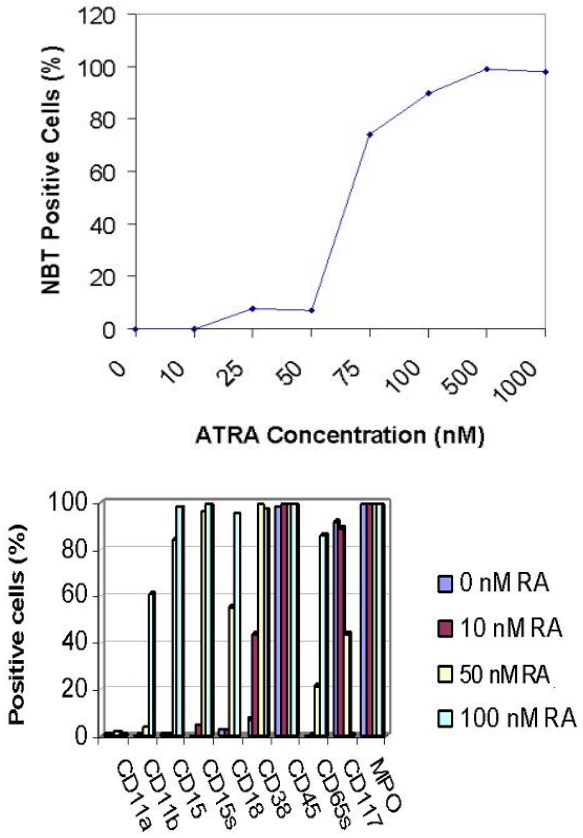

Figure 2. Differentiation response of AP1060 cells to ATRA. The cells were exposed to the indicated ATRA concentrations for 5 days. The percentage of terminally-differentiated cells was measured by NBT and a surface antigen panel.

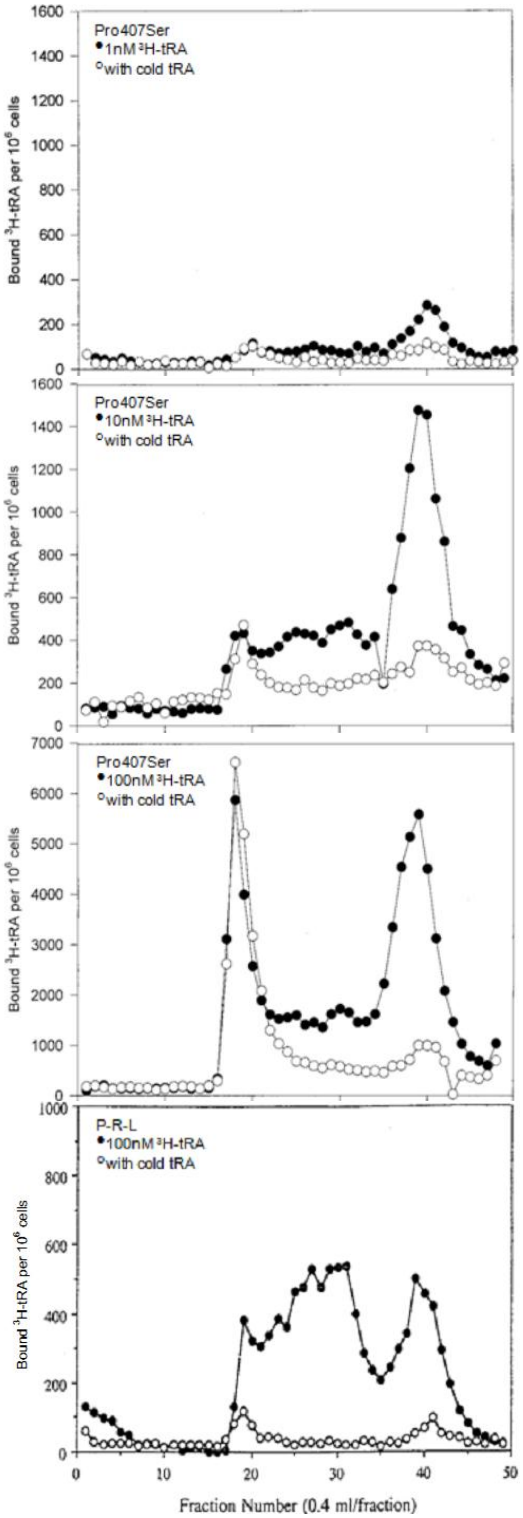

Figure 3. FPLC analysis as described in Methods. (•) [^{3}H]t-RA alone; (o) [^{3}H]t-RA plus 200-fold excess of unlabeled RA. The mutant (Pro407Ser) completely lacked RA binding activity at 1 nM RA, but bound ligand in a monomeric/dimeric form at 10 and 100 nM RA in contrast to the multimeric complexes presented by wild-type L-form (P-R-L).

In separate comparisons of the genes expression with the two different concentrations and time course, the initial ratio of treated to control was set as < 0.8 and > 1.25 (0.8 < t/c > 1.25). 2768 and 3003 genes at a t/c > 1.25 respectively was up-regulated after 12hr and 2623 and 3971 after 24 hr 10nM or 100nM RA exposure.

At a ratio < 0.8, 2330 and 2706 genes were down-regulated at 12 hr and 2001 and 2670 at 24hr. As analyzed at successive 0.25 increments or 0.1 decrements in t/c, the number of up- or down-regulated genes fell off with a bell-shaped distribution, but the numbers were still substantial up to 121 or 536 at 12 hr and 299 or 974 at 24 hr although t/c > 2.5 (Figure 4). Response to ATRA reflected the fact that alterations in expression proceed as a function of time, with increasing time; there was a progressive recruitment of sequences over the 24 h treatment.

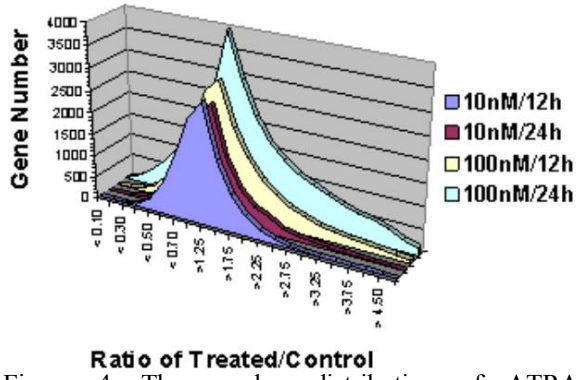

Figure 4. The number distribution of ATRA modulated genes. Screening the genes base on treated/control ratio (t/c) > 1.25 or < 0.8, and on successive 0.25 increments or 0.1 decrements at t/c.

The overall differences in profile of gene expression in 10nM and 100nM, and hence in mechanism of differentiation stimulated by ATRA, were impressive. We selected all genes from the database that were same altered in expression (0.8 < t/c > 1.25) at any time points for 100nM treatment but not for 10nM treatment, and that there were 9 gene cluster in this population of 1550 sequences. 730 among the 1550 sequences present differential expression pattern (Table 1). Other 820 sequences, which present same expression pattern in both 10nM and 100nM, were further dissected according to the criteria that the differential expression genes must show the ratio of the induced to sub-induced (100nM treated/control/10nM treated/control) more than 1.5 or less than 0.6. 240 in upregulated and 120 in downregulated genes set were selected based on these criteria.

In order to further understand the reprogramming of the RA-induced differentiation, we delved more deeply into the differences between the induction and sub-induction by defining gene database into 26 functional gene classes. The class of genes involved in signaling was by far the largest functional class, consisting of 329 members. This class was broadly defined both molecules that may interacted with components of signal transduction pathways (e.g., E-cadherin) and effectors molecules that alter transcription (e.g., fos) as well as signaling components (e.g., kinases) themselves. Within this subclass, 68 genes increased in expression, and 64 were repressed. The 132 genes that encode components of signaling pathways that altered at inducing and sub-inducing concentration, and over the time course of RA treatment. It is interesting that the big differences in genes expression exist between the induced and sub-induced. Thus, extensive alternations in large number of signaling pathways and their components characterize the RA response. The Cluster and TreeView were used to display the profile of the differential expression of these genes (Fig. 5). The results are shown in figure with red representing increased expression, green representing decreased expression, and the magnitude of change depicted by the intensity of color.

Table 1. Profile of the 1550 genes modulated in the induced treatment (100nM) by comparing with same genes set in the subindued (10nM). Upregulaed: Treated/control (t/c) > 1.25; No change: t/c > 0.8 & < 1.25; Downregulated: t/c < 0.8.

10nM \ 100nM	Downregulated	No Change	Upregulated
Downregulated	492	77	31
No Change	203		325
Upregulated	8	96	318

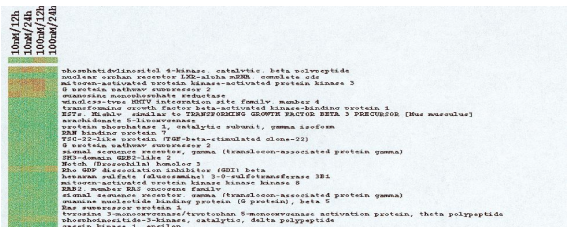

Figure 5. Treeview and Cluster analysis of the alerted expression of signal transduction subset. Red representing increased expression, green representing decreased expression, and the magnitude of change depicted by the intensity of color.

The second functional class of sequences investigated was a subset of 156 genes in the database involved in regulation of cell cycle progression (e.g., cyclins, cyclin-dependent kinases, Rb, myc (Fig. 6). By analysis of the Euclidean distances for this gene subject for the induced and sub-induced, it was first determined that for the functional class of genes responsible for cell cycle progression and arrest, the profile of expression in response to 10nM was less like that initiated by 100nM.

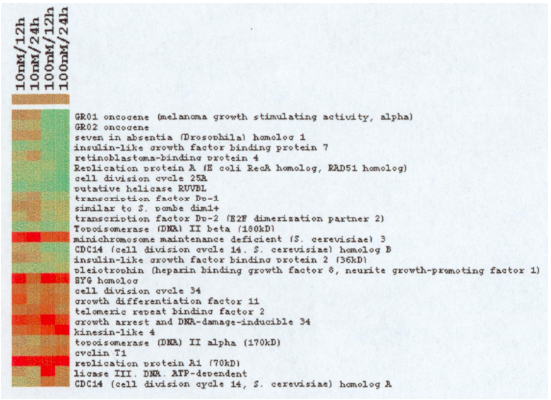

Figure 6. Difference in cell cycle genes set recruited by ATRA. Treeview and Cluster program were used to display sequences that were either induced (red) or suppressed in expression over 48 hours.

Figure 7. Alternation in transcription genes in response to ATRA. The differential expression between the induced and Subinduced cells was analyzed by Treaview and Cluster.

In the third class related to transcription, 20% genes were up-regulated and 33.9% were down-regulated by 100nM RA whereas respectively 11% and 3.6% by 10nM RA over 24 hours (Fig. 7). Most of sequences did not respond to 10nM RA. This illustrates that the transcription s that responded to 10nM and 100nM RA overlapped only to a very limited degree. In the fourth class, cytokines and growth factors, a different profile of genes expression was shown in Fig. 8. In the most interesting gene class, apoptosis, there is no any overlap between 10nM and 100nM RA treatment (Fig. 9). Finally, these data reflect the clear differences in mechanism of comeout treated by 10nM and 100nM RA.

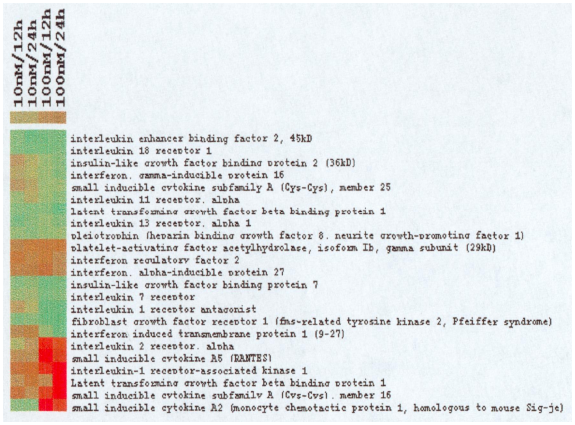

Figure 8. The differential expression in cytokine gene class between 10nM and 100nM ATRA treatment displayed by Treeview and Cluster.

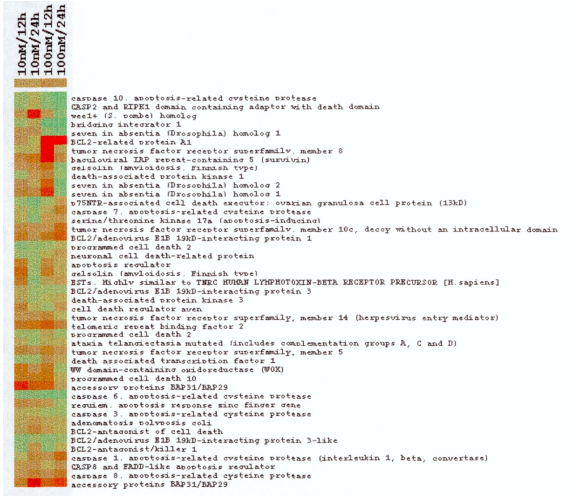

Figure 9. Recruitment of changes in gene expression in response to ATRA. This display all sequences related to apoptosis among 9256 genes printed on chip with Treeview and Cluster.

4. Discussions

One of the least understood aspects of the RA-mediated response of APL cells is the nature of the target genes that are activated leading to differentiation. Isolating the target genes has been an interesting topic. In the past few years, several other groups have tried on NB4 cell line that is inducible with RA and on the specimens of APL patients (Liu 2000; Ki-Hwan 2002; Morikawa 2003; Gutierrez 2005). However, It is impossible to screen the

subsets of genes related to the differentiation although a function assay was used to identify such genes (Khanna-Gupta 2007).

In Ap-1060 cells, a point mutation harbors in RARa portion of PML-RARa that generates an amino change from proline to serine (P407S). It confers AP1060 cell an unique character, abrupt response to ATRA, which was confirmed by either immunophenotype, NBT test or the transcription activity of mutant PML-RARa (P407S)(21). AP1060 cell line allows us to differentially analyze the transcriptional regulation and possible role in APL cells. We have used microarry technology for dissection of the reprogramming, which takes place when RA regulates the pathway of cell cycle arrest, apoptosis balance and terminal differentiation, by comparing induced with sub-induced. The distribution (Fig. 4) of the genes modulated by 10nM or 100nM RA reflected that these genes expression considerably overlapped in induction and sub-induction. In further comparing these genes in table 1, 1550 genes were found to be up- or down-regulated, but only 47.6% genes present a differential expression. These indicate that sub-inducing RA concentration produce substantial changes in gene regulation. But the integration and interaction of the gene modulation by 10nM is not enough to initiate reprogramming cell's differentiation.

This reprogramming is implicated the complicated procedure how pathways of cell cycling and cell death are coordinated to maintain differentiation. In four functional classes we have presented, genes involved in signal transduction, cell cycling, and apoptosis, there are a lot of significant modulations in expression of many genes that are generally considered fundamentally important in pathways of cell maturation, and many of these distinguish the induced-response from sub-induced response. Thus, the integration and interaction of modulation of large numbers of genes reflect the regulatory and functional circuits that determine the probability of a cell's behavior, such as continuation of proliferation, or cell cycle arrest accompanied by differentiation and/or apoptosis. Table 2 reflects such kind of modulation.

Many studies suggested that the initiation of differentiation required the transcriptional activation of specific genes leading to proliferation arrest and cell cycle exit (Rousselot 1999). Supporting this notion, most of G-protein family (RAB 13, Rho, and Rap1) were up-regulated whereas RAB interaction factor and G protein pathway suppressor were down-regulated. Along these same lines, genes favoring G1-S/G2-M transition, such as Mitogen-activited protein kinases (MAPK) and Transforming growth factor were repressed during inducing RA treatment. The fact that most of these modulations take place only at 100 nM RA treatment suggests that the sub-induced situation results from the modulations at 10nM that can not drive cells out off cell cycle (Table 2).

Table 2. The genes modulated by ARTA display the reprogramming map of cell differentiation.

			10nM RA		100nM RA	
			12h	24h	12h	24h
proliferation arrest and cell cycle exit	G protein family	RAB13 ↑	0.9	0.41	1.67	2.4
		RAB 36 ↑	0.88	0.87	1.26	1.7
		RAB interaction factor ↓	1.3	1.3	1..2	0.69
	G1-S/G2-M transition	M-phase phosphoprotein 1 ↑	1.1	1.3	1.3	2.69
		Growth arrest-specific 2 ↑	1.0	0.9	0.84	1.77
Cross-talking	G proteins – MAPK	in cooperative or antagonistic manner				
	TNF-INF – IL	TNF 11 ↑	0.87	0.95	1.1	2.2
		Interferon 2-inducible protein ↑	1.1	1.3	5.95	7.1
		IL 1 receptors ↑	0.61	1.0	0.8	1.8
		IL receptor 1 angonistor ↓	1.1	0.9	0.13	0.2
	cAMP related pathway	cAMP-dependent protein kinase	1.1	0.56	1.8	1.9
		cAMP responsive element modulator	0.7	0.55	0.54	1.44
Apoptosis and survival		BCL2-related protein A2 ↑	1.1	0.83	6.1	13.4
		Seven in absentia homologies (SIAH)↓	1.1	1.1	0.48	0.31
Differentiation		CEBPB ↑	1.6	1.9	8.7	5.7
		STAT3 ↑	1.97	1.18	7.1	13.4
		GATA Binding protein 2 ↓	2.1	1.3	0.72	0.70

A cross-talk between G-proteins and/or MAPK regulates various cellular functions in a cooperative or antagonistic manner (Matozaki and Sebolt-Leopold 2000; Pruitt 2001). Another cross-talk between Tumor necrosis factor and Interleukins may exist in RA-induced differentiation, in which TNF 1, 11 and IL receptors (e.g., IL-1R, IL-2Rα) were up-regulated and TNF receptor associated factor 3 (TRAF), TRAF interacting protein whereas IL receptor 1 angonistor, IL 8, IL6 were down-regulated. The role of TNF-related apoptosis-inducing ligand (TRAIL) has been confirmed in RA-induced apoptosis in APL (Altucci 2001). TRAF proteins are involved in signaling by members of the IL-1R/Toll receptor family or IL-15R and IL17R (Wajant 2001). TRAIL activation was a late event during RA-inducing differentiation ((Altucci 2001). Therefore, the early expression of IL1 may mediate the pathway related to TRAIL. Remarkably, TRAF proteins have also been shown to be involved in a functional MAPK cascade. Recently, FOXO3A, a target of TRAIL, was regarded as a key molecule for ATRA-induced granulocytic differentiation and apoptosis in APL(Yasuhiko 2010).

It is interesting detecting the increased expression of cAMP related protein such as cAMP phosphodiesterase, cAMP-dependent protein kinase and cAMP responsive element modulator. The sythestic treatment of CPT-cAMP and RA drives RA resistent NB4 subline, which contains a mutation (P407L) in PML-RARa, differentiation. Activation of cAMP-protein kinase pathway by cAMP correlated with the induction of differentiation and apoptosis (Kitamura 1997; Duprez 1996; Srivastava 2000).

Apoptosis antagonist, BCL2-related protein A2 was sharply up-regulated. Seven in absentia (Drosphila) homologies (SIAH-1, 2, and 3), in which SIAH-1, a p53-p21Waf-1 inducible gene, can induce apoptosis and promote tumor suppression (Roperch 1999), were repressed. These reflect the balance between apoptosis and differentiation to maintain the cell survival and maturation process.

The transcription factor C/EBP (CCAAT /enhancer binding protein) is a target gene of PML/RARα, and is crucial for induced myeloid differentiation in APL (Duprez and Truong 2003**;** Rego 2009). Here we found both of CEBPδ and GATA2 present opposite expression, up-regulated and down-regulated, separately in the induced and sub-induced RA treatment. CEBPβ presented 3-5 fold increasing modulation. Of note, GATA2 appears critically involved the survival and growth of multi-potential progenitors.[46] It was reported that the promyelocytic leukemia protein (PML) could complex with GATA2 and potentiated its transactivation capacity (Tsuzuki 2000).

The different profiles of gene expression induced by the inducing and sub-inducing RA concentration exhibited the differing kinetics of transcription activation. Our results indicated that the recombinant mutant PML/RARα bound [^{3}H] RA to saturation at 10nM, but presents monomeric comparing to dimeric form at 100nM, and in a transfection reporter vector assay using a direct-repeat RA response element/promoter, the transcriptional activation RA dose-response curve precisely followed the AP-1060 cell differentiation (Sun 2004). It imply that the transcription was activated by the protein interactions initiated by 100nM RA. Furthermore, Retinoid X receptors (RXR) that interact with RAR or PML/RARα to form functional hetrodimer shown differential expression in which RXRα was up-regulated by sub-inducing RA concentration and RXRγ presented the decreasing pattern in inducing RA treatment. Thus, the alerted population of nuclei receptors or transcription factors, which associate or dimerize with RARα or PML/RARα, may affect their target transcription. Therefore, there could be a same competition mechanism for them to bind to CoA or CoR molecular (Perez 1993). The dissociation of corepressors from this mutant PML/RARα was not found at both of inducing and sub-inducing RA treatment (Sun 2004). It may be the possible explanation that the different dimers or multimeric complexes between RARα or PML/RARα and other transcription factors posse the different affinity with tRA so that their interactions were disrupted by different kinetics.

In summary, we have established a new cell system, AP-1060, for RA-induced differentiation research, in which the mutation (P407S) in RARa portion of PML-RARa coffers this cells the abrupt response to ATRA. On the base of this cells, the large-scale profiling of gene expression was performed to characterize the response of APL cell to RA. The 730 genes were identified in differential expression between induced cells and sub-induced cells. It suggests that the AP1060I cell line provides a system for dissecting genes with different regulatory mechanisms and for identifying key differentiation-controlling genes, and that the pathway involved in RA-response may be the target for treating the patients who resistant to RA.

Corresponding Author:
Dr. Yiwu Sun
Department of Medicine
Brookdale University Hospital & Medical Center
Brooklyn, NY11212, USA
E-mail: yiwu_sun@hotmail.com

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