Life Science Journal

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Marsland Press

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Website:
http://www.sciencepub.net

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Phone: (347) 321-7172

Life Science Journal   2011   Volume 8,   Number 4,   Part 2   ISSN:1097-8135

ISSN 1097-8135

9 771097 813002

MARSLAND PRESS
Multidisciplinary Academic Journal Publisher

Life Science Journal

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Acta Zhengzhou University Oversea Version
(*Life Sci J*)

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CONTENTS

I

Mathematical Treatment for the Pollutant Dispersion Considering the Ground as an Absorber-Reflector Surface for the Pollutant

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Abstract: A two – dimensional steady – state, mathematical modeling has been presented for the pollutant released from an elevated source in an inversion layer. The study presents a treatment for computing the pollutant concentration distribution under a physically realistic boundary condition which considers the ground as an absorber-reflector surface for the pollutant simultaneously. The wind speed is parameterized in terms of vertical height using the power law profile. The partial differential equation describing the advection-diffusion of pollutants has been solved using separation of variables method. An upper boundary condition which assumes the presence of capping inversion is taken into consideration. The mathematical formulation for the pollutant concentration distribution obtained in the present treatment is given in terms of Bessel and Gamma functions.

Introduction

Mathematical modeling is a tool for establishing various aspects in air pollution like; emission control legislation, impact assessment, and emergency preparedness etc. [1]. The analytical solution of the advection-diffusion equation bears significant importance since all influencing parameters are expressed in mathematically closed form [2].From this modeling; it is possible to investigate dispersion from continuous point source given appropriate boundary and initial conditions.   The model that is commonly used worldwide for regulatory purposes is the general Gaussian Plume Model (GPM).This model is based on various a assumptions:(1) the mean wind speed and eddy diffusivities do not have spatial variation (2)the ground surface is a perfect reflector for the pollutant (3)the diffusion in the vertical direction is unrestricted ,i.e., it is not capped by an inversion which tends to reflect back the pollutant hitting the inversion base[3]. In this respect, gradient transport theory is one of the analytical techniques that can overcome these short comings by inclusion of some of the above-mentioned physical processes, which will be more appropriate for treatment of atmospheric dispersion.

The analytical solution for the standard conditions of the advection-diffusion equation is obtained only by making some particular assumptions about the eddy diffusivities (homogeneous turbulence) and considering stationary conditions [4-6].

In the present work, we will present an analytical solution for the advection-diffusion equation which describes pollutant dispersion from a continuous elevated point source. In this formulation, the steady state condition is taken into consideration under the following postulates:

The down-wind speed profile is parameterized as a power-law depending on the vertical height (z) above ground level [2].

The pollutant dispersion remains confined to a layer capped by an inversion lid at the top, which serves as an impermeable upper boundary layer for the pollutant [7].

The inclusion of the ground surface as a reflector-absorber for the pollutants at the same time. This assumption is taken into consideration as an appropriate-realistic lower boundary condition.

2. Mathematical Description of the Problem

Considering a Cartesian coordinates system in which the x-axis coincides with the direction of the average wind and z is the vertical axis, then the steady-state of a contaminant released from a point source is described by the following partial differential equation[8];

$$u\ \frac{\partial \chi}{\partial x} + v\ \frac{\partial \chi}{\partial y} + w\ \frac{\partial \chi}{\partial z} = \frac{\partial}{\partial x}(k_x \frac{\partial \chi}{\partial x}) + \frac{\partial}{\partial y}(k_y \frac{\partial \chi}{\partial y}) + \frac{\partial}{\partial z}(k_z \frac{\partial \chi}{\partial z}) \quad (1)$$

Where χ denotes the average concentration, kx, ky, kz and u, v, w are the Cartesian components of the eddy diffusivities and wind, respectively. The crosswind integration of Eq.1,in stationary conditions and neglecting the longitudinal diffusion) leads to:

$$u\ \frac{\partial \chi}{\partial x} = \frac{\partial}{\partial z}(k_z \frac{\partial \chi}{\partial z}) \quad (2)$$

A power-law profile is used to describe the variation of wind speed u with height in the surface boundary layer, thus,u can be parameterized in terms of z as:

$$\frac{u(z)}{u(z_r)} = \left(\frac{z}{z_r}\right)^m \quad , 0 < m \le 1 \quad (3)$$

Where m is the power-law exponent which depends on thermal atmosphere stability (z_r) is the speed at a reference height z_r(usually z_r=10m).Eq. 3, can be written as:

$$u(z) = bz^m \quad (4)$$

Where $\quad b = \dfrac{u_r}{z_r^m}$

The eddy diffusion coefficient k_z is assumed constant k in our derivation. Then Eq. 2, reduces to:

$$b\, z^m \frac{\partial \chi_y(x,z)}{\partial \chi} = k \frac{\partial^2 \chi_y(x,z)}{\partial z^2} \quad (5)$$

3. Method of Solution

The second order partial differential eq. 5 will be solved using the method of separation of variables. The concentration distribution function $\chi(x,z)$ is separated as:

$$(6)$$
$$\chi_y(x,z) = X(x) \bullet Z(z)$$

Differencing partially with respect to both x and z, and substituting in Eq. 6, we get:

$$b\, z^m\, Z(z) \frac{dX(x)}{dx} = k\, X(x) \frac{d^2 Z(z)}{dz^2} \quad (7)$$

Dividing both sides on z^m k X(x) Z (z) we get two ordinary differential equation in the two variables x and z as:

$$\frac{1}{X(x)} \frac{dX(x)}{dx} = -\frac{\beta^2}{b} k \quad (8)$$

And
$$\frac{z^{-m}}{Z(z)} \frac{d^2 Z(z)}{dz^2} = -\beta^2 \quad (9)$$

Where β^2 is a constant.

3.1. Boundary conditions
(i) The modified approach adopted in this study is to assume that the ground is assumed to be partially reflector and partially absorber surface to the pollutant.

Accordingly, diffusive flux at the ground surface does not vanish ,i.e.,

$$k \frac{dZ(z)}{dz} =$$

$$v_d\, \chi_\circ \qquad \text{at} \quad z = z_\circ \qquad (10)$$

Where $\chi_\circ$ is the pollutant concentration at a reference height z_0, the roughness height, which is very close to the ground surface (z_0=0.3 m – 1.0 m)[9].
(ii) The pollutant is not able to penetrate through the top of the inversion / mixed layer located at height a ,i.e., the concentration vanishes at the height a;

$$\chi_y(x,z) = 0$$

At $\quad$ z = a $\qquad$ (11)

3.2. Solution of the differential equations
3.2.1. The horizontal equation in the variable x
Eq. 8, can be integrated to get the solution:

$$X(x) = \exp\left(-\frac{\beta^2 k}{b} x\right) \qquad (12)$$

3.2.2. The vertical equation in the variable z
Eq. 9, can be transformed to Bessel differential equation [10] as;

$$Z(z) = z^{1/2}[c_1 J_n(2\beta n z^{1/2n}) + c_2 Y_n(2\beta n z^{1/2n})] + A$$
$$13)$$

Where $J_n(2\beta n z^{1/2n})$ is the Bessel function of order n and first kind, while $Y_n(2\beta n z^{1/2n})$is the Bessel function of second kind. The order n is; $\quad n = \frac{1}{m+2}$

A is a parameter which is determined from the boundary conditions such that it tends to zero for large values of z. c_1 and c_2 are constants.
From the characteristics of Bessel differential equations solution, if n is a fraction, the solution can be expressed in terms of two Bessel functions of first kind and of orders n and –n. Thus, the solution of the differential equation given by Eq. 13, is reduced to:

$$Z(z) = z^{1/2}[c_1 J_n(2\beta n z^{1/2n}) + c_2 J_{-n}(2\beta n z^{1/2n})] + A$$
$$(14)$$

3.3. Determination of the constants
From the boundary condition Eq. 10,which is valid very close to the ground surface, i.e. small values for the variable z, the Bessel function $J_n(x)$for small values of x is approximated as [11].

$$\Gamma(p) = \int_0^\infty x^{p-1} e^{-x} dx \quad (15)$$

Where Gamma function $\Gamma(n+1)$ has the formula :

$$J_n(x) = \frac{x^n}{2^n \Gamma(n+1)} \qquad (16)$$

When substituting Eq. 15, on Eq. 14, we find that the constant c_2 should vanish, since the function $J_{-n}(z)$ has imaginary argument . By applying the boundary condition Eq. 10, we get

$$kc_1 \frac{(\beta n)^n}{\Gamma(n+1)} = \nu_d \chi_\circ$$

Then the constant c_1 can found as:

$$c_1 = \frac{\nu_d \chi_\circ \Gamma(n+1)}{k(\beta n)^n} \qquad (17)$$

Thus, Eq. 14 can be written in the form:

$$Z(z) = z^{1/2} \frac{\nu_d \chi_\circ \Gamma(n+1)}{k(\beta n)^n} J_n(2\beta n z^{1/2n}) + A \quad (18)$$

On substituting both Eq. 12, and Eq.18 in Eq. 6, we get the concentration distribution function as:

$$\chi_y(x z) = \exp(-\frac{\beta^2}{b} k\, x)\left[A + z^{1/2}\frac{\nu_d \chi_\circ \Gamma(n+1)}{k(\beta n)^n} J_n(2\beta\, n z^{1/2n}) \right]$$

(19)

4. Application of the Upper Boundary Condition

Is the boundary condition given by Eq.11, for large values of z. In this case A will be zero, and the boundary condition then reads:

$$\exp(-\frac{\beta^2}{b} kx)\left[z^{1/2}\frac{\nu_d \chi_\circ \Gamma(n+1)}{k(\beta n)^n} J_n(2\beta\, n z^{1/2n}) \right] = 0$$

at z=a   (20)

Then, we get

$$\beta = \frac{(3n+2)\pi}{8n\, a^{1/2n}} \qquad (21)$$

5. The Boundary Condition at the Point of Emission

It is well known that the pollutant concentration $\chi_y(x, z)$ both in air and on ground level is directly proportional to the source strength Q and inversely with both the source height h_s and the wind velocity at the emission point $u(h_s)$.These concepts can be expressed as:

$$\chi_y(x, z) = \frac{Q}{h_s\, u(h_s)} \qquad \text{at} \quad x=0, z=h_s \qquad (22)$$

Applying this condition, Then the constant A may have the formula:

$$A = \frac{Q}{b h_s^{m+1}} - h_s^{1/2}\frac{\nu_d \chi_\circ \Gamma(n+1)}{k(\beta n)^n} J_n(2\beta\, n h_s^{1/2n})$$

(23)

The resulting expression for the concentration $\chi_y(x, z)$ can be obtained from Eq. 19,. When substituting the values of the constants A (Eq.23) and B (Eq.21).If we assume a Gaussian distribution for the contaminated plume in y-direction, the final formula of the total pollutant concentration $\chi\ (x, y, z)$ can be written as:

$$\chi\ (x, y, z) = \chi_y(x, z) \exp\left[-\frac{y^2}{2\sigma_y^2} \right] * \frac{1}{\sqrt{2\pi}\sigma_y}$$

(24)

6. Summary and Conclusions

In this work we present an analytical treatment to solve the advection – diffusion equation describing the atmospheric dispersion of pollutants released from an elevated point source. In the model, we take into account more realistic physical boundary conditions.

The ground is considered as reflector and absorber surface for the pollutants reaching it. Also, we take into consideration the existence of capping inversion layer at which the pollutants are not able to penetrate, and we assume that this layer is located at height a above the ground surface. The wind velocity profile is taken as power law variation with the height z in the vertical direction.

The solution of the differential equation is obtained in terms of Bessel function of the first kind. Due to the very limited data concerning atmospheric dispersion under the same group of boundary conditions adopted in our study, specially, the reflectivity and absorptive of the ground surface for the pollutants, the present model could not be validated by comparison its result with either experimental data or another models. We think when suitable data becomes available, namely, absorption and reflection of ground surface for the pollutants, deposition velocities of the pollutants under consideration, inversion layer heights and the source characteristics, we are confident to point out that the results of the present model can be validated easily.

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10/2/2011

Studies on Vibrio Infection in Cultured Freshwater Fish

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Abstract: During the course of this study 10 isolates were isolated from *M.capito* collected from several farms in Behera province .The morphological and biochemical characters of isolated bacteria were proved to be belong to *V.anguillarum* (2 isolates),*V.ordalii* (6 isolates) and *V. parahaemolyticus* (2 isolatea).The isolation of the 3 Vibrio sp. from internal organs of naturally infected *M.capito* indicated that isolates are able to induce infection in *M.capito* .The examined *M.capito* showed signs of septicemia in the form of hemorrhagic patches on the caudal peduncle area and base of the fins , superficial ulcers, ascites and congestion of internal organs .Up on injection of *V.ordalii* in eels both the clinical signs and postmortem lesions were more severe than that observed in naturally infected *M.capito* .The histopathological changes were severe hyperplasia of secondary gill lamellae, hepatocyts necrosis, activation of melanomacrophage centers and bacterial colonization in the ellipsoid of the spleen .The vaccinated eels respond positively to the injected *V.ordalii*   bacterin with relative level of protection of 100% .To the best knowledge of the authors it is the first time to isolate *V.ordalii* from *M.capito* in Egypt .Moreover, isolation of *V. parahaemolyticus* is an alarm not only as fish pathogen but also as human hazard .
[Safinaz G.M. Ismail, W.D. Saleh and Mona Zaki. **Studies on Vibrio infection in cultured freshwater fish.** Life Science Journal. 2011; 8(4):155-162] (ISSN: 1097-8135). http://www.lifesciencesite.com.

Keywords: Vibrio; Infection; Freshwater Fish

1. Introduction

One of the main factors affecting fish production and efficiency is the fish diseases and especially that resulted from bacterial diseases which are responsible for heavy mortality among wild and cultured fish **(Saad., 2002)**

Vibrio species are gram negative bacteria affected all type of fish of either marine or freshwater fish allover the world in the different areas of Asia, America ,Australia ,Africa and Europe **.(Toranzo and Barja 1993 ,.and Austin and Austin., 1999).**

Genus **Vibrio** comprises more than 45 species, most of which are which are widely distributed in the marine environment. Bacteria of this genus constitute the dominant intestinal microbiota of a wide range of marine fish **) Sakata et al., 1980 ;Onarheim and Raa. (1990).**

Fish affected by **Vibriosis** suffered from severe congestion at the base of the fins, erosion of the fins, excessive mucoid secretion on gills, severe congestion of gills, hemorrhagic ulcerations, linear hemorrhages over different parts of the body and severe congestion or hemorrhagic protrusion of the anal opening **(Toranzo et al., 2005)** . Morovere ,the most important postmortem lesions were congestion of internal organ and distention of gall bladder **) Xio et al., 2005 and Reader et al., 2007(**

In Egypt ,production of fish has significantly increased during the last ten years, due to the improvement in culture techniques specially in

Oreochromis niloticus farming . However,   disease outbreaks have bean reported with economic losses: The outbreaks of Vibriosis were common problems among cultured marine and freshwater fish which have occurred at various stages of cultured and caused serious economic losses (**Rasheed.,1989 a.)**

Vibrio spp **.**has   been   isolated   from freshwater environment and isolation rates increased with increase environmental temperature and organic pollution) Rhades et al 1986 ,.and Reham,Ali.(2009) The aim of this study was isolation and identification of *V .ordalii* that affect cultured *M.capito* and clarify the pathogenicity in cultured eel (*Anguilla Anguilla)***.**

2. Materials & Methods
Naturally infected fish

A total number of 80 *Mugil capito* (50± 5 mg)were collected moribund and alive from a private fish farm in Behera Province. Fish were subjected to clinical and microbiological examinations according to **Austin and Austin.,(1987)** and **Schaperculaus et al ., (1992)** .Isolation of Vibrio spp .was achieved from ulcers , liver , kidneys and spleen of naturally infected *M. capito* alive and freshly dead.

Experimental fish

A total of 130 apparently healthy eel *(Anguilla anguilla)* with an average weight of 40± 5 gm were obtained from natural sources in Behera province .

They were Kept in glass aquaria provided with aerated dechlorinated tap water and kept at temperature of 22± 1 C . with continuous aeration according to **Innes (1966)** . The fish were fed on commercial diet containing 40% crude protein at the level of 5% of body weight according to **Eurell et al ., (1978).** They were used for evaluation of the pathogenicity of isolated Vibrio Spp . Ten eels were random collected and submitted for bacteriological examination to verify the absence of *Vibrio ordalii*.

Primary isolation was don from internal organ of examined *M.capeto* according **to Eleonor., et al (1997**) ,on Tryptcase soya agar (TSA) .

Isolation and identification of the isolated bacteria.

Primary isolation was done on trypticase soya agar (TSA) supplied with different concentrations of sodium chloride (1.5-8%) according to **Eleonor et al (1997),** incubated for 24 hours at 30C .The recovered suspected colonies were picked up and purified for further identification according to culture, morphological and biochemical characterization

Morphological characters, colonial and growth feature on TSA as well as biochemical were used for identification of isolated bacteria according to **Berge's(1982) and Whitman. (2004).**

Moreover the API.20E system (Analytab products, Plainview New York) was also used for biochemical characteristics of all suspicious isolates.

Detection of the pathogen city of isolated bacteria in eel *(Anguilla anguilla).*

Medial lethal dose 50 (LD$_{50}$)

Medial lethal dose 50 (LD$_{50}$) for the isolate (N 10) was estimated in *A. anguilla* according to **Reed and Muench-(1938**). Graded doses ranged from 10^{-1} to 10^{-7} CFU /ml was used. A total number of 80 apparently healthy eels (40+ 5 gm) were grouped into 8 groups (10 eels / group). The first seven groups were injected intra peritoneal (I.P.) with one ml of specified bacterial concentrations. The eight groups was injected I.P with one ml of sterile saline and served as control. Mortalities were recorded for 7 days post injection. Freshly dead fish were submitted for bacterial isolation and re-isolation and identification of tested bacteria was done to verify specificity of mortality.

Experimental infection:

A total of 40 eels (40 + 5 g) was allotted to four equal groups . Fish of the first three groups were injected I. m with 0.2 ml of 0.5 dose of LD$_{50}$ according to **Shehate et al., (1988)** . The fourth group was injected with 0.2 ml of sterile saline and served as control. Infected and control groups were kept under daily observation for two weeks. Both clinical sings and mortalities were recorded.

All freshly dead eels were submitted to bacterial isolation and *V. ordalii* isolated was re-identified to verify the specificity of mortality.

Histopathological and ultra changes were carried out from organs of experimentally infected eels according to **Culling, (1983).**

Evaluation of potency of prepared vaccine against *V.ordalli* were done according to the method described by **Sakai et al., (1984)** and **Badran and Eissa, (1991)**. The formalin inactivated bacterin were mixed with an equal volume of 0.85% sterile saline and adjusted to Macfarland's No.5 (approximately 6×10^{8} cells/ml) .

Twenty eels were injected with 0.2 ml bacterin / fish (IP) . Twenty eels was also injected with 0.2 ml (IP) sterile saline control. After 2 weeks the injected eels received booster dose from bacterin (Same dose) and control group injected with 0.2 ml sterile saline.

Blood collection was carried out after 28 days post injection for serum collection ,The antibody titer was evaluated by microagglutination test according to **Badran and Eissa (1991)** .

After 28 days both infected and control groups were injected with 0.2 ml of virulent isolate of *V. ordalii* previously adjusted to 6×10^{8} cfu/ ml .

Clinical signs and mortality were observed for one week. The potency of bacterin was examined by calculating the relative level of protection (RLP) by the following formula:

RLP =　% 1- mortality of vaccinated eels x100 /
　　　　　% mortality of control

According to **Newman and Majnarich, (1982)**.

All groups of eels in this study were anesthetized with a solution containing 1 gm of benzocaine (ethyl aminobenzoate) in 10 ml ethanol prior to injection.

3. Results

Results of clinical examination of naturally infected fish:

The clinical signs in *Mugil capito* , were hemorrhagic patches on the caudal peduncle area and base of fins as well as superficial hemorrhagic ulcers at the abdominal wall (Fig.1). The postmortem changes in *Mugil capito* were characterized by deep seated muscle lesions, enlargement and congestion of the spleen which became cherry red in colour and losses its sharp edges .Moreover, ascites and corneal opacity were also noticed in some examined fish (Fig. 2)

Isolation and identification of Vibrio species :

Attempts to isolate Vibrio spp. from different organs (kidneys, liver and spleen) of

naturally infected *M. capito* gave ten isolates that grow on trypticase soya agar with different concentration from NaCl (1.5% to 8%). The colonies appeared after 24 hrs post – incubated at 30C.*Colonies* were of medium size (2-3 mm in diameter) and creamy in color . They proved to be Gram – negative, motile rods and gave presumptive identification of Vibrio species .

Biochemical characterization of isolates :

The biochemical and growth characteristics of the isolates using traditional tests indicated that all the isolates were positive for oxidase and motility tests. Variable results were obtained in case of lysine decarboxylase, arginine dihydrolase and ornithine decarboxylase .Moreover, all isolates gave positive results in case of sucrose fermentation except isolate No.9 and 10 . The other tested sugars gave variable results . Further biochemical characterization of 10 isolates, was carried out by using the API 20E system . All tested isolates were positive for sodium pyruvaate (VP) (except   N9 and N10), gelatin liquefaction and tryptophane (except N9 and N10) Negative results were obtained for tryptophane (IND) (except N9 and N10), sodium thiosulphate (H2S) and orthonitrophenyl galactoside (except isolates N1 and N2). Variable results were observed for arginine |(ADH), lysine (LDC), ornithine (ODC), sodium citrate (Cit) and urea (URE).

Concerning the results of sugar fermentation by using API20E system, the tested isolates gave positive results for glucose, mannitol, sucrose, sorbitol and rhamnose except (N2).Variable results were noticed in case of other sugars

According to both morphological and biochemical characters of Vibrio species, the tested isolates were identified as *V.anguillarum* (2 isolates), *V.ordalii* (6 isolates) and *V.parahaemolyticus* (2 isolates) .

Pathogenicity assay

Due to the unique isolation *of V.ordalii* for the first time in Egypt according to the best knowledge of the authors , the most virulent one which proved by developing rapid and severe clinical and MP lesions was used for further experimental studies in *Anguilla Anguilla* .

The results of determination of the virulence of selected *V. ordalii* isolate by calculation of the lethal concentration 50 (LD50) showed that the LD50 of *V. ordalii* was $10^{2.4}$ bacterial cells / ml.

Experimental infection with *V.ordalii*:

This experiment was done to determine the nature of experimental infection of selected *V.ordalii* isolate in eel (*A. Anguilla*).

After 2 day post injection, the eels became sluggish and the escape reflex was too weak . The clinical signs were characterization by server

hemorrhages over the body, in some cases these hemorrhages covered the whole body surface and congestion of the head region (Figs.3+4). Haemorrhagic ulcers were recognized at the body surface of infected eel.

The postmortem lesions were in the form of server congestion of the liver which some times became edematous. Enlargement of spleen which became cherry red and loss its sharp edges in addition to severe congestion of the kidneys and inflammation of the intestine (Fig 5). Fifteen eels were died during the course of experiment.

Evaluation of antibody titers of *Vibrio ordalii* in eel :

The antibody response in *A anguilla* infected with *V.ordalii* was detected 28 days post-injection of bacterin. The detected antibody titer was 2^5.

The injected *A.anguilla* were firstly examined to verify their freedom from Vibrio species infection and proved to be free. The results revealed that fish vaccinated with prepared bacterin gave complete protection when challenged with 10^4 cells /ml of live bacteria, were the relative protection level reached 100% .

The re-isolation of injected bacteria was positive in case of freshly dead infected eel.

Hstopathological changes :

Histopathological sections from different organs of injected eels with *V. ordalii* revealed the following results.

Gills:

The major histopathological changes were characterized by severe hyperplasia and filamental thickening   adhesion   with   mononuclear   cell infiltration (Figs. 6). There were also thrombus formation in the bronchial artery. There was also slight oedema and diffuse lymphocytic infiltration in the gill arch and epithelial hyperplasia at the base of the secondary lamellae .

Liver:

The lesion in the liver were characterized by extensive   areas of oedematous, vacuolated and atrophied hepatocytes. There were hepatocytic cell necrosis in between swollen cells and normal hepatocytes (Fig.7), as well as thrombus formation.

Kidneys :

The kidneys showed hyper activation of the melanomacrophage centers. The melanomacrophge centers were seen around and within the tunica media of the long arterioles . The interstitial tissues of the kidney showed depletion and cell necrosis (Fig.8).

Skin and underlying musculature :

The skin showed excessive melanosis in the dermis with multifocal slight dermal oedema (Figs .9). Leucocytic infiltration of muscle was observed .Oedema and extensive haemorrhages as leucocyte

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infiltration were noted between muscle fibers as well as muscle necrosis .

Spleen:

In most samples, the spleen was extensively colonized by *V. ordalii* Bacteria accumulated mainly in the ellipsoide .The spleen was hyperaemic and the number of macrophages and neutrophils increased considerably in the periellipsoidal area and red pulp. These macrophages were hypertrophied and contained phagocytosed erythrocytes, bacteria, melanin granules and cell debris. .

Ultra changes :

The ultra changes in liver of eel previously injected with *V.ordalii* were pronounced and clear. Electron micrograph of liver in case of infected eel revealed vacuolation of hepatocyte with server glycogen deposition (Fig.10) . Moreover, server endoplasmic dilatation and server glycogen deposition were observed .

4. Discussion

The Vibrio, *V. alginolyticus, V. anguillarum, V. ordalii* are fish pathogens. All are associated with acute bacterial septicemia or chronic focal lesions in infected fish. Generally, Vibriosis in fish accompanies with some other stress or physical trauma but some strains, especially of *V.anguillarum, V.ordalii* and *V.* salmonicida appear to be highly infectious primary pathogens (Robert et al., 1975).

During the course of this study 10 isolates from several outbreaks of Vibriosis among cultured *Mugil capito* were isolated. The isolates were submitted to complete morphological, cultural and biochemical examination by using both tube biochemical method and AP120E system.

The morphological and biochemical characters of isolated bacteria were proved to belong to *V.anguillarum* (2 isolates), *V.ordalii* (6 isolates) and *V. Parahaemolyticus* (2 isolates) .The identification of the previously mentioned Vibrio species were based on the data published by **Grisez et al. (1991**) and the criteria of the manufacturer of AP 120E system . Moreover, the negative results of arginine dihydrolase, reduction of NO_2, Voges-Proskauer production and utilization of both arabinose and sorbitol distinguished *V. ordalii* from *V. anguillarum*.

Also, the positive results of lysine decarboxylase, ornithine decarboxylase, citrate simmons and indole and negative results of Vp and utilization of sucrose and melibiose distinguished the *V. parahaemolyticus* from *V.ordalii* and *V. anguillarum* **(Austin and Austin, 1987, Grisez et al. 1991 and Saeed, 1995).**

The isolation of both *V. anguillarum* and *V.ordalii* from internal organs of naturally infected *Mugil capito* (namely spleen , kidneys and liver) indicated that both Vibrio species are pathogen and able to induce infection in *Mugil capito* . Moreover, the isolation of Vibrio species from internal organs may be attributed to the ability of bacteria to produce septicemia as well as bacteriaemia.

Chart and trust (1984) and Davease et at. (1985) reported that *V. anguillarum* and *V. ordalii* were well known to be the primary pathogens to fish. While**, Austin and Austin (1987)** pointed out that seven Vibrio fish pathogens as follows: *V. alginolyticus, V. anguillarum, V. carchariae, V. cholerae, V. damsela and V. ordalii.* While, **, Noel et al. (1996), Benediktsdottir et al. (1998) and Akhlaghi (1999)** had reported isolation of different types of Vibrio (namely , *V.anguillarum , V. alginolyticus , V . carchariae , V. cholera , V . damsela , V . ordalii , V salmonicida , V. parahaemolyticum* and *V. vulnificys* biotype 2) from different internal organs such as liver , spleen kidneys , muscular lesions of different infected fish species- . Moreover, **Ransom et al. (1984)** reported that *V. ordalii* induced pathogenesis in fish not particularly different from that of *V. anguillarum* but generally less server .They also added that the infection by *V. ordalii* may be occurred via ascending infection from the posterior gut or through the skin .

The isolation of the two isolates which were identified as *V. parahaemolyticus* is very interesting since the *V. parahaemolyticus* has been reported to be implicated in fish disease and human infection as fish food poisoning. Moreover, *V. parahaemolyticus* has been reported to be isolated from human disease situation **(Austin and Austin, 1987).**

Daniels et al. (2000) reported an outbreak of *V. parahaemolyticus* serotype $O_{3:} K_6$ infection in the United States .The same authors added that, the consumers should understand that raw or undercooked fish infected with *V. parahaemolyticus* can cause illness in from of gastroenteritis.

It is worthy to mention that, the less information about the susceptibility of eel to *V. ordalii* directed us to study the pathogenicity of this species in eels.

The result of LD_{50} proved that *V ordalii* (No. 10) used in the present study was highly virulent for eel. LD_{50} being estimated to be $10^{2.4}$ CFU/ fish.

Nordmo et al., (1997) recorded that LD_{50} of *V. ordalii* was 10^6 CFU/L fish in Atlantic salmon. The difference in the LD_{50} value in this study may be attributed to the difference in fish species , bacterial strain and environmental conditions .

The extensively colonization of *V.ordalii* in spleen ellipsoide directed us to believe that the spleen is the predilection sit for *V.ordalii* and organ of choice for isolation of the bacteria.

Fig.1 : *Mugil capito* **naturally infected with** *Vibrio ordalii* **showing hemorrhagic patches on the caudal peducle and base of fins**

Fig.2 : *Mugil capito* **naturally infected with** *Vibrio ordalii* **showing congestion and enlargement of spleen .**

Fig.3: Eel (*Anguilla Anguilla* **) experimentally infected with** *Vibrio.ordalii* **showing severe hemorrhages over the body .**

Fig .4:   Eel (*Anguilla Anguilla* **) experimentally infected with** *Vibrio.ordalii* **showing deep hemorrhagic ulcer .**

Fig.5: Eel *(Anguilla Anguilla* **) experimentally infected with** *Vibrio.ordalii* **showing congestion of internal organs .**
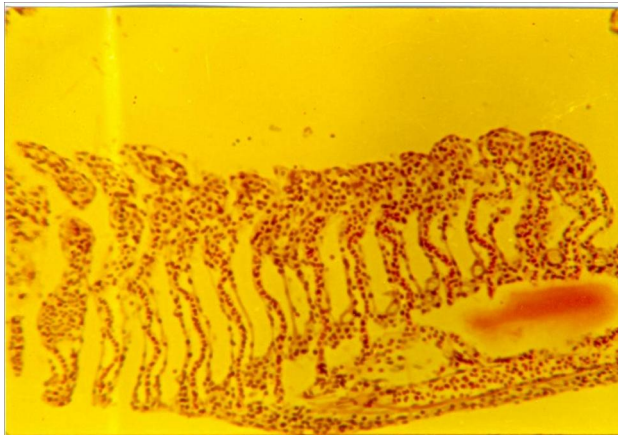
Fig6:Gills of eel experimentally infected with *Vibrio .ordalii* showing severe lamellar hyperplasia with filamental adhesion .(H&E.X160).
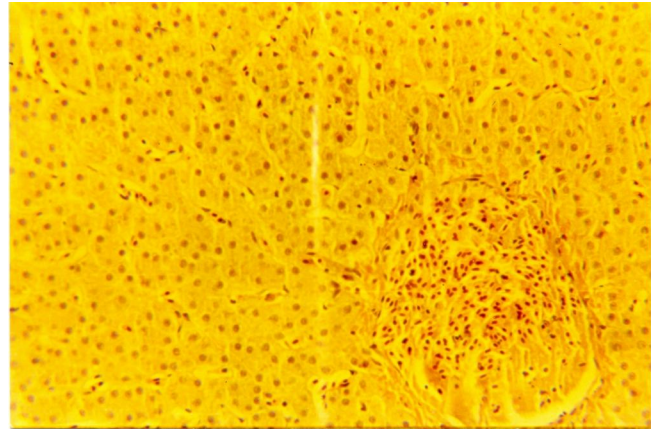
Fig.7: Liver of eel experimentally infected with *Vibrio.ordalii* showing thrombus formation .(H&E.X160).
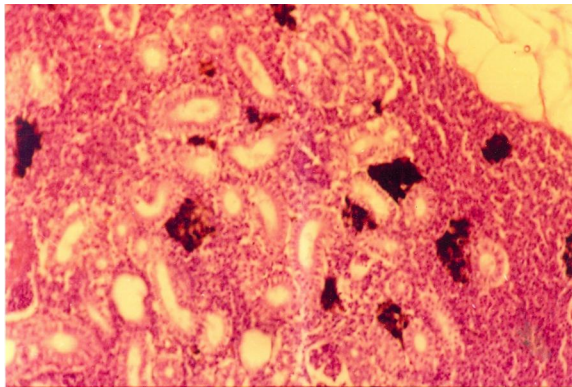
Fig.8:Kidneys of eel experimentally infected with *Vibrio.ordalii* showing hyper activation of the melanomacrophage centres .(H&E.X160).
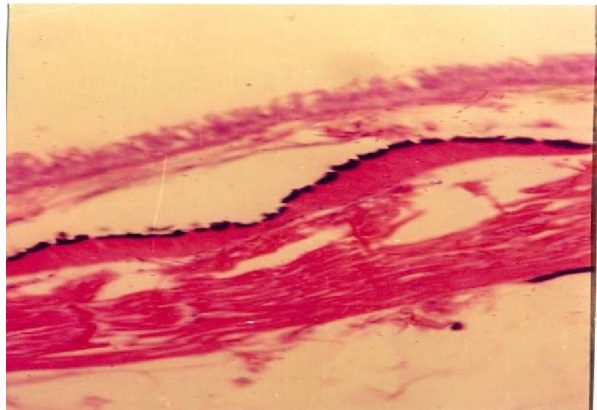
Fig.9: Skin of eel experimentally infected with *Vibrio ordalii* showing excessive melanosis in the dermis with multifocal slight dermal oedema.(H&E.X250).
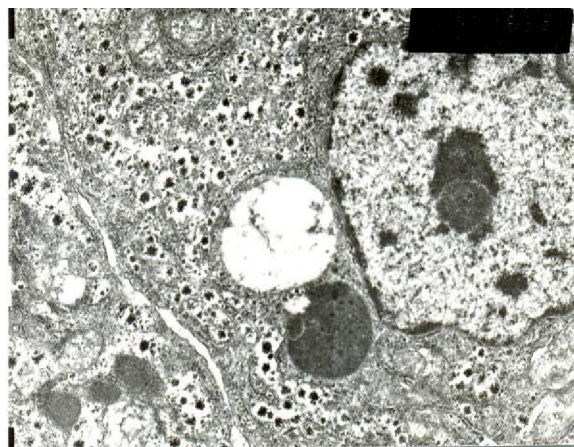
Fig .10: Electron micrograph of liver of eel experimentally infected with *Vibrio.ordalii* showing valuation of hepatocytes with severe glycogen deposition. (x10000).

The histopathological changes due to infection of *V.ordalii* varied and recognized in different organs . These changes may be attributed to the extensive bacterial multiplication and the secretion of cytotoxin, haemolysin and protease by *V.ordalii* (**Santos, et al 1991, kumar et al,. 2006 and Reham 2009)** Ultra structurally, the lesions in liver were not specific and frequently in the liver of fish exposed to toxic agents (**Ghadially, 1988**), The same conclusion was reported by **Lamas et al ., (1994)** in case of Rainbow trout experimentally infected with *V .anguillarum* .

The Relative level of protection (RLP) of vaccinated eel was 100%.This result proved that eel was respond positively to the formalized killed bacterin which prepared from *V. ordalii* isolated from *M. capito* in Egypt. *V. ordalii* isolated from *M.capito* succeeded to produce system infection in eel upon experimental infection .

The experimentally injected eels showed sings of septicemia in the form of sever hemorrhage of the body, hemorrhagic ulcers and congestion of internal organs . The recorded signs may by due to toxins produced by injected bacteria **Nordmo et al., (1997) and Reham(2009) .**

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10/2/2011

Clinicopathological Significance and Prognostic Importance of Circulating Plasma DNA Expression in Advanced Non-Small Cell Lung Cancer and its Efficacy as a Diagnostic Tool

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Abstract: Background/Aim: Lung cancer is one of the commonest neoplasms. So, there is a continuous need for the development and search for new prognostic markers which will aid in diagnosis and therapy. Circulating plasma DNA levels is over-expressed in many human cancers, including lung. The aim of this work is to study the expression of circulating plasma DNA in NSCLC and assessment of its utility as a diagnostic marker, and in evaluating its impact on therapeutic efficacy as well as correlation of these data with clinicopathologic findings and patient survival to assess its prognostic significance. Patients and Methods: The amount of plasma DNA was determined through the use of real-time quantitative polymerase chain reaction (PCR) amplification of the human telomerase reverse transcriptase gene (*hTERT*) in 41 patients with advanced non-small cell lung cancer (NSCLC) and 38 age-matched controls. All of the 41 patients with advanced NSCLC received platinum-based chemotherapy. The regimen was Gemcitabine 1000 mg/m^2 (day 1, 8) and platinol 70 mg/m^2 (day 1), the cycle was repeated at interval of 21 days for at least 3 cycles. About 3 to 4 weeks after chemotherapy, response was evaluated by restaging- computed tomography. Circulating plasma DNA levels was correlated with established clinicopathologic factors, response to therapy, progression free and overall survival, and lactate dehydrogenase (LDH) levels. Results: There was a significant correlation between circulating plasma DNA levels and stage (p=0.001), LDH levels (p=0.001), smoking status (p=0.02) as well as tumor status (p=0.004). Circulating plasma DNA levels were significantly inversely correlated with treatment response (p<0.001).   There was no statistical significant correlation when looking at the effect of age (p = 0.103), sex (p = 0.164), performance status (p = 0.267), pathological subtype (p = 0.26), and nodal status (p = 0.278) on the circulating plasma DNA levels. There was borderline statistical significant correlation between circulating plasma DNA levels and presence of distant metastases (p = 0.058). Circulating plasma DNA levels had also a highly significant relationship with shorter duration of PFS (p<0.001) and OS (p=0.0014).The mean circulating plasma DNA levels were 141.9 ng/mL (±56.3SD) in NSCLC patients and 69.9 ng/mL (±13.3SD) in controls, the difference being highly significant (p < 0.001). Conclusion: our results show that circulating plasma DNA levels is frequently over-expressed in primary NSCLC, and appears to be potentially useful marker for diagnosis. Overall, circulating plasma DNA levels was a significant predictor of survival and response to therapy. Circulating plasma DNA might be used as a new marker to stratify NSCLC patients for more optimal treatment modalities.
[Abd El Halim Abu-Hamar; Hanan Shawky; Ibrahim. S. Ibrahim And Abdel Khalek H.S. **Clinicopathological Significance and Prognostic Importance of Circulating Plasma DNA Expression in Advanced Non-Small Cell Lung Cancer and its Efficacy as a Diagnostic Tool]** Life Science Journal,. 2011; 8(4):163-170] (ISSN: 1097-8135). http://www.lifesciencesite.com.

Key words: Circulating plasma DNA, non-small cell lung cancer (NSCLC), diagnosis, clinicopathologic Study, prognosis, survival.

1. Introduction

Lung cancer is the leading cause of cancer death worldwide and NSCLC accounts for 80% of the cases[1].   The average 5-year survival in Europe is 10%, not much better than the 8.9% observed in developing countries[2]. The poor outcome is attributable to the absence of early detection plans, the frequency of metastases at diagnosis[3], and poor responsiveness to radiation therapy and chemotherapy[4]. However, survival of patients undergoing lung resection for small intrapulmonary cancers is greater than 80%[5]. As a consequence, there is a need to develop new tests that may facilitate earlier diagnosis and more effective treatment.

Diagnostic assays based on blood sample analysis are attractive because of the simplicity of sample collection. Accurate analysis of tumor markers in blood from cancer patients could have significant impact in facilitating the screening, diagnosis, and monitoring for disease recurrence after initial therapy[6].

With the introduction of PCR-based technologies in 1980s and refinements thereof, numerous molecular and biological markers on lung cancer tissues and exfoliated cancer cells have been investigated[7]. The finding that tumors are capable of shedding nucleic acids (DNA or RNA) into the blood

stream, which can be recovered from both serum and plasma and used as surrogate source of tumor DNA, has opened new areas in cancer diagnosis and prognosis in the past decade [8].

It is believed that plasma/serum DNA is of tumor origin because the genetic alterations are similar to those found in the corresponding primary tumors[9-11]. Thus, quantification of cell-free DNA in plasma/serum and characterization of specific molecular changes could be very useful in the management and screening of lung cancer.

To achieve maximum specificity and sensitivity, it is necessary to have a DNA concentration that does not overlap with the concentrations in control groups. It is clear that explicit cutoff values for DNA concentrations cannot be established at present because most of the published studies differ in the assays used. Three studies used real-time PCR for defining explicit DNA cutoff values[4,6,12], but all used different genes for the amplification. It was found that higher cutoff values increased the specificity of the assay but at the cost of sensitivity and vice versa. In a study by **Leon _et al._**[13], 61% of lung cancer patients had higher circulating DNA concentrations [above the cutoff value of 50 μg/L; mean (SE), 164 ± 44 μg/ml]; DNA concentrations decreased in 75% of these patients after therapy.

Sozzi _et al._[14], demonstrated in their analysis of circulating tumor DNA in plasma at diagnosis and during follow-up of lung cancer patients that, DNA concentration in the follow-up plasma samples (mean, 34 μg/L) was significantly lower than before surgery (mean, 345 μg/L) and was comparable to the concentration detectable in the control group[14]. Total DNA was increased in patients with untreated cancer and in those with disease recurrence, with a sensitivity of 75% and specificity of 86%[14]. From these studies it can be concluded that no explicit cutoff has been established that can serve as a valuable tool for the diagnosis and follow-up of individuals.

In the current study, we evaluated the circulating plasma DNA levels in newly diagnosed, advanced stage NSCLC and assessing its utility as a diagnostic marker when compared with age-matched controls. In addition, evaluating its impact on therapeutic efficacy and correlating these data with clinicopathologic findings and patient survival to assess its prognostic significance.

2. Patients and Methods
Patient Characteristics & Inclusion Criteria:

A total of 41 patients with newly diagnosed, histologically confirmed advanced stage NSCLC and 38 age-matched controls (patients with benign pulmonary diseases, including 15 chronic obstructive pulmonary disease, 10 interstitial lung disease, 7 pulmonary tuberculosis, 2 sarcoidosis, and 4 bronchiectasis) treated at Clinical Oncology Department and Chest Department, Faculty of Medicine, Tanta University Hospital between May 2008 and March 2011 were studied.

All NSCLC patients were required to have advanced stage NSCLC, age less than 75 years and greater than 18 years, Eastern Cooperative Oncology Group performance status (ECOG) of 0 to 2, adequate cardiac function (EF > 60%), adequate bone marrow reserve, adequate renal and hepatic functions. Patients with NSCLC with non-malignant systemic disease that precluded them from receiving systemic chemotherapy (e.g. active infection, any clinically significant cardiac arrhythmia, or congestive heart failure) or patients who were pregnant were not eligible.

The following parameters were assessed at baseline: circulating plasma DNA levels, lactate dehydrogenase (LDH) level, bronchoscopy, ECOG performance status, weight, chest and abdomino-pelvic computed tomography (CT) scan, isotopic bone scan, ECG, echocardiography, and CT or magnetic resonance imaging (MRI) scan of the brain (if indicated), blood counts (Total leukocyte counts, hemoglobin, granulocytes, and platelets), and blood chemistry (renal and liver function tests).

Sample Collection and DNA Isolation:

A 7.5-mL sample of peripheral blood was collected in tubes containing EDTA, from patients at time of study entry as well as 6 months after the end of treatment from responders during follow-up period and from controls at the time of spiral CT examination, and stored at deep freeze Plasma separation and DNA extraction were performed as previously reported by **Chang _et al._**[15]. The DNA purified from 1 mL of plasma was eluted in a final volume of 50 mL of water. Testing of plasma DNA was performed by technicians with no knowledge of the patient or control status.

DNA Quantification in Plasma:

To quantify the circulating DNA in plasma, we used a real-time quantitative PCR approach based on the 5' nucleotide method. This methodology is based on continuous monitoring of a progressive fluorogenic PCR by an optical system. The PCR system uses two amplification primers and an additional amplicon-specific and fluorogenic hybridization probe, the target sequence of which is located within the amplicon. The probe is labeled with two fluorescent dyes. One serves as a reporter on the 5' end (VIC dye; Applied Biosystems, Foster City, CA). The emission spectrum of the dye is

quenched by a second fluorescent dye at the 3' end (TAMRA; Applied Biosystems). If amplification occurs, the 5' to 3' exonuclease activity of the AmpliTaq (Applied Biosystems) DNA polymerase cleaves the reporter from the probe during the extension phase, thus releasing it from the quencher. The resulting increase in fluorescent emission of the reporter dye is monitored during the PCR process.

Primers and probes were designed to specifically amplify the ubiquitous gene of interest, the *hTERT* single copy gene mapped on 5p15.33. The amplicon size of the *hTERT* gene was 98 bp (position 13059 to 13156, GenBank accession number AF128893). The sequences of the primers and of the probe were the following: primer forward, 5'-GGC ACA CGT GGC TTT TCG-3'; primer reverse, 5'-GGT GAA CCT CGT AAG TTT ATG CAA-3'; probe, VIC5'-TCA GGA CGT CGA GTG GAC ACG GTG-3' TAMRA.

Fluorogenic PCRs were carried out in a reaction volume of 50 mL on a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Fluorogenic probe and primers were custom synthesized by Applied Biosystems. Each PCR reaction mixture consisted of 25 μL of TaqMan Universal Master Mix (Applied Biosystems), 0.67 μL of probe (15 mmol/L), 0.45 μL of primer forward (10 mmol/L), 0.45 μL of primer reverse (10 mmol/L), and 18.43 μL of sterile water. DNA solution (5 μL) was used in each real-time PCR reaction. Thermal cycling was initiated with a first denaturation step of 50°C for 2 minutes and then 95°C for 10 minutes. The thermal profile for the PCR was 95°C for 15 seconds and 60°C for 1 minute. Data obtained during 50 cycles of amplification were analyzed.

Amplifications were carried out in 96-well plates in a GeneAmp 5700 Sequence Detection System. Each plate consisted of patient samples in triplicates and multiple water blanks as negative control. For construction of the calibration curve on each plate, we used a standard TaqMan Control Human Genomic DNA (Applied Biosystems) at 10 ng/μL with appropriate serial dilutions at 50, 5, 2.5, and 0.5 ng, and 250, 50, and 10 pg. Linear amplification down to the last dilution point representing 10 pg of target DNA was obtained in each experiment (correlation coefficient, 0.999 to 0.995; slope, 3.25 to 3.35).

All of the data were analyzed using the Sequence Detection System software (Applied Biosystems) to interpolate the standard amplification curve of DNA at a known quantity with amplification cycle threshold of the unknown target sample, thus obtaining the relative amount of DNA in the experimental sample

Treatment

All of the 41 NSCLC patients had received systemic chemotherapy. Chemotherapy was applied in the form of GC regimen which consisted of a 60-120 minute intravenous infusion of gemcitabine (1000 mg/m^2, day 1 and 8), and platinol (70 mg/m^2, days 1), by intravenous infusion over 6 hrs and the cycle was repeated every 3 weeks and continued for 6 cycles unless there was evidence of disease progression or unacceptable toxicity. Patients were pre-medicated with 8 mg of dexamethasone, 50 mg of diphenhydramine, and 50 mg of ranitidine given intravenously. In addition, pre- and post-chemotherapy hydration was applied with platinol to avoid cisplatin-induced nephrotoxicity. Prophylactic use of growth factors was not recommended.

Supportive care included blood transfusions, growth factors and the administration of antiemetics and analgesics, as appropriate. The protocol provided for a decrease in Gemcitabine and platinol dose in patients experiencing grade 4 hematological toxicity or grade 3 non-hematological toxicities. G-CSF support was allowed in case of prolonged leucopenia (> 7 days) or febrile neutropenia in the prior cycle.

Evaluation of Treatment Response

Tumor response assessments were performed after 3 cycles. Response to therapy was classified according to the RECIST guidelines[16]. Evaluation was done using chest computed tomography (CT) owing to its convenient diagnosis of target lesion progress and identification of emerging new lesions.

Toxicity Evaluation:

Toxicities were graded according to the National Cancer Institute-Common Toxicity Criteria (NCI-CTCAE ver. 2.0). Treatment period was defined as the period from the initiation of therapy to 3 weeks after the last day of administration of Gemcitabine and platinol.

Follow-up Evaluation:

Basically, CT evaluations were performed every 3 months. Assessment of blood counts, blood chemistry, weight, performance status, toxicity, and chest examination was done every 3 months. Follow-up visits were scheduled every 3 months in the first 2 years after cessation of treatment and every 6 months thereafter.

Statistical analysis:

Patients were followed up until October 2011. At the time of analysis, the mean follow-up for the entire group was 11 months (range, 3.00 to 36 months). Descriptive statistics were used to summarize patient characteristics and statistical

analysis of the results was performed using SPSS version 12.0. Overall-survival (OS) rates were calculated from the time of initial treatment to the time of the last follow-up visit or death using the Kaplan-Meier method[17]. Mean and standard deviation were estimates of quantitative data. Chi-square/ Fischer exact were tests of proportion independence. Kaplan-Meier method was used for estimating survival and log rank to compare curves[17].

3. Results
Patient characteristics:

The study included A total of 41 patients with newly diagnosed, histologically confirmed advanced stage NSCLC and 38 age-matched controls (patients with benign pulmonary diseases, including 15 chronic obstructive pulmonary disease, 10 interstitial lung disease, 7 pulmonary tuberculosis, 2 sarcoidosis, and 4 bronchiectasis) treated at Clinical Oncology Department and Chest Department, Faculty of Medicine, Tanta University Hospital between years 2008 and 2011. The age of patients with NSCLC ranging from 36 to 70 years at the time of diagnosis (mean age 55.3±7.5 years) while the mean age of the controls at the time of study entry was 57.3±7.6 years (range 37-72 years). They showed positive history of smoking in 34 (82.9%) patients with NSCLC and in 26 cases (68.4%) of the controls.

The majority of cases (63.4%) of NSCLC were T3 or greater, and node positive. The demographic data of the patients and controls and their relation to circulating plasma DNA levels were summarized in table (1). No statistically significant difference between the demographic characteristics of NSCLC patients and controls as regard to sex, smoking status, and age.

Baseline DNA concentrations were measured in plasma of 41 NSCLC patients and 38 age-matched controls. The mean circulating plasma DNA levels were 141.9 ±56.3 ng/mL in NSCLC patients and 69.9±13.3 ng/mL in controls, the difference being highly significant ($P < 0.001$). The mean DNA concentration was 2-fold higher in plasma from patients with NSCLC compared with age-matched controls (Table 1). Among the latter group, 37 (97.4%) of 38 age-matched controls presented DNA concentrations less than 104.5 ng/mL in plasma. Therefore, we defined these values as cutoff levels to differentiate between normal and elevated DNA. As determined by the Mann-Whitney rank sum test, plasma DNA concentrations were significantly higher ($P < 0.001$) in NSCLC patients than in age-matched controls. In plasma, Cox proportional hazards regression test revealed a significant trend ($P = 0.004$) towards higher DNA concentrations at advanced tumor stages.

Table (1): Demographic characteristics of patients and controls and their relation to cDNA expression

Characteristics	NSCLC group (41)		Control Group (38)		P Value
	No.	%	No.	%	
Sex					
Male	37	90.2	30	78.9	0.166
Female	4	9.8	8	21.1	
Smoking					
Smoker	34	82.9	26	68.4	0.135
Non Smoker	7	17.1	12	31.6	
Age in years					
Mean	55.3		57.3		
Median	55		56		
Std. Deviation	7.5		7.6		0.346
Range	36-70		37-72		
C- DNA levels(ng/mL)					
Mean	141.9		69.9		
Median	120.0		74.0		0.0001
Std. Deviation	56.3		13.3		
Range	40.8 - 235.6		40.8 – 105		

Circulating plasma DNA levels in correlation with clinico-pathological factors in NSCLC:

Table (2) summarizes the relation of circulating plasma DNA levels to the patient and tumor

characteristics. There was a significant correlation between circulating plasma DNA levels and stage, with a higher frequency of stage IV cancers had elevated Circulating plasma DNA levels ($P =0.001$).

There were also positive correlations between Circulating plasma DNA levels and smoking status (P =0.02), LDH level (P =0.001), as well as tumor status (P =0.004). There was no statistical significant correlation when looking at the effect of age (P = 0.103), sex (P= 0.164), performance status (P = 0.267), pathological subtype (P = 0.26), and nodal status (P = 0.278) on the circulating plasma DNA levels. There was borderline statistical significant correlation between circulating plasma DNA levels and presence of distant metastases (P = 0.058).

Table (2): Circulating plasma DNA levels in relation to patient and tumor characteristics

Characteristics	No. (41)	Circulating plasma DNA levels (ng/mL)			
		Range	Mean	Median	P value
Age in years					
>60	23	40.8 – 235.6	129.3	105.6	0.103
<60	18	96.4 – 225.6	158.3	131.1	
Sex					
Male	37	56.2 – 235.5	137.9	119.7	0.164
Female	4	40.8 – 235.6	179.5	220.7	
Smoking Status					
Smoker	34	56.2 – 235.6	151.1	122.9	0.02
Non Smoker	7	40.8 – 119.7	97.8	105.6	
ECOG Performance Status					
≤2	26	40.8 – 235.6	134.5	107.3	0.267
>2	15	115.1 – 225.6	155	126.5	
Histopathology					
Adenocarcinoma	10	40.8 – 235.3	124.8	113.2	0.26
Squamous cell carcinoma	31	56.2 – 235.6	174.6	122.2	
Stage					
III	24	40.8 – 235.6	119.3	104.6	0.001
IV	17	115.1 – 235.3	174.1	193.5	
T-stage					
T1 -T2	15	40.8 – 215.8	110.1	105.6	0.004
T3 -T4	26	93.3 – 235.6	160.4	131.1	
N-Stage					
N0-N1	16	40.8 – 235.3	129.9	113.2	0.278
N2-N3	25	56.2 – 235.6	149.7	123.1	
M-Stage					
M0	32	40.8 – 235.6	133.2	115.2	0.058
M1	9	120 – 225.6	173.3	196.6	
LDH Level					
<240	18	40.8-215.8	110.4	112	0.001
>240	23	58.1- 235.6	166.8	193.5	

Relationships between Circulating Plasma DNA Levels and Response to Treatment:

Overall treatment response rate for patients with NSCLC was 39% (16/41), and tumor control rate (overall response and stable disease) was 73.2% (30/41) according to the RECIST criteria (Table 3). Complete response was observed in 3 patients (7.3%).

All objective responses were confirmed at least 4 weeks after first observation. Circulating plasma DNA levels were significantly inversely correlated with treatment response (P < 0.001).

Overall, the median DNA concentration of responders during follow-up (75 ng/mL) showed a clear trend toward decreases.

Table (3): Relationships between Circulating Plasma DNA Levels and Response to Treatment

Response	No. (%)	Circulating plasma DNA levels (ng/mL)			
		Range	Mean	Median	P value
Complete response (CR)	3 (7.3%)	40.8 –117.5	94.3	103.5	
Partial response (PR)	13 (31.7%)				
Stable disease (SD)	14 (34.1%)	95.7-235.6	169.5	148	
Progressive disease (PD)	11 (26.8%)				
Objective response (CR+PR)	*16 (39%)*	*40.8 –117.5*	*94.3*	*103.5*	*0.00001*
No response (SD+PD)	*25 (61%)*	*95.7-235.6*	*169.5*	*148*	

Relationships to survival:

Median PFS and OS times for all patients with NSCLC were 8 months (95% confidence interval, 9.99 - 14.01; SE: 1.02) and 12.00 months (95% confidence interval, 6.43 – 9.57; SE: 0.8), respectively, (Figures 1, 2).
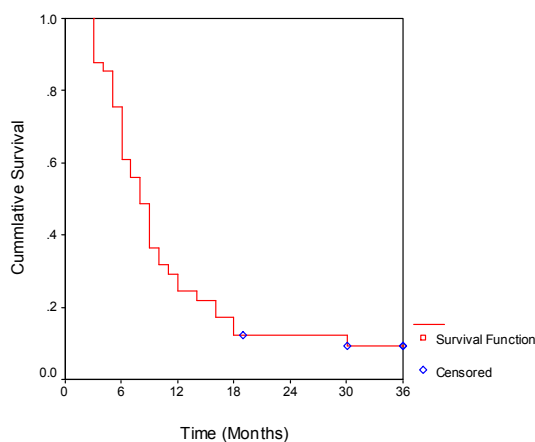
Figure 1. Kaplan–Meier curve of progression-free survival for all patients with NSCLC
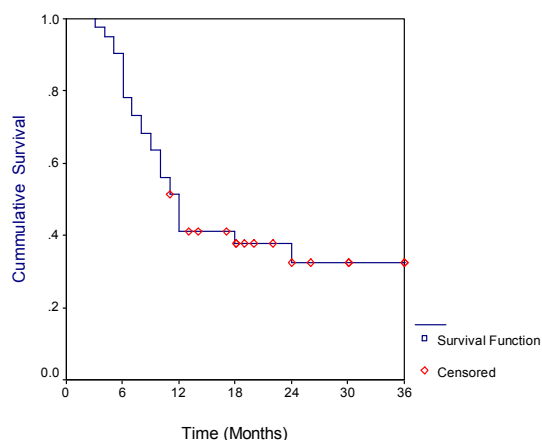
Figure 2. Kaplan–Meier curve of overall survival for all patients with NSCLC

To evaluate the prognostic significance of circulating plasma DNA levels, circulating plasma DNA levels were analyzed in relation to PFS and OS.

Circulating plasma DNA levels were significantly associated with a shortened PFS. Two-year PFS was 23.5% for patients with circulating plasma DNA levels ≤ 104.5 ng/mL (we defined these values as cutoff levels to differentiate between normal and elevated circulating plasma DNA levels) versus 4.2 % for patients with circulating plasma DNA levels > 104.5 ng/mL ($P < 0.001$) (Figure 3).
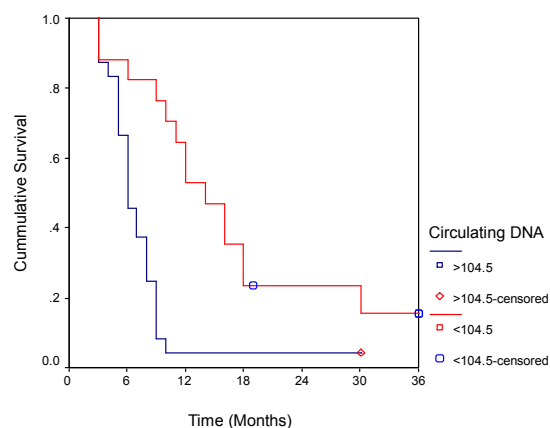
Figure 3. Progression free survival according to circulating plasma DNA levels

In terms of OS, The Kaplan–Meier survival curves demonstrate the better prognosis with circulating plasma DNA levels ≤ 104.5 ng/mL. Two-year OS was 58.4% for patients with circulating plasma DNA levels ≤ 104.5 ng/mL versus 14.3% for patients with circulating plasma DNA levels > 104.5 ng/mL ($P = 0.0014$) (Figure 4).
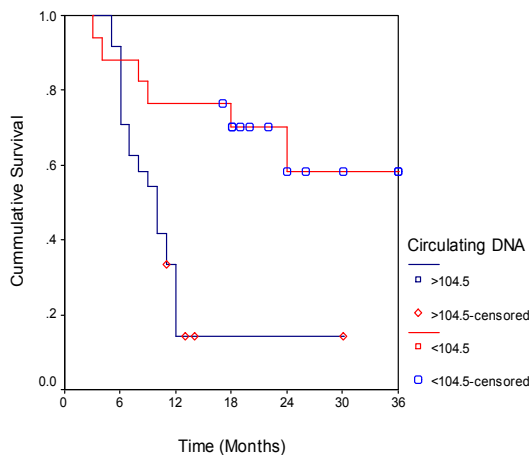
Figure 4. Overall survival according to circulating plasma DNA levels

4. Discussion

It is well recognized that tumor markers are not only of significance to the researcher in understanding tumor biology, but also to the clinician in treating patients with cancer[18]. Previous studies have reported significantly higher concentrations of circulating DNA in patients with various types of cancers, and have suggested the use of circulating DNA in cancer patients as a prognostic tool to monitor the effect of cancer therapy[13,19].

By using a simple colorimetric assay in a representative series of lung cancer patients and controls, we have demonstrated that a quantitative plasma DNA test is a valuable diagnostic tool to discriminate patients from age-matched controls. **Chang et al.**[15] in their study performed in a group of miscellaneous tumors confirmed these results[15].

Our results show that, mean circulating plasma DNA levels were 141.9 ±56.3 ng/mL in NSCLC patients and 69.9 ±13.3 ng/mL in controls, the difference being highly significant ($p < 0.001$). The mean DNA concentration was almost 2-fold higher in plasma from patients with NSCLC compared with age-matched controls. Among the latter group, 37 (97.4%) of 38 age-matched controls presented DNA concentrations less than 104.5 ng/mL in plasma. Therefore, we defined these values as cutoff levels to differentiate between normal and elevated DNA. Similar values were reported previously by **Kumar et al.**[20] in their study and could be of substantial benefit in clinical practice.

Our results showed that circulating DNA concentrations, using the 104.5 ng/mL as cutoff levels, was significantly associated with stage, smoking status, LDH level, as well as tumor status. There was no statistical significant correlation when looking at the effect of age, sex, performance status,

pathological subtype, and nodal status on the circulating plasma DNA levels. There was borderline statistical significant correlation between circulating plasma DNA levels and presence of distant metastases. On the other hand, an inverse relationship was found between circulating plasma DNA levels and response to chemotherapy. Circulating plasma DNA levels had also a highly significant relationship with shorter duration of PFS and OS.

Studies in patients with NSCLC have shown conflicting data about the prognostic significance of circulating plasma DNA levels, ranging from no prognostic significance, to adverse outcome. Disparity also exists with regard to variables such as clinical staging. In studies by **Fournie et al.**[21] and **Xie et al.**[22], plasma DNA was highest in patients with stage IV disease, whereas in other studies there was no such

association[4,6,12-14,23]. An association with age was reported in one study[4] but not in the other studies[6,12-14, 22,23,]. **Sozzi et al.**[4] found that no significant correlation was observed between plasma DNA concentrations and smoking intensity[4]. Similarly, no correlation has been established with histologic subtypes. **Xie et al.**[22] reported higher amounts of circulating DNA in NSCLC compared with SCLC, results in contrast to those reported by **Beau-Faller et al.**[23].

There are conflicting reports correlating the concentration of circulating DNA with survival. Some authors have reported no correlation between plasma DNA concentrations and PFS or OS[14,23], whereas other authors reported an association of plasma DNA with survival, lactate dehydrogenase[12,21], for a mixed group of SCLC and NSCLC patients[21], and for NSCLC patients only[12].

Overall, the median DNA concentration in our NSCLC responder patients during follow-up (75 ng/mL) showed a clear trend toward decreases, suggesting that quantification of plasma DNA might represent an approach to assess the efficacy of chemo-/radiotherapy[4]. **Gautschi et al.**[12] reported that tumor progression after chemotherapy was significantly associated with increasing plasma DNA concentrations.

In Conclusion, the presence of circulating tumor DNA in the plasma of lung cancer patients has sparked great interest because conventional diagnostic tests tend to be imperfect and more invasive, posing logistic difficulties for serial tumor sampling. Less-invasive techniques, such as blood tests, are attractive for screening, diagnosis, prognosis, surveillance for occult disease progression, identification of potential therapeutic targets, monitoring of tumor responses, and evaluation of

disease pathophysiology and biology. Moreover, levels of plasma DNA could help identify high-risk individuals for chemoprevention trials, and could be tested as a potential intermediate biomarker of the efficacy of intervention.

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10/5/2011

Some Potential Biological Predictors of Hypertension in Obese Male Rats

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Abstract: Obesity is a leading preventable cause of death worldwide. Patients with obesity are susceptible to hypertension which is a risk factor for all clinical manifestations of atherosclerosis, heart failure, coronary artery disease, stroke and renal disease. The aim of this thesis was to study some potential biological predictors of hypertension in obese male rats. Material and methods: 60 male albino rats were included in this study and classified into 2 main groups. Group 1(control group, n=30) and group 2 (obese group, n=30). Induction of experimental obesity was by feeding rats with high fat diet till reaching Lee index > 0.3. Lee index (cubic root of body weight (g) X 10 / naso-anal length (mm). All the following were measured at 3 months-interval (at the beginning of the experiment, after 3 months, after 6 months): systolic blood pressure (mmHg), serum adiponectin (μg/ml), serum uric acid (SUA) (mg/dl), HsC-reactive proteins (HsCRP) (μg/ml) and triglyceride (TG) (mg/dl). Results: Obesity resulted in significant increase of systolic blood pressure and significant decrease of adiponectin. It also induced a significant increase of SUA, HsCRP, and TG. There is significant negative correlation between adiponectin and body weight (r=-0.06 & P < 0.05) in obese group. Conclusion: Serum adiponectin, SUA, HsCRP, and TG are biological predictors of hypertension in obese male rats.

1. Introduction

Obesity is considered as a major obstacle in the maintenance of the human health system (1). Multivariate analysis identified obesity and current smoking as independently associated with uncontrolled blood pressure, both in patients with or without cardiovascular disease (2).

Many hormonal mechanisms have been participated in the regulation of appetite and food intake, storage patterns of adipose tissue, and development of insulin resistance as leptin's, ghrelin, insulin, orexin, cholecystokinin, as well as adiponectin (3). The adiponectin is a protein hormone mediator produced by adipose tissue and modulates a number of metabolic processes, including glucose regulation, fatty acid catabolism (4) and triglyceride clearance (5).

Adiponectin action is thought to modify many obesity-related diseases such as hypertension, type 2 diabetes, and coronary artery disease (6). It correlates negatively with adiposity, and bears an inverse relationship with insulin resistance, atherogenic dyslipidemia, and inflammatory markers (7).

The association of hyperuricemia with the presence of classical coronary risk factors and coronary artery disease (CAD) or myocardial infarction (MI) has been analyzed in many epidemiological studies. Numerous studies have revealed that hypertension, high body mass index (BMI), lipid disorders (especially raised triglycerides--TG level and low high dense lipoprotein cholesterol (HDL-C level), increased creatinine or insulin levels have caused hyperuricemia (8).

However, whether uric acid is an independent risk factor for cardiovascular mortality is still disputed there is still no well-established Pathophysiological link between hyperuricemia and the development of cardiovascular complications (9).

Acute ischemic stroke may trigger an inflammatory response that leads to increased levels of C-reactive protein (CRP). High levels of CRP may be associated with poor outcome because they reflect either an inflammatory reaction or tissue damage (10). Elevated preclinical atherosclerosis in young adults, partly mediated by inflammation (elevated C-reactive protein), high systolic blood pressure, and triglycerides. This association is most marked for those also born preterm (11).

The present work aimed to determine whether serum adiponectin, serum uric acid (SUA), high sensitive C-reactive protein (HsCRP) & serum triglycerides (TG) are potential predictors of hypertension in obese male rats. Also, to find out any association or correlation between those parameters and systolic arterial blood pressure.

2. Material and Methods:

Experimental animals:

The study was carried on 60 male albino rats. The rats were left for a week as a period of acclimatization during which all rats were fed a standard control diet prepared in the laboratory. The

body weight of each rat was recorded every 15 days. Rats were randomly distributed into 2 groups: a) control group receiving control diet (n= 30) b) High fat diet (HFD) – induced obesity group receiving high fat diet (n = 30).

Lee index (cubic root of body weight(g) X 10 / naso-anal length (mm) was assessed every 15 days till reaching Lee index significantly higher than 0.3 where rats were classified as obese (12).

Induction of experimental obesity by high fat diet was introduced by feeding rats with high fat diet. Diet were designed in our laboratory: the composition of control diet is (corn starch 480 g/kg b. wt, sucrose 100 g/kg b. wt, soyabean oil 50 g/kg b. wt, lard 120 g/kg b. wt, casein 190 g/kg b. wt). The composition of high fat diet (HFD) is corn starch 100 g/kg b. wt , sucrose 100 g/kg b. wt, soyabean oil 50 g/kg b. wt, lard 500 kg body wt, casein 190 g/kg b. wt) (13). High fat diets were weekly prepared and stored. The food was renewed every day for 24-week diet course (14).

Measurements:
All the following were measured at 3-months interval (the beginning, 3 and 6 months of the 24-week experimental period):
A) Anthropometric parameters: the body weight (BW) in gm and body length (nasoanal length) in mm were measured and used to determine: Lee index: Body mass index [BMI] Which equal cubic root of body weight (gm) X 10 / naso-anal length (mm)
B) Systolic blood pressure (SBP): was measured by Harvard 50-9331 Rectilinear Recording System, which is a rat tail Monitor.
C) Predictors of hypertension: Retro-orbital blood samples were taken from each rat. The serum was separated and stored at -20^0C for subsequent measurement of: adiponectin , SUA, HsCRP & TG.

Adiponectin was detected by Elisa kit from (B-Bridge International, Inc .USA) according to manufacturers instruction (15) & HsCRP was measured by Elisa kit supplied by (Immuno-Biological Laboratories, Inc. Minneapolis, USA) according to manufacturers instruction (16). SUA was measured according to **Fossati** *et al.* (17). TG was estimated by commercial available kit (18).

Statistical Analysis:
The data was encoded and entered using the statistical package SPSS Version 15. The data was expressed as means $\pm$ SD in each group. All data were analyzed by unpaired & paired student's t-test. Correlation between the continuous variables was assessed using Pearson's correlation coefficient.. The level of statistical significance was assumed to be P < 0.05.

3. Results

The results are shown in Tables (1- 3) and Figures (1-6).

Table 1 shows the significant increase in Lee index and SBP of the obese group, but Table 2 shows the significant reduction of serum adiponectin in control and obese groups after 6 months.

Table 2 shows also the significant increase in SUA and HsCRP in obese group at end of 6 months comparing to control and to the beginning. But, there is only significant increase in TG of the obese group comparing to control.

The % of reduction of adiponectin and the % increase of SUA, HsCRP & TG in obese group are illustrated in Table 3.

There is significant positive correlation between adiponectin & HsCRP (r=.+0.6 & P < 0.05) (Figure 5) in control group, but negative correlation between adiponectin and body weight (r= - 0.6 & P < 0.05) (Figure 6) in obese group. There were no correlations between the SBP and adiponectin, SUA or HsCRP in both studied groups.

Table 1: Lee index (BMI)(g/mm) And Systolic blood pressure (SBP) (mmHG) in control & obese groups

	At beginning		At 3 months interval		At 6 months interval	
	Control	Obese	Control	Obese	Control	Obese
Lee index	0.266+0.0097	0.273+0.001*	0.279+0.0012	0.288+0.006*#	0.282+0.001#	0.313+0.0015*#@
SBP	109.4+19	114.3+14.8	100.9+15	132.9+19*#	118.1+5.5 @	227.4+7.7*#@

Results are expressed as mean $^+$ SD
*Significant compared to corresponding value of control rats.
Significant compared to beginning value
@ Significant compared to 3 months value
P value < 0.05 is significant
P value > 0.05 is insignificant

Table 2: Predictors of hypertension: Adiponectin µg/ml, Uric acid mg/dl, High sensitive C-reactive proteins (HsCRP) (µg/ml), triglycerides (mg/dl) in control & obese groups

	At beginning		At 3 months interval		At 6 months interval	
	Control	Obese	Control	Obese	Control	Obese
Adiponectin	3+0.6	3.79+0.57*	3.97+1.01#	2.7+0.97*#	3.7+0.6#	2.4+0.5*#
Uric acid	2.7+0.3	1.6+0.6*	1.9+0.5#	2.5+0.6*#	1.8+0.3@	2.7+0.7*#
HsCRP	16.8+1.05	13.8+1.97*	13.5+1.3#	16.9+3.1*#	11.3+1.1#@	17.27+3.7*#@
Triglycerides	99.8+7	101.9+10.6	97.5+11.1	106.96+14.1	95.8+7.3	109.23+9.9*

Results are expressed as mean + SD
*Significant compared to corresponding value of control rats.
Significant compared to beginning value
@ Significant compared to 3 months value
  P value < 0.05 is significant
  P value > 0.05 is insignificant

Table 3: The percentage change in predictors of hypertension in obese group

Predictors of hypertension	Beginning	3 months	6 months
Adiponectin		- 29 %	- 37 %
Uric acid		+ 56 %	+ 69 %
HsCRP		+ 22%	+ 25.4
Triglyceride		+ 5%	+ 7.2%

HsCRP high sensitive C reactive proteins
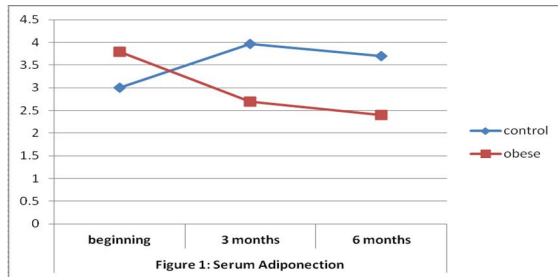
Figure (1): Serum adiponectin levels (µg/ml) in control and obese groups at the beginning of the study and after 3 and 6 months.
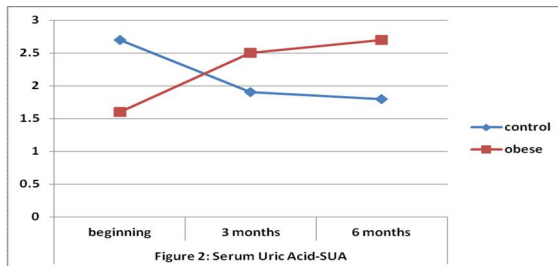
Figure (2): Serum uric acid levels (mg/dl) in control and obese groups at the beginning of the study and after 3 and 6 months.
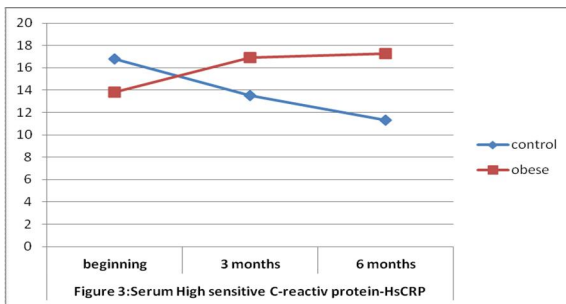
Figure (3): Serum high sensitive C-reactive protein levels (µg/ml) in control and obese groups at the beginning of the study and after 3 and 6 months.
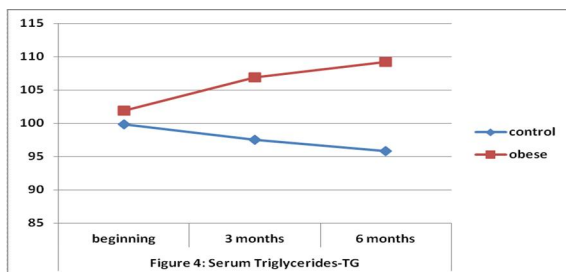
Figure (4): Serum triglyceride levels (mg/dl) in control and obese groups at the beginning of the study and after 3 and 6 months.
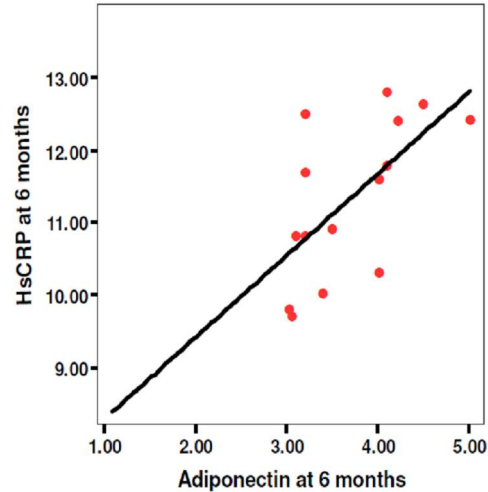
Figure 5: The correlation between HsCRP and adiponectin in control group at end of 6 months.
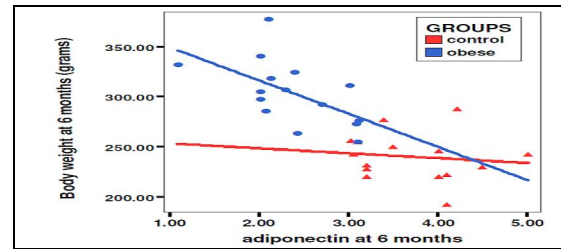
Figure (6): The correlation between adiponectin and body weight in control and obese groups at end of 6 months. (Significant correlation at P < 0.05)

4. Discussion

The present study shows that serum adiponectin, SUA, Hs-CRP and TG may be potential predictors of hypertension in obese male rats. The evidence is that, there is statistical significant decrease of adiponectin level in the obese group compared to the corresponding values in the control group after 3 and 6 months periods. Also, there are statistical significant increase of SBP, SUA and Hs-CRP after 3 and 6 months periods. The serum TG increases significantly after 6 months period.

Wang and Schere (19) related the association of low level of adiponectin with high blood pressure to 3 mechanisms: endothelial dysfunctions, increases renin angiotensin system (RAS) activity, and sympathetic nervous system (SNS).

The authors proved that adiponectin regulates endothelial nitric oxide synthase (eNOS) enzymatic activity and nitric oxide (NO) production by several mechanisms. Adiponectin exerts its effects through membrane-bound receptors and adaptor molecules (APPL1) in endothelial cells (19, 20). Adiponectin increases the stability and half-life of eNOS mRNA (21) and stimulates the phosphorylation of eNOS,

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which together lead to increase NO production. These results suggest that hypoadiponectinemia can cause decreased endothelium-derived NO production and subsequent endothelial dysfunction, ultimately contributing to the development of hypertension. Adiponectin stimulates phosphorylation of eNOS at Ser‑1177 in human endothelial cells through its ability to activate AMP-activated protein kinase signaling (22).

In addition to the above-mentioned signaling pathway, adiponectin promotes endothelial cell function through another mechanism. It promotes revascularization in response to tissue ischemia through endothelial cyclooxygenese-2 (COX-2)-dependent mechanism (23). Deletion of COX-2 in an endothelial specific manner results in suppression of adiponectin-induced revascularization response in ischemic muscle. In cultured endothelial cells, recombinant adiponectin treatment significantly increases COX-2 expression and promotes endothelial cell function (24).

Clinical studies shows that inhibition of RAS with angiotensin converting enzyme inhibitors and angiotensin II receptor blockers can decrease blood pressure and increase circulating adiponectin level in hypertensive patients (25).   Several mechanisms have been proposed to explain the stimulatory effect of angiotensin II receptor blocking on circulating adiponectin levels. Angiotensin II inhibits adiponectin production through angiotensin II receptor subtype 1, and angiotensin II receptor blockers may elicit their effect by inhibiting angiotensin II receptor subtype 1 signaling (19).

Angiotensin II infusion is associated with increased generation of reactive oxygen species (26) which may be one of the underlying reasons for the suppression of adiponectin production, because hydrogen peroxide has been shown to inhibit adiponectin expression (27).

Angiotensin II receptor blockers may increase adiponectin production directly by activating the nuclear receptor PPARγ. In 2004, 2 pioneering articles demonstrated that some angiotensin II receptor blockers act as partial peroxisome proliferators activated receptor-γ (PPARγ) agonists *in vitro* and *in vivo* (28 & 29).

SNS overdrive has been shown to suppress adiponectin expression. The treatment with norepinephrine synthesis inhibitors attenuated the suppression. β-Adrenergic receptor antagonist treatment releases this suppression, suggesting a local effect of sympathetic neurotransmitter signaling in adipose tissue (30).

Because adiponectin has been found in cerebrospinal fluid and administration of adiponectin centrally affects energy homeostasis, it is tempting to speculate that adiponectin may be involved in the regulation of SNS activity from the brain (31). Adiponectin affects SNS activity via central regulation (32).

Analysis of mutations in human adiponectin gene provides further information about the link between adiponectin and hypertension. Among several single-nucleotide polymorphisms of adiponectin gene, single-nucleotide polymorphism at position 164 has been associated with hypoadiponectinemia and high blood pressure in Japanese population (33).

The potential mechanisms involved with the association of hyperuricemia and hypertension include the following: 1.Decrease renal blood flow (decreased GFR) stimulating urate reabsorbtion, 2. Microvascular (capillary) disease that results in local tissue ischemia. 3.Ischemia with associated increased lactate production that blocks urate secretion in the proximal tubules and increased uric acid synthesis due to increased RNA-DNA breakdown and increased purine (adenine and guanine) metabolism,. 4. Ischemia induces increased xanthine oxidase (XO) production and increased SUA and ROS. These associations with ischemia and XO induction may help to understand why hyperuricemia is associated with preeclampsia and congestive heart failure (34).

The results of Lago *et al.* (35) coincide with our results. The authors suggested that visceral fat accumulation play an essential role in the development of the coexistent disorders in the metabolic syndrome (hyperlipidemia, diabetes, hypertension).

It was reported that, CRP is associated with components of metabolic syndrome, including abdominal obesity. Cytokine production by adipocytes might mediate the elevation of CRP levels. Adipose tissue secretes a number of cytokines, among which is interleukin 6 (IL-6). IL-6 regulates hepatic production of CRP (36).

Our results are in harmony with that of Kawamoto *et al.* (37) who proved that adiponectin was significantly lower in subjects with prehypertension and hypertension than those with normotension. Lower serum adiponectin were positively associated with prehypertension and hypertension. Serum adiponectin concentrations were inversely associated with blood pressure (BP) in the general male population.

Ohashi *et al.* (20) & Cohen *et al.* (38) proved that obesity, in particular, visceral fat accumulation, is implicated in the deregulated secretion of adipocytokines, which can contribute to the development of metabolic syndrome and cardiovascular diseases. Adiponectin is an adipocytokine that is exclusively secreted from

adipose tissue, but its plasma levels are reduced in obese subjects, especially those with visceral fat accumulation. Adiponectin has a variety of protective properties against obesity-linked complications, such as hypertension, metabolic dysfunction, atherosclerosis, and ischemic heart disease. Adiponectin exerts the beneficial effects on vascular disorders by directly affecting components of vascular tissue.

Our results also coincide with that of Tanimura *et al.* (39) who documented that Hypoadiponectinemia was positively associated with systolic blood pressure and the prevalence of hypertension

The results coincide with that of Waring & Esmail (40) who concluded that Dietary supplementation of 2 % oxonic acid causes a significant increase in blood pressure. Barbosa *et al.* (41) proved that, close correlation was found between the resultant increase in SUA concentration and rise in blood pressure. Tamakoshi et al (42) have shown a statistically significant positive correlation between CRP and body mass index, total cholesterol, triglycerides, LDL, fasting glucose, fasting insulin, uric acid , systolic blood pressure and diastolic blood pressure and a significant negative correlation of CRP with HDL-C.

Hs-CRP proved to be the strongest and most significant predictor of the risk of future cardiovascular events regardless of the LDL cholesterol level; the data indicate that use of pravastatin resulted in decreased levels of Hs-CRP in a manner largely independent of LDL cholesterol. The data raise the possibility that the addition of Hs-CRP to standard lipid screening will generate an improved method for identifying persons at high risk for future cardiovascular events, who would thus be candidates for primary-prevention interventions such as the use of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (43).

In conclusion, in this prospective evaluation of some biological predictors of hypertension in obese male rats, serum adiponectin, SUA, Hs-CRP and TG are proved to be significant predictor of the risk of future hypertension. Thus, these data raise the possibility that the addition of adiponectin, SUA and Hs-CRP to standard lipid screening will generate an improved method for identifying obese persons at high risk for future hypertension, who would thus be candidates for primary-prevention interventions.

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10/6/2011

Relationship between Iran and Europe Union on Context of Energy

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Abstract: This article is to explain the relationship between Iran and Europe union on context of energy in after Islamic revolution in Iran (1979). In the article, we tend to explain the changes that caused by the different presidents in relationship of Iran with Europe since Islamic revolution. The article is written in three main parts. In the first part, we are explained to issues like over Overview of Iran, Factor of U.S.A – Islamic Republic of Iran – oil, common interests and differences between Iran and Europe countries. In second part, we will explain the pragmatism viewpoint and policy of Hashemi Rafsanjani rather the Europe union. His foreign policy was based on restructuring and renovation of the oil industry and attracting investment, taking loans from the foreign countries and establishing the relations with Europe union. The important contracts have been established with Europe in context of oil and gas in different field of energy. In third part, we will investigate the presidency of Khatami which called the reformist government after the revolution of Iran, this period was one of the most significant for Iran because Iranian opened the door and relation with world especially with European countries, and this period was short time in foreign policy of Iran. There were so many differences between Iran and Europe which going to resolves by Khatami.
[Davod Kiany. **Relationship between Iran and Europe Union on Context of Energy**. Life Science Journal. 2011;8(4):177-185] (ISSN: 1097-8135). http://www.lifesciencesite.com.

Key world: foreign policy, energy, foreign relation.

1-Introduction

European countries have a long experience with Iran in political, cultural and economic era that is long. The most important element, which can explain European foreign policy towards Iran in the past century, was the energy interest. The oil in Iran was discovered by England companies. The history of modern Iran began with the discovery of oil in 1908, from this time, starting of the political and economical relationship between Iran and England on the basis of energy, all aspects of production, export, and sale were with Anglo-Iranian Oil Company, which was its first stage of communication (Fateh, 1979). The English companies were activated in this fields until 1953, because in this year Mohammed Mossadegh, primer ministry of Iran in that era overthrow by coup of U.S " CIA"s organization , which after this date the U.S companies entered in the energy sector of Iran, so after this time the Iranian oil were announced national. Overthrowing of Mohammad Reza Pahlavi government and beginning of Islamic government in Iran 1979 has changed the relationship between Iran and Europe's countries. Since Islamic revolution Iran has been an Islamic republic, governed mainly by its Shiite religious ulema. European relationship with Iran has been poor since the Iranian revolution of 1979 replaced the American supported shah with an Islamic theocracy.

After the revolution, new polices of Iran has been criticized by European countries, for following issues, the hostage of American embassy of U.S by Muslim student, human rights, its support for proscribed terrorist groups like Hezbollah in Lebanon and Hamas in Palestine, its enemy towards Israel and motto of export the revolution to neighbors countries, American hostage in Lebanon by Hezbollah which were tension between Iran and European countries.

After the revolution the Iran and Europe, whose passed three stages, which including, first stage that was from 1979 mainly the victory of new Islamic government. According to the majority of scholars this period called the "silence and black-out" foreign policy of European Union against Iran (Falahi, 2004). During the Iran and Iraq war, the relations between Iran and the European countries had a lot of fluctuations as well as. These relations, however, due to supporting Iraq by these countries were along with tensions. Moreover, because of supporting Iraq by America in the last year of the war, and its direct and indirect attacks to Iran, the relations between these two countries became gloomy. The second period was the "critical dialogue" between Iran and Europe, under the leadership of the Hashemi Rafsanjani who was moderate in foreign policy, Iran engaged in a process of economic and diplomatic rapprochement (Simon, 1998).The third stage was the period of presidency of Mohammad Khatami that was reformist in "comprehensive dialogue". In this article we will explain the relationship of Iran and European Union on the context of energy in after revolution till 2001 the end of presidency of Khatami.

2-Overview of Iran

The geopolitics is of important concerning international relations. In this way, it has a good geopolitics and geo-economics in world, which

definitely drive European interests in this country. There is no question that Iran belongs to the countries, which are of increased importance for Europe union. As Gawdat Bahgat(1999) argues, over the past several decades, history,geopgraphy and natural resources have contributed to the rise of Iran as a prominent regional power.(Gawdat,1999).Initially, it should be noted that Iran a central position in the Persian Gulf. The country borders Pakistan and Afghanistan to the east, Turkmenistan to the northeast, the Caspian sea to the north, Azerbaijan and Armenia to the northwest, the waters of the Persian gulf and gulf of Oman to the south, the strategic geographic position of Iran could not be easily ignored by global powers.

Iran has 930 million barrels of oil that means 10 percent of world oil is produced and holds 16 percent of world natural gas proven reserves. In particular, Iran which is the second largest oil producer of the organization of petroleum exporting countries (OPEC) has an economy which relies on oil export revenues. Iran almost has 40 producing oil fields, of which located onshore and offshore. The majority of Iran's oil reserves were located in the south-western Khuzestan province near the Iraqi border and the Persian Gulf. In addition, Iran has huge potential concerning natural gas as well, it contains an estimated 812 trillion cubic feet in proven gas reserves. It is second largest country in gas and is surpassed only by Russia. Iran's emergence as a global oil actor has a major impact on the world economy, and will continue to do so in the foreseeable future. Iran's nearness to the Strait of Hormuz universally recognized as the most important oil-shipping lane in the world, gives it further leverage over the global supply of oil. Iran located in Caspian Sea and five littoral Caspian states: Azerbaijan, Iran, Kazakhstan, Russian Federation, and Turkmenistan. The Caspian Sea region has become a central focal point for untapped oil and natural gas resources from the southern portion of the former Soviet Union (Maleki, 2007).

2-1 Common Interest of Iran & Europe Union

We, here, considered Europe as England, Italy, German and France. England has been existed since the first days of oil discovery. German had very good relations with Iran; also Imam Khomeini has lived in Nofel Loshato in France for many years. Italy is one of the civilizations in Rome and has an important place in the world .so, Europe was very important for Iran because:

These are the industrialized and advanced countries around the world;
France, England, German and Italy were members of the group 8;
France and England are the permanent members of the Security Council of UN;

These countries are present in international economical monetary organizations such as international monetary funds, World Bank, World Trade Organization, Economic Cooperation Organization and Development;
They are trading partnership of Iran;
They are against the unilateral world of U.S.A;
They determine the ways of economic political Organizations.

Iran is more important from viewpoint of Europe:
It is a developing country with oil and gas reserves;
It is prepare for an extend consuming market from viewpoint of agriculture;
Iran is the most important region for transferring the Energy from the central Asia to the Persian Gulf, Europe and East of Asia, also transiting the goods to Afghanistan, central Asia and Caucasian;
It is located in the center of Energy reserves of the world;
Iran is an active member of the Islamic Conference;
It is a member of the main EKO Organization;
It has the longest shores with energy sources in Caspian Sea;
It is located in the Persian Gulf near the important narrow pass of Hormoz;
Iran is a vital member in OPEC;
It has the second gas reserves in the world with 16% reserves and also has the third oil reserves about 10% around the world; it is the most secure and easiest as well as economic path for transit the energy from the central Asia to the Europe markets (Falahi, 2003).Europe is the serious trade partner of Iran. 40% of imports of Iran are from Europe. Since 1995, the rate of exportation of Europe to Iran was between 3.5 - 7 billion Euros. Most imported goods included machinery, chemicals and medicine. Exports from Iran are 36% of total exports. Most exports of Iran to Europe include the oil that is 80% of total exports of Iran (Falahi, 2003). Of course, because of political problems, now, china is one of the customers of oil which has a good relation with Iran.

2-2 Differences of Iran & Europe

Peace in Middle East: Iran don't formalize Israel regime and recognizes it as a usurper regime, and declares that Israel should be eliminated from the world map. The Israel's politics is to kill and attack to Palestinians for 50 years but Europeans are silent, thus Iran supports the rights of Muslims in the Philistine. Supporting the terrorism: Iran has shocked Israel, because of its influences on Lebanon and Palestine. Since Hezbolah in Lebanon has sent out Israelis from south of Lebanon and Hamas movement resisted against Israel in Palestine and Entefazeh doesn't give up, but Europe recognized these people movements that defend the people's right as Terrorism and Iran is supporter of terrorism. Human rights in Iran: Europe

believed that Iran doesn't respect the rights of women's situation, torturing people, execution, suppression the newspaper and religious minorities. Security problems: Europe believed that Iran seeks the human massacre and missiles and has another aim by nuclear energy, but Iran showed that there are no deviations up to now. Economic correction: Europe believes that Iran should make decision about economic releasing; this is the initial precondition to attract the important foreign investments from Iran (Dehshiar, 2004).

2-3 Factor of U.S.A – Islamic Republic of Iran – oil

When the U.S.A Embassy was seizures by the Muslim Students in 1979, the relations between Iran and U.S.A was declined and U.S.A forced its economic pressures on Iran and by orders of Carter – the president of U.S.A – importing the oil from Iran to U.S.A was stopped and all official assets of Iran in American Banks were taken into custody, the first act in aspect of political and economical relations was to stop purchasing the oil from Iran, because U.S.A has understood the status and the dependence of the country on oil. Although the policy of lack of exporting the oil to U.S.A was applied and about 12.5 billion Dollars for military equipments from U.S.A was canceled (Amirahmadi,1993), but the real date of U.S.A influence on Iran Oil Problems related to 1933 that their influences were extended in Middle East and the importance of the oil was revealed and also by taking Arabia Oil Concession and establishing the Aram co, the inference of U.S.A in the Middle East entered to a new stage (Raeesitoosi,1984) and some years later, by CIA coup and destruction of Mohammed Mosadegh in Iran in 1953, in practice all multilateral authorities of oil in Iran was given to U.S.A until 1979. By taking hostage by the Embassy, even the Europe Society asked Iran to release these citizens and informed that they wanted to execute the commercial boycotts against Iran and this subject caused to decrease purchasing oil from Iran (Razavi, 2001). However, after 444 days, and during the different events, the American citizens were released, but after this time, the economic-political oil relations between Iran and America converted to opposition and dispute. So Americans focused on oil and oil boycotts because they knew that oil plays an important role for Iran's survival.

2-4 the Effective Factors on Changes of the Foreign Policies in Iran

Some important events were occurred in the region such as death of Imam Khomeini in 1989. Secondly, the communist Soviet was broken up and the world converted to a pole that was U.S.A. third, Cold war was ended and a new regularity was dominant in the world. Forth, the statement of killing Salman Roshdi who acted with insolence towards Muslims in a book was

ordered by Imam Khomeini this order had a significant influence on foreign relations and policies of Iran. Fifth, it was the attack of Iraq to Kuwait in 1990.

In 1990, Saddam Hossein has attacked to Kuwait and asserted that Kuwait is one of provinces of Iraq because Kuwait had a lot of Gas and Oil Fields, so could occupied it during 24 hours, but U.S.A attacked Iraq under cooperation of Europe with Operations named Sahara Storm and took it back. In 1991, Petroleum Argus Publication wrote: "the main point of war in the Persian Gulf is that who wants to determine the oil price." Later, it was known as Blood and Oil War. Supposed that the main motive was to prevent the Soviet to access the oil mines in the region, because Iraq is in the east block and the other aim of U.S was a long domination in the Persian Gulf (Alhasani, 1995). Iran condemned the attack of Iraq to Kuwait and declared that it's impartial. Iraq has fired most oil marine wells and Kuwait platforms were burnt. So oil ministers of Iran could extinguish the oil wells of Kuwait because Iranians have experiences in field of extinguishing the oil wells during attacks of Iraq to Iran. Although, only the developed countries such as Canada and U.S had the technology of extinguishing these wells, but Iran also has done different services to accept the war refugees.

3- The foreign policy of Hashemi Rafsanjani with Europe on the energy 1989

The end of Iran-Iraq war (1989) is a turning point in foreign relations of Iran. In internal affairs, Iran focused on the economical and military reconstruction which had been weakened during the war. This required establishing intimate relations with the countries of the region and European Union. In terms of the Arab countries of the Persian Gulf Iran stated that their assisting Iraq during the war would be ignored and they would be interested in developing relations with them. This led to the improvement of Iran relations with these countries, so that the relations between Iran, Jordan, Saudi Arabia and Iraq was resumed and developed.

On the other hand, in order to resume the economy of the country, Iran needed to cooperate with the European countries. However, pronouncing the judgment of killing Salman Roshdi, created a serious crisis in foreign relations with Europe, so that the members of the Europe Union summoned all their ambassadors from Tehran in March 1989, simultaneously. But when Iran government announced that it will not interfere in this issue, these relations were resumed. Nevertheless, there still existed some tension-making issues in the relations of Iran and Europe countries, such as human rights and terrorism.

In addition, the relations of Iran and America recovered to some extent. George Bush, the father, described the policy of Hashemi Rafsanjani government

to be moderate and positive, and added: "presently, a change toward being moderate and rational has been observed in Iran quite obviously and hopefully this process goes on in this direction." Following this, the trade between Iran and U.S increased; and in 1993 it reached 4.9 billion dollars, so as the first commercial partner of Iran, America replaced Germany. Moreover, after ten years, the relations between Iran and the World Bank and the international monetary fund were resumed. Some of the factors which led to the improvement in the foreign relations of Iran are as follows: releasing the western and American hostages in Lebanon, and taking an intelligent position against occupying Kuwait by Iraq. The Arab and West countries welcomed the neutral policy of Iran, and failed the Iraq strategy which was based on coalescing Iran in order to oppose the west. While Iraq had been completely secluded, Iran was no more away from other countries. Besides the improvements in the foreign relations, occupying Kuwait had crucial effects on the internal issues of Iran as well. Iran had taken back its former occupied regions which was not able to retake during diplomatic discussions with Iraq.

On the other hand, collapse of the Soviet Union in 1991 and the independence of the central Asian countries and Caucasus, diverted Iran attention to these regions. In order to develop its relations with these countries, Iran tried a lot. For example, it revived the regional cooperation for development (RCD) under the new name of ECO. During this period, pragmatism approach dominated foreign relations of Iran. In addition to making attempts in reconstructing process, Iran stated that it would act according to the values, and cannot be indifferent about Islamic issues.

Rafsanjani served as President of Iran from 1989 to 1997. Rafsanjani has been described as a centrist and a "pragmatic conservative". He supports a free market position domestically, favoring privatization of state-owned industries, and a moderate position internationally, seeking to avoid conflict with the United States and the West.

Rafsanjani adopted an "economy-first" policy, supporting a privatization policy against leftist economic tendencies in the Islamic Republic. Another source describes his administration as "economically liberal, politically authoritarian, and philosophically traditional" which put him in confrontation with more radical deputies in the majority in the Majles of Iran. Rafsanjani advocated a free market economy. With the state's coffers full, Rafsanjani pursued an economic liberalization policy. He has been seen as flip-flopping between conservative and reformist camps since the election of Mohammad Khatami, supporting reformers in that election, but going back to the conservative camp in the 2000 parliamentary elections as a result of

the reformist party severely criticizing and refusing to accept him as their candidate.

After the war between Iran and Iraq, Mr. Hashemi Rafsanjani was restructuring and construction lines of Iran were drawn by orders of Imam Khomeini. Some plans of the government included restructuring of defensive status, completing the industries and defensive equipments and restructuring the industries, utilities and terminals and refineries which were damaged during the war. Other socioeconomic plans included privation, development and renovation of stock exchange of Tehran, creating the free business regions in the country, decreasing subsides, free business and control of the population (Ehteshami, 1999). The policy of Hashmi Rafsanjani that was known as an operative policy was based on construction of the country and he was titled as commander of constructing. The first plan of this government was two 5 years plans, the first 5 year plan and the second 5 year plan; this plan focused on development of the oil and gas and foreign investment for these fields. He remembered that: it is better the reserves of the country remained under the ground and mountains, because people need them, it is one of our nation glories to save our sources and use them for improvement and development our country (Heshmatzade, 2000).

His foreign policy was based on restructuring and renovation of the oil industry and attracting investment, taking loans from the foreign countries and establishing the rations with Europe countries because Iran was known as a fighter country. Oil was the connection bridge for Iran relations with other companies and countries although unit end of the war there were no investments in oil industries, so some great companies such as BP were entered to the oil market and could take about 18 million dollars loan from foreign countries and also could import some goods to Iran from U.S through Dubai. The government started its first economic plan from 1989 to 1993 that was a 5 years period with 147 billion dollars that it was appointed to supply about 103 billion dollars from selling and oil investment, 17 billion dollars from non-oil exportations, and 27 billion dollars from the foreign sources (Razaghi, 1997). Some of other plans included to relay on economic restructuring of infrastructure utilities of oil with aim of increasing the oil and gas exports, gaining the maximum foreign exchange for the country to reach an economic improvement and per capital income and people's welfare, and finally increasing the price of oil in OPEC.

The second 5 years plan was designed for 1994-1998 that was based on oil, gas, petrochemical for development the future of the country. Although Iran confronted the D'Amato Law in 1996, and was accused of disregarding to human right and supporting the international terrorism by U.S.A , but Iran could do

important actions in field of gas and oil, because between 55-80% of Iran's budget depends on oil (Maher,2000). The first action of the government and Ministry of Oil was to rebuild and restructure the oil infrastructure utilities, specially, restructuring the terminal and loading terminal of Khark and the great Refinery of Abadan. Thus the government invited the oil companies to reach its objectives. On the one hand, because of war between Iran and Iraq and role of Cooperation Council of Persian Gulf, the countries of the region were not optimistic about Iran, but policies of Mr. Hashemi was effective because his first action was to establish the relation with Saudi Arabia that played an important role in Iran's relationships. The authorities of Iran became informed from the new necessities to locate Iran in its real place and play a suitable role and have best relations with its neighbors (Ehteshami, 1999). Mr. Hashemi promised improvement and development of relations between the Persian Gulf countries: it is possible to create peace in framework of independence, territorial and national integrity for all countries located in the region and Iran tried to establish the peace and security in the region. According to Hashemi Rafsanjani: "we don't want to become the gendarme in the region and Iran cannot change the political plan of the region, so tries to change the balance of the new powers that U.S.A plays the main role there (Milani,1996), then Iran established its relationship with Saudi Arabia (Shahalam,2000), because Arabia has an important and vital role in the oil market and was one of the largest producers in OPEC and was the leader of Arabs. During Hashemi's period, the aspects of the foreign policies was changed from ultra-nation objectives to the nation ones, because during this period, the pattern of economic development of Iran was rendered and it was believed that if the government system in Iran which is an Islamic system could show that it is an successful and efficient model, the Muslims and other Islamic governments imitated it automatically .The base of the country constructions depended upon the oil because Iran was a single producer. Mr. Hashemi said: "our country has entered into the fourth stage; we decided to pass through this stage under protection of God and without alteration of the political independence in spite of obstructing by haughty states. So we want to render a pattern of an Islamic country to the world. What is we want is the economical-, scientific-, technological- and technical-independence. The way of development and improvement was based on the oil and foreign investment in fields of oil and gas, because from the beginning of the revolution up to end of war 1988, there was not any foreign investment in field of the oil and gas. Also, the oil utilities of terminals and refineries were damaged during the war and a part of oil utilities related to the first years of discovering the oil and gas

became worn out and on the other hand, according to the potentials, there was need to discovery and production the oil and gas fields that were potential intact. Cooperation between the producer and consumer was an important point. Restructuring the oil utilities needed the western technologies and it was necessary to start the political consistency of the region and good relations (Amirahmadi,1992) so in Iran, the most important tool for establishing the relations with western States and companies and also neighbor companies was the oil.

3-1 The Important Executive Projects of Energy

The most important restructuring projects and repair oil utilities were executed. A contract was concluded with an Italian company for establishing a part of the petrochemical utilities in Arak amounted 135 million dollars during 2.5 years. The contract about establishing the shipbuilding workshop and restructuring the excavating platform in the Caspian Sea was concluded between a Finland Company and the Natinal Oil Company in Iran amounted 66.6 million dollars and 960 billion rilas during the 40 mounts. Restructuring the oil terminal of khark and contract with a French company named ETPM amounted 220 million dollars during 27 months, also the contract with Interpoos company amounted 25 million dollars were planned . The joint venture between Steel Nippon Company from Japan and Toyo Menka Kayasha EOC Company to reconstruct the oil platform of Salman Plateau in Persian Gulf amounted 300 million dollars was done. Also other contracts were as follow:
A-Tiko Evil Company and Selko Shiken Company to produce the oil from two large oil fields in Persian Gulf.
B-ELF Company to develop the oil and gas field in south of Shiraz.
C-Shelemberger Company that has done a contractual project which its credits were supplied by a consortium from Iranian and French banks.
D-Italian Agip Company to develop the oil companies in Iran.
E-The Italian Micoprel Company to reconstruct the Nasr Platform in the Persian Gulf.
F-The Italian Saipem Company has established the pump house of gas amounted 36 million dollars since 1990.
G-The Canadian Companies such as STE and SPE were activated and Iran leased some equipment from Canada.
H-Sang Young Company in Korea to establish six crude reservoirs amounted 30 million dollars.
I-The Raema Repola Company in Finland has established the excavating platform in Caspian Sea under cooperation of a Russian Company.
J-the Total SFP Company for help to Iran about the oil field development. Iran has negotiated with many companies such as: England Oil Company, Deminex

Company in German, Petrofina Co. in Belgium, Gerhard Industries in U.S.A, Royal Group Duch Shelo in South Korea (Dewoo),Eni & Agip in Italy, Xapex with cooperation of Japan Nation Oil Company (J.N.V.C). Konoko and Emoko in U.S.A were activated in upstream projects in the Plateau of Persian Gulf. Iran improved to establish the new refineries. Snambrogti and ChiveDai Company established a consortium in Italy and Japan in form of a contract amounted 1,243 million dollars for establishing a refinery with capacity of 232,000 barrels in Bandar Abbas. Also a contact was concluded with the TPL Company and JJC Company in Japan amounted 1 billion dollars for establishing the refinery in Arak. Gas Refinery of Kangan was established under a contract between Engineering Iran Co. and Deiee Lim Co. amounted 200 million dollars. For petrochemical productions, Iran has concluded many important contracts with the foreign companies that are extra of our discussion.

One of oil policies of Iran was to sell more oil (1.5 million barrels per day) to the European refineries, so as the past times, Iran didn't want to sell its oil but tended to sell it through the oil merchants such as Fibro Energy Co. and Salum Brother – Su Mark Rich Co., these companies started to sell the crude oil and oil productions. Iran didn't tend to sell as self-selling or barter, and tried to open U.S.A doors. In 1992, about 1.3 oil of Iran was purchased by U.S.A Oil Companies and Exon was the greatest American purchaser. These transactions didn't depend on the trading Boycotts against Iran by U.S.A, because the oil didn't export to U.S.A directly. However, Iran has started from aspect of foreign policies and oil very well. Europe was the greatest trading partner of Iran and purchased about 60.7 % of total oil exports of Iran (Rahmani & Taeb.1996), but it wasn't long before the different factors of this policy were influenced by some events. One of important contracts was concluded between Siri Oil Fields and Total French Co. in 1995 that amounted 610 million dollars.

3-2 U.S.A – Persian Gulf "oil – Containment policy of Iran & Iraq & D'Amato Rules

U.S.A has many important and fundamental aims in Persian Gulf:

Prevention of spreading the human weapons;

Supply the benefits and interests of U.S.A and controlling the oil flow in Persian Gulf;

Prevention of Iran's power and risk of influence of Iran to other regions;

Encouraging the peace between Arabs and Israel;

After 11-September-2001, U.S.A determined Iran and North Korea as the focus of insurgence and aborted Iraq (Bagheri & SalemiGhamsari, 2002). Martin Endik, the Nation Security of U.S.A, proposed a new bilateral controlling policy and declared that Iran supported the

Terrorists and sought the human massacre and it should be introduced as an illegal country in the international relations area (Christen, M.2003). The bilateral control policy meant to control both Iran and Iraq by aim of creating equilibrium of power to U.S.A's advantages in the Persian Gulf. So, another policy was extending the protection-political and military power of U.S.A in the region under concluding the contract with Qatar and Arabia and creating a military station into these countries, also concluding a contract with Kuwait for 10 years and keeping the oil wells in the Persian Gulf.

U.S.A tried to control the oil of Iran and shock the Iranians and tried to prevent Europe and other countries to invest in field of gas and oil because many company (as above said) was active in fields of gas and oil. In 1996, the U.S.A President signed an approved law named D'Amato or ILSA that based on this approved all non-American companies which invested more than 20 million dollars per year in field of gas and oil in Iran and Libya were boycotted and punished economically. Later, this amount decreased to 40 million dollars. Contemporary the Energy Commissioner of the Europe Union declared that this law will create the serious and extended problems and difficulties for European Industry and most countries in the Europe Union opposed against these economical oil & gas boycotts against Iran. So attempts of U.S.A had no results and the mentioned companies have invested in Iran enormously. Of course, from viewpoint of Europeans, opposition against U.S.A means opposition against unilateralism of U.S.A (Kazempoor, 2002). Also, the old oil and gas industry in Iran needed to invest in field of know-how and technology (Jafarivaldani, 1998). On the one hand, Iran needed the European investments but many years later Europe and U.S.A was united and impacted Iran very much.

4- Periods of Khatami President, 1997

Khatami is regarded as Iran's first reformist president, since the focus of his campaign was on the rule of law, democracy and the inclusion of all Iranians in the political decision-making process. However, his policies of reform led to repeated clashes with the hard-line and conservative wing in the Iranian government, who control powerful governmental organizations like the Guardian Council, whose members are appointed by the Supreme Leader. Khatami lost most of those clashes, and by the end of his presidency many of his followers had grown disillusioned with him. As President, according to the Iranian political system, Khatami was outranked by the Supreme Leader.

Khatami presented the so called "twin bills" to the parliament during his term in office; these two pieces of proposed legislation would have introduced small but key changes to the national election laws of Iran and also presented a clear definition of the president's power

to prevent constitutional violations by state institutions. Khatami himself described the "twin bills" as the key to the progress of reforms in Iran. The bills were approved by the parliament but were eventually vetoed by the Guardian Council. Khatami's economic policies followed the previous government's commitment to industrialization. At a macro-economic level, Khatami continued the liberal policies that Rafsanjani had embarked on in the state's first five year economic development plan (1990–1995). A year into his first term as president of Iran, Khatami acknowledged Iran's economic challenges, stating that the economy was, "chronically ill and it will continue to be so unless there is fundamental restructuring."

For much of his first term, Khatami saw through the implementation of Iran's second five-year development plan. On 15 September 1999, Khatami presented a new five-year plan to the Majlis. Aimed at the period from 2000–2004, the plan called for economic reconstruction in a broader context of social and political development. The specific economic reforms included "an ambitious program to privatize several major industries Unemployment remained a major problem, with Khatami's five-year plan lagging behind in job creation. During Khatami's presidency, Iran's foreign policy began a process of moving from confrontation to conciliation. In Khatami's notion of foreign policy, there was no "clash of civilizations"; he favored instead a "dialogue among civilizations". Relations with the US remained marred by mutual suspicion and distrust, but during Khatami's two terms, Iran increasingly made efforts to play a greater role in the Persian Gulf region and beyond. President Khatami introduced the theory of Dialogue Among Civilizations as a response to Samuel Huntington's theory of Clash of Civilizations.

During 1979-1989 the foreign policy of Europe Union against Iran was quiet (Falahi, 2004). After 1992, the critical disputes have started. When Mr. Khatami was selected as the president of Iran in 1997, a new form of arguments were begun because a positive area was formed by corrections of Khatami and changing the international sights, particularly Europe. In 1998, the Council of Ministers of the Europe Union asked the Europe Commission to establish connections with Iran (Falahi,2004) and establish the cooperative areas in fields of Energy, trading, investment, human rights, and disarmament, environment (Farsaee,SH.2001). The most important section was the oil problems that were reviewed seriously. Domination of U.S.A over the Persian Gulf didn't include Iran, because Europeans tried to invest in field of oil and gas. Although the law of D'Amato prohibited, but Europeans were against this law, so Iran was forced to transact with Europe. Thus it was estimated that oil and gas in Iran needed for 8-9 billion dollars investment (Farhang, 1997), and it is the motive and attraction for European oil companies particularly for companies which confronted unemployment crisis. Under such conditions, the competitive market and mutual selling contracted was concluded. The deceased Mohsen Noorbakhsh, the president of the central Bank, said: the economic section in the country needs lack of debates in the foreign relations to achieve the aims." If the foreign policy of Iran doesn't act to remove the debates and a problem is created for selling the oil or importing the goods to Iran, all economical sections in the country confronts many difficulties (Jafarivaldani,2004). Removing the debates in relations between Iran and Europe was an important aspect. Iran referred Europe to spread the oil area and hoped that increases the production rate of the old fields by using the technical know-how of European companies and their advanced ways such as horizontal excavation, earthquake recording, thus the different contacts were concluded with European companies for developing the utilization from the oil and gas fields. For this reason, some diplomacies in Iran related to Europe named "Oil Diplomacy" (Jafarivaldani, 2004). One of problems in Khatami Periods was to fall down the oil price and it caused Iran was confronted many difficulties and didn't pay its debts on time. Iran should paid about 3 billion dollars of its short-time debts to Europe and Japan but didn't. Using Euro instead of Dollar for commercial transactions of Iran and Europe could fill the vacuum of dollar, because Iran was damaged and incurred a loss for decreasing the dollar value and transactions of oil by Euro between Iran and Europe.

In 1999, Iran declared that it has discovered many new oil areas in 30 oil areas of Azadegan Plateau, the Minister of Oil estimated the oil of this region about 24 billion barrels, 300-400 thousand barrels per day. In 2000, a contract was concluded between Iran and Japan to execute the oil development plans. In 1997, the oil field of Dar Khoeein near Abadan was discovered. In 2001, the ANI Italian Company concluded a contract about 1 billion dollars, about 40% of Dar Khoeein oil for Iran and about 60% of it for Italy that was 160 thousand barrels per day. The SEPSA Spain Company signed a 300 million dollars contract with Iran to complete the plans under cooperation of Total LFB (Farshadgohar, 2002). The first project was a mutual selling investment in 1988, when the oil Siri ALEF area was under the operations has been done by Total Co. in France and PETRONAS Co. in Malaysia and its capacity was 7000 barrels per day and beside it the project of Siri was started in 1999 with capacity of 120000 barrels per day. In 1999, Iran has concluded a contract with Canadian BVEB Co. as mutual selling for developing the Balal field with capacity of 80 million barrels per day.

In 1999, the A. French Co. and ANI Italian Co. concluded a 1 billion dollars contract for discovery of

the oil area in the south with capacity of 1.5 billion barrels per day in Khark.In 2000, Stat Norway Co. has signed a contract with National Oil Company in Hormoz area. In field of developing plans, ENGL was considered for south regions of Iran that amounted 500 million dollars. In 2001, Beliton BHB Co. started to develop the Frozen Esfandyar and increased the production rate from 50,000 to 150,000.In 2001, a contract was concluded between the National Oil Company and Sweden counselors of JVE and Sadra Co. for exploitation in Caspian Sea (Farshadgohar,2002). In 1999, Shell Co. declared that this company has been selected for developing the Norooz and Soroosh Oil Areas under a mutual selling contract, these fields were damaged during war between Iran and Iraq. It is appointed to conclude the contract of oil area "Ahvaz Bangestan" with an English BP Co. with investment amounted 950 million Dollars. Although the oil boycotts by U.S.A has informed in 1996, but in 1997 a consortium composed of Total French Co., Gas Prom in Russia, PETRONAS in Malaysia a contract has been concluded with Gas Field of South of Pars (Pars-Jonoobi) that amounted 2 billion dollars.

The largest gas field in Iran is the south Pars that is common with Qatar. This project has 25 independent phase that up to now about 5-7 phases are utilized. It is estimated that in Bandar Asaluyeh the gas about 3 billion square foot per day and liquid gas about 120,000 barrels per day are produced. The gas piping line to India and Pakistan will use this gas. It might these contracts sustain a loss to the national Iranian interests and many of them are adjusted as joint venture with other countries. Europe's position against U.S.A caused that Iran came out from isolation and rescued from surrounding and boycotts. But after 2001, and attack to Business Towers of U.S.A, America and Europe cooperated with each other and Iran was under pressure. For example, in 2003, 15 countries member of the Europe Union through a resolution warned to Iran that the negotiations of the Union with Iran provided to respect of human rights and prevent to produce nuclear weapons, terrorism and peace in the Middle East. Then, America and Europe united and started a conflict against Iran that is continued up to now.

Conclusion

Energy is political and economical goods and was an important factor that influenced on the foreign relations and foreign policy, Iran is producer of one product and each change in energy could influence on relations and politics of Iran. The oil consumers which are Europe and the industrial countries could be effective with lack of purchasing and breaking off the dependent relations. After the 8 years war between Iraq and Iran the infrastructures of oil and dependent affairs were ruined completely or some of them were out of order or ruined.

Then, according to the 8 years war Hashemi Rafsanjani attempted to force a peace policy because he started to act and operate these policies and strengthened his relations with Europe, so on the other hand, Iran needed to attract the foreign investment by the governments and European other countries in fields of oil and gas. So new investments were started to reconstruct the different regions such as the important terminal of Oil Platforms in Khark and refineries through invitation of some companies and foreign governments, it was satisfactory until when problems between Iran and U.S.A were appeared, D'Amato Law was approved by Bill Clinton - the president of U.S.A, and according to this law all oil companies which invest in Iran and amounted more than 20 million dollars should be punished. Although Europeans opposed against this law and European companies started to invest in Iran, but these relations were influenced by increasing the problems between Iran and U.S.A such as accusation to Iran about human massacre, don't respect the human rights, supporting the Islamic movements like as Hamas in Palestine and Hezbollah that called Terrorist Groups from viewpoint of U.S.A so Europeans pressured on Iran or prohibited to invest in Iran. This procedure has been continued until 1997, i.e. when the president Khatami was selected; because in Khatami's period by the civilization conversations relations between Iran and Europe entered to a new stage, since Iran was an important region for Europe and also Europe was very important for Iran. Khatami periods are very important for the foreign policies of Iran. Khatami even tended to negotiate with U.S.A and could decrease the pressures of Iran by Europe and U.S.A. he called his government as a Reform State and Home & Foreign Reformations. He started to invest with European companies and to conclude the mutual selling contracts. During his period, the 25 phase's project of South of Pars (pars Jonoobi) was established that is a common project with Qatar for producing the gas. After 11-September and Terrorist attacks to the Business Towers in U.S.A, again the pressures on Iran were intensified, and economic business and relations of Iran confronted the political problems.

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16/09/2011

Topical Antioxidant and Narrowband versus Topical Combination of Calcipotriol plus Betamethathone Dipropionate and Narrowband in the Treatment of Vitiligo

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Abstract: Vitiligo is a specific, common, often heritable, acquired disorder characterized by well-circumscribed milky-white cutaneous macules devoid of identifiable functional melanocytes because of multifactorial and overlapping pathogenic mechanisms. The basic defect in vitiligo is loss of melanocytes. Narrowband ultraviolet B (NB-UVB) is an emerging, effective and safe therapy for vitiligo. Because of defective calcium homeostasis in depigmented skin, the vitamin D-3 analogs (calcipotriol and taclacitol) have been used topically in vitiligo, where modulation of the local immune response on specific T cell activation occurs. A new topical product containing a combination of vegetal catalase (CAT) and superoxide dismutase (SOD) has been used in vitiligo. *In vitro* studies demonstrated the capacity of this complex to dramatically reduce the production of free radicals in vitiligo cell and even to restore a normal level of melanin in melanocytes of vitiligo. Patients and Methods: The current study comprised a total of 40 patients with different clinical varieties of vitiligo. They were recruited from the Outpatient Clinic of Dermatology and Venereology Department, Tanta University Hospitals. *The studied patients were divided into: G1*: Included 20 patients subjected to topical combination of calcipotriol plus betamethazone dipropionate ointment with NB-UVB on the left side of the patient and NB-UVB alone on the right side of the same patient. *G2:* Included 20 patients subjected to topical SOD/CAT gel with NB-UVB on the left side of the patient and NB-UVB alone on the right side of the same patient. Results: Comparison in the response of the treatment in GI and GII between right and left side revealed no statistically significant difference between the two sides. Comparison in the response in the left side in G I and G II showed no statistically significant difference in repigmentation between the two groups. There were no significant correlation between the results of the combination treatment plus NB-UVB in both groups and clinical criteria of vitiligo patients. There were statistically significant differences between distribution of sites of the lesions in vitiligo patients and treatment with topical applications plus NB-UVB regarding response of the treatment in the face and neck in G II. While in G I the face had excellent response but not statistically significant. Conclusions: The current study had shown that NB-UVB treatment alone is a moderately effective treatment for vitiligo. Betamethasone dipropionate / calcipotriol, when used in combination with NB-UVB were   found to be superior in efficacy than NB-UVB alone, but the results were not statistically significant, while SOS/CAT gel does not appear to add any incremental benefit to NB-UVB alone. It could be recommended that further studies should be performed on this subject.

1. Introduction

Vitiligo is an acquired depigmentary dermatosis characterized by sharply demarcated lesions heterogeneous in size and shape. The disease generally runs in a progressive course. The disease affects 1-2% of the world population, irrespective of age, gender, and skin color[1] . From therapeutic and prognostic viewpoint, vitiligo is broadly classified into two major subtypes, segmental vitiligo (SV) including focal lesions confined to a segment of the body that does not progress towards generalized disease; and non-segmental (NSV) vitiligo which comprises all generalized usually symmetrical forms, including acrofacial vitiligo[1,2]. The etiopathogenesis of vitiligo is still not fully understood, and the major theories include melanocyte destruction (autoimmune, neural andimpaired redox status) and melanocyte inhibition or defective adhesion [3]. It proposes that vitiligo is a primary melanocytorrhagy disorder with altered melanocyte response to friction and possibly other types of stress, including their indolent attachment and subsequent transepidermal loss [4] Calcipotriol is derived from 1-24-dihydroxy vitamine D3 and has the same mechanisms of action as other vitamin D derivatives, and these mechanisms involve both genomic and non-genomic pathways[5] . In regards to vitiligo, the non-genomic mechanism is involved. Vitamin D increases intracellular calcium concentration through hydrolysis of phosphatidyl inositol phosphate,

leading to production of diacylglycerol and inositol triphosphate with subsequent release of intracellular calcium stores. The intracellular calcium concentration regulates a number of cellular functions including proliferation and differentiation of melanocytes. A combination of topical calcipotriol and corticosteroids demonstrated effectiveness in repigmenting vitiligo, even in patients who were previous topical corticosteroids failures.[6]Abnormal antioxidant activity in peripheral blood mononuclear cells of vitiligo patients has also been observed, with increased SOD activity, and reduced CAT, glutathione and vitamin E levels. This variability in antioxidant levels was seen exclusively in subjects with active disease. These changes in antioxidants could be responsible for the generation of intracellular reactive oxygen species in vitiligo patients [7]. Topical applications of a combination of vegetal CAT and SOD has been used in vitiligo [8]. *In vitro* studies had previously demonstrated the capacity of a SOD + CAT complex to dramatically reduce the production of free radicals in vitiligo cell and even to restore a normal level of melanin in melanocytes of vitiligo.[9] It is not only important to remove epidermal hydrogen peroxide ($H2O2$), but also for a successful treatment against vitiligo.[10]The NB-UVB is an emerging, effective and safe therapy for vitiligo. [11] It is effective as PUVA, without side effects [12] . The road in the treatment for vitiligo looks promising as more and more knowledge about the disorder is being discovered, thus allowing doctors and pharmacists all around the world to develop future therapies [10] .

2. Patients and Methods

The current study comprised a total of 40 patients with different clinical varieties of NSV , diagnosed on the basis of the typical appearance of the skin lesions .The patients were recruited from the Outpatient Clinic of Dermatology and Venereology Department, Tanta University Hospitals, from March / 2010 to December / 2010 and follow up continued till March /2011. All patients were of Fitzpatrick skin type III– IV. The studied patients were treated as a part of an open - controlled right-left comparative study and divided into the following groups*: G1*, included 20 patients subjected to topical combination of calcipotriol (0.005%) plus betamethazone dipropionate (0.05%) ointment with NB-UVB and G2,included 20 patients subjected to topical SOD/CAT gel with NB-UVB.

Exclusion criteria:

Patients who had used any topical or systemic treatment or phototherapy during the 6 weeks prior to the incorporation in this study, pregnant or lactating women, patients with any significant systemic, psychiatric or other dermatological conditions that may compromise the result of the study, any contraindication to phototherapy as: skin cancer or lupus erythematosus , special emphasis on patient's occupations and exclusion of those with inevitable sun exposure, patients who did not complete sessions or the follow up and who react to an application of the ointment/gel .

All patients were subjected to the following:

-Complete history taking
-Thorough clinical and dermatological examination. The extent of the vitiligo was assessed and recorded.
-Colored digital photographs were taken for each patient before the first session as a baseline, before each visit at two weeks intervals, at the end of the treatment and monthly thereafter for the following 2 months during follow up period.
-All selected patients were instructed to avoid the use of any other vitiligo therapy (topical or systemic) during the whole duration of the study and follow up period. Informed written consent was obtained from all patients before starting treatment.

Pretreatment preparation of the patients:

- Application of the ointment or gel on the patient's anterior forearm as a thin layer over the entire affected site for 24 hours, to assess any reaction or sensitivity with the topical drug.
- The minimal erythema dose was calculated for all patients prior to the onset of the treatment. The treatment is initiated with 75 to 90% of this dose, varying according to the patient skin phototype (Table 1).

Table (1) :   Initial ultraviolet B radiation dose [13] .

Skin type	mj/cm^2	75% of dose(mj/cm^2)
I	20 – 30	19
II	25 - 35	23
III	30 -50	31
IV	45 – 60	37
V	60 – 100	50
VI	100 - 200	107

Treatment procedure:

GI: The patients instructed to apply topical

combination of calcipotriol/ betamethasone dipropionate ointment on the left side twice daily except on the day of NB-UVB session. GII: The patients instructed to apply topical SOD / CAT gel twice daily on the left side and from 2 hours before NB-UVB session.

-Each patient received NB-UVB (311-313 nm) session twice /week alone on the right side and with combination treatment on the left side.

-Post NB-UVB erythema usually appears 12 hours after the session. The dosage is gradually increased in order to minimize burn reactions to the UVR (Table 2).

Table (2): Ultraviolet B radiation according to degree of erythema [14]

Degree of erythema	Dose increment
0 (no erythema)	20%
1 (minimal erythema)	10%
2 (intense erythema)	Do not apply
3 (erythema and edema)	Do not apply
4 (erythema, edema and blisters)	Do not apply

Post treatment care:

After each session, the patients were instructed to avoid sun exposure as much as possible and to avoid skin rubbing or friction, a bland emollient like panthenol was prescribed to the patients if the irritation of the skin occurred and evaluation was done every 2 weeks for 48 sessions and for follow up period.

Assessment of the efficacy of the treatment:

-Physician's evaluation: clinical evaluations were done before treatment and two weeks after treatment sessions by three dermatologists.

-Patient's opinion: assessment of the over all vitiligo severity.

-Assessment of the mean value of the all evaluations.

-Digital image analysis of standardized colored photographs taken of each visit was used to determine the percentage repigmentation of the vitiligo lesions.

Treatment efficacy was categorized as:

Excellent: which was designated if the patient showed great improvement >75% as compared to pretreatment condition and no noticeable lesions on the skin or any complication from the treatment procedure. Moderate: 50%-75%. Mild: 25%-50%. No response or treatment failure. :   <25%

Statistical presentation and analysis of the present study was conducted, using the mean, standard error, unpaired student t-test,the Wilcoxon tests   and chi-square by SPSS V12.

3. Results

Clinical results:

Comparison between studied groups revealed no significant differences as regard age, gender, family history (FH) and different sites of the vitiligo lesions, but there was significant difference between two groups as regard duration of the disease.

Treatment results:

-The response of treatment of the studied groups was illustrated in (Table 3).

-Comparison in the response of the treatment in G I and GII between right side and left side revealed no statistically significant difference between the two sides (Tables 4 &5).

-Comparison in the response in the left side showed no statistically significant difference in repigmentation between the two groups (Table 6)

-There were no any significant correlation between the combination treatment plus NB-UVB in both group and clinical criteria of vitiligo patients as age, gender, duration of the disease and patients with positive and negative FH. There were statistically significant differences between sites of vitiligo and treatment in the left side in the face, neck in G II [P = 0.005] while in G I the face had excellent response but not statistically significant (Table 7).

Table 3: The response of the treatment of the studied groups

		Response									
		No		Mild		Moderate		Excellent		Tot	
		N	%	N	%	N	%	N	%	N	%
Group I	Right	4	20	4	20	4	20	8	40	20	100
	Left	4	20	2	10	5	25	9	45	20	100
Group II	Right	5	25	5	25	1	5	9	45	20	100
	Left	4	20	4	20	4	20	8	40	20	100
Total		17	21.25	15	18.75	14	17.5	34	42.5	80	100

Table 4: Comparison in the response of the treatment between right side and left side in group I

Group I	Response					
	Right		Left		Total	
	N	%	N	%	N	%
No	4	20.00	4	20.00	8	20.00
Mild	4	20.00	2	10.00	6	15.00
Moderate	4	20.00	5	25.00	9	22.50
Excellent	8	40.00	9	45.00	17	42.50
Total	20	100.00	20	100.00	40	100.00
Chi-square X^2	0.837					
P-value	0.840					

Table 5: Comparison in the response of the treatment between right side and left side in group II

| Group II	Response					
	Right		Left		Total	
	N	%	N	%	N	%
No	5	25.00	4	20.00	9	22.50
Mild	5	25.00	4	20.00	9	22.50
Moderate	1	5.00	4	20.00	5	12.50
Excellent	9	45.00	8	40.00	17	42.50
Total	20	100.00	20	100.00	40	100.00
Chi-square X^2	6.400					
P-value	0.093					

Table 6: Comparison in the response of the treatment in the left side between group I and group II

| Left	Group					
	Group I		Group II		Total	
	N	%	N	%	N	%
No	4	20.00	4	20.00	8	20.00
Mild	2	10.00	4	20.00	6	15.00
Moderate	5	25.00	4	20.00	9	22.50
Excellent	9	45.00	8	40.00	17	42.50
Total	20	100.00	20	100.00	40	100.00
Chi-square X^2	0.837					
P-value	0.841					

Table 7: Relation between site of the lesions and response of the treatment in the left side in studied groups

Group	Site		Left(Topical application + NB-UVB)					Chi-square	
			No	Mild	Moderate	Excellent	Total	X^2	P-value
Group I	Forearm	N	0	0	0	2	2	26.956	0.029
		%	0.00	0.00	0.00	10.00	10.00		
	Face	N	0	0	0	3	3		
		%	0.00	0.00	0.00	15.00	15.00		
	Leg	N	0	1	3	1	5		
		%	0.00	5.00	15.00	5.00	25.00		
	Trunk	N	0	0	0	2	2		
		%	0.00	0.00	0.00	10.00	10.00		
	Hand	N	2	0	2	0	4		
		%	10.00	0.00	10.00	0.00	20.00		
	Foot	N	2	1	0	1	4		
		%	10.00	5.00	0.00	5.00	20.00		
Group II	Forearm	N	2	1	1	1	5	32.750	0.005*
		%	10.00	5.00	5.00	5.00	25.00		
	Face	N	0	0	3	3	6		
		%	0.00	0.00	15.00	15.00	30.00		
	Leg	N	0	2	0	0	2		
		%	0.00	10.00	0.00	0.00	10.00		
	Neck	N	0	0	0	4	4		
		%	0.00	0.00	0.00	20.00	20.00		
	Trunk	N	2	0	0	0	2		
		%	10.00	0.00	0.00	0.00	10.00		
	Hand	N	0	1	0	0	1		
		%	0.00	5.00	0.00	0.00	5.00		

Digital photographs of treated patients included 4 photos:

1) Before treatment   **2)** After 3 months (24 session)   **3)** After 6 months (48 session)   **4)** During follow up

Digital photographs of patients treated in group I
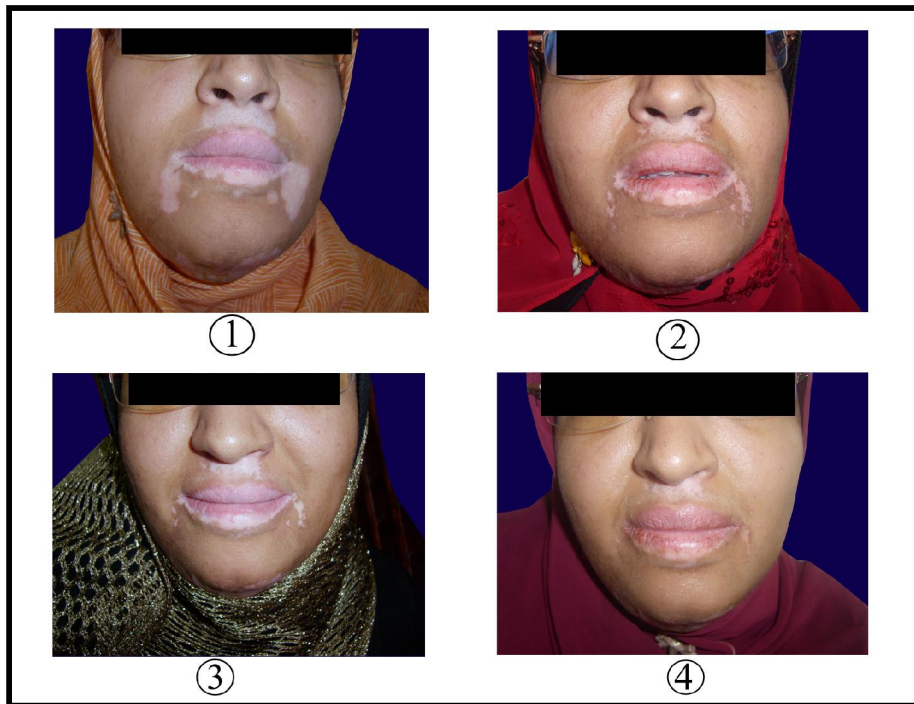
Photo1:.Excellent response in both sides of the face (>75% repigmentation)
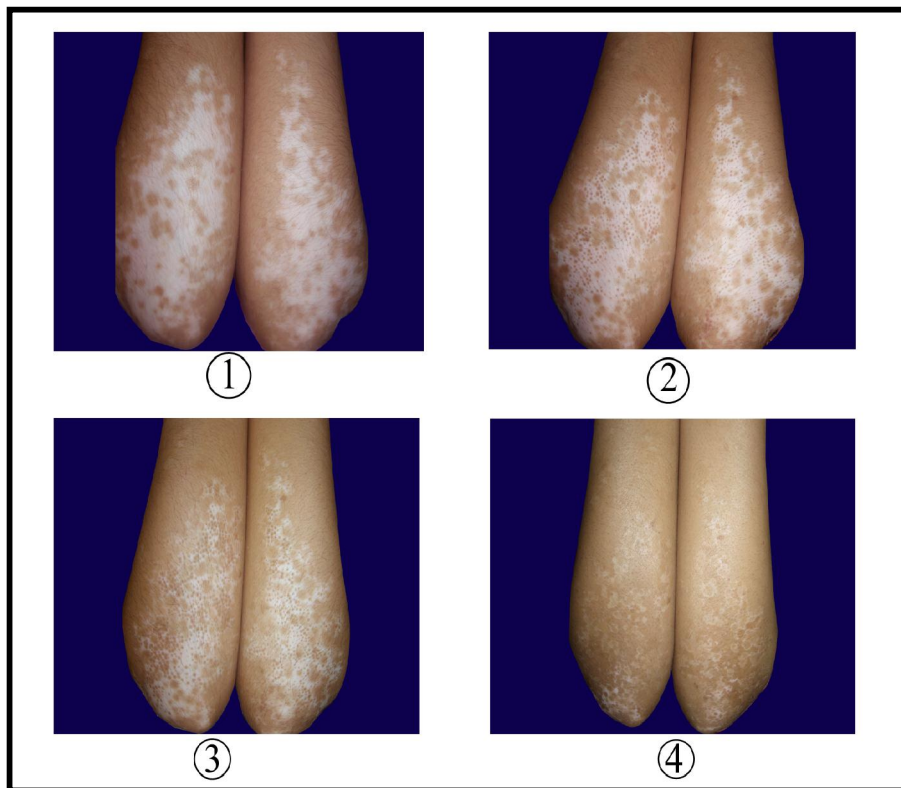
Photo2: Excellent response in both sides of the forearms (>75% repigmentation)
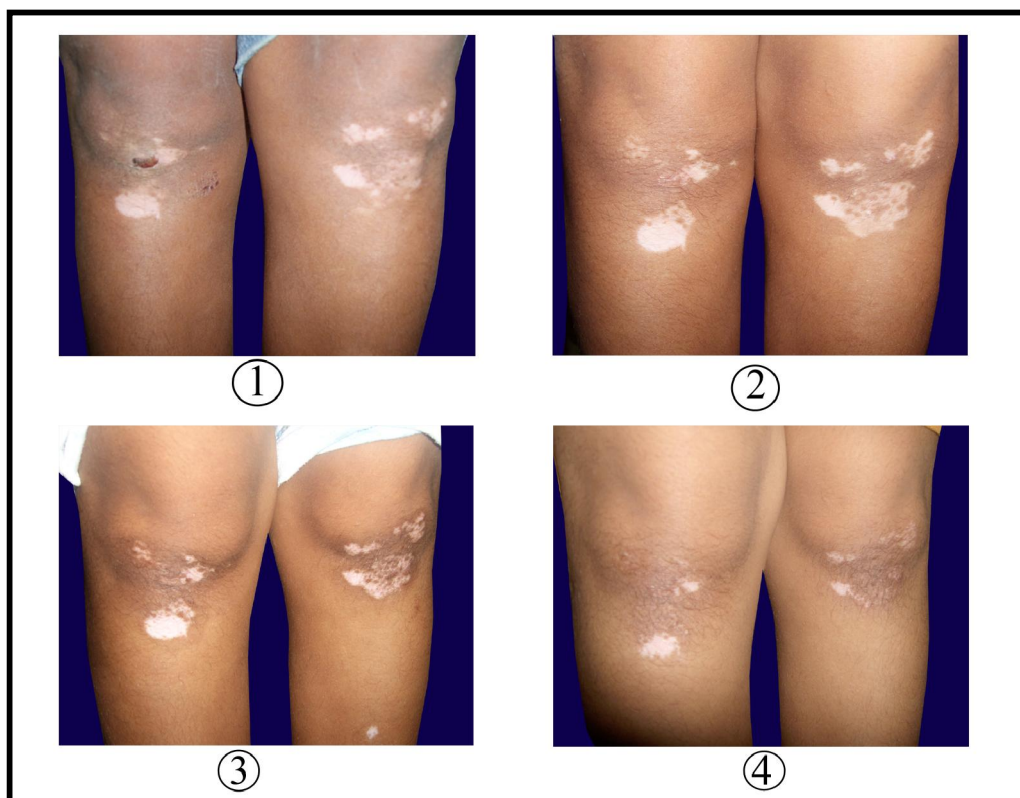
Photo 3: Moderate response in both sides of both knees (50%- 75% repigmentation)

Digital photographs of patients treated in group II

Photo 4 a: Excellent response of the left side of the face (>75% repigmentation)

Photo 4 b : Mild response of right side of the face (25% 50% repigmentation)

Photo 5:   No response in both sides of the wrist and hands (<25% repigmentation)

4. Discussion

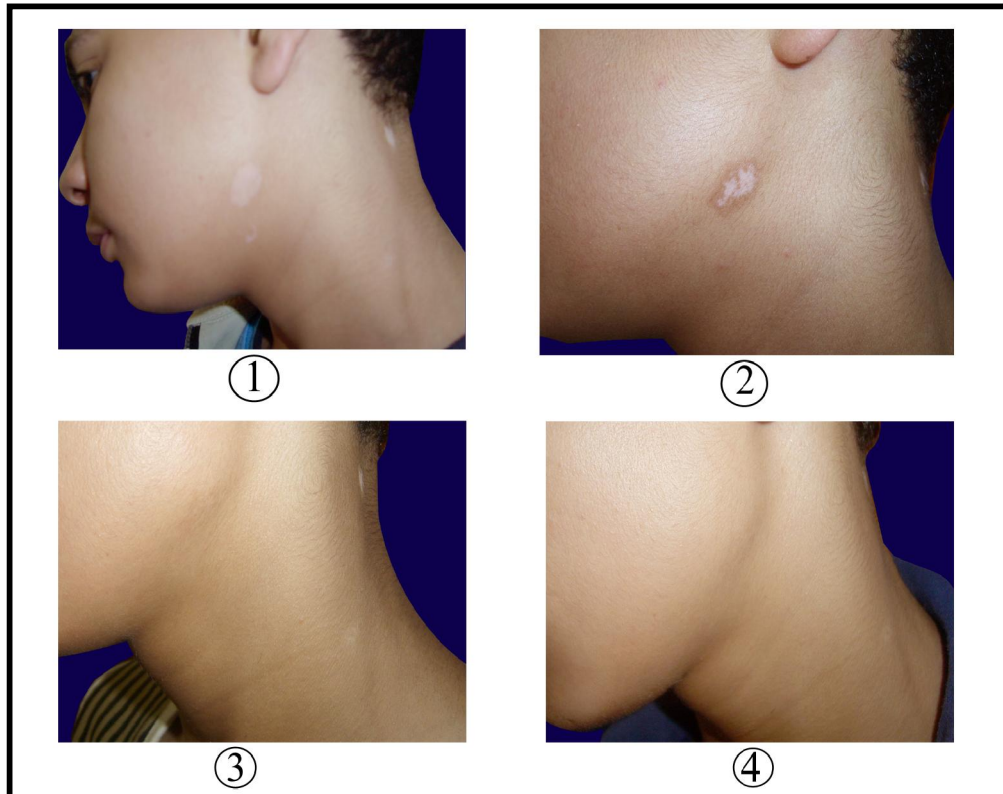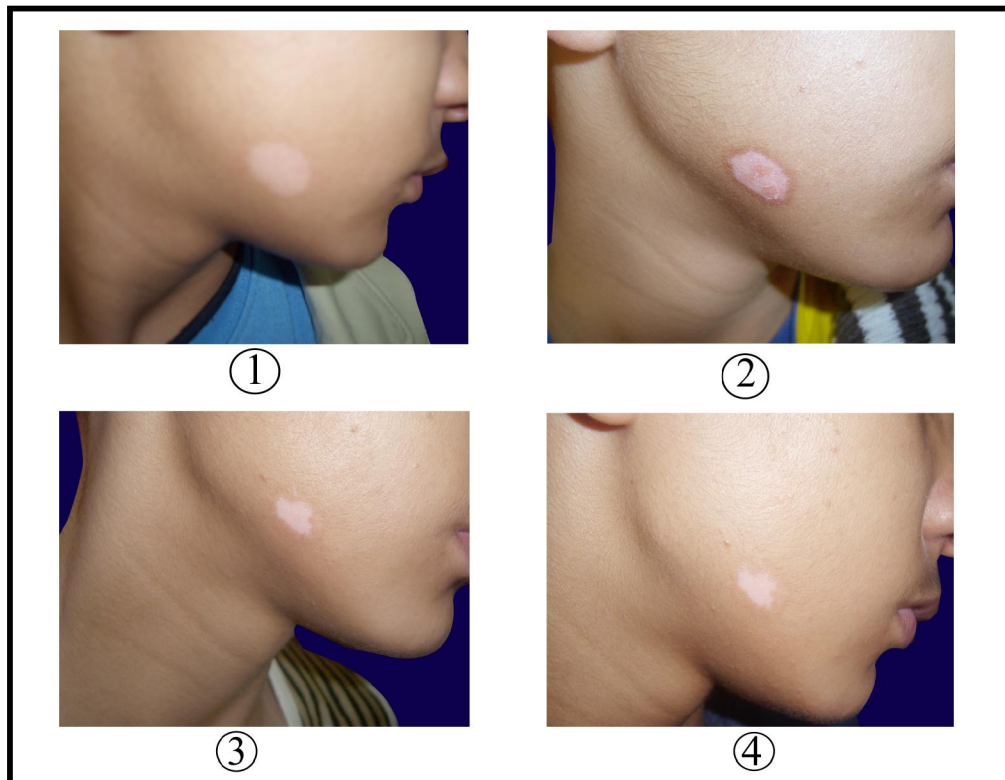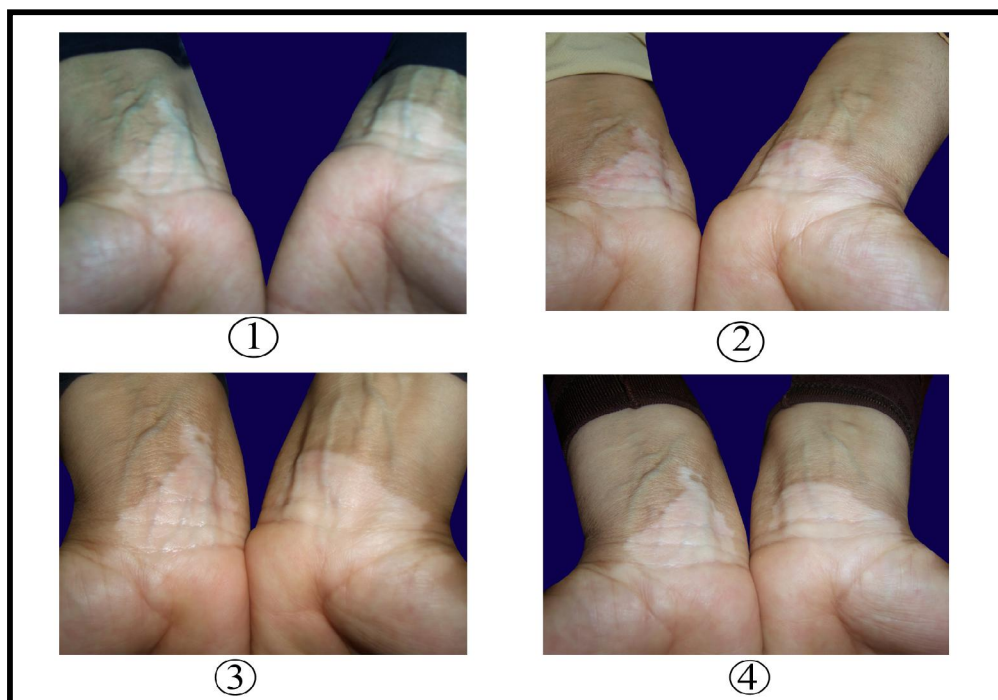The treatment of vitiligo is a tough challenge to dermatologists. A variety of therapeutic agents have been tried on vitiligo but none is uniformly effective. Potent topical corticosteroids and phototherapy is the mainstay of treatment. Response rates of 56% and 63%, respectively, have been reported with the above modalities of treatment. But these modalities are often required for a longer duration of time and they carry a potential risk for various side-effects [15].Vitiligo in the current study, affects both gender and can present at any age, this in agreement with the previous reports Kumaran et al., [15], Yuksel et al., [16]. In clinical practice, parents with vitiligo very often wish to know the risk of their children developing vitiligo [. In the current study, positive FH of vitiligo was seen in 15% in GI and 30% in GII. This agreed with the result of *Kumaran et al.,* [15] in which the negative FH was more with his patients than those with positive FH.

NB-UVB therapy has also been reported to be safe in childhood vitiligo [17,18]. The mechanism of NB-UVB-induced repigmentation involves the stabilization of the depigmenting process and the stimulation of residual follicular melanocytes. In particular, NB-UVB is probably involved in the upregulation of the melanogenesis and melanocytes migration [19]. In the current study; number of the patients treated with NB-UVB alone were 40 patients received NB-UVB only in their right side; 42.5% achieved excellent repigmentation, 12.5 % achieved moderate repigmentation, 22.5 % achieved mild repigmentation and 22.5 % achieved no repigmentation. During post treatment follow up for 2 months 12.5% of the patients, the repigmentation faded after stopping the treatment. While in a study reported by Kishan Kumar et al.[20],only 2% patients developed depigmentation of repigmented sites during the follow-up period of 6 months after one year treatment with NB-UVB. Also in a study by Sitek *et al.*[21], who assess the stability of NB-UVB-induced pigmentation on patients with generalized vitiligo, 16% experienced >75% stable repigmentation in 3 years after cessation of NB-UVB therapy . Chen *et al.* [22] , performed a retrospective study on 72 patients to examine the efficacy of NB-UVB in the treatment of vitiligo and found that only one patient show mild repigmentation, 9 patients showed 75%-100% repigmentation, 24 patients showed 50%-75% repigmentation and 20 patients showed 25% -50% repigmentation. Also Anbar *et al.,* [23] performed a study on 150 patients. 90% NSV and 10% SV and found that; in NSV 48% of patients had marked pigmentation, 27% of patients had moderate repigmentation and in 25% of patients had mild

repigmentation, but patients with SV had only mild repigmentation.

Topical corticosteroids have been used to treat vitiligo since 1970 with varying results [24]. The mechanism of action on vitiligo is supposed to be the suppression of direct or antibody-dependent cytotoxicity [25]. Glucocorticoids have also been implicated in the modulation of Th1/Th2 cytokine production, presumably by suppressing type 1 cells and/or by switching Th cells from Th1 to Th2 phenotype [26]. Calcipotriol is derived from 1-24-dihydroxyvitamine D3. Vitamin D increases intracellular calcium concentration, which regulates a number of cellular functions including proliferation and differentiation of melanocytes [5]. Calcipotriol has recently been shown to be helpful in repigmentation of vitiliginous lesions when used as a monotherapy or in combination with PUVA. There have been several reports of hyperpigmentation after combined use of calcipotriol and phototherapy in psoriasis [15]. Combination treatment with topical calcipotriol and topical steroids has been shown to be efficacious in psoriasis and the side-effects related to steroids use were also decreased. Combination therapy reduced the irritation and hyperpigmentation seen with calcipotriol used alone. The anti-inflammatory activity of corticosteroids may be responsible for this beneficial effect [15] . Similar findings, reported by Lebwohl *et al.,*[27] , Ruzicka and Lorenzl,[28]in patients with psoriasis. In the present study, in GI; left side of the patients had better repigmentation than right side but there was no statistically significant difference between both sides. In the left side about 45% of the patients achieved excellent pigmentation, 25% achieved moderate pigmentation , 10% mild pigmentation and 20% had no pigmentation, while in the right side about 40% of the patients achieved excellent pigmentation, 20% had moderate pigmentation, 20% had mild pigmentation and 20% had no pigmentation. In agreement with Kumaran *et al.,*[15] who divided patients with localized vitiligo into three treatment groups. In GI, patients applied betamethasone dipropionate cream 0.05% twice daily; in GII, patients applied calcipotriol ointment 0.005% similarly; and in GIII, patients applied betamethasone dipropionate in the morning and calcipotriol ointment in the evening. When used individually, the betamethasone dipropionate and the calcipotriol were found to be equally effective but the combination of the two, appeared to give a significantly faster onset of repigmentation along with better stability of the achieved pigmentation and with lesser number of side-effects .Chiavérini *et al.,*[29]performed a prospective, right-left comparative, open study and examined the efficiency of topical

calcipotriol as a monotherapy for the treatment of vitiligo. They concluded that; it was not effective .In a clinical trial, the combination of NB-UVB and calcipotriol showed no increase in efficacy, probably due to the fact that calcipotriol is rapidly degraded (>90%) by UVR [30]. In the current study during post-treatment follow-up, 3 patients in GI maintained their achieved pigmentation and the pigmentation continued to appear in their left side. In 3 patients the pigmentation started fading on stopping the therapy and one patients developed new lesions and the remaining 13 patients have stable pigmentation. In comparison to Kumaran *et al.,* [15] , they showed in their study that during post-treatment follow-up of 2 months, 26.6% in GI maintained their achieved pigmentation and in one of them the pigmentation continued to appear and complete repigmentation occurred in 20 weeks. In the remaining 55.6% patients the pigmentation started fading on stopping the therapy and 33.3% out of these 55.5% patients subsequently developed new lesions. In GII, 2 patients maintained their achieved pigmentation; in the remaining 66.7% it faded after stopping the treatment and 2 of these patients developed new lesions subsequently. In GIII, 90% of the patients who pigmented, maintained their achieved pigmentation. In only 9.1% , the pigmentation faded and multiple new lesions developed. In all the groups;the lesions with a diffuse type of repigmentation started to fade early. On comparing the patients in GIII to those in G I and II; the achieved pigmentation in vitiligo lesions of this group was much more stable .

Pseudocatalase is a bis-manganese III EDTA (HCO3)2 complex, capable of degradation of H_2O_2 to O_2 and H_2O after photo-activation with UVB or solar irradiation. After topical application of pseudocatalase preparation, a reduction of the H_2O_2 peak was detected *in vivo*. So, topical UVB-activated pseudocatalase can be successfully used for removing epidermal H_2O_2 in vitiligo.[31] In the present study, topical SOD/CATgel used with NB-UVB therapy . In GII: right side of the patients had better repigmentation than left side but there was no statistically significant difference between both sides. In the left side   about 40% of patient achieved excellent pigmentation   , 20% achieved moderate pigmentation , 20% achieved mild pigmentation and 20% had no pigmentation, while in the right side about 45% achieved excellent pigmentation , 5% achieved moderate pigmentation , 25% had mild pigmentation and 25% had no pigmentation .In agreement with   Schallreuter and Rokos,[32]who studied the efficacy of this formulation SOD/CAT gel in the removal of reactive oxygen species they reported that the combination does not have the

capacity to reduce H_2O_2. In order to test the clinical efficacy of the combination they treated 6 patients with facial vitiligo over 4 months with the application of the formulation twice daily together with solar exposure for at least 30 minutes over 4 months and they did not notice any significant repigmentation. But low patient numbers and short treatment duration affected the efficacy of this study .Another study in which combination of SOD/CAT was used in the literature came from Kostovic *et al.,*[33] .Their study included patients applied the gel containing SOD /CAT twice a day and received NB-UVB 3 times per week. 15.79% of the patients showed more than 75% repigmentation, 31.58% showed 26%-50% repigmentation and 5.26% showed 1%-25% repigmentation, where as no repigmentation was recorded in 5.26% of the patients .Sanclemente *et al*.,[34] compared the effect of topical 0.05% betamethasone valerate versus CAT/ SOD and concluded that; vitiligo repigmentation with topical CAT/SOD at 10 months is similar to repigmentation with topical 0.05% betamethasone valerate. Besides these, there is also another study which suggested that topical pseudocatalase was not effective in vitiligo. In this study, the efficacy of topical pseudocatalase mousse that contained pseudocatalase, calcium chloride, manganese chloride and sodium bicarbonate in a base enclosed in an aerosol canister pressurized by butane , applied twice daily to the hands and face of vitiligo patients in combination with twice-weekly NB-UVB phototherapy, was assessed and this treatment was not shown to be effective [35] . However, the pseudocatalase formulation used in this study was different from that in other studies [36]. Schallreuter *et al.,* [37] reported successful treatment of vitiligo with topical application of pseudocatalase and calcium followed by short term UVB light exposure. According to their study, repigmentation occurred in the majority of the cases after 2 to 4 months treatment. In all patients, active depigmentation was arrested. None of the patients developed new lesions or recurrence of the disease during 2 years follow up. During the follow up in GII, one patients maintained their achieved pigmentation; in 2 patients the pigmentation faded after stopping the treatment and 2 patients developed new lesions and the remaining 15 patients had stable pigmentation.

No association was found in this study between response to treatment and age gender of patients, FH and duration of disease. This has been confirmed by several other studies [38-40]. Although some studies report such an association with the age of the patient that reported that children respond faster to NB-UVB with lesser number of exposures and cumulative dose of NB-UVB.[20] Although AL Mokadem et al., [41]

found a significant negative correlation between duration of the disease and clinical response .In addition; Anbar et al., [23] and De Francesco et al., [42] reported that short duration of the disease was associated with better results.

The current study found in GI; the best results obtained with the face in the left side than the foot and acral parts, also in GII; the face in the left side had the best results than other parts of the body. In agreement with Gamil et al., [19] the best results were found for facial lesions, with the trunk and proximal limbs having good to moderate repigmentation, but the hands and feet were resistant to the combination treatment. Also Schallreuter et al., [43] in a retrospective study of 71 children with vitiligo found that more than 75% repigmentation was achieved in 66 of the 71 children on the face/neck, 48 of 61 children on the trunk, and 40 of 55 children on the extremities after NB-UVB activated pseudocatalase daily treatment for 8-12 months. The therapy had no side-effects .The favorable results obtained on the face may be due to stimulation of the melanocytic reservoirs in the hair sheaths by NB-UVB, as repigmentation occurred in a perifollicular pattern and was not seen in lesions with white a melanotic hair.[18] In addition, the face in particular is a body site with a great number of pilosebaceous units, which are activated by NB-UVB. The lower repigmentation rates in the acral regions may be attributed to lack of hair follicles. The darker skin phototype, as seen in Egyptian patients with higher initial and total cumulative NB-UVB doses and more frequent exposure, are predisposing factors to good response to NB-UVB in vitiligo [44]. Treatment responses of vitiligo lesions could be different due to the disease duration, gender, age of the patients, their skin phenotype and location of the lesion. Therefore, we suggest that it is necessary to plan other further studies with properly matched-patients on disease duration and gender ratio, and to further study the relationship between clinical parameters of the patients and response to treatment. The adverse effects in the present study were minimal like erythema burning, irritation and none of the patients required discontinuation of therapy. The adverse effect profile observed in the current study was similar to that reported in the other literatures. All these studies, including this one, clearly establish the safety profile of NB-UVB therapy.

Conclusions

The treatment of vitiligo is a tough challenge to dermatologists. A variety of therapeutic agents have been tried on vitiligo but none is uniformly effective. Recent advances in the pathogenesis of vitiligo have contributed to find better treatments; however progression of the disease and partial or lack of complete repigmentation still occurs in a good number of patients. The current study had shown that NB-UVB treatment alone was a moderately effective treatment for vitiligo. Betamethasone dipropionate / calcipotriol, when used in combination with NB-UVB were found to be superior in efficacy and safe than NB-UVB alone, but the results were not statistically significant .SOD/ CAT gel does not appear to add any incremental benefit to NB-UVB alone. So, further evaluation of the combination in large scale should be undertaken. Many studies should be performed to determine which treatment is the best for vitiligo. Since there is no consensus on the pathogenesis of vitiligo, a treatment to completely cure vitiligo does not exist. More randomized controlled trials on the treatment of vitiligo are necessary.

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Vascular Endothelial Growth Factor (VEGF) and Transforming Growth Factor β1 (TGF- β 1) as Predictors of Hepatocellular Carcinoma in HCV Related Liver Cirrhosis

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Abstract: Hepatocellular carcinoma (HCC) is one of the most serious complications of liver cirrhosis. Therefore, evaluation of biomarkers that predicts early the occurrence of HCC in patients with hepatitis C virus (HCV) induced liver cirrhosis is of great clinical value from the diagnostic and prognostic points of view. **Aim**: The aim of this work was to study serum levels of TGF-β1 and VEGF in cirrhotic HCV patients with and without HCC. **Subjects and methods**: This research was conducted on 30 patients with chronic HCV and liver cirrhosis (Group I), 30 patients with HCC on top of HCV induced liver cirrhosis (Group II) and 20 healthy controls. .Serum TGF- β1 and VEGF were measured by ELISA. **Results**: Mean VEGF and TGF- β1 levels were significantly higher in patients (Groups I and II) than controls. Furthermore, their values were significantly higher in HCC cases (Group II) than in those with liver cirrhosis (Group I). Significant positive correlations were noticed between each of TGF- β1 and VEGF and Child Pugh score ($p<0.05$). Moreover, statistically significant positive correlations were observed between size of hepatic focal lesions and each of TGF -β1 and VEGF in group II patients ($p<0.05$)
Conclusion: Serum TGF-β1 and VEGF reflect well the degree of hepatic dysfunction. Their serial measurements might be of diagnostic and predictive value for occurrence of HCC in patients with chronic HCV induced liver cirrhosis.
[Ayman El Shayeb , Akram Deghady , Abdel-Aziz Belal and Salah Eldin-Eldesoky **Vascular Endothelial Growth Factor (VEGF) and Transforming Growth Factor β 1 (TGF- β 1) as Predictors of Hepatocellular Carcinoma in HCV Related Liver Cirrhosis**]. Life Science Journal. 2011;8(4):198-204] (ISSN:1097-8135). http://www.lifesciencesite.com.

1. Introduction

HCC is the most common primary malignant liver tumor. It has a fulminant course and a poor prognosis [1]. Many etiological factors have been linked to the occurrence of HCC like liver cirrhosis, chronic hepatitis B or C infection and alcohol intake [2].

Development of HCC is related to chronic necroinflammatory liver process. The time elapsed between acquiring hepatitis C virus (HCV) infection and HCC development varies between 10-15 years[3]. Moreover, 97% of patients with chronic HCV and HCC have liver cirrhosis[4]. Factors that predispose to HCC among HCV infected individuals include male gender, old age, HBV and HIV coinfection and heavy alcohol intake as well as HCV genotype and quasi species [5-7]

Serum concentration of a variety of cytokines and cytokines antagonists are elevated in patients with liver disease[8]. Some have been incriminated in the occurrence of liver cancer. TGF- β1 over expression in transgenic mice is associated with 60% incidence of hepatoma[9]. TGF- β1 is an important mediator which plays a role in the development, growth and progression of HCC[10].

Tumor angiogenesis is important for growth and spread of cancer and is controlled by angiogenetic factors. HCC is a hypervascular tumor with rich blood supply; therefore, circulating angiogenesis markers have been studied not only as diagnostic but also as predictors and prognostic markers in cancer patients [11]. VEGF is the most potent, directly acting mediator of angiogenesis in both physiological and pathological conditions [12].

Aim of the work

This study was planned to evaluate serum VEGF and TGF- β1in patients with chronic HCV induced liver cirrhosis with or without hepatocellular carcinoma and their correlation with the size of hepatic focal lesion as determined by triphasic CT.

2. Subjects and Methods

Sixty chronic HCV patients were divided into two groups according to history, examination, ultrasound and biopsy whenever possible;
Group I: 30 chronic HCV patients with liver cirrhosis.

Group II: 30 patients with HCC on top of chronic HCV and hepatic cirrhosis (confirmed by triphasic CT study).

Moreover, 20 healthy Egyptians were included as controls (Group III)

Informed written consent was obtained from all those who were included in this study. The research protocol was approved by the ethics committee of the Faculty of medicine, Alexandria University

Exclusion criteria

Patients with chronic HBV infection or diabetes mellitus as well as those with cardiovascular, chest or renal diseases, alcoholics and those suffering from fever or autoimmune disease were not enrolled in the study.

Beside complete blood picture, ESR and liver function tests

All patients and controls were subjected to the following

1. HBs Ag (13) and Anti-HCV antibodies by ELISA. [14]

2. Serum HCV RNA level was done for cases with positive anti-HCV Ab using quantitative polymerase chain reaction (The Cobas Amplicor HCV Monitor [TM] test, Roche molecular systems, Banch burg, NJ, USA) [15].

3. Estimation of serum alphafetoprotein by Chemiluminescence (Immulite 1000, Siemens, Germany)

4. VEGF was determined in patients and controls sera using VEGF ELISA kit (Peninsula inc. USA) [16].

5. Patients' and controls' serum TGF- β1 was measured using Human TGF beta ELISA Kit (Abcam Company –USA) [17].

6. Abdominal ultrasonography

7. Triphasic CT abdomen (Siemens, Germany) after oral water and IV contrast administration and examination in the hepatic arterial phase (HAP), portal venous phase (PVP) and delayed phase was performed for cases of HCC

Statistical analysis

Data were collected, revised and transferred into statistical package for social science (SPSS/ version 10). Results were expressed as means and standard deviation. Statistical tests used in this study were student t test, F test and Pearson correlation. A level of 5% was considered as the cutoff level of significance.

3.Results

Results revealed that group I patients included 5 Child A patients (Mean score 5.6), 12 Child B (Mean score 8.03) and 13 Child C (Mean score 11.76). Besides, in group II 15 patients were Child B (Mean score 8.46) and 15 Child C (Mean score 11.86)

All group I and II patients were HCV positive (HCV Ab +ve and confirmed by PCR for HCV-RNA). None of them had HBsAg.

Tables I, II and III show the blood picture, liver function tests and α-fetoprotein findings in the three studied groups

Table I: Hematological findings in the three studied groups (Mean± SD).

	RBCs(million/μl)	WBCs(x10^3 /μl)	Platelets(10^3 /μl)
Group I (n=30)	4.14±0.80	6.56±1.6	161.2±67.53
Group II (n=30)	3.7±0.82	6.76±2.2	119.4±44.1
Group III (n=20)	4.96±0.34	5.69±1.58	251.60±57.5
F	17.9	2.11	32.45
P value	0.000*(I,II) (I,III) (II,III)	0.000* (II,III)	0.000* (I,II) (II,III), (I,III)

*Significant at p≤0.05

Table II: Liver function tests among the three groups (Mean±SD)

	ALT(U/l) (ULN=40)	AST(U/l) (ULN=40)	S.Bilirubin (mg/dl)	S.albumin gm/dl	Prothrombin time(sec)
Group I (n=30)	48.33±15.23	71.86±16.97	3.29±.155	2.99±0.32	18.6±2.8
Group II (n=30)	69.70±26.27	113.66±39.20	4.05±2.42	2.83±0.42	19.43±2.34
Group III(n=20)	20.45±5.1	15.35±3.71	0.82±0.14	4.51±0.44	13.14±0.71
F	41.19	84.04	20.88	121.72	50.49
P value	0.001*(I,II), (I,III),(II,III)	0.000*(I,III) (I,II) (II,III)	0.000*(I,III) ,(II,III),	0.000* (I,III) (II,III)	0.000* (I,III) (II,III)

* ULN= upper limit normal   *Significant at p≤0.05

Table III: Mean serum α-fetoprotein in the three studied groups (Mean±SD).

1.	α-fetoprotein (ng/ml)
Group I (n=30)	41.13±32.06
Group II (n=30)	467.79±388.45
Group III (n=20)	5.9±1.8
F	31.9
P value	0.000* (I,II) (II,III) (I,III)

*Significant at p≤0.05

Regarding TGF-β1, the mean serum levels were significantly higher in groups I and II than in group III and in group II than in group I. Furthermore, mean serum VEGF was significantly elevated in patients with liver cirrhosis and HCC than in controls and also higher in HCC cases than those with liver cirrhosis (Table IV).

Table IV: Mean serum TGF-β1and VEGF in the three studied groups (Mean±SD).

	TGFβ1(pg/ml)	VEGF(pg/ml)
Group I (n=30)	345±182.01	260.2±120.4
Group II (n=30)	868±164.6	981.06±34.1
Group III (n=20)	30.50±8.82	127.3±35.15
F	199	113.5
P value	0.000* (I,II) (II,III) (I,III)	0.000* (I,II) (I,III) (II,III)

- Significant at p≤ 0.05

In patients with liver cirrhosis (Group I), the mean serum TGF-β1 was significantly higher in Child C patients than in Child A and B ones and also in Child B cases than in Child A ones. As regards VEGF, its mean was significantly higher in Child C cases than in Child A and B ones, while no significant difference was found between Child B and Child A patients (Table V).

Table V: VEGF and TGF-β1in group I patients (Mean±SD).

	TGF-β1 (pg/ml)	VEGF(pg/ml)
Child A	73.7±30.17	166.6±10.47
Child B	304.16±52.91	203.66±23.48
Child C	487.65±151.04	348.38±138.81
F	28.58	10.40
P	0.001* A&B, A&C, B&C	0.001* A&C, B&C

- Significant at p≤ 0.05

In patients with HCC (Group II) the mean serum TGF-β1 and VEGF levels were significantly higher in Child C patients than in Child B ones (Table VI).

Table VI: VEGF and TGF-β1in group II patients (Mean±SD).

	TGF-β1 (pg/ml)	VEGF(pg/ml)
Child B	773.33±153.09	726±204.6
Child C	960.8±119.6	1236.13±253.16
T	3.96	6.06
P	0.001*	0.001*

- Significant at p≤ 0.05

As regard correlation studies, a significant positive correlation was detected between TGF-β1 and VEGF on one hand and Child Pugh score on the other hand in groups I and II (p<0.05) (Figs.1 and 2). Furthermore, a significant positive correlation was observed between VEGF and TGF-β1 in the same groups (r= 0.74 and 0.47, respectively) (p= 0.000 and 0.00, respectively)
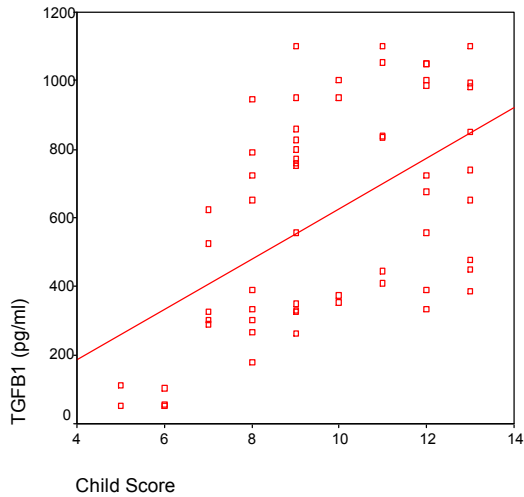
Figure 1: correlation between TGF-β1 and Child Pugh score

Figure 2: Correlation between VEGF and Child Pugh score.

In group II patients (HCC cases) triphasic CT revealed solitary hepatic focal lesion in 28 patients and multiple hepatic lesions in 2 patients. The lesions showed blush enhancement in the arterial phase with washout at the venous and delayed phases (Figs. 3 and 4). Furthermore, a significant positive correlation was observed between lesion size and each of VEGF and TGF-β1 in group II patients (r= 0.62 and 0.40, respectively) (p= 0.000 and 0.02, respectively).
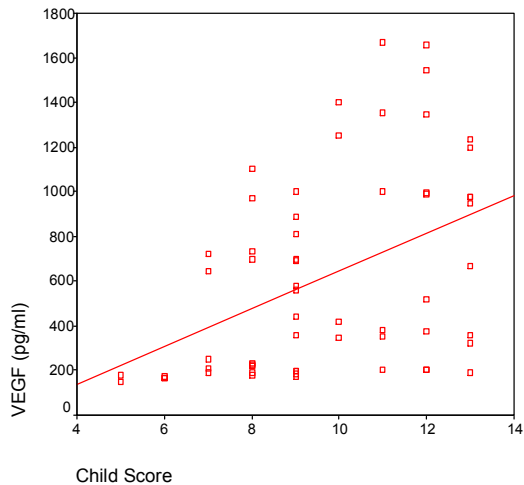

Figure 3: Shrunken liver showing established cirrhotic changes with 3cm hepatic focal lesion noted at right lobe that expressed moderate homogenous blush enhancement at HAP phase

Figure 4: Shrunken liver with established cirrhotic changes and medium sized 4x 4cm expanding hepatic focal lesion affecting area VIII of right hepatic lobe that expressed mild heterogeneous capillary blush enhancement at the late HAP

4. Discussion

Association between chronic HCV infection and HCC has been established. HCV is likely to predispose to HCC via viral proteins as well as enhanced hepatocyte turnover that happens in an attempt to replace infected hepatocytes which have attacked by immune cells [2].

Alphafetoprotein (α-fetoprotein) has been utilized as a marker for HCC, despite its low sensitivity and positive predictive value. Moreover, it has been estimated that up to 30% of HCC patients have normal α-fetoprotein levels [18]. Therefore, novel biomarkers are needed to be used for early detection of cases with HCC. With the advance of cellular and biological techniques, many molecular markers have been studied such as angiogenic factors [19].

VEGF is a well known angiogenic 46k glycoprotein which accelerates vascular permeability and has a role in proliferation of endothelial cells [20]. Various types of human cancer secrete VEGF, and its expression by tumor is closely linked to tumor progression, prognosis and even metastases [21,22]. Production of VEGF is regulated by oxygen, steroid hormones and protein C agonists [23].

In the present study, serum VEGF was significantly higher among patients than controls. Furthermore, it was more significantly elevated in HCC cases than in those with liver cirrhosis. Similar results were reported by **Abdel Haleem et al**. [24] and **Abdelmoaty et al**. [25]. Moreover, **Jerzy et al.,** recorded elevated circulating levels of VEGF and its receptors in patients with liver cirrhosis [26].

In the current work, Child class C patients had significantly higher serum VEGF than Child class A and B ones. Moreover, a significant positive correlation was noticed between VEGF and Child Pugh score. These results were in agreement with those of **Jerzy et al**. [26] and **Abdelmoaty et al**. [25].

Poon et al, reported that serum VEGF level is a predictor of microscopic venous invasion in HCC, suggesting that it may be useful as a biologic marker of tumor invasiveness [27]. Similar results were obtained by Chao et al who concluded that preoperative serum VEGF is a significant independent predictor of tumor recurrence [28]. Furthermore, **Poon et al.,** reported that high serum VEGF is predictor of poor outcome after resection of HCC [27].

In agreement with the previous studies, the present research revealed a significant positive correlation between serum VEGF and tumor size. This positive correlation could be attributed to the fact that angiogenesis is essential for tumor growth and invasion. This was supported by Folkman **et al** who clarified that neovasculature facilitates shedding of tumor cells into surrounding blood vessels [29].

Later on, this finding was supported by Jinno **et al** who recorded elevated serum VEGF in HCC patients with distant metastases [30].

Salgado et al., showed that platelets are able to store circulating VEGF [31]. It has been postulated that platelet adhering to circulating tumor cells may be activated to release VEGF. They also suggested that fast growing tumors may release thrombopoietic cytokines in addition to VEGF. **Hino et al.,** reported that HCC could express thrombopoeitin which could be a mediator in inducing thrombocytosis [32].

However, such correlation between VEGF and platelets could not be detected in this study. This can be attributed to the fact that most patients had thrombocytopenia as a result of liver cirrhosis and splenomegaly. So, high serum VEGF in included patients could be attributed to its production by tumor cells.

VEGF has been linked to hepatic dysfunction and this was proved in the current work by the presence of a significant positive correlation between VEGF & Child Pugh score. It could be suggested that elevated VEGF may contribute to enhanced hepatic fibrosis through induction of proliferation of hepatic stellate and sinusoidal cells [26].

Geert's et al., found that angiogenesis is increased in the mesenteric microvasculature in animal models with portal hypertension and cirrhosis. They also reported high VEGF in the mesentery of animal models suggesting its contribution to portal hypertension [33].

This observation was previously reported by **Fernandez et al.,** who found decreased intestinal neovasculature, splanchnic blood flow and porto-systemic collaterals in portal hypertensive rats following administration of anti- VEGF receptor 2 monoclonal antibodies [34].

In the present study, patients with HCC had significantly higher TGF-β1 than cirrhotic patients. Furthermore, a significant positive correlation was noticed between serum TGF-β1 and Child Pugh score.

Neuman et al., recorded that serum TGF-β1 could reflect the degree of fibrosis in HCV patients [35]. Moreover, **Flisiak et al.,** suggested the possible use of plasma TGF-β1 as a good marker of liver function impairment [36]. **Sacco et al.,** reported elevated serum TGF-β1 in HCC patients in 23% of cases with normal α-fetoprotein [37]. In the present work, α-fetoprotein was normal in 2 patients proved to have HCC by triphasic CT. These patients had elevated TGF-β1.

This elevation in HCC cases could be attributed to its production by the tumor. This was in accordance with the present results by the positive correlation between tumor size and TGF-β1.

Moreover, Okumoto et al, showed overexpression of TGF-β1 in HCC tissues which correlated well with carcinogenesis and tumor progression [38].

In clinical cases where high TGF-β1 could be correlated with tumor, attempts to decrease or inhibit TGF-β1 action by blocking its receptors may be used to treat advanced or metastatic disease [39].

In the current work, a significant positive correlation was found between TGF-β1 and VEGF. This finding was in accordance with **Chun et al.,** who clarified that TGF-β1 can activate macrophages to express angiogenic mediators such as VEGF [40].

Conclusion

Serum TGF-β1 and VEGF reflect well the degree of hepatic dysfunction. Their serial measurements might be of diagnostic and predictive value for occurrence of HCC in patients with chronic HCV induced liver cirrhosis.

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10/24/2011

Design of Steel Column Using LRFD Method

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Abstract: In this paper a procedure for designing column with slender sections was established. A column design curve for slender sections was established by applying a reduction factor, Q, to the LRFD column design curve. A stability analysis was conducted to study the effect of plate local buckling on flexural column buckling. A finite element model of an axially loaded I-column was developed using shell elements. Material and geometric nonlinearities were incorporated. Geometric imperfections similar to the first buckling mode with amplitude of 1/775 of column length, L, were applied. The analysis was carried out using the general purpose finite element program ANSYS. A wide range of plate width-to-thickness ratios and column slenderness ratios was studied. Column sections were grouped into three Groups: Group 1; sections with slender unstiffened plate elements, Group 2; sections with slender stiffened plate elements, and Group 3; sections composed of slender stiffened and unstiffened elements. The buckling loads for 144 I-column configurations made of steel 37, 44 and 52, and were compared to respective values adopted by the AISC-LRFD and Euro-Code3 specifications.

1. Introduction

The load carrying capacity of compression members composed of slender plate elements is probably less than the overall buckling strength based on the slenderness ratio of the entire cross-section. This is because local buckling may occur in one of the plate elements that make up the cross-section. Therefore, the buckled element will not support its proportionate share of column load, thus the cross-section efficiency is reduced [1].

It is evident that studying the buckling of uniformly compressed plates is essential for the determination of column load when slender plate elements are used. A brief description of previous research work established to determine the bucking resistance of columns with thin-walled elements is presented in the following sections.

1.1 Behavior of Plates under Edge Compression:

The behavior of plates in compression is similar to columns and the basic elastic buckling stress for plates corresponding to Euler equation for columns was derived [1,2] as:

$$F_{cr} = k\pi^2 E/\{12(1-\mu^2)(b/t)^2\} \qquad (1)$$

Where k is plate buckling coefficient depending on boundary conditions and loading configuration, μ is Poisson's ratio, E is the elastic modulus, b is the plate width and t is the plate thickness. Examination of critical stress recorded from experiments showed that for low b/t ratios, strain hardening is achieved and F_{cr} exceeds the yield stress, F_y. The actual strength of

plates for large b/t ratios exceeds F_{cr} given by Equation (1), i.e. they exhibit post-buckling strength.

1.2 Post Buckling Strength and Effective Width of Plates:

When a thin plate is axially loaded, it will buckle in regular waves when the stress reaches F_{cr}, but it will not collapse due to material ductility. If plate edges parallel to load were kept straight by supports, the plate will exhibit post-buckling strength. The central portion of the plate will exhibit excessive lateral deflections with increasing load and can hardly participate in carrying the load. Stresses will be continually redistributed so that stresses are increasing at edges and kept almost constant at the central portion. The resulting non-uniform stress distribution can thus be replaced by an equivalent uniform stress applied on an effective width of the plate. The post-buckling strength of plates was first described [3] by the effective width concept introduced by **Dawson and Walker** in **1972**. Both the American and British design specifications for cold-formed sections adopted a semi-empirically computed effective width to describe the post buckling behavior of plates.

A generalized imperfection parameter written in terms of F_y and F_{cr} was used in the derived expressions. Results were confirmed by comparison with test data. **Lind et al.** [4] reviewed the data basis for the effective width formulas used in slender sections design. A simple effective width formula that yields correct results in view of experimental evidence rather than mechanical analysis was introduced based on statistical approach. Results were adopted by the **Canadian**

standard S-146 (1974). **Horne and Narayanan** [5] established a design method for stiffened slender plates subjected to compression. Usami [6] studied the problem of elastic post-buckling behavior of plates in combined compression and bending.

1.3 Elastic and Inelastic Buckling of Plates:

Lind [7] established a numerical procedure to compute the elastic local buckling load of plate assemblies by solving an eigen value problem of an ordinary differential equation by the Newmark numerical approach. The method is applicable to slender sections subjected to constant compressive force and moment. **Sherbourne and Korol** [8] showed that upper bound of plates buckling load in the intermediate stage of elastic-plastic interaction for moderate b/t ratios can be determined by the intersection of plastic mechanism with elastic post-buckling strength. The accuracy of such estimate was verified by comparison with test data that showed that buckling load in the inelastic range was slightly dependent on imperfection amplitude. **Dawe et al.** [9] proposed a set of orthotropic material properties, derived semi-empirically for predicting inelastic buckling of stiffened and unstiffened plates. **Dawe et al.,** utilized the proposed orthotropic material properties in an analytical technique to predict the elastic and inelastic buckling load of hollow structural sections [10]. Effects of manufacturing process and interaction between adjacent plates were included in the formulation. Results were in good agreement with test data.

1.4 Numerical Buckling Analysis of Plate Assemblies:

The interaction between local and Euler buckling of thin-walled compression members was solved numerically by the finite element and finite strip methods. The finite element solution conducted by Gallagher incorporating material and geometric nonlinearities provided successful results in simulating nonlinear and post buckling behavior of plates. **Hancock** [11] extended the finite strip method to include the nonlinear membrane stiffness resulting from the interaction of geometric imperfections with local and post buckling phenomena of plates.

1.5 Design Specifications for Slender Compression Members:

For axially loaded compression members of cross sections having coincident shear center and centroid and composed of slender plate elements, the design strength, P_u, specified by the **AISC-LRFD** specifications [12] is given by:

$$P_u = \phi A_g F_{cr} \qquad (2)$$

For $\lambda_c (Q)^{1/2} \leq 1.5$, $F_{cr} = Q (0.658^{Q\lambda_c^2}) F_y$ (3)
For $\lambda_c (Q)^{1/2} > 1.5$, $F_{cr} = (0.877/\lambda_c^2) F_y$

Where $\lambda_c = (Kl/r\pi) (F_y/E)^{1/2}$ column slenderness parameter based on gross section properties
(Kl/r)= Slenderness ratio of the column
Q = reduction factor to account for local buckling of slender plates
$= Q_a Q_s$
Q_a = reduction factor for stiffened elements.
Q_s = reduction factor for unstiffened elements.
ϕ = strength reduction factor for compression members, 0.85
A_g = gross cross section area

As discussed in Sec. 1.2, plate elements in compression possess post buckling strength. The AISC accounts for plate local buckling and post buckling strengths by applying the factors Q_a and Q_s is computed as the ratio of the effective area at a stress equal to ΦF_{cr} to the gross area of the cross section. The effective area is computed utilizing an effective width formula for stiffened elements based on Winter work [13].

The Euro-Code3 based on the **LRFD** approach [14] specifies the following design buckling resistance for compression members:

$$P_u = X \beta_a A_g F_y /\gamma_m \qquad (4)$$

Where β_a = 1, for compact and non-compact sections
= Effective area / gross area, for slender sections (corresponding to Q_a factor adopted in the AISC-LRFD)
γ_m = partial safety factor for buckling strength, 1.1
X = $1/\{\phi +(\phi^2-\beta_a\lambda_c^2)^{1/2}\} \leq 1.0$
ϕ = $0.5[1 +\alpha \{\lambda_c(\beta_a)^{1/2}-0.2\} + \beta_a\lambda_c^2]$
α = imperfection factor dependent on shape and axis of bending.

Similar to the AISC-LRFD, the Euro-Code3 accounts for local buckling of slender plate elements by introducing the reduction factor β_a based on the effective width concept. Unlike the AISC specifications, both stiffened and unstiffened elements are treated similarly by computing the effective width for a maximum edge stress equal to the material yield stress rather than the design compressive stress. Therefore the effective width computed by the Euro-Code will be conservative compared to the AISC and

will not require iterative solution.

Similar to the Euro-Code3, the Egyptian Code based on the allowable stress design, **ASD** [15] and **LRFD** [16] accounts for local buckling of slender plate elements by applying the effective width concept. The allowable compressive load, P_a, specified for members with slender plate elements composed of mild steel (St. 37) is given by:

$$P_a = A_{eff}\, F_{cr} \tag{5}$$

For $l/r \leq 100$ $F_{cr} = 1.4 - 65 \times 10^{-6}\,(l/r)^2$ t/cm^2 (6)
For $l/r > 100$ $F_{cr} = 7500/(l/r)^2$ t/cm^2

Where   l/r = governing slenderness ratio based on gross section properties.
A_{eff} = effective area based on effective width concept for stiffened and unstiffened plates.

The limit load computed was compared to the nominal buckling load adopted by the **AISC-LRFD** and Euro-Code3. Based on the study conducted herein, a set of design strength equations for columns composed of slender plate elements were established utilizing the effective width concept and the design strength formulas for non-compact members proposed by the same author [17].

2. Problem Description:
2.1 Geometric Configuration:

The column section considered herein is composed of an I-shaped section with flange and web width of 30 and 42 cm respectively. The column length, L, was assumed 3.0 m. Plate thicknesses were selected such that three Groups of slender sections were investigated. Group 1: sections composed of slender flanges at which C/t_f (Figure 1) exceeds $23/(F_y)^{1/2}$ [2] and non-compact web with d_w/t_w equals to $64/(F_y)^{1/2}$ [2]. Group 2: sections composed of slender web (i.e. d_w/t_w exceeds $64/(F_y)^{1/2}$) and non-compact flanges. Group 3: sections composed of slender flanges and web. Tables 1, 2 and 3 in Appendix A list the geometric configuration for Group 1, 2 & 3 sections considered; respectively.

2.2 Boundary Conditions:

Two boundary conditions configurations (Figure 1) were applied to mimic short and moderate length columns at which local plate buckling influences the column overall buckling. Case A; two hinged column with buckling length equals to L to represent short columns with small slenderness ratio. Case B; Fixed free column with buckling length of 2L to represent moderate columns with larger slenderness ratio. The slenderness ratio for each case based on section

geometry and boundary conditions was listed in Tables 1, 2 & 3 in Appendix A.

2.3 Material Non Linearity:

A nonlinear stress-strain relation was adopted in the stability analysis to account for residual stresses [18]. The column strength curve adopted by the Column Research Council, CRC, was used with the tangent modulus theory to derive [17, 18] the stress-strain curve for steel 37, 44 & 52. Derivation of the adopted constitutive relation is illustrated in a previous research work by the same author [17]. The proportional limit stress, F_{pl}, was assumed equal to 0.5 F_y as per **CRC** [17, 18].

2.4 Geometric Imperfections:

Based on Koiter's buckling theory [17, 19, 3 & 11], an initial geometric imperfection having the shape of the first buckling mode of the perfect column was applied. Such imperfection configuration was used because it represents the worst possible imperfection that significantly affects the column load carrying capacity. The imperfection shape includes local buckling of thin-walled flanges and web.

3. Finite Element Modeling:

A finite element model for the column was constructed using **ANSYS** [20] shell elements. Flanges and web were modeled by plastic shell element, Shell43, in ANSYS element library. In order to incorporate material and geometric nonlinearities, the shell element selected possesses plastic, stress-stiffening and large deformation capabilities [20]. Figure 2 illustrates the three-dimensional column model used in the analysis. The first buckling mode was obtained for each column configuration by solving an Eigen-value problem by the general purpose finite element program, **ANSYS** [20]. An imperfection-amplitude of L/775 [17] was considered compared to an adopted value of L/1550 in the **AISC-LRFD** specifications [18]. Figure 3 illustrates the first buckling mode obtained for a column section of Group 1 with hinged-hinged boundary condition. Local buckling of thin-walled flange plates was dominant. However, if non-compact plate elements were used, a flexural half sine wave buckling mode about the minor axis would have been obtained.

4. ANALYSIS PROCEDURE:

A stability analysis was conducted to obtain the column limit load. The stability analysis is essentially a static analysis at which the column was loaded incrementally till failure. At each load step, equilibrium equations were solved iteratively till convergence was achieved by the modified **Newton-Raphson** technique [20,21]. Load increments were computed by the Arc-

Length option [20,21] to determine the limit load at which the column loses its stability. Non-uniform stress distribution was obtained during loading due to the existence of geometric imperfections and the application of non-linear material and geometry.

5. Comparison Of Finite Element Results With Specifications Designs:

The limit load stress obtained from the finite element solution for all column configurations studied herein was compared to the design compressive stress computed by the **AISC-LRFD** [12] and **Euro Code3** [14] specifications (Eqs 3 & 4 respectively). The following sections discuss the results obtained for sections of Groups 1, 2 & 3.

5.1 Sections of Group 1:

For sections of Group 1, C/t_f was varied from $0.7(E/F_y)^{1/2}$ to $1.15(E/F_y)^{1/2}$ whereas d_w/t_w was kept below the non-compact limit of $1.4(E/F_y)^{1/2}$. The ratio of flange area, A_f, to web area, A_w, ranged from 0.2 to 0.5.

Results indicated that in all cases the design compressive stress determined by AISC and Euro-code3 was less than FE results. Figures 4 and 5 depict the average computed compressive stress for each C/t_f ratio for short and medium columns respectively. Compressive stresses were normalized by the material yield stress. The AISC was more conservative than Euro-code3 when compared to FE results. For short columns, the design compressive stress recommended by the AISC was sharply reduced from 0.8 to 0.5 of FE limit load stress when C/tf was increased from $0.7(E/F_y)^{1/2}$ to $1.15(E/F_y)^{1/2}$ (see Fig 4). Similarly, for medium columns (Fig 5), the AISC design compressive stress was reduced from 0.90 to 0.75 of FE limit load as C/t_f increased. On the other hand, the Euro-Code3 recommended design compressive stress which took almost a constant value of 0.9 the FE limit load. This indicated that the AISC formulas overestimated the effect of local buckling on flexural buckling as C/t_f ratio increased and L/i ratio decreased.

5.2 Sections of Group 2:

For sections of Group 2, d_w/t_w was varied from $1.55(E/F_y)^{1/2}$ to $2(E/F_y)^{1/2}$ whereas the flange ratio C/t_f was kept below the non-compact limit of $0.5(E/F_y)^{1/2}$. Results indicated that both the AISC-LRFD and Euro-Code3 provided a good estimate of FE results. The ratio of design compressive stress computed by AISC-LRFD or Euro-Code3 was almost not affected by the variation in d_w/t_w ratio.

For short columns, the ratios of AISC-LRFD to FE and Euro-Code3 to FE results were almost constant with an average value of 0.90 and 0.93 respectively (Fig 6). Similarly, the ratios of AISC-LRFD and Euro-

Code3 design compressive stress to FE results were constant, however, unlike short columns, the Euro-Code3 was slightly more conservative (Fig 7). This indicated that the recommended design compressive stress adopted by the Euro-Code3 and AISC-LRFD based on effective width concept was in good agreement with FE results.

5.3 Sections of Group 3:

For sections of Group 3, d_w/t_w was varied from $1.55(E/F_y)^{1/2}$ to $2(E/F_y)^{1/2}$ and for each value of d_w/t_w, the value of C/t_f was varied from $0.7(E/F_y)^{1/2}$ to $1.15(E/F_y)^{1/2}$. In most cases, the AISC-LRFD was more conservative than Euro-Code3 compared to FE results. It was noticed that the web width-to-thickness ratio had an insignificant effect on results. For short columns and similar to sections of Group1, the ratio of Euro-Code3 design compressive stress to FE limit load stress was almost constant with a mean value of 0.9 (Figs 4 & 8).

On the other hand, the design compressive stress recommended by the AISC-LRFD was sharply reduced from 0.9 to 0.5 of FE limit load stress (See Fig.8). A similar behavior was noticed for sections of Group 1 (Fig. 4). A similar behavior was noticed for medium columns, however, the AISC design compressive stress was in a better agreement with FE results (Fig 9). The ratio of AISC-LRFD design stress to FE limit load stress ranged from 0.9 to 0.7 when C/t_f increased from $0.7(E/F_y)^{1/2}$ to $1.15(E/F_y)^{1/2}$, whereas the ratio of Euro-Code3 design stress to FE limit load stress increased from 0.8 to 0.97.

The above discussion indicated that the AISC-LRFD overestimates local buckling effect on column buckling particularly for short columns with cross section containing slender unstiffened elements with high width-to-thickness ratio. This is mainly attributed to the application of the conservative stress reduction factor, Q_s (Sec. 1.5). The Euro-Code3, however, provided a better estimation of local buckling effect. In all cases, the effect of local buckling on the column carrying capacity is significantly reduced when slenderness ratio of the column increases and elastic buckling controls.

6. Column Design Curve For Members With Slender Plate Elements:

Based on the above discussion, the effective area approach adopted in the Euro-**Code3** [14] and **ECP-ASD** [15] specifications was adopted to account for the effect of slender plate local buckling on overall buckling of columns. The column design curve of compression members composed of slender plate elements was based on applying a reduction factor, Q, to the LRFD column curve proposed [17] for columns with non-compact sections. The reduction factor, Q is the ratio of the effective reduced area of the section,

A_{eff}, to the actual gross area, A_g. The effective width, b_e, of a slender plate is computed as per the **ECP-ASD 2010** [15] as follows:

$$b_e = \rho \, b \qquad (7)$$

Where
$$\rho = (\lambda_p - 0.20)/\lambda_p^2 \text{ if } \lambda_p > 0.673 \qquad (8\text{-}a)$$
$$\rho = 1.0 \qquad \text{if } \lambda_p \leq 0.673 \qquad (8\text{-}b)$$
$$\lambda_p = \{(b/t)(F_y/K)^{1/2}\}/44 \qquad (9)$$

b = appropriate flat width of slender plate element (Fig. 1)
= C for outstanding flanges
= d_w for webs
ρ = reduction factor to account for local buckling
λ_p = plate slenderness parameter
= $(F_y/F_{cr})^{1/2}$
F_{cr} = Elastic critical buckling stress of plates (Eq. 1) [7] K = plate buckling coefficient [7].
= 0.425, for uniformly compressed with unstiffened edges
= 4, for uniformly compressed plates with stiffened edges.

The effective width, b_e, is computed as follows:

$$b_e = 0.63\, t\, (E/F_y)^{1/2}\, [\, 1-0.13\, (E/F_y)^{1/2}/(b/t)] \qquad (10)$$

Equation 10 is applicable to plate elements with unstiffened edges such as flanges, angles, and plates projecting from rolled or built-up sections of compression members. For plate elements with stiffened edges and substituting K by 4 in Eq. 9, b_e is computed as follows:

$$b_e = 1.92\, t\, (E/F_y)^{1/2}\, [\, 1-0.385\, (E/F_y)^{1/2}/(b/t)] \qquad (11)$$

The design compressive load, P_u, of compression members with slender plate elements can be written as follows:

$$P_u = \phi\, A_g\, F_{cr} \qquad (12)$$

For $\lambda_c \leq 1.1$ $F_{cr} = Q\, F_y\, (1-0.384\, \lambda_c^2) \qquad (13)$

For $\lambda_c > 1.1$ $F_{cr} = 0.648\, Q\, F_y/\lambda_c^2 \qquad (14)$

Where   Q   = A_{eff}/A_g
A_{eff} = effective area based on effective width of slender plate elements as per Eqs. 10 & 11.
ϕ = strength reduction factor, 0.8.
λ_c = $(l/r\pi)(F_y/E)^{1/2}$ column slenderness parameter
l/r   = governing slenderness ratio of the column.

The design compressive stress computed by Eqs 13 & 14 with the application of the strength reduction factor, ϕ, was compared to that computed by FE solution (Sec. 5), AISC-LRFD and Euro-Code3 in Tables 1, 2 & 3 (see Appendix A) for sections Groups 1, 2 & 3; respectively . The average design stress computed for each width-to-thickness ratio was also plotted for comparison in Figures 4 to 9.

7. Comparison of Proposed Column Ultimate Design Load Curve with Aisc-Lrfd Curve:

The ultimate load, P_u, computed by the proposed Eqs 12, 13 & 14 was compared to that obtained by the AISC-LRFD formulas for slender compression members. For Group 1 sections, the flange width to thickness ratio, C/t_f, was varied from $0.7(E/F_y)^{1/2}$ to $1.15\ (E/F_y)^{1/2}$. Since the reduction factor, Q, proposed herein is computed as the ratio of A_{eff} to A_g, it will be dependent on the ratio of the flange gross area to web gross area, A_f/A_w. Therefore, the factor A_f/A_w was varied from 0.1 to 0.5 to cover a wide range of practical cases. Figure 10 illustrates a comparison of column design curve computed by the proposed method (Eq 12) with that adopted by the AISC-LRFD (Eq 2). The comparison showed that for all values of C/t_f and A_f/A_w, the proposed equations are conservative compared to AISC-LRFD for long columns with $\lambda_c \geq 1.1$. This was attributed to the fact that the proposed column design formula adopts higher factor of safety for elastic buckling. On the other hand, the AISC-LRFD neglects the effect of local buckling in the elastic buckling region.

For columns with $\lambda_c \leq 1.1$, the proposed method assumes higher design load compared to the AISC-LRFD especially for high values of C/t_f and A_f/A_w ratios. However, for $A_f/A_w \geq 0.4$ and $C/t_f \leq 0.85$ $(E/F_y)^{1/2}$, the proposed method is more conservative.

For Group 2 sections, the d_w/t_w ratio was varied from $1.55(E/F_y)^{1/2}$ to $2.0(E/F_y)^{1/2}$ whereas A_f/A_w was varied within the practical range of 1 to 4. Although the effective area approach is adopted in the proposed method and AISC-LRFD for Group 2 sections, comparison of design curves shows that the proposed method is more conservative with an average ratio of 0.92 for short columns and 0.68 for long columns (Fig. 11). This is attributed to the fact that the effective web width in the proposed method was based on the yield stress F_y (Eq. 11) whereas the AISC-LRFD uses the actual stress ϕF_{cr} in computing the effective width of unstiffened elements thus higher values of b_e will be provided. On the other hand, the proposed design curve is conservative in the elastic buckling range compared to the AISC-LRFD.

For Group 3 sections, the C/t_f ratio was varied from $0.7(E/F_y)^{1/2}$ to $1.15(E/F_y)^{1/2}$ whereas the d_w/t_w ratio was varied from $1.7(E/F_y)^{1/2}$ to $2(E/F_y)^{1/2}$ and A_f/A_w ratio was varied in the practical range of 0.5 to 2.0. The ratio of column ultimate design load curve determined by the proposed method to that adopted by the AISC-LRFD is illustrated in Fig 12. It is shown that the proposed design curve was conservative compared to AISC-LRFD for long columns with $\lambda_c \geq 1.1$. For short columns with $\lambda_c \leq 1.1$, the proposed method is also conservative compared to AISC-LRFD for sections with $C/t_f \leq 1.0\ (E/F_y)^{1/2}$.

8. Comparison of Proposed Column Ultimate Design Load Curve with Euro-Code3 Curve:

The proposed design curve is compared to Euro-Code3 column design curve based on the LRFD method. Since the Euro-Code 3 column design curve possess a flat plateau at small value of λ_c whereas the proposed design curve is parabolic, a noticeable reduction was observed in the ratio of the two design curves at λ_c equals 0.2 as shown in Figure 13.

Figure 13 depicts the column design curve for a wide range of C/t_f and A_f/A_w ratios. For Group 1 sections, comparison of design curves showed that the proposed method is always conservative compared to the Euro-LRFD with an average ratio of 0.86 for columns with $\lambda_c \leq 1.1$ and an average ratio of 0.60 for columns with $\lambda_c \geq 1.1$. This was attributed to the fact that the proposed method assumes higher factor of safety for column buckling.

For Group 2 sections, the d_w/t_w ratio is varied from $1.7(E/F_y)^{1/2}$ to $2.0(E/F_y)^{1/2}$ whereas the A_f/A_w ratio is varied from 1 to 4. Column design curves determined by the proposed method are compared to the Euro-LRFD in Figure 13. Comparison shows that the ratio of design load computed by the proposed method to that computed by the Euro-LRFD is neared from 0.86 for columns with $\lambda_c \leq 1.1$ to 0.7 for columns with $\lambda_c \geq 1.1$.

For Group 3 columns, the C/t_f ratio is varied from $0.7(E/F_y)^{1/2}$ to $1.15(E/F_y)^{1/2}$, d_w/t_w ratio is varied from $1.7(E/F_y)^{1/2}$ to $2(E/F_y)^{1/2}$, whereas A_f/A_w is varied from 0.5 to 2.0. Results depicted in Fig. 15 showed that the proposed method is conservative compared to Euro-LRFD in all cases with an average ratio of 0.75 in the inelastic buckling range and 0.5 in the elastic buckling range.

9. Summary and Conclusions:

In this work, a proposed design method for the design of columns with slender plate elements is established based on the LRFD approach. Columns are classified into three Groups, Group 1: columns with slender unstiffened plate elements, Group 2: columns with slender stiffened plate elements and Group 3: columns with slender stiffened and unstiffened plate elements. A finite element model is constructed for axially loaded columns covering the three Groups of slender sections. The critical load obtained from stability analysis incorporating material and geometric nonlinearities and geometric imperfections is compared to the design compressive stress adopted by the AISC-LRFD and Euro-Code3 specifications. The comparison shows that the effective width concept adopted in the Euro-Code3 to account for local plate buckling provides a good representation for buckling of columns with thin-walled plate elements. On the other hand, it is shown that the AISC greatly underestimates the buckling resistance of columns with slender unstiffened plate elements having high flat width-to-thickness ratio. Therefore, the proposed method is established based on the effective area approach adopted in the Euro-Code3 and ECP-ASD specifications. Direct comparison of column design curves determined by the proposed approach with that adopted in Euro-Code3 specifications, showed that the proposed method is always conservative compared to the Euro-LRFD specifications. Comparison with AISC-LRFD showed that the proposed method is conservative for Group 1 sections with $A_f/A_w \geq 0.4$ and $C/t_f \leq 0.85(E/F_y)^{1/2}$ and for Group 3 sections with $C/t_f \leq (E/F_y)^{1/2}$. The proposed method is also conservative for Group 2 sections compared to the AISC-LRFD. This conclusion is considered satisfactory since it is illustrated that the AISC-LRFD underestimates the critical load especially for high C/t_f ratios. On the other hand, the ratio C/t_f seldom exceeds the limit $0.85(E/F_y)^{1/2}$ in practice.

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Appendix A: Comparison of Finite Element Results with AISC-LRFD, Euro-Code3 and Proposed method for Design of Thin-walled Columns.
Tables 1, 2 and 3 list the geometric configuration, slenderness ratio and steel grade of 144 column cases with sections Group 1, 2 & 3 respectively. The finite element critical load stress in t/cm^2 was listed with the design buckling stress in t/cm^2 obtained by the AISC-LRFD, Euro-Code3 and proposed method for each column.

Table 1 Comparison of Results for Group 1 Sections

C/t_f	Sections Composed of Mild Steel 37							
	20.71		25.14		29.58		34.02	
A_F/A_w	0.509		0.419		0.356		0.310	
L/i	48.74	97.48	51.26	102.52	53.66	107.32	55.95	111.9
F_cr FE	1.912	1.327	1.887	1.240	1.906	1.159	1.956	1.049
AISC	1.650	1.211	1.451	1.074	1.253	0.945	0.983	0.775
Euro	1.686	1.159	1.607	1.078	1.557	1.015	1.524	0.962
Propos	1.565	1.010	1.481	0.893	1.429	0.796	1.395	0.723
C/t_f	Sections Composed of Steel 44							
	19.17		24.65		30.12		35.60	
A_F/A_w	0.509		0.396		0.324		0.274	
L/i	48.74	97.48	52.07	104.14	55.19	110.38	58.15	116.36
F_cr FE	2.243	1.429	2.195	1.297	2.229	1.171	1.881	0.997
AISC	1.893	1.318	1.589	1.126	1.230	0.916	0.899	0.719
Euro	2.173	1.313	2.124	1.203	2.076	1.108	2.029	1.024
Propos	1.789	1.030	1.665	0.857	1.596	0.747	1.553	0.667
C/t_f	Sections Composed of Steel 52							
	20.529		25.359		30.190		35.021	
A_F/A_w	0.419		0.339		0.285		0.246	
L/i	51.25	102.5	54.41	108.82	51.25	102.5	54.41	108.82
F_cr FE	2.814	1.458	2.796	1.318	2.814	1.458	2.796	1.318
AISC	2.070	1.319	1.674	1.117	2.070	1.319	1.674	1.117
Euro	2.609	1.319	2.533	1.203	2.609	1.319	2.533	1.203
Propos	2.075	0.894	1.962	0.771	2.075	0.894	1.962	0.771

Table 2 Comparison of Results for Group 2 Sections

d_w/t_w	Sections Composed of Mild Steel 37							
	45.85		50.29		54.72		59.16	
A_F/A_w	0.788		0.864		0.940		1.017	
L/i	44.28	88.56	43.51	87.02	44.28	88.56	43.51	87.02
F_cr FE	2.010	1.488	1.976	1.516	2.010	1.488	1.976	1.516
AISC	1.855	1.395	1.834	1.413	1.855	1.395	1.834	1.413
Euro	1.900	1.347	1.877	1.355	1.900	1.347	1.877	1.355
Propos	1.791	1.278	1.761	1.276	1.791	1.278	1.761	1.276
d_w/t_w	Sections Composed of Steel 44							
	43.818		49.295		54.772		60.249	
A_F/A_w	0.813		0.915		1.017		1.118	
L/i	44.01	88.02	43.07	86.14	44.01	88.02	43.07	86.14
F_cr FE	2.351	1.634	2.303	1.676	2.351	1.634	2.303	1.676
AISC	2.130	1.536	2.094	1.564	2.130	1.536	2.094	1.564
Euro	2.201	1.495	2.172	1.515	2.201	1.495	2.172	1.515
Propos	2.045	1.360	2.004	1.364	2.045	1.360	2.004	1.364
d_w/t_w	Sections Composed of Steel 52							
	45.89		50.72		55.55		60.38	
A_F/A_w	0.966		1.067		1.169		1.271	
L/i	42.67	85.34	41.97	83.94	41.38	82.76	40.89	81.78
F_cr FE	2.964	1.926	2.925	1.969	2.917	2.005	2.936	2.036
AISC	2.604	1.802	2.576	1.820	2.554	1.829	2.537	1.837
Euro	2.693	1.703	2.671	1.726	2.654	1.744	2.642	1.759
Propos	2.481	1.447	2.454	1.472	2.434	1.493	2.421	1.510

Table 3 Comparison of Results for Sections Group 3

d_w/t_w	Sections Composed of Mild Steel 37															
	45.85				50.29				54.72				59.16			
C/t_f	20.7	25.1	29.5	34.0	20.7	25.1	29.5	34.0	20.7	25.1	29.5	34.0	20.7	25.1	29.5	34.0
A_F/A_w	0.56	0.47	0.39	0.34	0.62	0.51	0.43	0.38	0.67	0.56	0.47	0.41	0.73	0.60	0.51	0.44
L/i	47.5	49.9	52.1	54.2	46.6	48.7	50.8	52.8	45.7	47.7	49.6	51.5	44.9	46.9	48.7	50.5
F_cr FE	1.836	1.792	1.782	1.767	1.804	1.724	1.701	1.693	1.762	1.674	1.639	1.618	1.726	1.635	1.592	1.569
AISC	1.659	1.459	1.259	0.988	1.657	1.465	1.265	0.992	1.632	1.455	1.270	0.995	1.611	1.433	1.258	0.998
Euro	1.626	1.535	1.483	1.446	1.583	1.488	1.428	1.385	1.548	1.445	1.379	1.335	1.519	1.408	1.336	1.288
Propos	1.504	1.411	1.364	1.316	1.455	1.356	1.292	1.251	1.415	1.307	1.238	1.194	1.382	1.267	1.194	1.143
L/i	95	99.8	104.1	108.4	93.2	97.4	101.6	105.6	91.4	95.4	99.2	103	89.8	93.8	97.4	101
F_cr FE	1.369	1.287	1.207	1.090	1.405	1.326	1.245	1.122	1.435	1.327	1.183	1.046	1.472	1.328	1.191	1.061
AISC	1.235	1.098	0.966	0.790	1.255	1.117	0.982	0.803	1.273	1.133	0.997	0.813	1.276	1.147	1.009	0.822
Euro	1.160	1.077	1.018	0.967	1.158	1.078	1.017	0.967	1.158	1.074	1.013	0.966	1.156	1.068	1.005	0.958
Propos	1.000	0.884	0.794	0.720	0.990	0.876	0.790	0.716	0.981	0.865	0.780	0.712	0.972	0.855	0.771	0.706
d_w/t_w	Sections Composed of Steel 44															
	43.818				49.295				54.772				60.249			
C/t_f	19.2	24.6	30.1	35.6	19.2	24.6	30.1	35.6	19.2	24.6	30.1	35.6	19.2	24.6	30.1	35.6

| A_F/A_w		0.583	0.454	0.371	0.314	0.583	0.454	0.371	0.314	0.583	0.454	0.371	0.314	0.583	0.454	0.371	0.314
L/i		47.17	50.20	53.04	55.73	47.17	50.20	53.04	55.73	47.17	50.20	53.04	55.73	47.17	50.20	53.04	55.73
F_{cr}	FE	2.135	2.061	2.018	1.908	2.135	2.061	2.018	1.908	2.135	2.061	2.018	1.908	2.135	2.061	2.018	1.908
	AISC	1.907	1.602	1.239	0.904	1.907	1.602	1.239	0.904	1.907	1.602	1.239	0.904	1.907	1.602	1.239	0.904
	Euro	1.988	1.877	1.810	1.767	1.988	1.877	1.810	1.767	1.988	1.877	1.810	1.767	1.988	1.877	1.810	1.767
	Propos	1.702	1.563	1.486	1.437	1.702	1.563	1.486	1.437	1.702	1.563	1.486	1.437	1.702	1.563	1.486	1.437
L/i		94.34	100.4	106.1	111.5	94.34	100.4	106.1	111.5	94.34	100.4	106.1	111.5	94.34	100.4	106.1	111.5
F_{cr}	FE	1.493	1.369	1.238	1.051	1.493	1.369	1.238	1.051	1.493	1.369	1.238	1.051	1.493	1.369	1.238	1.051
	AISC	1.359	1.163	0.944	0.729	1.359	1.163	0.944	0.729	1.359	1.163	0.944	0.729	1.359	1.163	0.944	0.729
	Euro	1.363	1.243	1.149	1.071	1.363	1.243	1.149	1.071	1.363	1.243	1.149	1.071	1.363	1.243	1.149	1.071
	Propos	1.036	0.856	0.741	0.662	1.036	0.856	0.741	0.662	1.036	0.856	0.741	0.662	1.036	0.856	0.741	0.662
d_w/t_w		**Sections Composed of Steel 52**															
		45.89				50.72				55.55				60.38			
C/t_f		20.53	25.36	30.19	35.02	20.53	25.36	30.19	35.02	20.53	25.36	30.19	35.02	20.53	25.36	30.19	35.02
A_F/A_w		0.570	0.462	0.388	0.334	0.570	0.462	0.388	0.334	0.570	0.462	0.388	0.334	0.570	0.462	0.388	0.334
L/i		47.43	49.97	52.38	54.69	47.43	49.97	52.38	54.69	47.43	49.97	52.38	54.69	47.43	49.97	52.38	54.69
F_{cr}	FE	2.507	2.432	2.305	2.148	2.507	2.432	2.305	2.148	2.507	2.432	2.305	2.148	2.507	2.432	2.305	2.148
	AISC	2.093	1.709	1.237	0.935	2.093	1.709	1.237	0.935	2.093	1.709	1.237	0.935	2.093	1.709	1.237	0.935
	Euro	2.294	2.181	2.109	2.060	2.294	2.181	2.109	2.060	2.294	2.181	2.109	2.060	2.294	2.181	2.109	2.060
	Propos	1.834	1.695	1.611	1.555	1.834	1.695	1.611	1.555	1.834	1.695	1.611	1.555	1.834	1.695	1.611	1.555
L/i		94.86	99.94	104.8	109.4	94.86	99.94	104.8	109.4	94.86	99.94	104.8	109.4	94.86	99.94	104.8	109.4
F_{cr}	FE	1.649	1.511	1.323	1.113	1.649	1.511	1.323	1.113	1.649	1.511	1.323	1.113	1.649	1.511	1.323	1.113
	AISC	1.438	1.216	0.949	0.755	1.438	1.216	0.949	0.755	1.438	1.216	0.949	0.755	1.438	1.216	0.949	0.755
	Euro	1.453	1.339	1.245	1.166	1.453	1.339	1.245	1.166	1.453	1.339	1.245	1.166	1.453	1.339	1.245	1.166
	Propos	0.894	0.759	0.670	0.606	0.894	0.759	0.670	0.606	0.894	0.759	0.670	0.606	0.894	0.759	0.670	0.606
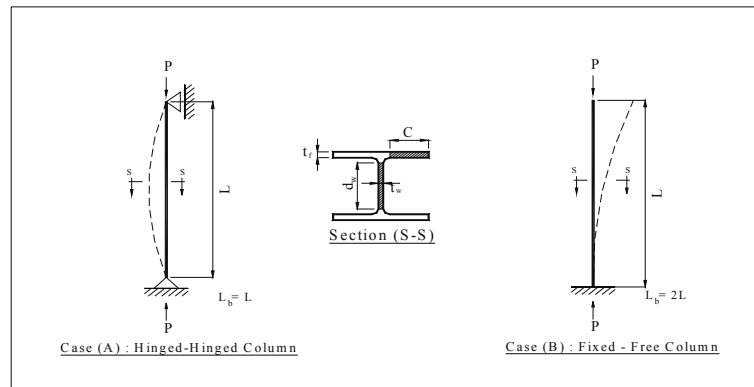
Figure 1 Geometric Configuration and Boundary Conditions

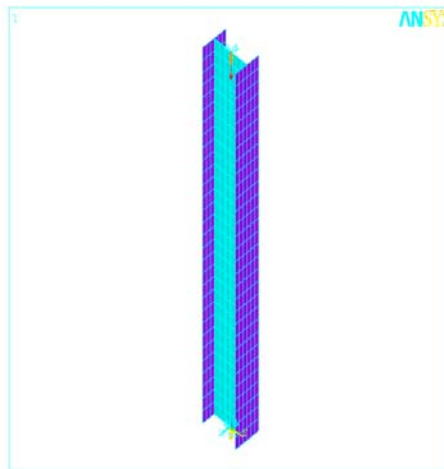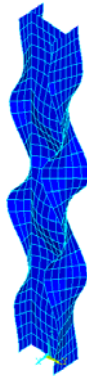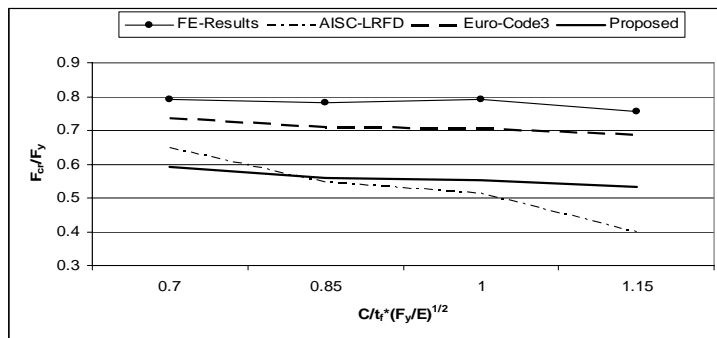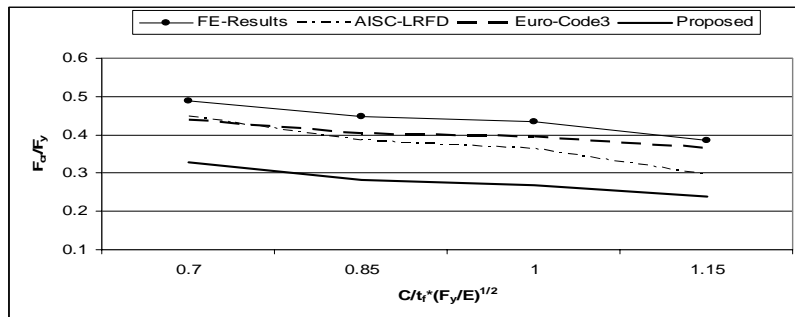**Figure 2 Finite Element Model of Axially Loaded Column**
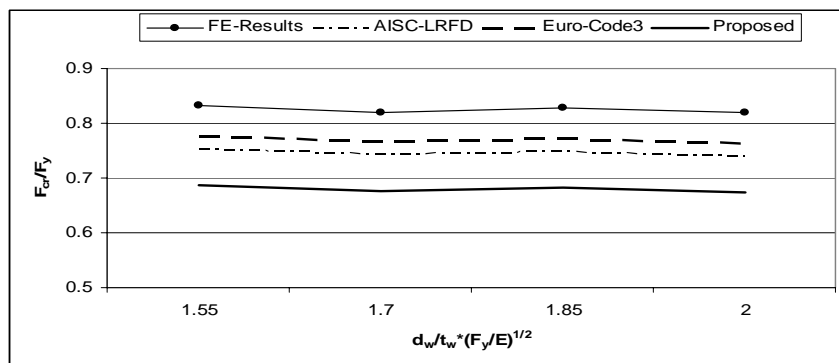
Figure 3 First Buckling Modes for Hinged-Hinged Column of Group 1

Figure 4 Comparison of results for short columns of Group 1 sections

Figure 5 Comparison of results for medium columns of Group 1 sections

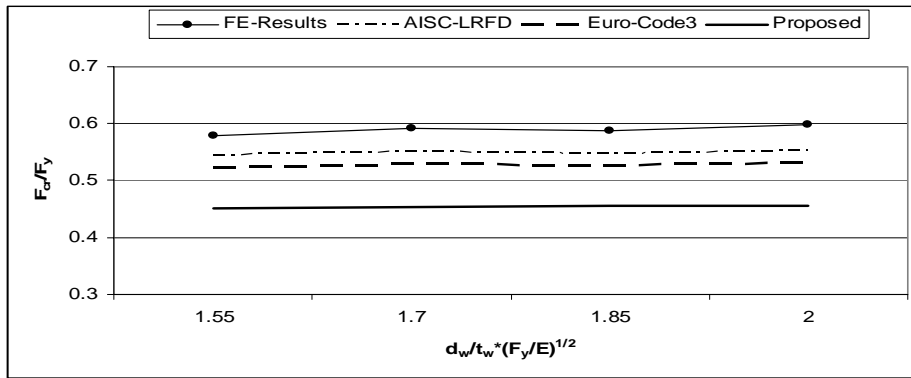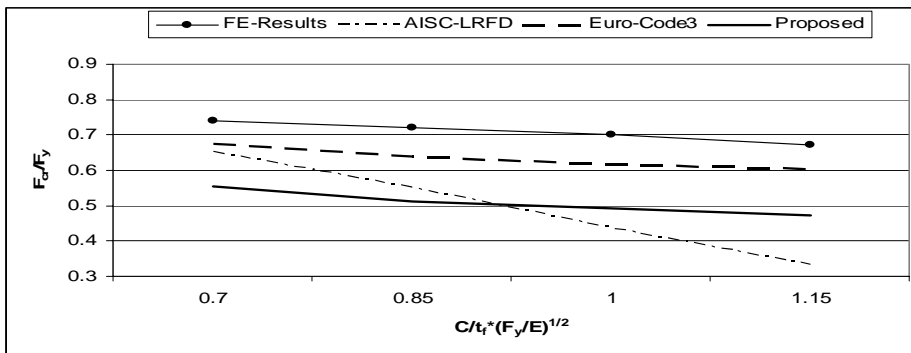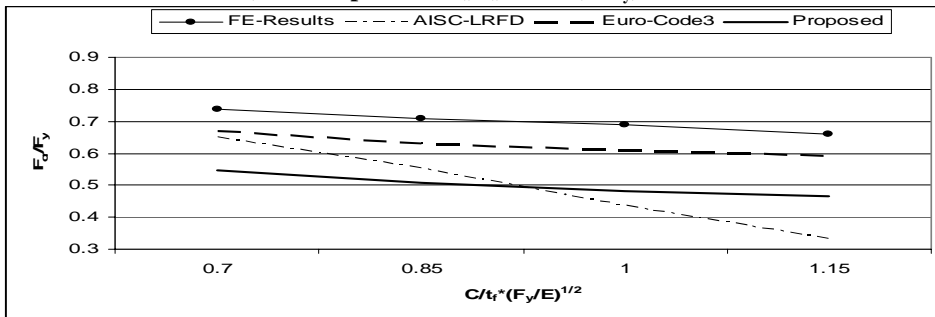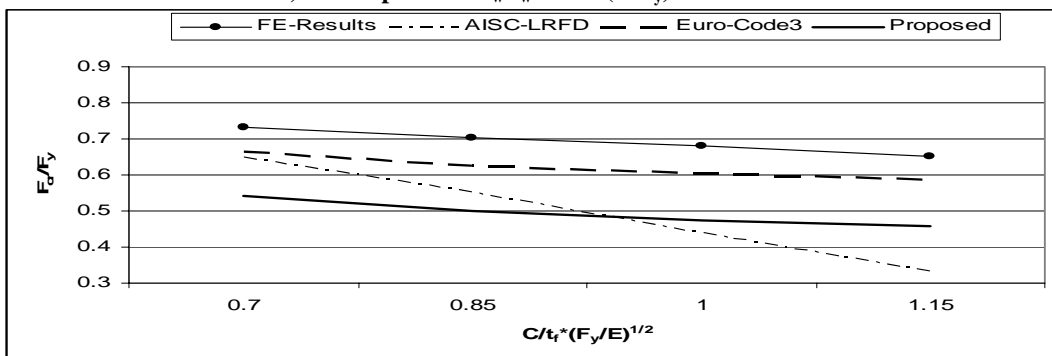**Figure 6 Comparison of Results for short columns of Group 2 sections**

Figure 7 Comparison of Results for medium columns of Group 2 sections

i)   **Group 3 with $d_w/t_w = 1.55(E/F_y)^{1/2}$**

ii)   **Group 3 with $d_w/t_w = 1.70(E/F_y)^{1/2}$**

iii) **Group 3 with $d_w/t_w = 1.85(E/F_y)^{1/2}$**

Figure 8 Comparison of results for short columns of Group 3 sections

iv)   Group 3 with $d_w/t_w = 2.0(E/F_y)^{1/2}$
Figure 8 (Continued)

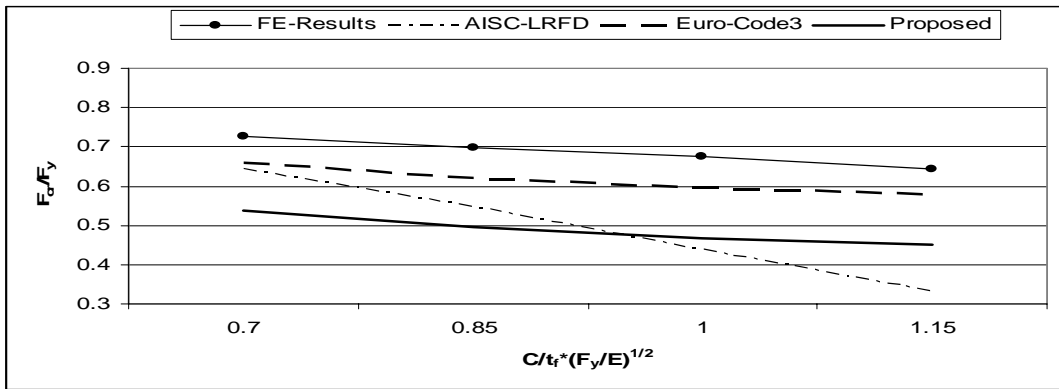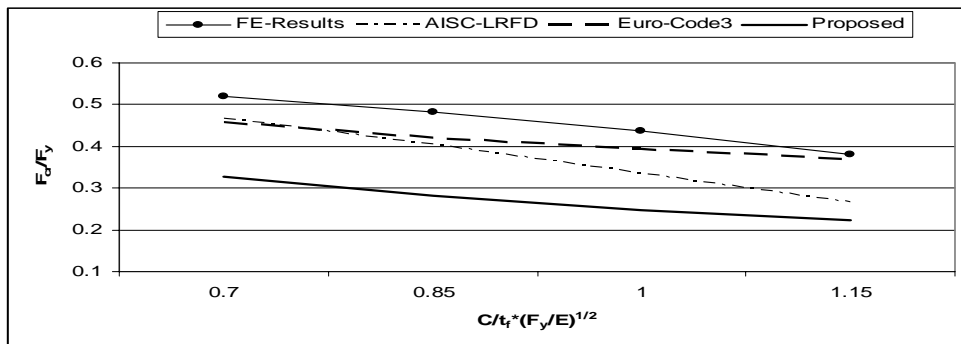i)   Group 3 with $d_w/t_w = 1.55(E/F_y)^{1/2}$

ii)   Group 3 with $d_w/t_w = 1.70(E/F_y)^{1/2}$
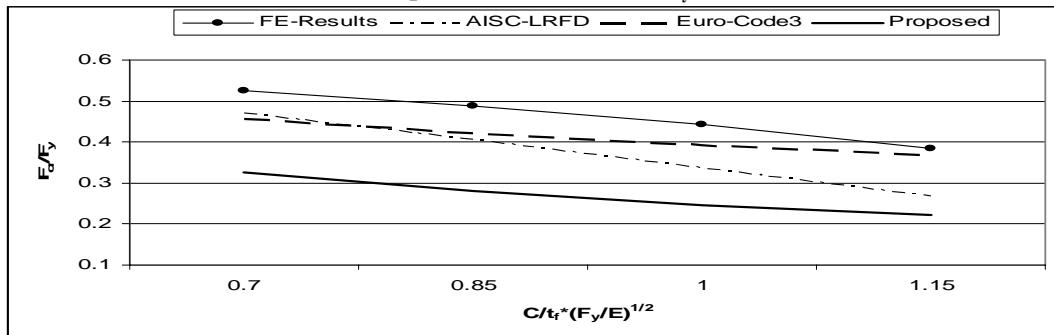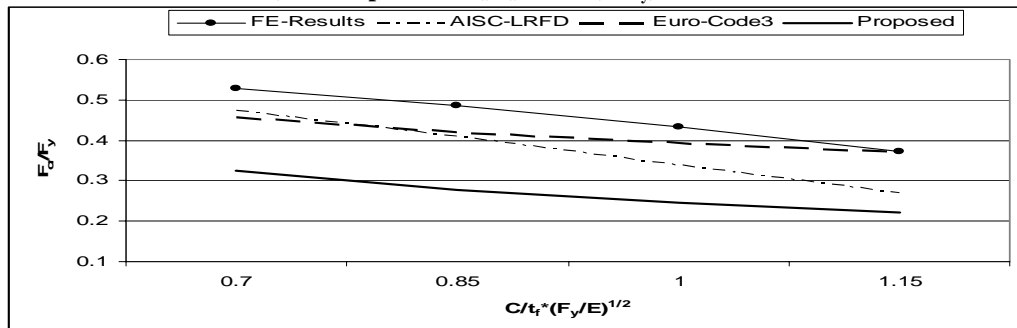

iii) Group 3 with $d_w/t_w = 1.85(E/F_y)^{1/2}$

Figure 9 Comparison of results for medium columns of Group 3 sections

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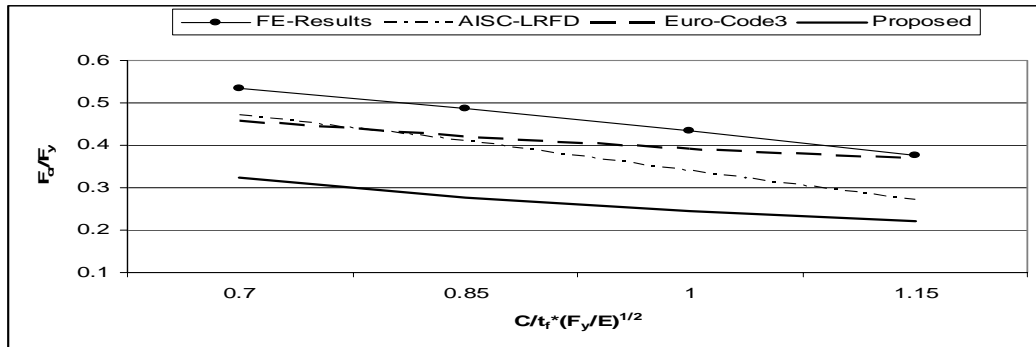
iv) Group 3 with $d_w/t_w = 2.0(E/F_y)^{1/2}$
Figure 9 (Continued)
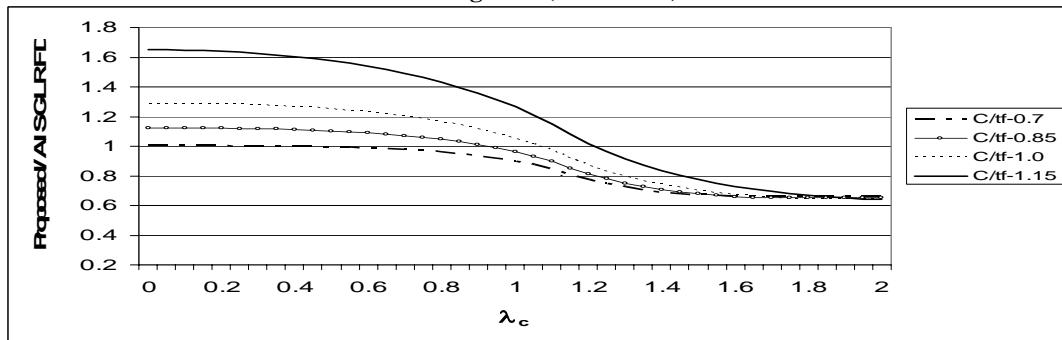
a) Af/Aw = 0.1
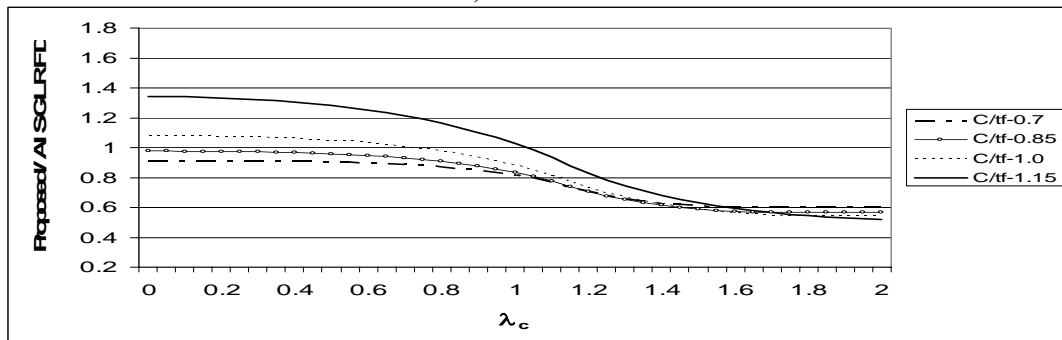
b) $A_f/A_w = 0.5$
Figure 10 Comparison of Proposed Column Design Curve (Eq 12) with AISC-LRFD Design Curve (Eq 2) for Group 1 Sections
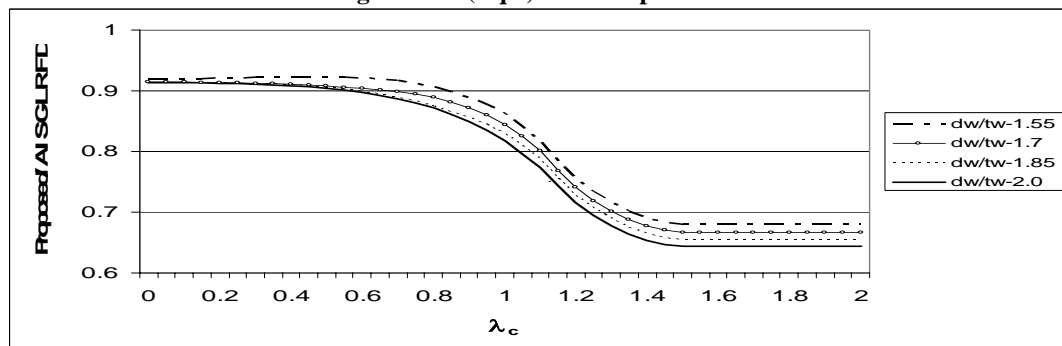
a)   Af/Aw = 1
Figure 11 Comparison of Proposed Column Design Curve (Eq 12) with AISC-LRFD Design Curve (Eq 2) for Group 2 Sections
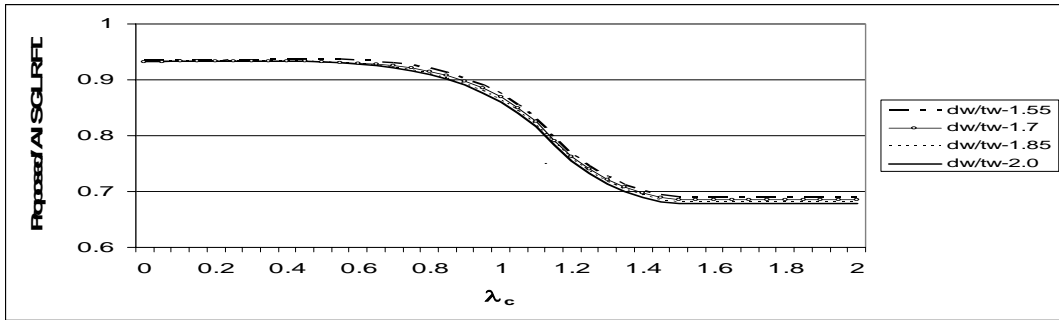
b) Af/Aw = 4
Figure 11 (Continued)
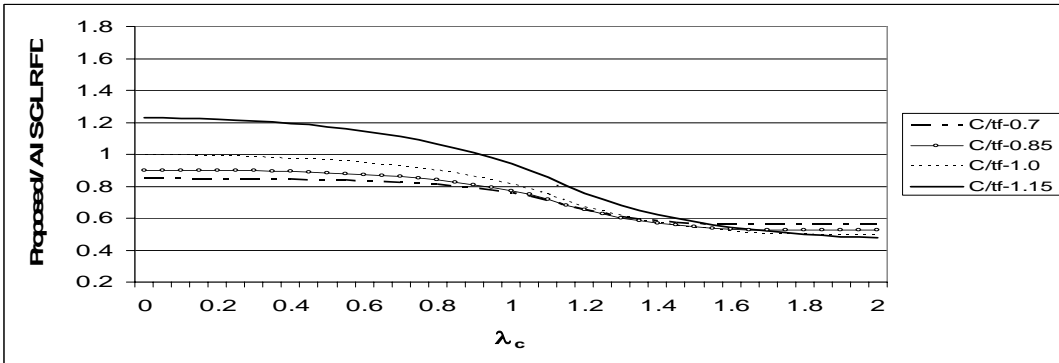
a) $A_f/A_w = 0.5$, $d_w/t_w = 1.7(E/F_y)^{1/2}$

b) Af/Aw = 2.0, dw/tw = 1.7(E/Fy)1/2

c) Af/Aw = 0.5, dw/tw = 2.0(E/Fy)1/2
Figure 12 Comparison of Proposed Design Curve (Eq 12) with AISC-LRFD
Design Curve (Eq 2) for Group 3 Sections

d) $A_f/A_w = 2.0$, $d_w/t_w = 2.0(E/F_y)^{1/2}$
Figure 12 (Continued)

a)   Af/Aw = 0.1

b) $A_f/A_w = 0.50$
Figure 13 Comparison of Proposed Column Design Curve (Eq12) with Euro-LRFD Design Curve (Eq 4) for Group 1 Sections

a) Af/Aw=1
Figure 14 Comparison of Proposed Column Design Curve (Eq 12) with Euro-LRFD Design Curve (Eq 4) for Group 2 Sections

b) $A_f/A_w = 4$
Figure 14 (Continued)

a) $A_f/A_w = 0.5$, $d_w/t_w = 1.7(E/F_y)^{1/2}$

b) Af/Aw = 2.0, dw/tw = $1.7(E/Fy)^{1/2}$
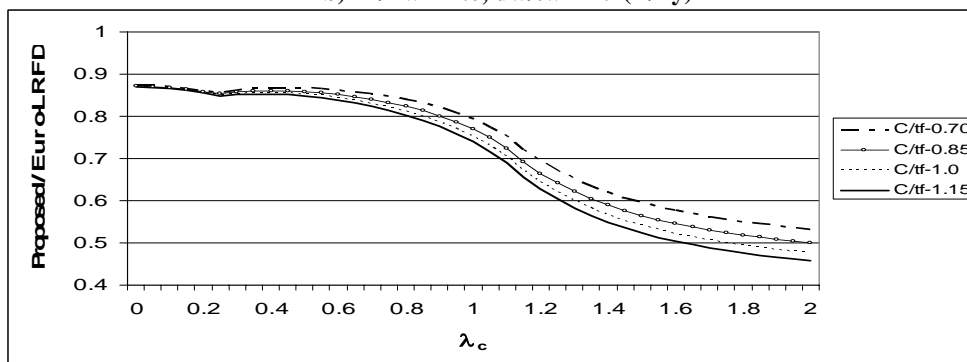
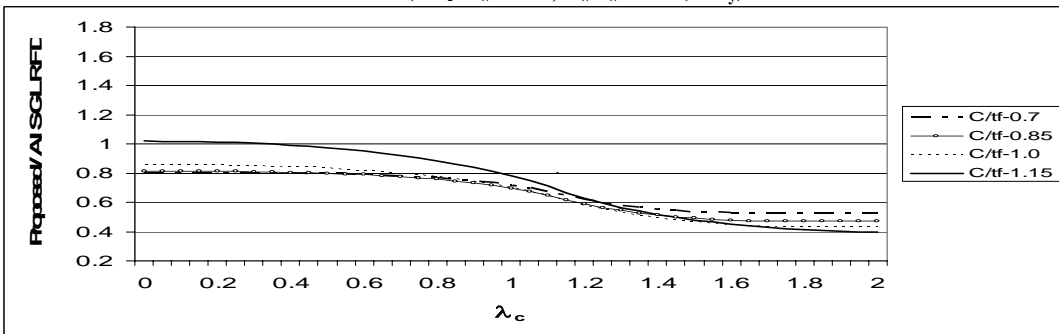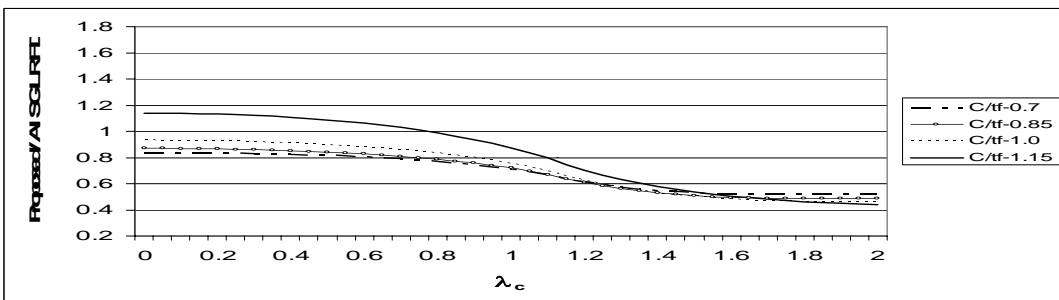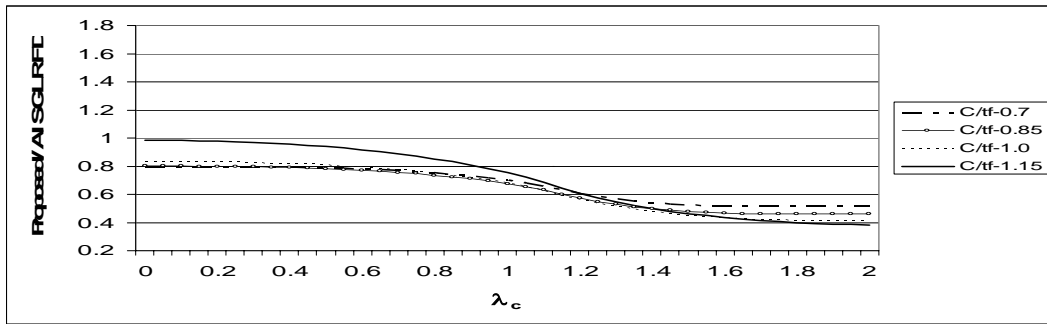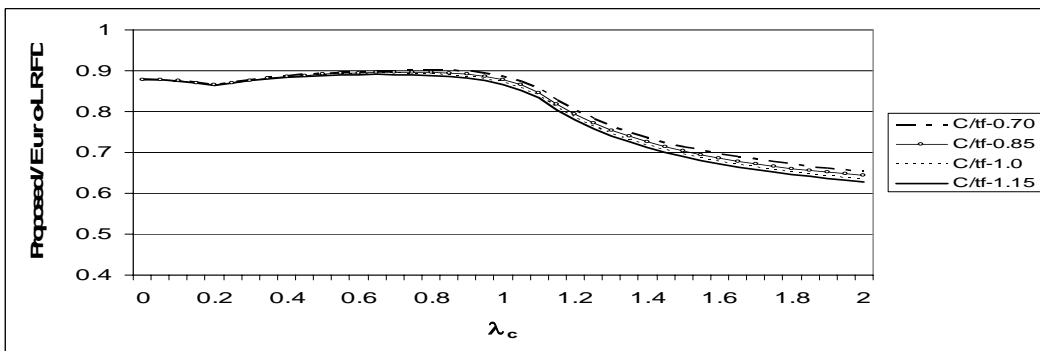c)   Af/Aw = 0.5, dw/tw = $2.0(E/Fy)^{1/2}$
**Figure 15 Comparison of Proposed Design Curve (Eq 12) with Euro-LRFD
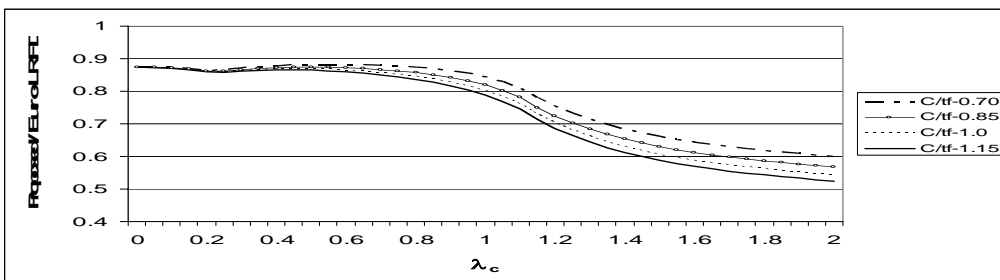Design Curve (Eq 4) for Group 3 Sections**

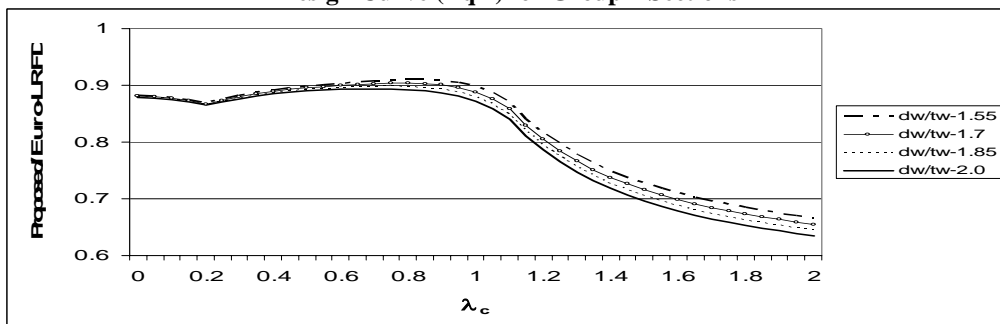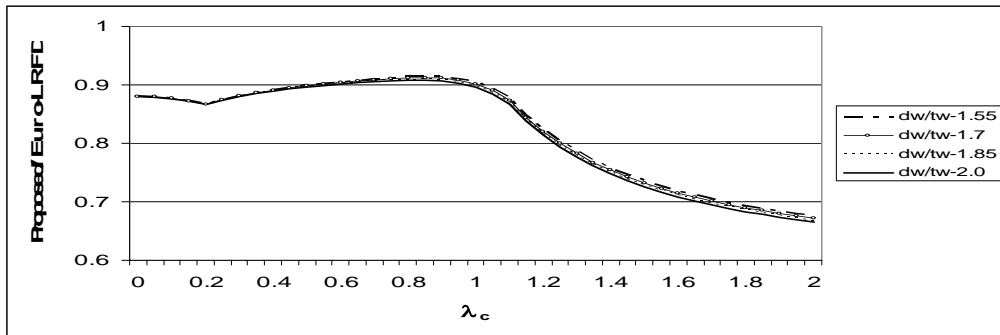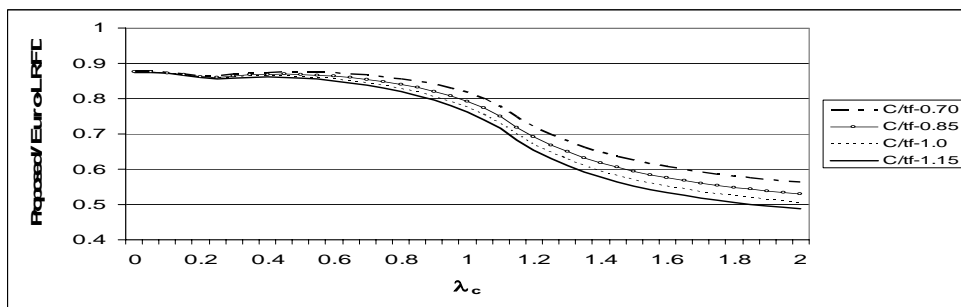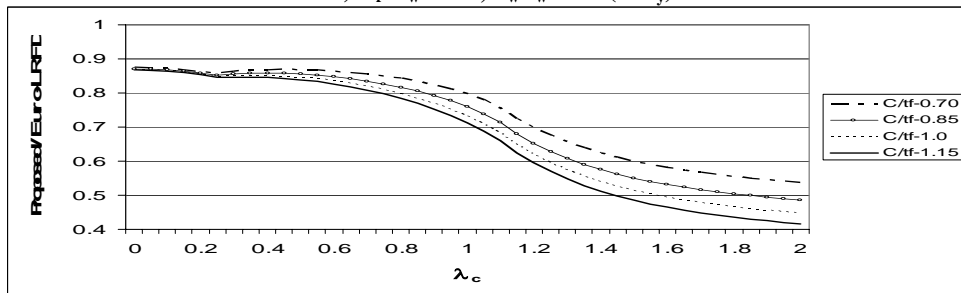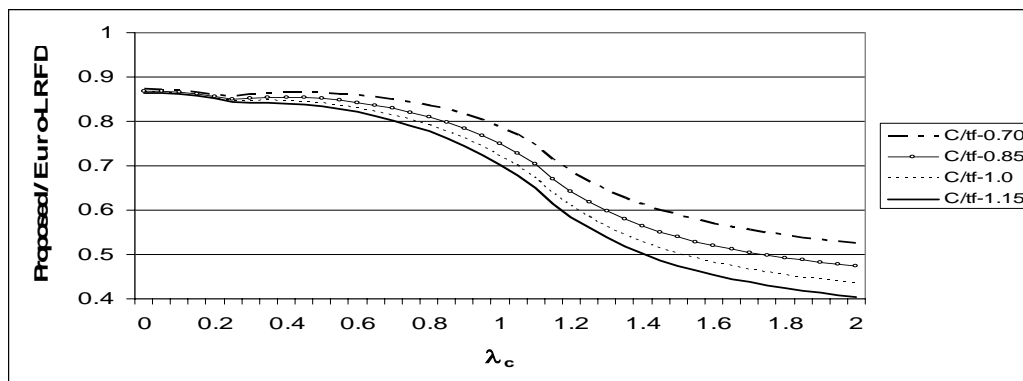**d) $A_f/A_w = 2.0$, $d_w/t_w = 2.0(E/F_y)^{1/2}$**
Figure 15 (Continued)

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10/24/2011

Ki-67 Expression in Gingival Overgrowth: An Immunohistochemical Study

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Abstract: Ki-67 is a well-recognized nuclear proliferation marker. Considering that an unusual cell proliferation may have a role in the pathogenesis of gingival overgrowth with different etiologies. The study involved 4 patients with cyclosporine induced gingival overgrowth (CGO), 6 patients with phenytoin induced GO (PGO) and 5 patients with hereditary gingival fibromatosis (HGF). Healthy tissue samples without clinical signs of periodontal inflammation were also included as control samples. Immunohistochemistry against the proliferation antigen Ki-67 was performed and optical density measured and compared in both epithelium and connective tissue. Ki-67 was expressed both in the epithelium and corium of the four studied groups. The expression patterns of Ki-67 were significantly higher ($p<0.00$) in CGO, while no significant difference between HGF and PGO groups was detected and both showed lower values than CGO. Control group showed the significantly lowest mean of Ki-67 level and the expression was mainly in the basal layer of epithelium. In conclusion; increased cell division may have a role in the pathogenesis of gingival overgrowth induced by cyclosporine and phenytoin or inherited as HGF as reflected by increased expression of Ki-67.

Keywords: Cyclosporine; phenytoin; hereditary gingival fibromatosis; gingival hyperplasia/pathogenesis; Ki-67

1. Introduction

Gingival enlargement may be caused by a variety of etiologic factors. Some drugs such as cyclosporin A; the drug of choice in preventing transplant rejection; and phenytoin; the most commonly used drug for managing epileptic seizures; are commonly associated with the adverse effect of gingival overgrowth (Rateitschak et al., 1983; Bulut et al., 2004; Lin et al., 2007). Gingival overgrowth can also be inherited as an autosomal dominant disorder, or occasionally as an autosomal recessive mode of inheritance, in a condition known as hereditary gingival fibromatosis (HGF) (Singer et al., 1993; Coletta and Graner, 2006). Both types of gingival overgrowth (drug induced or familial) are characterized histologically by thickened, parakeratinized epithelium with elongated rete-pegs and increased extracellular matrix within the connective tissue (Mariani et al., 1993; Martelli et al., 2000; Vardar et al., 2005).

Squamous cell carcinomas may arise in some cases of drug induced gingival hyperplasia although it has been long thought that these conditions are not related to tumorigenesis (Varga and Tyldesley, 1991; McLoughlin et al., 1995; Saito et al., 1999). Oral cancers and increased proliferative activity of oral tissues have been analyzed for many years by monoclonal antibodies to specific antigens such as Ki-67 (Zoeller et al., 1994). Ki-67 is a proliferation associated antigen that serves as a marker for estimation of tissue growth as it is present in the nuclei of proliferating cells located in G1, S, G2, and M phases of the cell cycle and absent in quiescent cells lagging in G0 phase, suggesting a role for Ki-67 in the early steps of rRNA synthesis (Schlter et al., 1993; Buduneli et al., 2007). The mean rate of ki-67 positive cells in phenytoin-induced gingival overgrowth is proven to be more than 10% of immune-stained sections, which is comparable to that of dysplastic oral mucosae (Saito et al., 1999).

As for the HGF, although they usually represent a totally benign condition, yet one case of epithelial dysplasia of the overgrown tissue has been reported (Gunhan et al., 1995). HGF epithelial cells demonstrated higher proliferation rates than normal gingivae and increased expression of proliferation markers as proliferating cellular nuclear antigen (PCNA) and Ki-67 of HGF mesenchymal fibroblasts has been detected *in vitro* (Saygun et al., 2003; Martelli et al., 2005).

The present study aimed to evaluate the state of imbalance in homeostasis of the proliferative activity of gingival epithelium and connective tissue cells by comparing the immunohistochemical expression of a commonly used proliferation marker, Ki-67, in cyclosporine and phenytoin gingival overgrowth as well as cases of HGF.

2. Material and Methods
Study Population

Gingival biopsies were collected from 15 subjects (seven females and eight males with age ranges from 10-32 yrs) with moderate to severe gingival overgrowth (GO) during gingivectomy procedures. The study involved 4 patients with cyclosporine induced gingival overgrowth (CGO), 6 patients with phenytoin induced GO (PGO) and 5 patients with hereditary gingival fibromatosis (HGF). Diagnosis was based on patients' history and clinical examination to differentiate between different causes of gingival overgrowth shown in figure 1 (A, B and C).

Healthy tissue samples without clinical signs of periodontal inflammation were also obtained from the marginal gingiva of four unrelated HGF patients (two males and two females, aged 16 - 28 yrs) when the subjects underwent routine dental treatment (tooth extraction for orthodontic reasons or crown-lengthening procedures). All patients signed a consent form after being advised of the nature of the study.
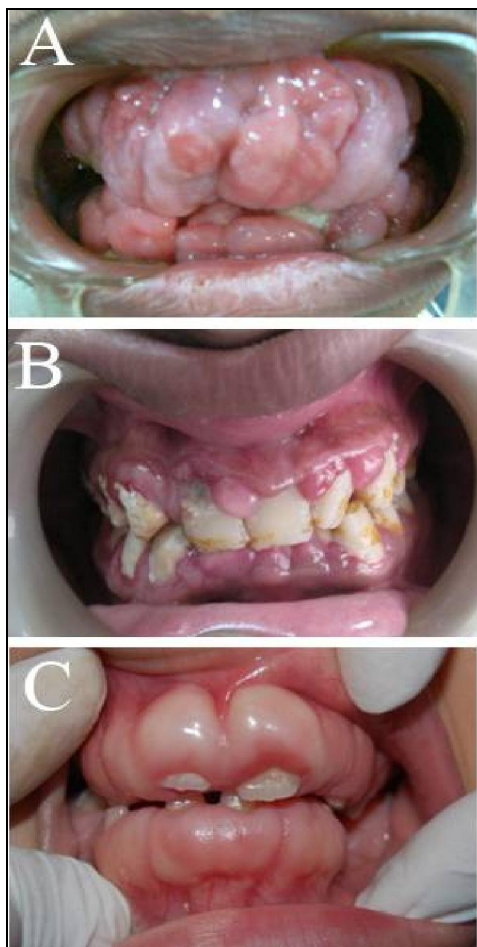
Figure (1). Clinical view of three patients with severe gingival overgrowth: A: CGO, B: PGO and C: HGF

Tissue Processing

As previously described by **Buduneli *et al.*, (2007),** tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections with 5-µm thickness were cut at the central region of each specimen to obtain maximum standardization of the cutting surface. One of the sections was stained with hematoxylin and eosin to evaluate the histopathologic presentation of gingival enlargement.

For Ki-67 staining, sections were deparaffinized by passing through xylene and alcohol, and rehydrated in 96% ethanol, then immersed in 3% hydrogen peroxide to block endogenous peroxide activity. The sections were incubated with a mouse anti-human Ki-67 antibody (Zymed, CA, USA) at 4°C overnight. Normal serum was used as a negative control. Subsequently, the standard streptavidin–biotin–peroxidase complex method was performed using SP kit (Zhongshan Goldenbridge Biotechnology, Beijing, China) for immunohistochemical detection of the proliferation marker Ki-67. Reaction products were visualized by immersing the sections for 5 min in diaminobenzidine solution. Nuclei were lightly counterstained with hematoxylin. Each step was followed by thorough washes with phosphate buffered saline (PBS).

Assessment of immunostaining

Ordinary light microscope was first used to detect the positive and negative immunostaining and localization of the positive reaction within the tissues. Image analyzer computer system (Leica Qwin 500 image analyzer computer system, Wetzlar, Germany) was used to measure the optical density (OD) of the immunostained sections. Five sections were used for each subject and three fields of a gingival section were chosen randomly for the analysis of Ki-67 staining using a magnification of (x400) so that a total of 15 microscopic fields were analyzed for each subject.

Statistical Analysis

Data were presented as mean and standard deviation (SD) values. One-way Analysis of Variance (ANOVA) was used for comparison between the four groups. Tukey's post-hoc test was used for pair-wise comparison between the groups when ANOVA test is significant. Paired t-test was used to compare between Ki-67 levels in epithelium and connective tissue. The significance level was set at $P \leq 0.05$. Statistical analysis was performed with PASW Statistics 18.0 (Predictive Analytics Software) for Windows (IBM Company, Chicago, IL, USA).

3. Results

Histopathology and Immunohistochemistry

As shown in H&E stained sections in figure 2 (A&B), the histopathological features did not differ greatly between different cases of drug induced GO (CGO and PGO). They shared a common histopathology of a significant papillary hyperplasia and parakeratinized epithelial layer with acanthosis and deep ridges penetrating into the underlying connective tissue, with wide variable levels of inflammatory cell infiltration and chorion fibrosis.

Connective tissue alterations were more marked in specimens from HGF group manifested by increased amount of collagen fiber bundles and fewer fibroblasts. Mild chronic inflammatory infiltrates were also frequently observed in the subepithelial connective tissue samples. These changes are shown in fig (3).

Nuclear immunoreactivity for Ki-67 antigen was easily identified, and nuclei with a clear brown color, regardless of the intensity of staining, were interpreted as positive, but this positive reaction was more marked and widely distributed within epithelial cells than within connective tissue cells. In healthy control tissues, Ki-67-positive cells were observed only in the basal cell layer of epithelium while the majority of gingiva samples from the CGO group showed deep and widely distributed Ki-67 positive cells throughout epithelial layers. The PGO and HGF groups showed almost similar expression of Ki-67 antigen which was mainly located in the basal and suprabasal layers of the epithelium. In the lamina propria, Ki-67 expression was observed in fibroblasts of hyperplastic gingival tissues mainly in tissue sections belonging to the CGO, while the control gingiva revealed week immunostaining of fibroblasts. These findings of the immune stained sections are shown in figure (4).
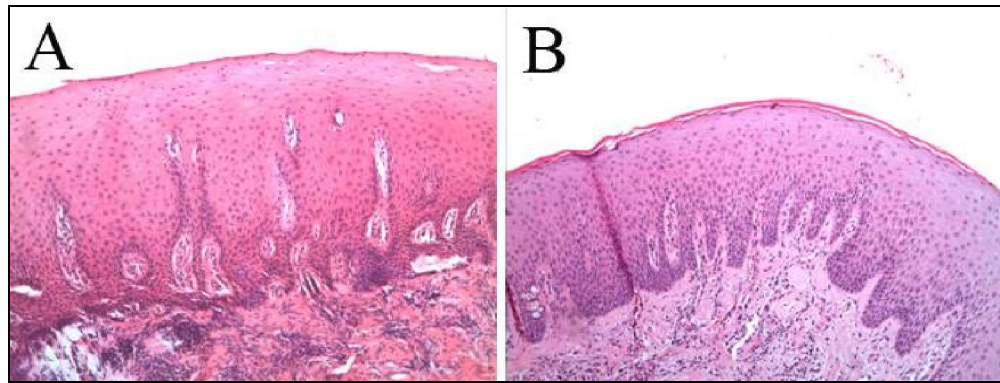
Figure (2). Histopathologic presentations of drug induced GO: A - Section from CGO showing irregular acanthosis and chorion fibrosis with marked inflammatory cellular infiltrate. B - Section from PGO group showing acanthosis with mild parakeratosis and papillomatosis with chorion fibrosis and lymphomononuclear infiltrate (H&E; original magnificationx100).
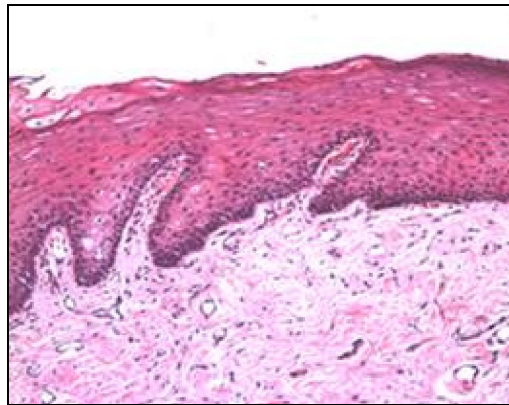
Figure (3). Histopathologic presentation of HGF showing dense connective tissue predominantly consisting of thick and irregularly arranged collagen fibers underlying a well structured epithelium (H&E; original magnification x100).
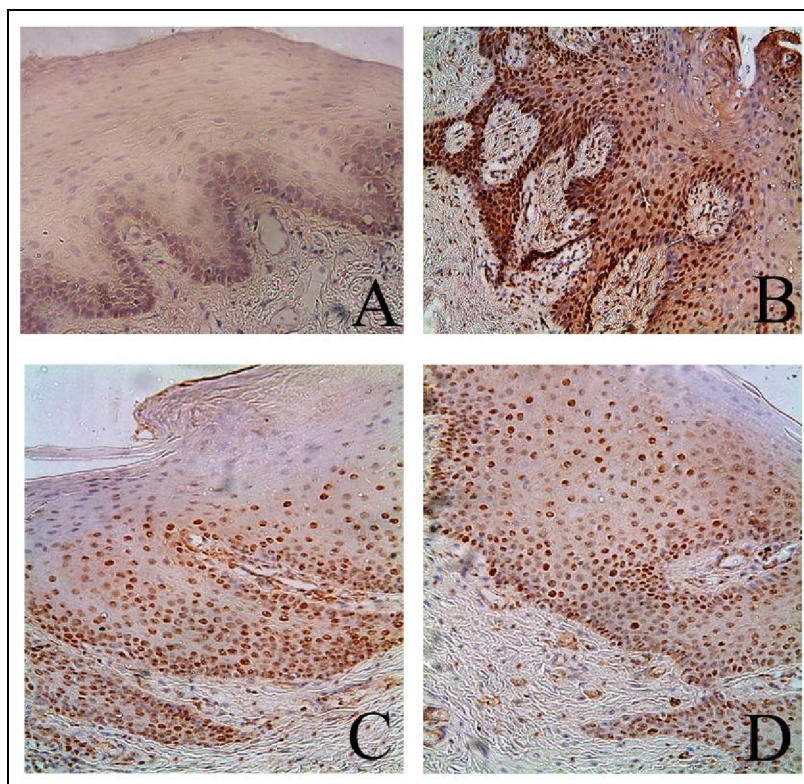
Figure (4). Ki-67 antigen expression in tissue samples of all study groups.
A: Ki-67 antigen –positive nuclei observed mainly in the basal layer of control gingival epithelium. **B:** Deep immune staining of the nuclei of almost all layers of the hyperplastic epithelium in CGO. **C:** immune stained section of PGO and **D:** HGF showing comparable patterns of Ki-67 antigen expression in the basal and suprabasal layers of the epithelium. Note that in all sections connective tissue expression of Ki-67 antigen is less marked than epithelium (immunostaining; original magnification x200).

Optical Density

As shown in table (1), Ki-67 was expressed both in the epithelium as well as the corium of the four studied groups with the epithelium showing the significantly higher means of Ki-67 OD than connective tissue in CGO and PGO groups as well as control tissue samples at P values of 0.003, 0.006 and 0.014; respectively. As for HGF group, there was no significant difference in the mean values of Ki-67 OD between epithelium and connective tissue (P=0.747).

As shown in table (2), The OD of Ki-67 in the keratinocytes within CGO group showed the significantly highest mean of Ki-67 level (73.4±4.4) at a P value <0.001. There was no significant difference between HGF and PGO groups; both showed lower values than CGO, while control tissue samples showed the lowest mean of Ki-67 level (33.4±11.8). Similar findings were detected in the corium of the test and control groups with the significantly highest mean of Ki-67 seen in the corium of CGO group (60.4±0.4).

Table (1): The mean, standard deviation (SD) values and results of paired t-test for comparison between Ki-67 OD levels in epithelium and connective tissue within each group

	Control	HGF	PGO	CGO
Epithelium	33.4±11.8	46.7±2.9	50.9±2.1	73.4±4.4
Connective tissue	16±7.9	45±10.6	30.9± 9	60.4±0.4
P-value	0.014*	0.747	0.006*	0.003*

*: Significant at P ≤ 0.05

Table (2): The mean, standard deviation (SD) of optical density values and results of ANOVA test for comparison between Ki-67 levels in the four groups

	Control	HGF	PGO	CGO	P-value
Epithelium	33.4±11.8[c]	46.7±2.9[b]	50.9±2.1[b]	73.4±4.4[a]	<0.001*
Connective tissue	16±7.9[c]	45±10.6[b]	30.9±9[b]	60.4±0.4[a]	<0.001*

*: Significant at $P \leq 0.05$, Means with different letters are statistically significantly different according to Tukey's test

4. Discussion

Although it has been thought that drug-induced gingival hyperplasia is not related to tumorigenesis, recent case reports have shown that squamous cell carcinoma may arise in gingival hyperplasia induced by cyclosporine (Varga and Tyldesley, 1991) and phenytoin (McLoughlin et al., 1995) and also as unusual histologic finding with HGF (Gunhan et al., 1995).This possible implications between the pathogenesis of GO and tumorigenesis suggested the aim of the present study which was the examination of the expression of a tumor-related marker, Ki-67, in hyperplastic gingival tissues induced by cyclosporine and phenytoin as well as cases of HGF and compare them to healthy control tissues.

Currently, more than 15 drugs have been identified as possible causative agents of gingival overgrowth. However, phenytoin and cyclosporine are more commonly involved (Lin et al., 2007; Silverstein et al., 1997). One property that is common for these two different classes of drugs is that they directly affect cellular calcium metabolism. Since cellular production of collagenase is modulated by calcium influx, fibroblasts from patients treated with these drugs may produce an inactive form of collagenase, being responsible for an increase in the extracellular matrix (Brunet et al., 1996). Combined with this reduction in extracellular matrix degradation; enhanced proliferation of keratinocytes and/or resident fibroblasts were reported (Saito et al., 1999; Nurmenniemi et al., 2001). These previous findings align with the histopathologic changes and the significant increase in the optical density of Ki-67 staining reported in the current study within the epithelium and corium of both cyclosporine and phenytoin induced GO groups when compared to the control tissue samples.

Nurmenniemi *et al.,* (2001) also reported a significant increase in numbers of Ki-67–labeled cells in patients with CGO compared to healthy controls. Saito *et al.,* (1999) found that mean rates of Ki-67–positive cells in PGO were significantly higher as well than healthy tissues. These conflicts with a previous study reported that the acanthosis observed in cyclosporine-treated patients is not caused by enhanced keratinocytes proliferation but rather by prolonged cell life caused by an antiapoptotic effect of cyclosporine (Niimi et al., 1990). Bulut *et al.,*(2004) revealed that epithelial proliferation rates may be unchanged in renal transplant patients with CGO when compared to healthy controls.

As for HGF, most attention has been focused on the proliferative potential of mesenchymal fibroblasts. In the present study, both epithelium and connective tissue cells were studied and no significant difference was found in the proliferative potential of epithelium and connective tissue as reflected by Ki-67 optical density which was higher than control tissues and comparable to that of PGO group. In corroboration, a study with 12 different cell lines from patients of a Brazilian HGF family demonstrated a significantly higher proliferation rate of HGF fibroblasts compared to fibroblasts from normal gingivae (de Andrade et al., 2001). On the other hand, Saygun *et al.,*(2003) suggested that the underlying mechanism of HGF are not involved with increased cellular proliferation and that lack of proliferation is caused by unfavorable cellular environment lacking key nutrients caused by excessive extracellular matrix deposition.

Findings from the current study confirmed that increased cell division and proliferation may have a role in the pathogenesis of drug-induced gingival overgrowth as well as HGF, however; several factors, including age, genetic predisposition, pharmacokinetic variables and plaque-induced inflammatory changes are believed to be important in the onset and severity of gingival overgrowth. Accordingly, further studies with larger sample size will provide more conclusive data on the possible role of enhanced proliferative activity of cells in the pathogenesis of gingival overgrowth.

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10/24/2011

Evaluation of Bone Turnover in Children with Chronic Renal Failure in Egypt

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Abstract: Background: Renal osteodystrophy is a multifactorial and universal disorder of bone metabolism in advanced chronic kidney disease. It is a spectrum of bone mineral changes that could range from the high turnover lesions of secondary hyperparathyroidism to the low turnover lesions of adynamic bone disease. Objective: to evaluate the bone turnover, estimated by the measurement of some serum biochemical markers and bone mineral density in children with chronic renal failure either on conservative therapy or regular hemodialysis. Methods: The study included 35 children suffering from chronic renal failure, 20 out of them on regular hemodialysis (group I) & the other 15 on conservative therapy (group II). Each group was subdivided into three subgroups according to iPTH values. In addition to 20 apparently healthy children served as a control group. All children underwent thorough history taking, physical examination, routine, specific laboratory& radiological investigations as serum Ca, P, ALP, iPTH , β_2-microglobulin & DEXA scan. Results: both of groups I &II had significant increase in SBP, DBP, serum β_2-microglobulin and iPTH than the controls. Meanwhile, no statistical significant differences in serum β_2-microglobulin & iPTH levels were found between groups I & II. BMD was measured using DEXA scan revealed that osteopenia was found in 50% group I and 53% of group II. The frequencies of LTBD estimated by iPTH in groups I & II were 20% and 27%, respectively. Meanwhile, the HTBD frequencies were 60 % in the both groups. Children with CRF in the subgroups with high iPTH had significantly higher SBP and DBP than those with low iPTH either in group I or II. Serum β_2-microglobulin showed a significant increase in high iPTH subgroup than low iPTH subgroup only in group I. iPTH correlated positively with SBP, DBP & β_2-microglobulin. Meanwhile, negatively with Ca & BMD Z-score in groups I & II. Conclusions: Maintenance of normal bone turnover may be important in prevention of irreversible bone disabilities and CVD. The preserving of normal BMD is a challenge for pediatric nephrologists so, continuous and regular monitoring systems by combination of iPTH, serum Ca, ALP, β_2-microglobulin & BMD Z-score could be early, accurate and non invasive assessment of the skeletal system in children with CRF.
[Mohamed Bahbah; Ali El-Shafie; Nagy Abou El Hana; Mohsen Deeb; Seham Khodeer; Azza Abdu-Allah and Hossam Hegran**. Evaluation of Bone Turnover in Children with Chronic Renal Failure in Egypt**. Life Science Journal. 2011; 8(4): 227-235] (ISSN: 1097-8135). http://www.lifesciencesite.com.

Key words: Bone turnover, β_2- microglobulin, iPTH, chronic renal failure

1. Introduction

Renal osteodystrophy is a multifactorial and universal disorder of bone metabolism in advanced chronic kidney disease (CKD)[1]. It is a spectrum of bone mineral changes that could range from the high-turnover lesions of secondary hyperparathyroidism to the low-turnover lesions of adynamic bone disease [2]. Despite the fact that bone biopsy is the gold standard for the diagnosis of renal osteodystrophy, it has not been routinely performed mainly because it requires an invasive procedure to obtain a bone sample and needs special equipment and expertise [3].

Hyperparathyroidism is a common finding in patients with renal insufficiency [4]. Parathyroid hormone (PTH) is considered a uremic toxin responsible for many of the abnormalities of the

uremic state and bone disease [5]. The interpretation and significance of intact PTH (iPTH) levels in the individual patient is further complicated by skeletal resistance to PTH in chronic renal failure, it is confirmed in the epiphyseal cartilage growth plate of uremic rats. Therefore, additional tests or new markers of bone remodeling are needed, that allow a correct and dynamic non invasive diagnosis of bone turnover. These biochemical markers have to be compared and/or combined with iPTH plasma values to evaluate their potential interest [6]. β_2-microglobulin is a low molecular weight protein produced by all nucleated cells at a constant rate. It is freely filtered by the glomerulus, reabsorbed and catabolized by proximal tubular cells [7]. β_2-microglobulin was proposed as a potential bone

growth factor. Studies evaluating the microglobulin effects on bone have been performed utilizing different experimental models on cells obtained from different animal species and using varying doses of β_2- microglobulin. In these studies the bone cells undergo a series of developmental stages such as proliferation, differentiation and apoptosis [8].

Radiological studies are of little diagnostic utility, because biochemical changes precede radiological changes. They are useful as the first step in the study to detect vascular calcifications and amyloidosis due to β_2- microglobulin and in symptomatic and at risk patients to detect vertebral fractures [9]. Bone densitometry: dual energy x-ray absorptiometry (DEXA) is the standard method to determine bone mineral density. It provides information on changes in bone mineral content, but not on the type of underlying bone disease. It is useful for follow up of bone mass or for the study of bone mass changes in the same patient [10].

Aim of the work was to evaluate the bone turnover, estimated by the measurement of some serum biochemical markers and bone mineral density in children with chronic renal failure either on conservative therapy or regular hemodialysis

2. Subjects and Methods

The present study was carried out on 55 children from Tanta and Menoufiya Universities Hospitals, divided into 3 groups: group I (dialysis group) included 20 children (8 males & 12 females) with mean age ±SD 13.7± 2.3 years with end stage renal failure (ESRF), on regular hemodialysis therapy. This group was subdivided into three subgroups according to iPTH values; subgroup with low iPTH levels which was less than the target range, subgroup with iPTH levels within the target range and subgroup with high iPTH levels which was above the target range. The target range defined according to the NKF/KDOQI guidelines. Group II (conservative group) included 15 children (6 males & 9 females) with mean age ±SD 12.7 ±4.6 years. Also, they were subdivided into three subgroups according to iPTH values as before in group I where the target range defined according to the NKF/KDOQI guidelines. The cut-off values of 150 pg/mL and 300 pg/mL represent low-turnover and high-turnover disease respectively [11]. The values between150-300 pg/mL could be considered within the "safe" limits of iPTH ("controlled" ROD). In addition to 20 apparently healthy children, age and gender matched served as a control group. All patients were given calcium-based phosphate binders and calcitriol. Exclusion criteria included malignancy, history of parathyroidectomy, sever trauma & biochemical evidence of obstructive jaundice.

All subjects were subjected to thorough clinical examination including weight, height, BMI and blood pressure. Laboratory & radiological investigations as serum Ca, P, ALP, iPTH, β_2-microglobulin, & DEXA scan. Informed consents were obtained from all participants' parents.

Samples collection and preparation

Immediately before a dialysis session, 5 ml of venous blood were withdrawn and the sample divided as follow: 2 ml of whole blood were allowed to clot, the serum separated in a refrigerated centrifuge, and stored at -20 °C for later determination of iPTH. The other 3 ml of whole blood were allowed to clot, centrifuged & serum sample were divided in two aliquots, one of them kept immediately at-20 ^{0}C for determination of β_2-microglobulin & the other aliquot for determination of total and direct bilirubin, albumin, urea, creatinine, Ca, P ,and ALP.

Laboratory Methods:

- Total & direct bilirubin, albumin, urea,creatinine, Ca, P and ALP assayed on Synchron Cx9 (Beckman Instrument. Inc. Fullerton, California USA.).
- iPTH assayed by the DAI Intact PTH Immunoassay (IBL GESELLSCHAFT. HAMBURG,GERMANY) which is a two-site enzyme-linked immunosorbent assay for the measurement of the biologically intact 84 amino acid chain of PTH. Two different goat polyclonal antibodies to human PTH have been purified by affinity chromatography to be specific for well defined regions on the PTH molecule.
- β_2-microglobulin was assayed by indirect solid phase enzyme immunometric assay (ELISA, ORG 5BM, ORGENTEC DIAGNOSTIKA Gmbh). The microplate is coated with   highly purified anti-β_2 microglobulin antibodies where any present β_2-microglobulin bind to the immobilized antibodies. With the addition of anti-h- β_2 microglobulin-hoseradish peroxidase conjugate, it recognizes β_2-microglobulin molecules bound the immobilized anti-β_2 microglobulin forming the sandwich complexes.

Radiological investigations:

Bone mineral density (BMD): BMD at lumbar spinal region (L2-L4) was measured in all children using DEXA(Challenger envision osteodensitometer). BMD was classified according to Bakr [12] , on the basis of BMD Z-score which were calculated from the following equation: Z-score = [BMD (g/cm^3) of the patient—BMD predicted for age and sex/SD for BMD (age, sex and height matched)]. A patient was considered osteopenic if the Z-score was <–1.0. If the Z-score was≤ –2.5 the patient was classified as

having severe osteopenia.

The statistical analysis was undertaken using SPSS software (version 17; SPSS Inc., Chicago, IL, USA). Descriptive statistics in the form of mean and standard deviation for parametric data were used. ANOVA test for comparison between three groups having quantitative variables normally distributed followed by LSD (least significant difference). Kruskal-Wallis test for comparison between three groups not normally distributed having quantitative variables. Pearson correlation coefficient (r) was used to test correlation between two quantitative variables. The level of significance was set at 0.05.

3. Results and Discussion:

Renal failure is a growing problem that involves a large part of the population and has a great social impact, with often incapacitating complications, mainly related to mineral bone disorders referred to as renal osteodystrophy [13]. Changes in mineral metabolism and bone structure are linked to abnormalities in the metabolism of calcium, phosphate, vitamin D, and parathyroid hormone levels [11].

In the current work, the obtained results revealed a presence of a statistical significant difference between group I and the control group regarding BMI. While, no statistical significant difference was found neither between group II and the control groups nor group I and group II regarding BMI. In a study done by **Gupta et al.,** [14], they found that Kuwaiti patients with ESRF had a lower body mass index when compared with the controls**.**

In the present study both groups of chronic renal failure had statistical significant increase in SBP, DBP, serum β_2-microglobulin and iPTH than the control group. Meanwhile, no statistical significant increase in SBP, DBP, β_2-microglobulin and iPTH levels was found between both groups of chronic renal failure. In a study done by **Michelis et al.,** [15] they found that the salivary and serum β_2 microglobulin concentrations were 90.7% higher in CKD patients compared with healthy controls. In healthy individuals, β_2-microglobulin is synthesized at a constant rate, but retention of it occurs in renal failure [16].

In the present study, the BMD at lumbar spinal region (L2-L4) was measured using DEXA revealed the presence of statistical significant difference between both groups of renal failure and the control group. While no statistical significant difference was found between both groups of renal failure as regard the same parameter. DEXA Z-score results revealed

that osteopenia was found in 53% of group II and 50% of group I. This high frequency is probably related to the high rates of bone growth and the remodeling process that are characteristic of the immature skeleton.

Ziolkowska et al.,[17] reported that 48.4% of children with CRF were osteopenic. One-third of these patients were treated conservatively while two-thirds were on dialysis. **Bakr** [12] demonstrated that osteopenia was present in about 62% of 21 children with predialysis CRF and 59% of 44 children with ESRF. In a study done by **Gupta et al.,** [14], they found that the ESRF Kuwaiti patients had a lower BMD than the controls.

In most clinical settings, it is not necessary to identify the specific form of renal osteodystrophy but rather determine if bone turnover activity is high or low. In children with CKD stage 5, a combination of serum PTH and calcium can distinguish between high (eg, osteitis fibrosa cystica) and low (eg, adynamic bone disease) turnover bone disease [9]. The high PTH subgroup represents high turnover bone disease (HTBD) and low PTH subgroup which represent low turnover bone disease (LTBD), while normal PTH group represent controlled ROD.

In the present work, the frequencies of LTBD estimated by serum iPTH in groups I & II were 20% and 27%, respectively. Meanwhile, the frequencies of HTBD estimated by serum iPTH were 60 % in both groups I & II. The other 20 % of group I and 13 % of group II could be considered within the "safe" limits of iPTH ("controlled" ROD). **Avila-Diaz et al.,** [18] reported that there were 20 (48.8%) children with PTH <150 pg/ml were classified as having LTBD; the remaining 21 (51.2%) children were classified as having no LTBD. In previous reports, the prevalence of LTBD by biopsy in children undergoing dialysis was 27% in Poland [19] & 29% in Turkey [20].

In the current study, there was no significant difference between high and low PTH subgroups as regard duration on dialysis in group I. Meanwhile, there was a significant differences between high and low iPTH subgroups in group II regarding duration of renal impairment, this may be attributed to longstanding high calcium intake and over treatment with 1,25-dihydroxyvitamin D. This finding agreed with **Avila-Diaz et al.,** [18].

The clinical relevance of LTBD in children undergoing dialysis treatment or on conservative management is related mainly to growth retardation. In the present study, no significant statistical

differences in height and weight were found between the three subgroups of groups I & II. This may explained by the wide range of age at the onset of renal failure, which has a significant effect on linear growth. In accordance with the present study, *Avila-Diaz et al*., [18] reported that LTBD group has lower weight and height than HTBD group but not statistically significant.

The present results revealed that only patients with CRF in the subgroup with high iPTH had significantly higher SBP and DBP levels than the patients in the subgroup with low iPTH either in group I or II. The results of the current study run parallel with the results of the *Ávila-Diaz et al.,*[18]. Moreover, the present results revealed a significant positive correlation between iPTH levels and DBP & SBP among groups I & II. Elevated PTH is associated with a greater prevalence and incidence of CV risk factors and predicts a greater likelihood of prevalent and incident disease, including mortality. PTH represents an important new CV risk factor that adds complementary and independent predictive value for CV disease and mortality. As well, this may be attributable to PTH-induced increased intracellular Ca^{++} affecting vascular endothelial function that leads to increased vascular tone and stiffness [21]. Another explanation stated that the increase in phosphorus, calcium, inflammatory mediator and uraemia levels have been observed to promote smooth muscle cells transforming into osteogenic lineage cells. These cell produce collagen matrix, which is later mineralized [9].

The current study shows that low iPTH subgroup has significant elevated serum calcium than high iPTH subgroup in groups I & II. Meanwhile, no significant statistical differences as regard serum phosphorus in the three subgroups either in group I or group II. These obtained results run parallel with those of study carried by *Salusky et al.*, [22]. The present results supported the *Ávila-Diaz et al.,*[18]. hypothesis who has defined patients with LTBD, by PTH <150 pg/ml and total Ca >10 mg/dl and patients without LTBD, as defined by PTH >150 pg/ml and total Ca <10 mg/dl. Furthermore, there were significant negative correlations between iPTH level and serum calcium among groups I & II which is comparable to the results of *Inaba et al*., [23]. So, determination of serum calcium considered to has a high discriminatory value between LTBD and HTBD.

In the present study, patients with LTBD had lower levels of ALP than those with HTBD in both groups I &II. The same results were obtained by

Piscitelli et al., [24] & *Avila-Diaz et al.,* [18] found that ALP in children with LTBD was significantly lower than those without LTBD. As well, the obtained results revealed a significant positive correlation between serum iPTH and ALP in group I but not group II.

As regard serum β_2-microglobulin, the obtained results showed a significant increase in serum β_2-microglobulin in high iPTH subgroup than low iPTH subgroup of group I but not group II which were partially comparable to the results of *Ferreira and DrÜeke*[6] who observed that patients with HTBD have higher serum levels of β_2-microglobulin than patients with normal bone or LTBD. Also, a significant positive correlation was found between serum iPTH and β_2-microglobulin in groups I & II. This association of high serum β_2-microglobulin with high serum markers of bone turnover suggests that β_2-microglobulin could be either a direct or an indirect activator of bone cells or at least another marker of bone cell activity. So, determination of serum β_2-microglobulin could be considered to have a discriminatory value between HTBD and LTBD.

In the present study, group II showed no statistical significance in Z-score between high and low iPTH subgroups which run parallel with the results of *Andrade et al*., [2] . On the other hand, a statistical significant difference in Z-score was found between high & low iPTH subgroups of group I which were similar to that of *Bakr* [12]. In the present study, Z-scores at lumbar spines were significantly negatively correlated with iPTH in groups I & II and with ALP in group I but not in group II. Besides, positively correlated with Ca in group I but not in group II. On the other hand, no statistical significant correlations were found between Z-scores at lumbar spines and duration of renal impairment & dialysis, BMI, weight, height, phosphorus or ß2-microglobulin. *Waller et al.,* [25] found that in patients with CRF the BMD Z-score did not correlate with any biochemical markers such as serum iPTH, Ca, ALP and P. The inconsistencies in the results as regard relation between DEXA findings and other biochemical parameters of bone turnover are possibly due to small patient's number and varying patient's characteristics as well as differing therapeutic management strategies affecting bone turnover.

It seems likely that the maintenance of normal serum PTH levels is an important factor in preserving normal BMD which supported by the current results which showed normal BMD in patient with normal PTH value in groups I & II which could be explained by adequate treatment with calcitriol.

Disturbances involving phosphate excretion, vitamin D3 metabolism, hypocalcemia , increased PTH, and acid base disturbances are known pathophysiological factors in patients with ROD . These factors lead to the loss of bone mass, destruction of bone micro architecture, and subsequently increase bone turnover due to increased bone formation and resorption. This could explain the correlation observed between Z-score values and some biochemical markers of bone activity, as DEXA measures the amount of mineral in the scanned area [12]. Measuring of BMD Z-score alone is considered of variable value as HTBD showed more osteopenia than LTBD but not reach significance in group II.

Table (1): Comparison between the studied groups regarding the clinical data, biochemical variables and BMD Z-score

Parameter	Group 1 (N=20) X± SD	Group II (N=15) X± SD `	Control group (N=20) X± SD	P- value
Age (years) ∞	13.77±2.39	12.73±4.68	12.4±3.56	>0.05 for all
Weight (kg) ∞	32.22±9.04	43.27±14.11	42.95±13.41	P1<0.001** P2>0.05* P3<0.05
Height (cm) ∞	136±12.08	136.8±28.89	147.1±13.95	>0.05 for all
BMI (kg/m2) ∞	17.12±3.12	17.34 ±1.9	19.33±3.6	P1<0.05* P2>0.05 P3>0.05
SBP (mmHg) ∞	126.7±14.35	129.33±15.79	115±19.6	P1<0.05* P2<0.05* P3>0.05
DBP (mmHg) ∞	84.25±9.9	88.66 ± 9.53	73.5±6.09	P1<0.001** P2<0.001** P3>0.05
GFR (ml/min/1.73 m2) ∞	9.62±1.31	20.84±10.95	238.22±78.38	P1<0.001** P2<0.001** P3>0.05
Total calcium (mg/dl) ∞	8.73±1.33	9.03±1.24	9.45±0.40	P1<0.05P2>0.05 P3>0.05
Phosphorus (mg/dl) ∞	4.98±1.05	5.24±0.78	4.74±0.54	P >0.05 for all
Intact PTH (pg/ml) ♦	791.9±689.75	546.34±532.91	36.8±14.56	P1<0.001** P2<0.001** P3>0.05
ALP (IU/L) ∞	255.15±139.9	172.4±101.2	142.2±56.63	P1<0.001** P2>0.05 P3<0.05*
ß2-microglobulin (mg/l) ∞	6.99±3.48	6.77±3.44	0.98±0.37	P1<0.001** P2<0.001** P3> 0.05
BMD Z-score♦	-1.63±1.13	-1.63±0.51	-0.03±0.28	P1<0.001** P2<0.001** P3 >0.05

♦Kruskal walls test   ∞ ANOVA TEST   N= number
P1 between control and dialysis group   P2 between control and conservative group   P3 between dialysis group and conservative group   P>0.05 = not significant   P< 0.001**= highly significant   P<0.05 = significant
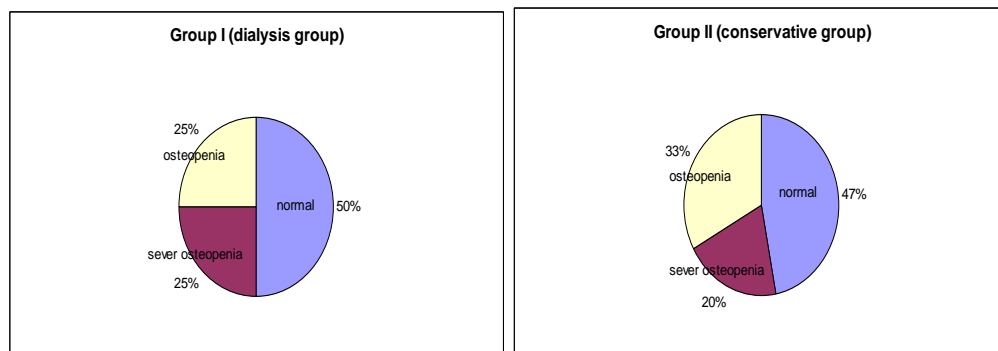
Fig. (1): Frequency of the osteopenia degree in group I& group II

Table (2): Clinical characteristics, biochemical variables, bone mineral density Z-score of group I classified according to iPTH values:

Parameter	Low iPTH (N=4) X $\pm$ SD	Normal iPTH (N=4) X $\pm$ SD	High iPTH (N=12) X $\pm$ SD	p- value
iPTH (pg/ml) ♦	86$\pm$49.81	219.7$\pm$46.5	1217.9$\pm$567	P1 <0.05* P2 <0.001** P3 <0.001**
Duration of dialysis (months) ♦	43.5$\pm$29.13	19.25$\pm$19.2	32.91$\pm$20.98	P> 0.05 for all
Weight (kg) ∞	31.62$\pm$7.8	33$\pm$13.8	35.25$\pm$9.56	P>0.05 for all
Height (cm) ∞	132.08$\pm$12.1	134.7$\pm$12.3	143$\pm$12.35	P>0.05 for all
BMI (kg/m2) ∞	16.4$\pm$2.2	16.89$\pm$4.57	17.4$\pm$3.0	P>0.05 for all
SBP (mmHg) ♦	115$\pm$5.7	118.7$\pm$11.8	133.3$\pm$13.7	P1 > 0.05 P2 <0.05* P3 > 0.05
DBP (mmHg) ♦	75$\pm$5.77	80$\pm$8.16	88.75$\pm$9.07	P1 >0.05 P2 <0.05* P3>0.05
Total calcium (mg/dl) ∞	10.62$\pm$0.27	9.37$\pm$0.61	7.89$\pm$0.85	P1 < 0.05* P2 <0.001** P3 <0.005**
Phosphorus (mg/dl) ∞	5.07$\pm$2.02	4.97$\pm$0.45	4.95$\pm$0.86	P>0.05 for all
ALP (U/L) ♦	72$\pm$31.02	207.75$\pm$37.86	332$\pm$117.21	P1 <0.001** P2 < 0.001** P3 <0.05
ß2-microglobulin (mg/l) ♦	3.8$\pm$1.17	4.97$\pm$2.33	8.72$\pm$3.25	P1 >0.05 P2 < 0.001** P3 <0.05*
BMD Z-score♦	-0.82$\pm$0.39	-0.72$\pm$0.35	-2.19$\pm$1.12	P1 >0.05 P2 <0.05* P3 <0.05*

♦ Kruskal wallies test   N= number
P1 between low & normal   ∞ ANOVA test
P2 between low & high   P3 between normal & high
P>0.05 = not significant   P<0.05 = significant

Table (3): Clinical characteristics, biochemical variables, bone mineral density Z-score of group II classified according to iPTH values:

Parameter	Low iPTH (N=4) Mean ± SD	Normal iPTH (N=2) Mean + SD	High iPTH (N=9) Mean + SD	p- value
iPTH (pg/mL) ♦	27.52+35.01	150+70.71	865+455.88	P1 >0.05 P2 <0.001** P3 <0.05*
Duration of renal impairment (months) ∞	42+15.49	43+16.97	18.44+13.48	P1 >0.05 P2 < 0.05* P3 <0.05*
Weight (kg) ♦	31.77+14.67	37+4.24	38.5+14.88	P>0.05 for all
Height (cm) ∞	129.11+29.11	162+28.2	141.5+31.68	P> 0.05 for all
BMI (kg/m2) ∞	16.05+1.36	17.94+2.24	17.78+1.96	P> 0.05 for all
SBP (mmHg) ∞	115+17.32	125+7.07	136.66+12.24	P1 >0.05 P2 < 0.001** P3 >0.05
DBP (mmHg) ∞	77.5+9.57	90+0.0	93.33+6.12	P1 >0.05 P2 < 0.001** P3 >0.05
Total calcium ∞ (mg/dL)	10.8+0.14	9.550+.07	8.13+0.41	P1 <0.001** for all
Phosphorus (mg/dL) ∞	5.5+0.91	4.95+0.07	4.96+0.72	P>0.05 for all
ALP (IU/L) ♦	57.5+9.57	225+106.06	211.77+86.44	P1 <0.001** P2 <0.001** P3 >0.05
ß2-microglobulin♦ (mg/l)	4.29+2.34	6.4+0.28	7.96+3.73	P> 0.05 for all
BMD Z-score ♦	-1.27+0.55	-0.80+0.14	-1.96+1.87	P>0.05

♦Kruskal wallies test   P1 between low & normal   ∞ ANOVA test
P1 between low & normal   P2 between low & high   P3 between normal & high   P3 between normal & high
P>0.05 = not significant   P< 0.001**= highly significant   P<0.05* = significant

Table (4): Correlation between DEXA Z score and clinical& laboratory data in group I&II

Parameter	group I		group II	
	R	**P**	**r**	**P**
Duration of renal impairment and dialysis	0.15	P >0.05	0.05	P >0.05
BMI (kg/m2)	-0.36	P >0.05	0.08	P >0.05
Weight (kg)	-0.19	P >0.05	-0.33	P >0.05
Height (cm)	-0,07	P >0.05	0.38	P >0.05
SBP (mmHg)	-0.65	P <0.001**	-0.31	P >0.05
DBP (mmHg)	-0.62	P<0.001**	-0.29	P >0.05
Total calcium (mg/dL)	0.78	P <0.001**	0.34	P >0.05
Phosphorus (mg/dL)	- 0.15	P >0.05	-0.19	P >0.05
ß2-microglobulin (mg/l)	-0.29	P >0.05	-0.23	P >0.05
iPTH (pg/mL)	-0.61	P <0.001**	-0.68	P <0.001**
ALP (IU/L)	a-0.48	P <0.05*	-014	P >0.05

r: Pearson correlation coefficient   P>0.05 = not significant
P< 0.001**= highly significant   P<0.05* = significant

Table (5): Correlation between iPTH and clinical& laboratory data in group I&II

parameter	group I		group II	
	r	*P*	*r*	*P*
BMI (kg/m2)	0.01	P >0.05	0.26	P >0.05
Duration of renal impairment and duration of dialysis	0.19	P >0.05	-0.29	P >0.05
Weight (kg)	-0.01	P >0.05	-0.28	P >0.05
Height (cm)	-0,01	P >0.05	-0.39	P >0.05
SBP (mmHg)	0.63	P <0.001**	0.55	P <0.05*
DBP (mmHg)	0.71	P <0.001**	0.56	P <0.05*
Total calcium (mg/dl)	-0.73	P <0.001**	-0.76	P <0.001**
Phosphorus (mg/dl)	-0.17	P >0.05	-0.32	P >0.05
ß2-microglobulin (mg/L)	0.79	P <0.001**	0.57	P <0.05*
ALP (IU/L)	0.81	P <0.001**	0.44	P >0.05

r: Pearson correlation coefficient   P>0.05 = not significant   P< 0.001**= highly significant
P<0.05* = significant

Conclusions: Maintenance of normal bone turnover may be important in prevention of irreversible bone disabilities & CVD. The preserving of normal BMD is a challenge for pediatric nephrologists so continuous and regular monitoring systems by combination of iPTH, serum Ca, ALP, β_2-microglobulin & BMD Z-score could be early, accurate and non invasive assessment of the skeletal system in children with CRF.

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10/24/2011

Positive urine culture of patients with urinary tract infection and antibiotic response of microbes isolated from the in great oil hospital of Ahvaz (ministry of oil)

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Abstract: Urine culture is a diagnostic test to detect bacteria in urine is performed and the identification of microbes that cause urinary tract infections. Urinary tract infection (UTI) is common in women and children, and all except the urinary tract, the urethra is sterile. E-coli are the most common germs. In a 1024 study by the antibiogram were 10,132 people suffering from urinary tract infection. Of these, 222 patients (21.7%) men and 801 women (78.3%) women with positive urine culture were 124 people (12.1%) were diabetic and 21 were women and 898 I and 103 patients (87.8%) were non-diabetic, 83.4% of patients in the outpatient and other inpatient wards have had positive urine cultures.
[Sarami Abdollah, Habibi Hasan, Bahrevar Naser, Alinejad Mastaneh**, Positive urine culture of patients with urinary tract infection and antibiotic response of microbes isolated from the in great oil hospital of Ahvaz (ministry of oil) .** Life Science Journal. 2011;8(4):236-238] (ISSN:1097-8135). http://www.lifesciencesite.com.

1. Introduction

Most people instead of the literal word of urinary tract infection bladder infection and a bladder infection that can cause inflammation and often feel the need to eliminate symptoms such as frequent urination and dysuria associated with, who used. This condition, also called cystitis among women 20 to 50 years old who are sexually active, is relatively common.

But bacteria can infect any part of the urinary tract. Urinary excretion of urine from the kidneys, which can be initiated through tubes called ureters to the bladder, where urine collects that, can be drawn. The urethra, which it was, ends with a short tube that carries urine out of the body.

When bacteria reach the intestines, the rectum and the urethra into the bladder is elevated, infection may occur. Bacteria can infect the bladder and cause cystitis or they can develop without symptoms, they are amplified. In each of these cases, the bacteria may travel up the ureters and kidneys are infected. Kidney infections are dangerous and may lead to premature delivery or other adverse effects.

1.1. Risk factors for urinary infection

A. Sex
B. Using birth control pills
C. Low estrogen levels
D. Mark cutter intermittent or persistent urinary tract
E. Diabetes and cancer, for example, due to reduced resistance to infections.
F. Urolithiasis

1.2. Symptoms of urinary tract infection (symptom)

Fever, chills, abdominal and leg cramps - frequent urination - an urgent need to evacuate

1.3. Signs (sign)

Dark urine or no residual particles in urine - a foul-smelling urine and blood in urine Urinary tract infections are divided into two groups: infections, superficial and deep infections. Superficial infections of the urinary tract or mucous surface coating affects more than ninety percent of these infections includes device.

While deep tissue was infections deep involvement of the kidneys, prostate and testicular cause. The deep infections, patients usually have high fever and bad general condition.

There are basically two categories of symptoms: symptoms of irritation and inflammation of the urinary tract infection varies depending on location, and general symptoms of infection in the body, including anorexia, nausea, vomiting, etc.. In addition, symptoms in children and adults vary, but common symptoms of urinary tract infections, there should be aware of all the people, the symptoms include:

a) *Dysuria and frequency due to bladder irritation and inflammation caused by infection, as well as low tolerance of the bladder in children and adults may sometimes be able to lead to urinary incontinence.*

b) *Discoloration of the urine cloudy or bloody urine, usually patients to panic, if superficial and simple bladder infection may also cause rupture of capillaries in the mucosal inflammation and blood-red blood and urine can be quite the treatment of infections immediately improved.*

c) *Fever, chills and nausea and vomiting in patients under one year of urinary tract infections and deep infections, kidney, prostate and testis in adults is common.*

d) Reduction and growth in children. Mothers in the presence of such symptoms should see a doctor to check for urinary tract infection. 5. Flank pain deep in the kidneys and severe infections with fever, chills and nausea and vomiting.

e) Difficulty in urination and urinary retention, bladder infections and prostate and urethra may be detected. The study was conducted in Finland, the risk of urinary tract infection in women of the fermented milk products like yogurt, fruit juices that are like seeds, berries, barberry, blueberries are used, compared with other women was very low.

The researchers say that so many Hungarians urinary infections in women by the bacteria in the intestinal tract there are, and foods mentioned above may affect the bacteria in the stool and prevent infection of the urethra into the urine. Also believe that the live bacteria in yogurt are replaced by harmful bacteria in the intestinal tract and causes the bacteria will move toward the bladder.

1. Materials and Methods

1.1. Objective:

To identify the most common microbes found in the urine of patients admitted in different wards of the hospital and their antibiotic resistance is the best treatment to begin. In a 1024 study by the antibiogram were 10,132 people suffering from urinary tract infection. Of these, 222 patients **(21.7%)** men and 801 women **(78.3%)** women with positive urine culture were 124 people **(12.1%)** were diabetic and 21 were women and 898 and 103 patients **(87.8%)** were non-diabetic, 83.4% of patients in the outpatient and other inpatient wards have had positive urine cultures. In part were 5. 6%, Outpatient Clinic 5.83%, children 2.7%, infant 5.1%, ccu% **1.2** had.

2. Results

2.1. The prevalence of bacteria

Most the age group was:

At risk over 65 years (23.4%)
30-34 years (12.1%)
38-39 years (10.81%)
Lowest in the 0-4 years (1.8%)

In this experiment, the error coefficient, $\alpha = 0.05$ There was no significant association between diabetes and urinary tract infection (Table1).

Table1. Contamination by bacteria and the percentage

No.	bacteria	No. Sample	%
1	E-coli	744	72.7
2	Klebsiella	151	14.8
3	C. p. Staphylococcus	39	3.8
4	Pseudomonas	24	2.3

Significant relationship between gender and urinary tract infection is even $\alpha = 0.01$ urinary tract infection in women is common agreement that the world of study.

2.2. Strains isolated on the basis of sensitivity and antibiotic resistance

A. **E-coli**
Highest Sensitivity to co-trimoxazole(100%)Nitrofurantoin (92.1) and amikacin (90.9) and the highest resistance to ampicillin (81.3) and nalidixic acid (39.7) respectively.

B. **Klebsiella**
The highest response in vitro to amikacin (87%) ciprofloxacin (81%) and ceftizoxime (75%) and highest resistance to cephalexin - Ampicillin and nitrofurantoin

C. **Pseudomonas**
Co-trimoxazole highest sensitivity (100%) and amikacin (75%) and highest resistance to nitrofurantoin (96%) nalidixic acid **(95.5),** and bacterim were 91%.

D. **Staphylococcus coagulase-positive**
Most sensitive to nitrofurantoin (% 91.2) - 73% and Bacterim- amikacin 75% and 100% greater resistance to tetracycline and "ampicillin (100%) were reported.

E. **Proteus**
Most laboratory response to amikacin, ciprofloxacin 100%, 90% and 100% more resistant to cephalexin and nitrofurantoin was 80%.

F. **Entero bacter**
Highest Sensitivity to amikacin, 100% - 100% nalidixic acid, ciprofloxacin and gentamicin % 90.5 and the highest resistance to tetracycline, and cephalexin was 50%.

G. **Citrobacter**
The highest sensitivity to ciprofloxacin and gentamicin 88% and the highest resistance to the bacterim and 33% nalidixic acid 33%.

H. Streptococcus

Highest susceptibility to amikacin and nalidixic acid 100% and highest resistance to ciprofloxacin were the 83.3% and Bacterium and 78. 3%.

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10/2/2011

Peripheral Blood Smudge Cells Percentage in De Novo CLL: A Comparison with Other Established Laboratory Prognostic Markers

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Abstract: Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in the Western world. The timing as to when a patient will require treatment is highly unpredictable. Thus, there has been great interest in identifying prognostic markers that can be used to distinguish patients who may have an aggressive form of CLL and might benefit from early intervention. Recently developed molecular markers are costly and often require a high level of technological expertise. Recent data give evidence for the prognostic relevance of peripheral blood smudge cells percentage in CLL. In our study, we investigated the prognostic potential of smudge cell percentage in 180 de novo CLL patients referring to the National Cancer Institute, Cairo University, Egypt and correlated the smudge cell percentage with established prognostic markers; including age, sex, ZAP 70 and CD 38 expression, pattern of marrow infiltration, Beta-2 microglobulin, Lactate dehydrogenase and lymphocyte doubling time. Our results showed that smudge cells percentage correlated inversely with markers of bad prognosis and correlated positively with hemoglobin and lymphocyte doubling time, which confer a better prognosis. We concluded that peripheral blood smudge cells percentage could be used as a simple, inexpensive and independent prognostic marker that can predict the outcome and survival in de novo CLL patients.

1. Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most common type of adult leukemia in Western countries. Each year, 15,000 to 19,000 individuals are diagnosed with this disease in the United States [1]. It shows a remarkable heterogeneity, with some patients having an almost normal lifespan, while others having only several years of survival in spite of intensive chemotherapy [2]. With the widespread use of automated blood cell counters and flow cytometric immunophenotyping, up to 80% of patients with CLL are diagnosed at an early stage of the disease [3]. However, approximately 50% of patients with early stage disease have accelerated disease progression, while many of the rest survive for more than a decade without even requiring therapy [4]. The prognosis of CLL patients had been accordingly linked to many factors, the most important of which are the immunoglobulin heavy chain gene mutation status, leukemic cell expression of CD38 and ZAP 70 associated protein, chromosome analysis by fluorescence in situ hybridization (FISH) and others, which identify patients with biologically aggressive disease and shorter survival time [5]. Unfortunately, most of such recently developed prognostic tests are costly and often require a high level of technologic expertise [6] and despite this progress, many patients have limited access to these laboratory tests, which require highly sophisticated instruments and a high degree of technical expertise and are costly to perform. In addition, because of the technical complexity of some of the assays, a considerable effort is necessary to ensure reproducibility between the laboratories [7-9].

Smudge cells are ragged lymphoid cells found mainly in peripheral blood smears of CLL patients and which are ruptured during smear preparation of virtually all CLL patients. For nearly a century, smudge cells were thought to be merely an artifact of slide preparation [10]. The interpatient variability in the percentage of smudge cells on a peripheral blood smear is well recognized and is independent of the absolute lymphocyte count and also of the staining technique [11].

It has been discovered that smudge formation is related to the content of the cytoskeletal protein vimentin present in leukemic cells [12]. It was shown that CLL patients with high vimentin content have a low percentage of smudge cells. In addition, it was found that high vimentin expression is associated with a

shortened time to initial therapy in early-stage CLL [5]. Because vimentin expression was found to be a prognostic factor in early-stage CLL, we hypothesize that there could accordingly be an association between smudge cell percentage and prognosis in patients with CLL.

Aim of work:

This study was performed to investigate the possible prognostic implication of the percentage of peripheral blood smudge cells, as an easy and cheap technique in de novo CLL patients, through comparing it with other established laboratory prognostic tests.

2. Patients and Methods:

Our study included 180 CLL patients referring from the Medical Oncology Department to the Clinical Pathology Department, Egyptian NCI, during the period from May 2007 to Dec 2009. These un-selected patients were newly diagnosed after fulfilling the diagnostic criteria of CLL according to the National Cancer Institute-sponsored Working Group [NCI-WG] guidelines [13]. At diagnosis, these patients were subjected to complete blood count (CBC), bone marrow aspiration/biopsy (BMA/BMB), flowcytometric immunophenotyping (IPT), serum beta-2 microglobulin (ß2M), serum lactate dehydrogenase enzyme (LDH) and smudge cells percentage in peripheral blood smears. Lymphocyte doubling time (LDT) was concluded after two check points evaluating the absolute lymphocytic count; the 1st when patients achieved maximum response of treatment (CR-PR-SD) and the 2nd after 12 months. Correlations between smudge cells percentage and all of the above-mentioned laboratory prognostic tests were explored.

Hemograms were done on Cell-Dyn-3700 automated cell counter. Absolute lymphocytic counts (ALCs) were revised and calculated from peripheral blood smears. Leishman-stained BMA smears were examined for percentages of mature (± immature) lymphocytes. Bone marrow biopsy (BMB) cores were fixed, decalcified, processed, embedded, sectioned and H&E-stained according to the well known routine techniques [14]. BMB sections were histologically examined for lymphoid infiltration patterns.

CLL diagnosis was confirmed by immunophenotypic analysis performed on Partec-III flowcytometer using a panel of McAbs [Dako, Denmark and Santa Cruz Biotechnology, USA] including CD3, CD4, CD20, FMC7, HLA-DR, and Kappa light chain conjugated with fluorescein isothiocyanate (FITC); CD5, CD23, CD10, CD22, CD79b, CD8 and Lambda light chain conjugated with phycoerythrin (PE) and CD19 conjugated with phycoerythrin-Cyanine 5(PE-Cya5). For all of these markers, results were expressed as a percentage of cells showing positive surface expression (when the marker was identified in ≥20%). As prognostic markers, the cytoplasmic expression of ZAP-70 and the surface expression of CD38 were further determined in (CD5/CD19) positive B-CLL cells by using anti-ZAP-70-FITC and anti-CD38-PE McAbs [BD Bioscience, Mountain view, California]. ZAP-70 expression was considered as positive when identified in ≥20% of the gated (CD5/CD19) positive B-cells [15], while CD38 expression was considered as positive when identified in ≥30% of the gated (CD5/CD19) positive B-cells [16]. Serum ß2M was estimated on the fully automated Axyum, Abbott, USA, by micro-ELISA technique. ß2M ≤ 3.4μg/ml was considered normal and ß2M > 3.4μg/ml was considered elevated. LDH was estimated on Synchron CX-9-PRO, Beckman Coulter, Inc., USA, by the spectrophotometric technique. LDH ≤ 480 U/L was considered normal and LDH >480 U/L was considered elevated.

Assessment of Pb smudge cells percentage:

From an EDTA blood samples, smears were freshly prepared by the manual wedge method using a clean glass-slide with frosted edges. For each case, two stained peripheral blood smears were examined simultaneously in a blinded manner by two hematopathologists and the mean of their readings was obtained. Smudge cells were identified a broken cells with disrupted nuclear membrane and without intact cytoplasm; accordingly, a total of 300 intact lymphoid cells and smudge/basket cells were counted on each smear. Then, the percentage of smudge cells was calculated through dividing the smudge/basket cells count by the sum of intact lymphoid cells and smudge/ basket cells counts times 100.

Statistical methods:

Data was analyzed using SPSSwin statistical package version 17 (SPSS inc., Chicago, IL). Chi-square test was used to examine the relation between qualitative variables. For quantitative data, comparison between two groups was done using Mann-Whitney test (non parametric t-test).

Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA). Spearman-rho method was used to test correlation between numerical variables. P-value<0.05 was considered significant.

3. Results:

Our study was carried out on 180 patients with de novo CLL, 126 males (70%) and 54 females (30%). Their age ranged from 35 to 79 years with a median of 57 years and a mean of 57.9 ± 8.3 years. Table (1) describes the data of the numerical laboratory parameters of the 180 patients included in the study. It shows that the peripheral blood smudge cells percentage was ranging from 3 to 75 with a median of 27 and a mean of 30.1 ± 19.7

Table (1): Descriptive data for the numerical laboratory parameters of the 180 patients included in this study

Parameter	Patients number = 180				
	Median	Minimum	Maximum	Mean	±SD
Hb (gm/dl)	10	5.1	15.3	9.8	2.5
TLC (x10³/Cmm)	80.8	6.6	514	105	89.1
Pb (L) %	83	38	99	80.1	13.8
Pb (IL) %	0	0	12	1.2	2.9
Pb ALC (x10³/Cmm)	66.23	5.02	493.44	90.08	82.55
BMA (L) %	79	12	97	75.1	15.8
BMA (IL) %	0	0	9	1	2.1
Serum B2M (mg/L)	3.7	1.6	7.3	4	1.5
Serum LDH (U/L)	572	282	1538	632	261
Pb SCs %	27	3	75	30.1	19.7

Hb= hemoglobin**, TLC**= total leucocytic count**, L**= lymphocyte, **IL**= immature lymphocyte**, ALC**= absolute lymphocytic count, **BMA**= bone marrow aspiration, **ß2M**= beta 2 microglobulin, **LDH**= lactate dehydrogenase, **Pb**= peripheral blood, **SCs**= smudge cells

Figure (1) shows a peripheral blood smear with high smudge cells percentage (70%) and figure (2) shows another smear with low smudge cells percentage (4%) in 2 de novo CLL patients.
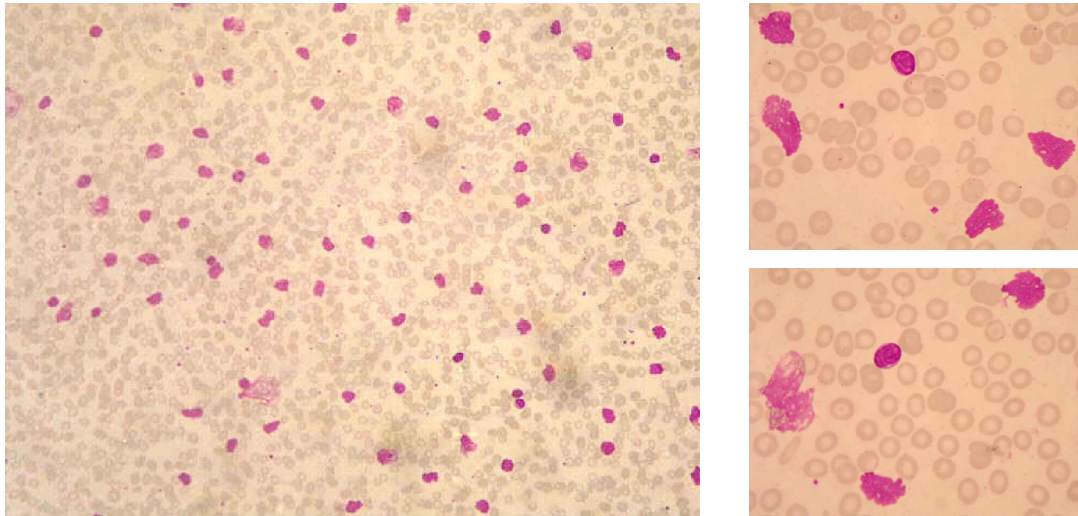
Fig. (1): Peripheral blood smear shows high smudge cells percentage (70%) in de novo CLL patient, Leishman's stain, x20, x100 and x100, respectively.

Table (2) shows the results of categorical laboratory parameters obtained in this study. They included immunophenotyping for common prognostic markers [namely ZAP 70 and CD38], LDT and BM biopsy infiltration pattern. On the flowcytometer, 83 patients (46.1%) showed positive ZAP 70 expression, 58 patients (32.2%) showed positive CD38 expression and 33 patients (18.3%) showed ZAP70/CD38 co-expression. Twenty five patients (13.9%) were ZAP 70 negative and CD38 positive, 50 patients (27.8%) ZAP 70+ CD38- and 72

patients (40%) were both ZAP70 and CD38 negative. On BM histological examination, 95 patients (52.8%) showed diffuse pattern of lymphoid infiltration, 11 patients (6.1%) showed nodular pattern and 74 patients (41.1%) showed mixed pattern of lymphoid infiltration. Out of the 180 patients, 61 were missed and could not

be followed for more than 12 month, while, 119 patients could be followed for estimating the lymphocyte doubling time (LDT). Thirty nine patients (32.8%) had LDT less than 12 months and 80 ones (67.2%) had LDT more than 12 months.

Fig. (2): Peripheral blood smear shows low smudge cells percentage (4%) in de novo CLL patient, Leishman's stain, x20, x100 and x100, respectively.
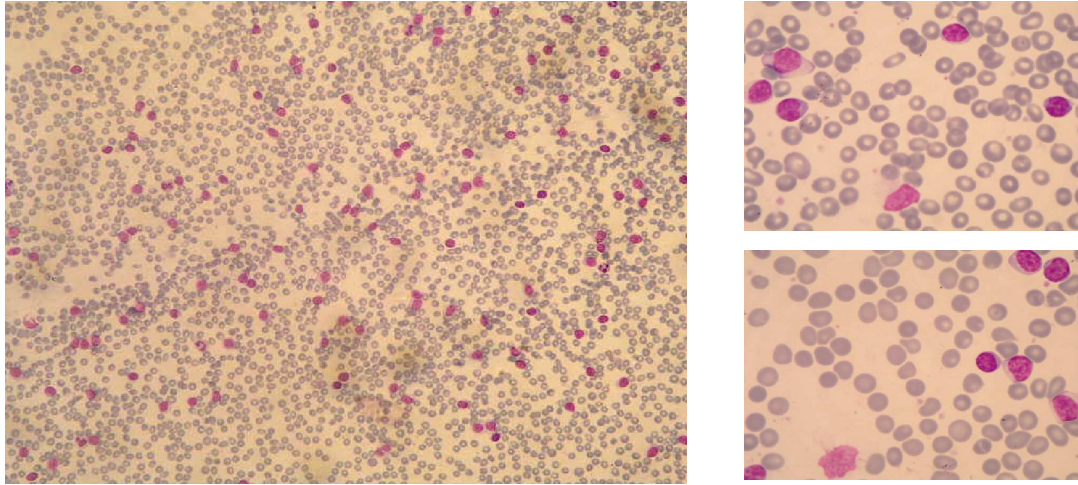
Table (2): Results of categorical laboratory parameters of the 180 patients including: (a) IPT for prognostic markers (b) BM biopsy pattern and (c) LDT.

Parameter		Patients number = 180	
		Positive	Negative
(a)	CD38 expression	58 (32.2 %)	122 (67.8%)
	ZAP-70 expression	83 (46.1%)	97 (53.9%)
	CD38/ ZAP-70 co-expression	33 (18.3%)	147 (81.7%)

		Diffuse	Nodular	Mixed
(b)	BM biopsy infiltration pattern	95 (52.8%)	11(6.1%)	74(41.1%)

		Patients number (valid) = 119*	
(c)	Lymphocyte Doubling Time	< 12 month	≥ 12 month
		39 (32.8%)	80 (67.2%)

IPT= immunophenotyping, **BM**= bone marrow and **LDT**= lymphocyte doubling time
*Out of total 180 patients, 119 were valid to be followed and 61 were missed and could not be followed

Peripheral blood smudge cell % was lower with high ZAP 70 expression, high CD38 expression, high ZAP70/CD38 coexpression and diffuse pattern of marrow infiltration (P<0.001 for each).

Peripheral blood smudge cell % was negatively correlated with Beta-2 microglobulin level (P=0.029), LDH level (P=0.002), bone marrow lymphocyte % (P<0.001) and bone marrow immature lymphocytes % (P=0.008). It was also negatively correlated with TLC (P=0.035) and peripheral blood absolute

lymphocytic count (P=0.039).

Peripheral blood smudge cells % was positively correlated with Hb (P<0.001) and LDT (P<0.001). There was no correlation with peripheral blood lymphocyte % (P=0.287) peripheral blood immature lymphocyte % (p=0.061), age (P=0.82) or sex (p=0.566). Table (3) shows different correlations between peripheral blood smudge cells percentage and numerical laboratory parameters included in this study.

Table (3): Correlations between the peripheral blood SCs percentage and other numerical laboratory parameters

		Peripheral blood					BMA		Serum	
		Hb	TLC	L %	IL %	ALC	L %	IL %	ß2M	LDH
Pb SCs	r-value	0.406	- 0.158	- 0.08	- 0.140	- 0.154	- 0.288	- 0.198	- 0.374	- 0.322
%	*p*-value	**0.001**	**0.035**	0.287	0.061	**0.039**	**0.001**	**0.008**	**0.001**	**0.001**

Pb= peripheral blood, **SCs**= smudge cells, **Hb**= hemoglobin, **TLC**= total leucocytic count, **L**= lymphocyte, **IL**= immature lymphocyte, **ALC**= absolute lymphocytic count, **BMA**= bone marrow aspiration, **ß2M**= beta 2 microglobulin, **LDH**= lactate dehydrogenase

4. Discussion:

The appearance of smudge cells on a peripheral-blood smear is a characteristic feature of CLL, with virtually all patients demonstrating at least some degree of smudging [5]. Since their description in 1896 by **Gumprecht** on blood smears of patients with lymphocytic leukemia, smudge cells which are also known as Gumprecht or basket cells, were thought to be just an artifact of slide preparation resulting from the fragility of CLL cells [17]. In 1959 **Heinivaara** made two important observations, first that the percentage of smudge cells was not dependent simply on the degree of lymphocytosis or the slide stain method and second that smudging appeared to be patient specific [5].

Studies demonstrated that smudge cells formation is inversely correlated with CLL B cell content of vimentin, a cytoskeletal protein critical for rigidity and integrity of lymphocytes [17]. High vimentin expression has been shown to be associated with poor prognosis and metastatic potential in breast [18] and colon cancer [19].

In the present study, we hypothesized that the calculated smudge cells percentage on a blood smear would have prognostic value in CLL, based on the studies that proved that the percentage of smudge cells inversely correlates with vimentin expression, which by its turn was proven to confer bad prognosis [17].

While clinical staging systems have been used to stratify patients into risk categories, they lack the ability to predict disease progression or response to therapy. Recent advances in the understanding of the biology of CLL have led to the identification of numerous cellular and molecular markers with potential prognostic and therapeutic significance. We correlated the percentage of peripheral blood smudge cells with such established markers of prognosis. Several studies demonstrated that age and gender [20], ZAP 70 [21,22], CD38 [23,24], Beta-2 microglobulin [25], serum lactate dehydrogenase [26], lymphocyte doubling time [27] and bone marrow infiltration pattern [22] are important prognostic factors in CLL.

Our results showed that lower smudge cells percentage was correlated with markers of bad prognosis, such as ZAP 70 expression (P<0.001), CD38 expression (P<0.001) and ZAP70/CD38 coexpression (P<0.001). We agree in this respect with the results reported by **Nowakowski et al.,** [17] and **Johansson et al.,** [6]. It was also correlated with high B-2 microglobulin level (P=0.029), high LDH level (P=0.002), diffuse pattern of marrow infiltration (P<0.001), high TLC (P=0.035), high peripheral blood absolute lymphocytic count (P=0.039), high bone marrow lymphocyte % (P<0.001) and high bone marrow immature lymphocyte % (P=0.008).

On the other hand, higher peripheral blood smudge cells percentage was correlated with higher Hb (P<0.001) and lymphocyte doubling time of more than 12 months (P<0.001), which confer a better prognosis in CLL patients.

To the best of our knowledge, no other studies correlated peripheral blood smudge cells percentage with Beta-2 microglobulin, LDH level, pattern of marrow infiltration, absolute lymphocytic count, immature lymphocyte % or lymphocyte doubling time.

Further investigation to define a precise cut off for the peripheral blood smudge cells percentage might be a helpful parameter to discriminate the lower bad prognostic values from the higher good ones, which might have different impacts on the clinical outcome.

We conclude that peripheral blood smudge cells percentage, estimated by microscopic examination of routine blood smears, could be used as a simple, inexpensive and independent prognostic marker that can predict the outcome and survival in de novo CLL patients.

Acknowledgements

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10/24/2011

First record of *Benedenia sciaenae* (Monogenea: Capsalidae) infecting the brown-spotted grouper fish *Epinephelus chlorostigma* (Family: Serranidae) from the Red Sea in Egypt

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Abstract: *Benedenia* (Capsalidae) is a genus of important oral and cutaneous fluke parasite of aquarium, cultured and marine fish. In the present study, the morphological and morphometric characterization of *Benedenia sciaenae,* a monogenean parasite infecting the gills of the brown-spotted grouper fish *Epinephelus chlorostigma* were described by means of light microscopy as a first description from *Epinephelus chlorostigma*. 215 out of 290 (74.1%) fish samples were found to be infected with this ectoparasitic capsalid causing pathogenic and epizootic events. The adult worm is flattened, elongated with an anterior adhesive organ enclosing two anterolateral adhesive structures, each one possesses three lobes which aids for adhesive secretions while the enlarged posterior end enclosing haptor. The adult worm measured about 0.52 -0.67 (mean 0.59 ±0.03) mm in total length and 0.33 – 0.49 (mean 0.38 ±0.02) mm in width. Haptor width measured 0.25-0.29 (mean 0.26 ± 0.02) mm; its hard parts consist of two pairs of hamuli and the accessory sclerites. The anterior hamulus measured 0.027-0.034 (mean 0.31±0.002) mm long while the posterior one measured 0.030-0.040 (mean 0.036±0.002) mm and each of the accessory pieces measured 0.032-0.044 (mean 0.040±0.002) mm long. Results showed that the general morphology of the present *Benedenia* sp. resembles that of *B. sciaenae* described previously in Turkeys from *Argyrosomus regius* fish host with the dimensions of body more or less similar. Also, there were significant correlations ($P \leq 0.05$) between fish length, weight and parasite abundance per fish. Number of monogeneans was increased with host size and age to fish of intermediate length and weight, and then it decreased probably because changes in size of gill filaments affect their attachment capability, enhancing the possibility of being detached by respiratory currents.
[Kareem Morsy; Sayed Abdel-Monem; FathyAbdel-Ghaffar; Abdel-Rahman Bashtar; Ali Al-Ghamdi and Rania Abdel-Gaber **First record of *Benedenia sciaenae* (Monogenea: Capsalidae) infecting the brown-spotted grouper fish *Epinephelus chlorostigma* (Family: Serranidae) from the Red Sea in Egypt].** Life Science Journal. 2011; 8(4):245-252] (ISSN: 1097-8135). http://www.lifesciencesite.com.

Key words: *Benedenia sciaenae* – Monogenea – Capsalidae - *Epinephelus chlorostigma*- Red Sea – Light microscopy.

1 Introduction

Monogenea is a class of platyhelminthes parasitic mostly on external surfaces and gills of freshwater and marine fishes (Whittington *et al*., 2004). Boeger and Kritsky (2001) recognized 53 families based on morphological characters. Most of monogeneans are highly host specific (Hargis, 1955 and Lawler, 1981), which aids in the specific identification of worms from a particular host. Benedeniide monogeneans belonging to the family Capsalidae are pathogenic to fish under culture (Thoney and Hargis, 1991; Chisholm *et al.,* 2004). Some of these large capsalids are known to be concealed on their hosts, a phenomenon first reported by Van Beneden (1856**)** for *Benedenia sciaenae* from *Sciaena quila* (Sciaenidae) of the Belgian coast. Several taxonomic and morphological studies have provided some knowledge about geographical distribution, host range of Benedeniinae (Whittington and Horton, 1996; Egorova, 1997; Whittington *et al*., 2001a). Tokşen *et al.* (2007) studied the Infestation

of *Benedenia sciaenae* van Beneden, 1856 of Cultured Meagre (*Argyrosomus regius* as a new host record) in Turkeys, they described this parasite belonging to Benedeniinae due to the presence of one pair of accessory sclerites and two pairs of hamuli. *Benedenia seriolae*, has been a longstanding pathogen of species in intensive culture in Japan (Whittington *et al*., 2001b). Approximately 20% of the total production costs for farmed Carangidae species in Japan are spent to control *B. seriolae* (see Ernst *et al*., 2002). Ogawa *et al.*(1995) study the development, infection rate and pathogenicity of *Benedenia hoshinai* which infects the Japanese striped knifejaw *Oplegnathus fasciatus* cultured in a net pen in Nagasaki, he found that the intensity of infection was estimated to be 80-220 worms per fish causing ulcers at the infection sites which may decrease the growth and productivity of the infected fish leading to death. Also he found that the number of worms on the host sharply decreased as they grew bigger than 1.5 mm long, suggesting that most small

worms detached themselves from the host before reaching that size.

The present investigation aims to study the prevalence of natural infection with monogenean parasites in addition to their morphological and morphometric characterization by means of light microscopy. Also, the relationship between length, weight of infected fish and the extent to which they are parasitized by this monogenean was studied to determine if the frequency and intensity of parasitism changed over time.

2 Material and Methods

A total of 290 specimens of *Epinephelus chlorostigma* (Forsskal, 1775) fish (family: Lutjanidae Valenciennes 1828) (size range: 10 – 25 cm, mean 18.5 ± 7.15cm; body weight 100 – 250 g, mean 205 ± 20 g) were caught from the coasts along Gulf of Suez and Hurghada city, Red Sea, Egypt. Samples were obtained at irregular intervals in 2010-2011. Fish were identified according to Randall (1983) and their modern names follow Froese and Pauly (2011). They were immediately transported in water tanks to the parasitology laboratory, Zoology Department, Faculty of Science, Cairo University. To prevent the loss of mobile and temporary ectoparasites, the captured fish were kept alive in aquaria filled with the same water source and examined within few hours. Firstly, the characteristics of the host such as standard length and weight were noted. Skin surface, fins and gills were then examined by naked eyes and with the help of dissecting microscope for any attached parasites, lesions or external changes. After removing opercula and exposing gill arches, each gill was removed carefully from the fish, immersed in normal saline to remove any excess gill mucus. Monogenean parasites were recovered with a Pasteur pipette using a dissecting binocular microscope. The monogeneans were fixed in 4% formalin and the worms were washed with distilled water to remove excess fixative. Worm identification was confirmed by mounting specimens on slides in drops of ammonium picrate glycerine under cover slips, and examining hard parts using light microscopy. For permanent whole mount preparation, some of the fixed and flattened specimens were stained with acid carmine followed by washing in a ascending alcohol series and then cleared in clove oil, xylene and then mounted with Canada balsam (Ergens and Dulmaa, 1969). For each manogenean parasite, the sclerotized parts of the haptor were drawn using Camera Lucida and measured using an ocular micrometer calibrated against a stage micrometer slide according to Gussev, 1985 (Bychovskaya –Pavlovskaya *et al.*, 1962). Ten

specimens were measured for the range and the mean ± standard deviation (SD). Prevalence, mean abundance and measurements followed the guidelines of Bush *et al*. (1997). In order to determine if number of parasites present was related to fish length and weight, correlation test was performed to determine if there is any association between these parameters and parasite load. The total and lengths of the fish were measured in centimeter (cm) using a measuring board. Fish samples were weighed to the nearest gram (g) using weighing balance. To satisfy the assumption of the statistical analyses used, all infection data number of parasites per length and weight of host were analyzed by t test at 95% confidence level to achieve homoscedasticity or linearity. Values of p<0.05 were considered as statistically significant.

3 Results

Two hundred and fifteen (215) Out of 290 *E.* chlorostigma fish with a prevalence of 74.1% were infected with the ectoparasitic *Benedenia* sp. The parasite have flattened, elongated symmetrical body tapering anteriorly and enlarging to haptor level posteriorly (Figs. 1,2). The total body length was 0.52 -0.67 (mean 0.59 ±0.03) mm while its width at mid level of testes measured 0.33 – 0.49 (mean 0.38 ±0.02) mm. Two pairs of eye spots were present anterior to pharynx (Fig. 3), the anterior pair being smaller than the posterior one. Pharynx measured about 0.08 – 0.21 (mean 0.10 ±0.02) mm in diameter. Two ellipsoidal equatorial testes were observed at the mid part of the body, each measured 0.12 – 0.18 (mean 0.16±0.02) mm in diameter. Ovary pretesticular and oval in shape. Vetellaria follicular occupying almost the entire available space of the body proper. Two anteroventral attachment organs were observed anteriorly and measured 0.046 – 0.068 (mean 0.06 ± 0.003) mm for each (Fig. 3). Posteriorly, the worm is armed with a disc-like haptor supplied with hooks (Figs.1,2). These haptor was oval, aseptate, the basic arrangement comprises a saucer-shaped attachment organ measured 0.25-0.29 (mean 0.26 ±0.02) mm and armed with three pairs of median sclerites that are usually large and 14 small hooklets at the periphery of the haptor proper and a thin membranous marginal valve around the edge (Figs. 3,4). Median sclerites comprise a central pair of accessory sclerites located anteriorly and two pairs of ventrally directed hamuli as anterior and a posterior pairs (Figs.4,5). Accessory sclerites (1 pair) stout, scoop-shaped with pointed tips directed anteriorly measuring 0.032-0.044 (mean 0.040±0.002) mm long (Fig. 6). Anterior hamuli long, slender, curved posteriorly, but straight at anterior end and measure 0.027-0.034 (mean

0.31±0.002) mm long. Posterior hamuli slender, with smoothly recurved posterior terminus measuring 0.030-0.040 (mean 0.036±0.002) mm . Marginal hooklets arranged radially in haptor region and measured 0.002-0.005 (mean 0.003±0.001) mm (Fig. 7). Line diagram of the worm body, haptor, sclerites, hamuli and hooklets was shown in (Fig. 8).

The highest prevalence of infection was obtained in the examined fish species within the standard length (15 – 20cm) and weight (70 – 95g) ranges as shown in (Figs. 9, 10). The parasites number was lowest in the smallest and lightweight fishes (13.0 – 17.0 cm, 20 – 60g). An increase was recorded in fishes having 18.00 – 23.00 cm body length and 62 – 85g weight, followed by a decline in larger and heavyier fishes (more than 25 cm, 100 g). Overall, the results of this experiment supported the hypothesis that increased fish length and weight are correlated with an increased number of parasites per fish.

Taxonomic summary
Type-host: *Epinephelus* chlorostigma (Family: Serranidae).
Site of infection: infecting the gills of fish host.
Type-locality: Gulf of Suez and Hurghada city, Red Sea, Egypt
Prevalence: 290 fish samples were examined for monogenean parasites, 215 (74.1%) fish were infected.
Materials deposited: Slides were deposited at Zoology Department museum, Zoology Department, Faculty of Science, Cairo University, Egypt.
Etymology: The specific genus name relates to the phenomenon first reported by Diesing (1858) for *Benedenia* sp.

4. Discussions
Gill filaments and gill lamellae of the host fish act as an important and predominant source of food and provide a relatively safe shelter for gill monogeneans as well as other ectoparasites dwelling the surface of the host gills. The gill apparatus possesses numerous attachment sites that accommodate the haptoral elements of the invading monogenean worms and provide these parasites with components of the gill tissues (blood, epithelial cells or mucous). The Benedeniinae are the largest of nine capsalid subfamilies, includes genera with an aseptate, apapillate haptor and a pair of discrete testes. Thirty three nominal benedeniine genera were previously reported worldwide and no records of Benedeniinae worm in Egypt, these are *Benedenia* (Diesing, 1858); *Allobenedenia* (Yamaguti, 1963); *Allometabenedeniella* (Velasquez**,** 1982); *Dioncopseudobenedenia* (Yamaguti 1965);

Lagenivagino pseudobenedenia (Yamaguti, 1966); *Oligoncobenedenia* (Yamaguti, 1965); *Pseudallobenedenia* (Yamaguti, 1966) and *Tareenia*, (Hussey, 1986) *Allobenedenia* and *Allometabenedeniella* were transferred to the *Trochopodinae* by Price, 1936; *Menziesia* Gibson, 1976; *Benedeniella* Johnston, 1929, *Calicobenedenia* Kritsky and Fennessy, 1999 and *Trimusculotrema* Whittington and Barton, 1990 are considered to belong in *Entobdellinae* Bychowsky, 1957. Morphological characteristics of the described worm here resembled that of the monogenean, *Benedenia* sp described by Tripathi (1957) who reported that heavily infested fish showed excessive mucus secretions on the body surface. The morphology of the anterior ends of the majority of benedeniine genera is similar because they bear characteristic anterior attachment organs (Whittington and Barton, 1990; Whittington and Horton, 1996).

Whittington *et al.* (1994) have reported that the anterolateral region of each attachment organ is divided into three adjacent zones. The morphology of the disc – shaped, sucker-like anterior attachment organs suggests that these structures are capable of generating suction. the arrangement of the three adhesive zones on each side of the head among monogeneans appears to be a relatively common morphological feature (Kearn, 1994).

Among the capsalids, the number of the anterior attachment zones on each side of the head could be of taxonomic importance. The basic, cup-like suctorial haptor of adult capsalids help in the secure attachment to rough surfaces, this haptor is composed of one pair of median sclerites and two pairs of ventrally directed hamuli, an anterior and a posterior pair. There is evidence from some species that median sclerites and hooklets do effect mechanical attachment of the parasite to host tissue, but in other species, the capacity of the capsalid haptor to generate suction has contributed appreciably to their expansion across a wide range of host surfaces (Kearn, 1994). The arrangement of musculature external to the haptor and the way these elements interact play a major role in how the haptor generates suction. Kearn (1994) concluded that the presence of a marginal valve is critical to maintaining suction. There is unsubstantiated evidence that *Trimusculotrema* species, which lack a marginal valve and also lack large median sclerites in the haptor, may use an adhesive to attach to host ray skin. Heavy infection of *B. epinepheli* caused not only haemorrhagic and abrasive lesions, but also mortalities in cultured marine fish due to severe necrosis of the gill tissues possibly resulting in suffocation (Leong, 2001).The present described species resembles *Benedenia sciaenae* Van Beneden,

1856 reported from cultured meagre (*Argyrosomus regius*) in Turkey in its general morphology but with nearly large dimensions in the present study.

From the present study, the absence of parasites on small sized fish may be due to the small size of scales in fishes, where the parasites cannot maintain proper hold onto the body of the host. Moreover, a decrease in the parasites burden seen in the very large fishes is observed. One reason for this might be the random selection of specimens that is; more of the juveniles and sub-adults were examined as compared to the adult fishes. Another reason for it may be due to the development of acquired immunity as reported by (Etchegoin and Sardella, 1990; Tasawar and Khurshid, 1999; Tasawar and Naseem, 1999; Tasawar and Shazad, 2001; Akinsanya *et al*., 2007). However, as reported by Roberts *et al.,* (2000), bigger fishes provides larger surface area for infection than smaller ones. It is therefore a plausible explanation that the big fishes provide a good ground for the parasites to multiply over time. An increase in size is a reflection of increase in length which is considered a measure of age (Boxshall, 1974; Torres *et al*., 1977; Kabata and Whitaker, 1981; Etchegoin and Sardella, 1990). The higher parasitism observed in sub-adults over juveniles might be as a result of change in diet of the fish from weeds, seeds, phyto and zooplanktons as juveniles to insect larvae, snails, crustaceans, worms and fish as adulthood is attained (Anderson & Gordon, 1982). Another possible reason for the higher parasitism in the sub –adults over juveniles may be that of activity. The sub-adults as expected would be more active than the juveniles and probably even adults. As such, are able to compete better for food than the other age groups which mean that more contact with food and hence a higher tendency of getting infected with parasites.

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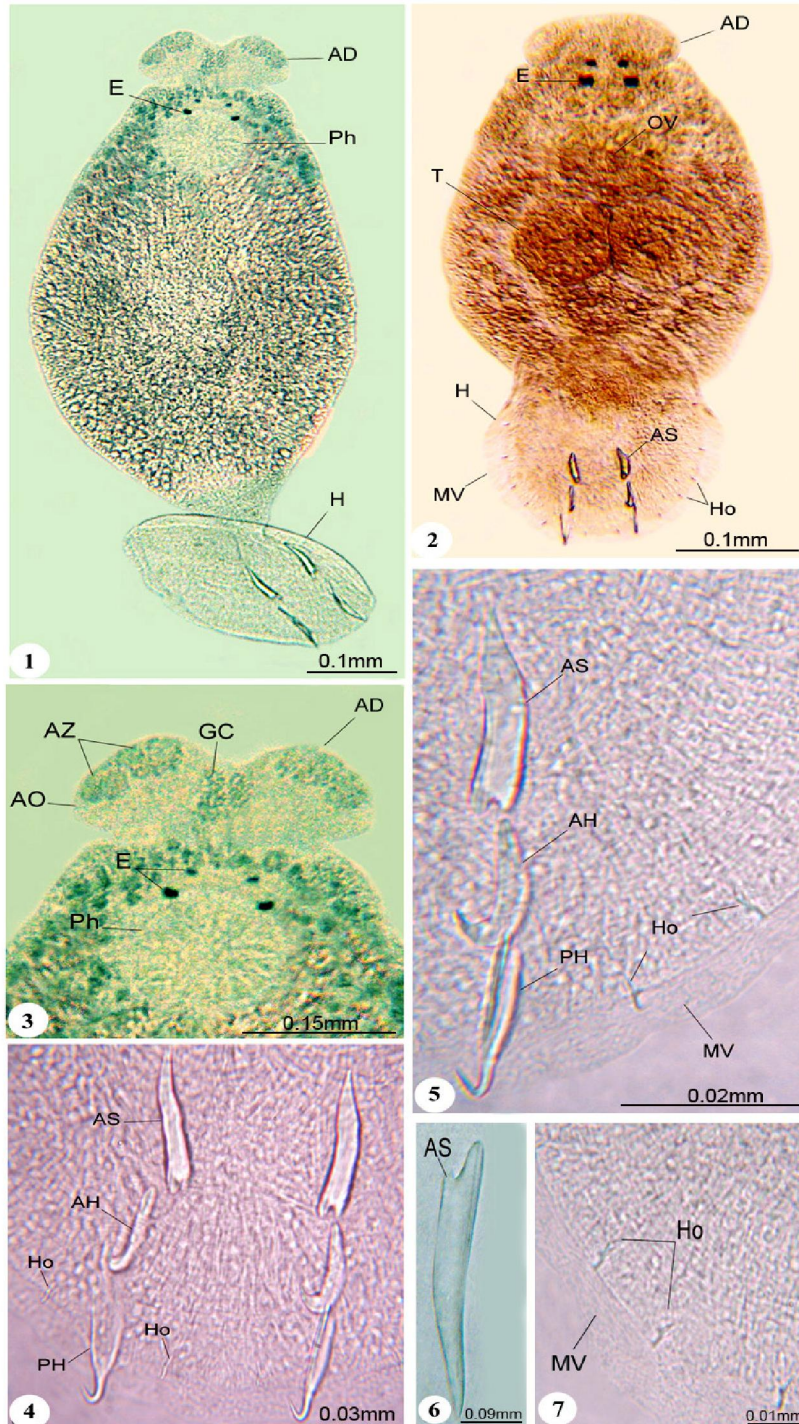
Figs.1-7 Photomicrographs of the adult *Benedenia sciaenae* showing: *1*, *2* the adult worm with a flattened body consist of the anterior adhesive organs (AD), two pairs of eyes (E), large pharynx (Ph), the haptor region (H), two large equatorial testes (T) and a pretesticular ovary (O). The haptor (H) is bordered by a marginal valve (MV) and contains one pair of accessory sclerites (AS) and two pairs of hamuli. The periphery of haptor is supported by a large number of hookelts (Ho). *3* A high magnification of the anterior body region of the worm showing the anterior attachment zones (AZ), each consists of anterior attachment organs (AO). A zone of gland cells (GC) also seen between these organs. *4, 5* High magnifications of the haptor region showing haptoral elements as one pair of accessory sclerites (AS) located anteriorly, two pairs of hamuli, an anterior pair (AH) and a posterior one (PH).

Haptor is bordered by a marginal valve (MV) supported with hooklets (Ho). *6* The accessory sclerite (AS). *7* The marginal valve (MV) with tl
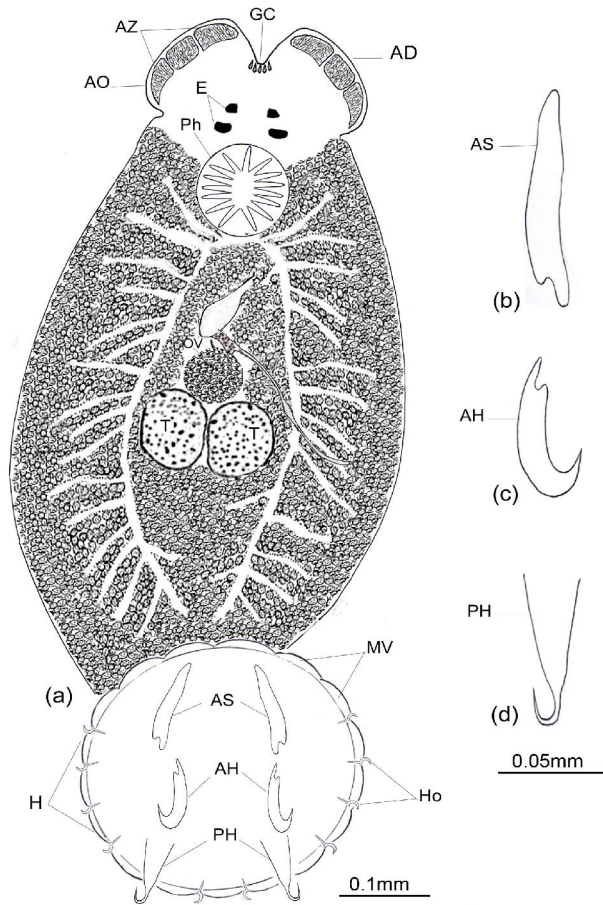
Fig.8. Schematic drawings of the adult Benedenia bohari showing the structure of its body. *a* The adult worm with anterior adhesive organ (AD) containing two pairs of eyes (E), the anterior attachement zones (AZ) and organs (AO) with its gland cells (GC) and pharynx (Ph). The haptor (H) is composed of one pair of accessory sclerites (AS), two pairs of hamuli anterior (AH) and posterior ones (PH), b-c High magnifications of the *b* Accessory sclerite (AS). *C* Anterior Hamulus (AH). *d* Posterior Hamulus (PH).
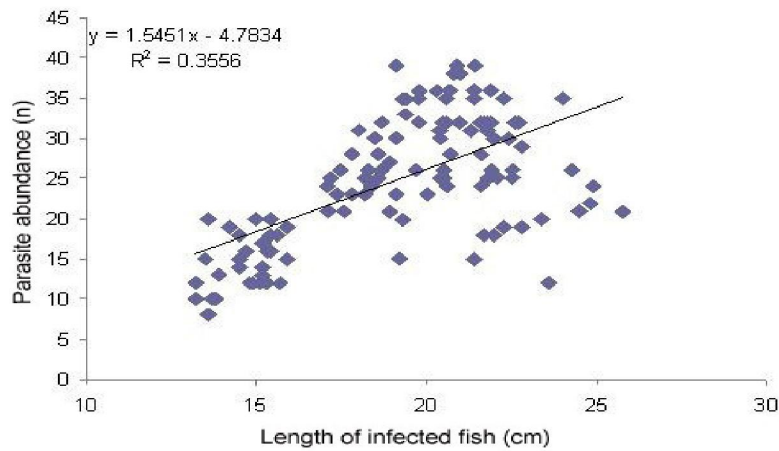
Fig. 9. Relationship between the number of parasites per fish as a function of fish length. Positive correlation was observed, the number of parasites increased by increasing length of the infected fish till a limit where at high fish length, the parasite abundance decreased.
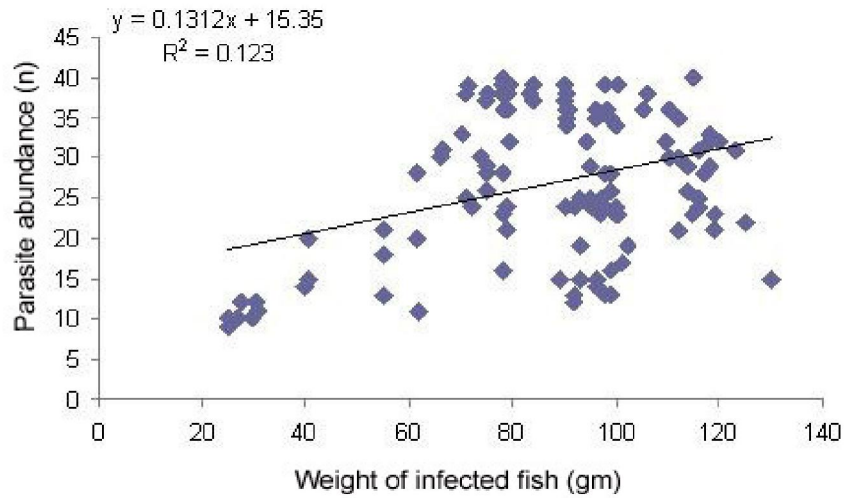
Fig. 10. Relationship between the number of parasites per fish as a function of fish weight. Positive correlation was observed between parasite abundance and weight of the infected fish till a limit where at high fish weight the number of parasites decreased.

Synergistic Effect of Ischemic Preconditioning, Postcoditioning and Xanthine Oxidase Inhibition on Cardiac Tissue apoptosis of Hepatic Ischemic-Reperfused Male Rats

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Abstract: Accumulating evidences have recently documented that hepatic ischemic mechanical preconditioning, postconditioning or ischemic pharmacological preconditioning had protective effects on the liver, which were associated with a reduction in oxidative stress, inflammation and endogenous antioxidant preservation. However, assessment of cardioprotective effects of remote hepatic ischemic preconditioning, postconditioning or pharmacological preconditioning is unclear and needs further investigations. The aim of this study was to investigate the remote effect of hepatic ischemia/reperfusion (IR) on cardiac tissue. And to investigate whether hepatic ischemic preconditioning (IPC), postconditioning (IPO) and/or pharmacological preconditioning by xanthine oxidase inhibitor (allopurinol) (Allo) may extend a beneficial synergistic effect to protect the cardiac tissue. Forty male Albino rats were divided into 5 experimental groups: group I: sham-operated controls, group II: Hepatic I/R, group III: IPC+ I/R+ IPO, group IV: Allo + I/R, group V: Allo+IPC+I/R+IPO. Serum interleukin-6, cardiac malondialdehyde (MDA), reduced form of glutathione (GSH), Bax and Bcl-2 mRNA expressions were measured at the end of experiment. Results revealed that IPC, IPO and/or Allo treatment significantly reduced the levels of IL-6, MDA, Bax mRNA and Bax/Bcl-2 ratio and significantly increased the levels of GSH and Bcl-2 mRNA. In conclusion: IPC, IPO and Allo treatment may act synergistically to protect cardiac tissue against oxidative stress and mitochondrial injury during hepatic I/R.
[Hanan A. Mubarak **Synergistic Effect of Ischemic Preconditioning, Postcoditioning and Xanthine Oxidase Inhibition on Cardiac Tissue apoptosis of Hepatic Ischemic-Reperfused Male Rats**] Life Science Journal,. 2011; 8(4): 253-262] (ISSN: 1097-8135). http://www.lifesciencesite.com.

Key words: hepatic –ischemia- preconditioning- cardiac- apoptosis - allopurinol

1. Introduction

Warm hepatic ischemia-reperfusion (I/R) injury is a significant medical problem in many clinical conditions such as liver transplantation, hepatic surgery for tumor excision, trauma and hepatic failure after hemorrhagic shock. Partial or, mostly, total interruption of hepatic blood flow is often necessary when liver surgery is performed. This interruption of blood flow is termed "warm ischemia" and upon revascularization, when molecular oxygen is reintroduced, the organ undergoes a process called "reperfusion injury" that causes deterioration of organ function. Ischemia reperfusion results in cellular damage and tissue injury associated with a complex series of events **(1)**.

Ischemia-reperfusion injury is a multifactorial process that results in the accumulation of reactive oxygen species (ROS) that initiate tissue injury and stimulate a cellular cascade leading to inflammation **(2)**. The inflammatory process is secondary to endothelial activation and dysfunction, adherence and activation of neutrophils and platelets **(3)**, and the activation of complement **(4)** and T cells **(5)**. The proinflammatory process results in cell death and in severe cases leads ultimately to organ failure **(2)**.

Ischemic cell death is a consequence of irreversible mitochondrial injury **(6)**. Previous studies reported an association between mitochondrial dysfunction caused by reactive

oxygen species (ROS) and both necrotic and apoptotic cell death **(7)**. Oxidative stress and mitochondrial dysfunction are considered key mediators of cardiomyocyte apoptosis associated with post-I/R cardiac damage **(8)**.

Hepatic IR results in proinflammatory process and release of cytokines, chemokines, and adhesion molecules that are identified in peripheral tissues including the heart and kidney resulting in cell death and in severe cases leads ultimately to organ failure **(9,10)**. However, remote effects of hepatic I/R injury on the heart tissue need further clarification.

Accumulating evidences have documented that hepatic ischemic mechanical preconditioning, postconditioning or ischemic pharmacological preconditioning had protective effects on the liver, which were associated with a reduction in oxidative stress, inflammation and endogenous antioxidant preservation **(11-13)**. However, assessment of cardioprotective effects of remote hepatic ischemic preconditioning, postconditioning or pharmacological preconditioning, against hepatic ischemia-induced cardiac tissue insult, is unclear and needs further investigations.

Accordingly, the objective of this study was to test the hypothesis that hepatic IR had remote effects on cardiac tissue oxidative/anti-oxidative status and cardiac tissue apoptosis. And to investigate whether hepatic ischemic preconditioning, postconditioning and/or xanthine

oxidase inhibitor (allopurinol) preconditioning may extend a beneficial synergistic effect to protect the cardiac tissue against hepatic ischemia-induced apoptosis.

2. Material and Methods
Experimental Design:

Forty male Albino rats belonging to local strain weighing between 180-250 gm were obtained from the Animal House of Faculty of Medicine, Cairo University and included in this study. The animals were housed in wire mesh cages at room temperature with 12:12h light-dark cycles and maintained on standard rat chow and tap water. Veterinary care was provided by Animal House Unit of Faculty of Medicine, Cairo University. The rats were divided randomly into 5 experimental groups of 8 rats each.

Group I, Control group (sham-operated rats):

Rats were anaesthetized with thiopental sodium (Eipico co., Egypt) 40 mg /kg body weight. A midline laparotomy & liver exposure for 2.5 hours were performed with no further surgical manipulations (14).

Group II (I/R): hepatic ischemia reperfusion group:

Rats underwent the same surgical procedure as sham operated rats but with the induction of hepatic ischemia / reperfusion (I/R) injury as follows: 30 minutes (min) of ischemia by clamping the hepatic pedicle using a non traumatic microvascular clip, followed by 2 hours of reperfusion (15).

Group III (IPC+ I/R+ IPO): Hepatic I/R in combination with ischemic pre conditioning (IPC) & postconditioning (IPO):

Rats were subjected first to mechanical IPC by 10 min of ischemia followed by 10 min of reperfusion (11). Then hepatic pedicle was occluded again for 30 min. Then mechanical IPO was performed by 30 seconds of reperfusion followed by 30 seconds of re-occlusion for 3 cycles (16). Finally reperfusion was maintained for 2 hours.

Group IV (Allo + I/R): Allopurinol preconditioning group:

Rats were injected intraperitoneally with xanthine oxidase (XO) inhibitor, allopurinol from Galaxo Smith Kline (S.A.E), in a dose of 50 mg/kg body weight, twice, 18 hrs and one hour before the induction of hepatic I/R procedure (17).

Group V (Allo+IPC+I/R+IPO): Hepatic I/R in combination with allopurinol preconditioning (Allo), ischemic preconditioning (IPC) & postconditioning (IPO):

Rats were pretreated with allopurinol the same as in group IV, then hepatic I/R was performed in combination with ischemic pre & post conditioning the same as described in group III.

At the end of the experimental procedure, blood samples were obtained from retro-orbital vein for detection of serum interleukin-6. Then, all rats were sacrificed and the hearts were rapidly excised for further detection of: Malondialdehyde (MDA), reduced form of glutathione (GSH), Bax and Bcl-2 mRNA expressions.

Measurement of serum IL-6

Serum were examined for IL-6 level by ELISA technique by kit supplied from Quantakine (R&D system) (USA) according to manufacturers instruction (18).

Measurement of MDA in heart tissue

Malondialdehyde was measured by (MDA colorimetric Assay Kit from Oxis International, Inc. Foster City, CA 94404 USA). To measure the MDA concentration (19), 100 mg of heart tissue in 1 mL PBS (phosphate buffered saline) at pH 7.0, was homogenized with micropestle in microtube. 20 % TCA (trichloroacetic acid) was added to heart homogenate to precipitate the protein, and centrifuged. Supernatants were collected and thiobarbituric acid (TBA) solution was added to the supernatants. After boiling for 10 minutes in water bath, the absorbance was measured. Concentration of MDA in supernatants of heart homogenate was calculated using the standard curve of MDA standard solution (0; 0.625; 1.25; 2.5; 5.0 nmol/mL).

Measurement of reduced form of glutathione (GSH) in heart tissue

GSH concentration was measured from heart homogenate in phosphate buffer pH 8.0 and then 5% TCA was added, to precipitate heart protein. After centrifugation, dithiobisnitrobenzoate (DTNB) solution was added to the supernatants of heart homogenate, and incubated for 1hour. The absorbance was measured. Concentration of GSH in heart tissue was calculated using the standard curve of GSH standard solution (0; 10; 20; 40; 50; 100 mg/mL) (20). The heart protein concentration was calculated by using standard curve of bovine serum albumin (BSA) solution.

Polymerase chain reaction (PCR) detection of Bax and Bcl2 gene expression in heart tissue

Total RNA was extracted from heart tissue homogenate using RNeasy purification reagent (Qiagen, Valencia, CA) according to manufacturers instruction then cDNA was generated from 5 μg of total RNA extracted with 1 μl (20 pmol) antisense primer and 0.8 μl superscript AMV reverse transcriptase for 60 min at 37 °C. For PCR, 4 μl cDNA was incubated with 30.5 μl water, 4 μl (25mM) MgCl2, 1 μl (10 mM) dNTPs, 5 μl 10×PCR buffer, 0.5 μl (2.5 U) Taq polymerase and 2.5 μl of each primer containing 10 pmol. Primer

sequences were as follows: Bax forward primer 5 CTGAGCTGACCTTGGAGC-3, reverse primer 5-GACTCCAGCCACAAAGATG-3; Bcl2 forward primer 50GGAGGGCACTTCCTGAG-30 and reverse primer 5-GCCTGGCATCACGACT-3. The reaction mixture was subjected to 40 cycles of PCR amplification as follows: denaturation at 95 °C for 1 min, annealing at 67 °C for 1 min and extension at 72 °C for 2 min. PCR products were electrophoresed on 2% agarose stained with ethidium bromide and visualized by ultraviolet transilluminator. Semiquantitation was performed using gel documentation system (BioDO, Analyser, Biometra, Gottingen, Germany). According to the amplification procedure, relative expression of each studied gene (R) was calculated according to the following the formula: densitometrical units of each studied gene/densitometrical units of b-actin.

PCR detection of b-actin

Presence of RNA in all samples was assessed by analysis of the 'house-keeping' gene b-actin. Complementary DNA was generated from 1 mg total RNA extracted with avian myeloblastosis virus reverse transcriptase for 60 min at 37 1C. For PCR, 4 ul complementary DNA was incubated with 30.5 ul water, u ml 25mM MgCl2, 1ml deoxyribonucleotide triphosphates (10mM), 5 ul 10x PCR buffer, 0.5 ul (2.5 U) Taq polymerase and 2.5 ul of each primer containing 10pM. b-actin primers (forward 5-TGTTGTCCCTGTATGCCTCT-3; reverse 5 TAATGTCACGCACGATTTCC-3). The reaction mixture was subjected to 40 cycles of PCR amplification, denaturation at 95 C for 1min, annealing at 57 C for 1min and extension at 72 C for 2min.

Statistical analysis:

The data were statistically analyzed using the statistical package SPSS (version 15). Values were expressed as mean $\pm$ standard error (M$\pm$SE). Statistical analysis was performed by ANOVA (analysis of variance) and multiple comparison Post-Hoc Tests to determine significant differences between groups. Correlations were done to test for linear relations between variables using Pearson correlation test. The level of statistical significance was set at $p \leq 0.05$.

3. Results:

This study examined the effects of ischemic mechanical preconditioning (IPC), post conditioning (IPO) and allopurinol (Allo) preconditioning or a combination of them on the extent of cardiac tissue oxidative/anti-oxidative status and apoptosis due to hepatic ischemia (for 30 minutes) followed by two hours reperfusion (I/R).

Hepatic I/R resulted in a significant (p< 0.05) increase in serum IL-6 (64.875 ± 4.335 pg/ml),

cardiac MDA (3.338±0.404 μmol/mg protein), Bax mRNA (1.163±0.099), and Bax / Bcl-2 ratio (4.808±0.582) and induced a significant (p< 0.05) decrease in cardiac GSH (17.325 ±1.514 μmol/mg protein) and Bcl-2 mRNA (0.273±0.042) compared to the corresponding values of sham controls (28.737 ± 2.161 pg/ml, 0.875 ±0.025 μmol/mg protein, 0.116±0.011, 0.075±0.009, 40.075±2.223 μmol/mg protein, , 1.633±0.115 and), respectively (Table 1, 2 and Figure 1).

In I/R group the increases in serum IL-6 level & cardiac Bax / Bcl-2 ratio (64.875 ± 4.335 pg/ml, 4.808±0.582 respectively) were suppressed by either IPC+IPO (54.425 ± 3.607 pg/ml, 1.449±0.087 respectively), Allo (41.425 ± 1.764 pg/ml, 0.935±0.113 respectively) or IPC+IPO+Allo (46.613 ±2.049 pg/ml, 0.621±0.041 respectively) without any synergistic effect.

In addition, the increases in cardiac MDA & Bax mRNA (3.338 ± 0.404 μmol/mg protein, 1.163±0.099 respectively) were suppressed by IPC+IPO (2.988 ± 0.175 μmol/mg protein, 0.716±0.033 respectively) or Allo (2.688 ± 0.239 μmol/mg protein, 0.589±0.023 respectively) alone. However, IPC+IPO+Allo appeared to have a synergistic effect in further suppressing the increases in MDA (1.686 ± 0.141 μmol/mg protein) and Bax mRNA (0.479±0.036) (Table 1, 2 and Figure 1).

Meanwhile, the reductions in cardiac GSH and Bcl-2 mRNA observed in I/R group (17.325 ±1.514 μmol/mg protein, 0.273±0.042 respectively) were decreased by IPC+IPO (24.988 ± 1.430 μmol/mg protein, 0.499±0.014 respectively) or Allo (27.400 ± 1.173 μmol/mg protein, 0.678±0.057 respectively) alone. However, IPC+IPO+Allo appeared to have a synergistic effect in further decreasing the reductions and increasing the levels of both GSH (34.438 ± 1.524 μmol/mg protein) and Bcl-2 mRNA (0.778±0.045) (Table 1, 2 and Figure 1).

Correlations between the measured variables were shown in figures (2-7).

Cardiac MDA concentrations showed significant positive correlations with serum IL-6 levels (figure 2), cardiac Bax mRNA and Bax / Bcl-2 ratio (figure 3) (r = 0.696, 0.785, 0.590 respectively and p<0.05) in the five studied groups. In contrast it showed significant negative correlations with cardiac GSH (figure 4) and Bcl-2 mRNA (r = -0.721, -0.737 respectively and p<0.05).

Cardiac GSH had significant positive correlation (r = 0.751   p<0.05) with Bcl-2 mRNA level but significant negative correlations with serum IL-6 (figure 5), cardiac Bax mRNA and Bax / Bcl-2 ratio (figure 6) (r = -0.722, -0.813, -0.757 respectively   and p<0.05) in the five studied groups.

Finally, serum IL-6 level was positively correlated with cardiac MDA, Bax mRNA level and Bax / Bcl-2 ratio (figure 7) (r = 0.696, 0.737, 0.746 respectively   and p<0.05) and was negatively correlated with cardiac GSH and Bcl-2 mRNA level (r = -0.722, -0.717 respectively   and p<0.05) in the five studied groups.

Table-1: Effect of hepatic ischemia (30 min) followed by two hours reperfusion (I/R) on serum interleukin-6 (IL-6 in pg/ml), cardiac malondialdehyde (MDA in µmol/mg protein) and reduced form of glutathione (GSH in µmol/mg protein) in the five studied groups.

Groups n=8 Parameters	Group I (Sham control rats)	Group II (I/R)	Group III (IPC+I/R+IPO)	Group IV (Allo+I/R)	Group V (Allo+IPC+ I/R+IPO)
Serum IL-6 (pg/ml)	28.737 ± 2.161■▲□#	64.875 ± 4.335*▲#	54.425 ± 3.607*▲	41.425 ± 1.764*■□	46.613 ±2.049*■
Cardiac MDA (µmol/mg protein)	0.875 ±0.025■▲□	3.338 ± 0.404*#	2.988 ± 0.175*#	2.688 ± 0.239*#	1.686 ± 0.141■▲□
Cardiac GSH (µmol/mg protein)	40.075±2.223■▲□	17.325 ±1.514*□▲#	24.988 ± 1.430*■#	27.400 ± 1.173*■#	34.438 ± 1.524■▲□

Results are mean ±SE

n: number of male rats in each group   I/R: ischemia reperfusion
IPO: ischemic preconditioning   IPC: ischemic postconditioning
Allo: allopurinol preconditioning   *   Significant compared to sham control value
(p<0.05)
■ Significant compared to I/R value (p<0.05)   □ Significant compared to IPC+I/R+IPO value
(p<0.05)
▲Significant compared to Allo/IR value (p<0.05)   #   Significant compared to Allo+IPC+ I/R+IPO
value (p<0.05)

Table-2: Effect of hepatic ischemia (30 min) followed by two hours reperfusion (I/R) on cardiac Bax mRNA, Bcl-2 mRA and Bax / Bcl-2 ratio in the five studied groups

Groups n=8 Parameters	Group I (Sham control rats)	Group II (I/R)	Group III (IPC+I/R+IPO)	Group IV (Allo+I/R)	Group V (Allo+IPC+ I/R+IPO)
Cardiac Bax	0.116±0.011■▲□#	1.163±0.099*□▲#	0.716±0.033*■ #	0.589±0.023*■	0.479±0.036*■□
Cardiac Bcl-2	1.633±0.115■▲□#	0.273±0.042*▲#	0.499±0.014*#	0.678±0.057*■	0.778±0.045*■□
Cardiac Bax / Bcl-2 ratio	0.075±0.009■□	4.808±0.582*□▲#	1.449±0.087*■	0.935±0.113■	0.621±0.041■

Results are mean ±SE
n: number of male rats in each group   I/R: ischemia reperfusion
IPO: ischemic preconditioning   IPC: ischemic postconditioning
Allo: allopurinol preconditioning   *   Significant compared to sham control value
(p<0.05)
■ Significant compared to I/R value (p<0.05)   □ Significant compared to IPC+I/R+IPO value
(p<0.05)
▲Significant compared to Allo/IR value (p<0.05)   #   Significant compared to Allo+IPC+ I/R+IPO
value (p<0.05)
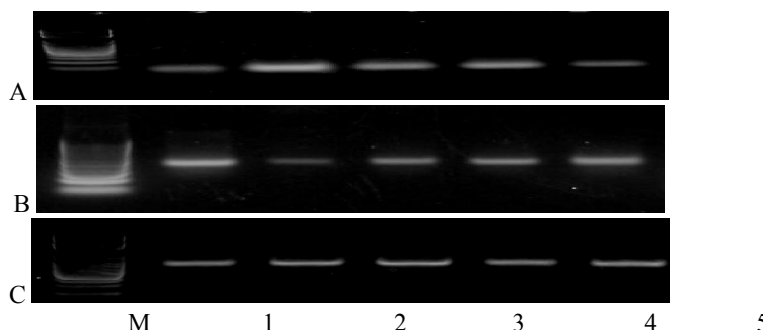
Figure-1: Agarose gel electrophoresis of PCR products Bax (A), Bcl2 (B) and b- actin (C) in the five studied groups.
Lane M, PCR marker with 100 bp ladder   Lane 1, control group (sham-operated rats)

Lane 2, hepatic ischemia reperfusion group (I/R)   Lane 3, allopurinol preconditioning group (Allo+I/R)
Lane 4, hepatic I/R in combination with ischemic preconditioning (IPC) & postconditioning (IPO) group (IPC+I/R+IPO)
Lane 5, hepatic I/R in combination with allopurinol preconditioning (Allo), ischemic preconditioning (IPC) & postconditioning (IPO) group (Allo + IPC+I/R+IPO)
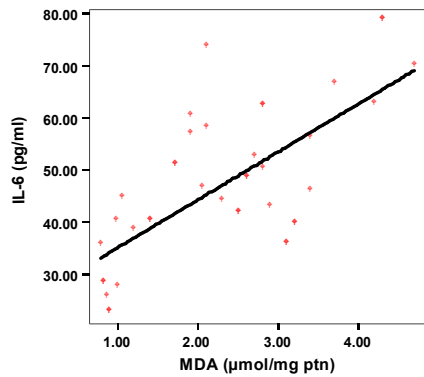
Figure (2): Positive correlation between cardiac malondialdehyde (MDA in µmol/mg protein) and serum interleukin-6 (IL-6 in pg/ml) in all rats of the five studied groups
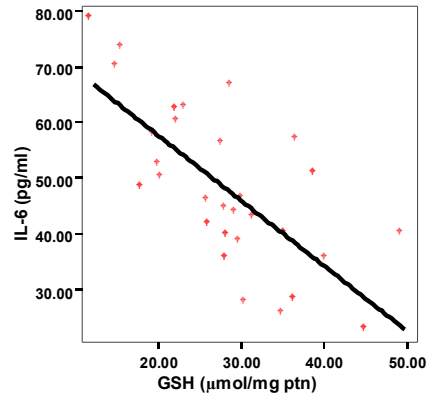
Figure (5): Negative correlation between cardiac reduced forms of glutathione (GSH in µmol/mg protein) and serum interleukin-6 (IL-6 in pg/ml) in all rats of the five studied groups
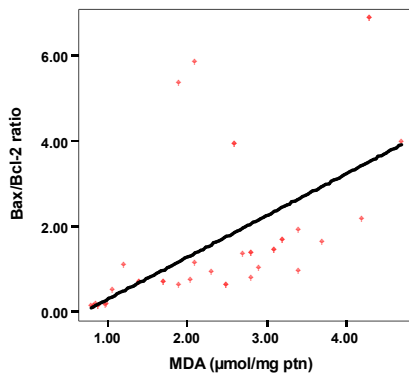
Figure (3): Positive correlation between cardiac malondialdehyde (MDA in µmol/mg protein) and Bax/Bcl-2 ratio in all rats of the five studied groups
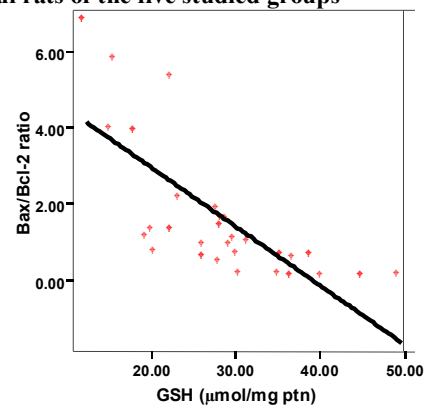
Figure (6): Negative correlation between cardiac reduced forms of glutathione (GSH in µmol/mg protein) and Bax/Bcl-2 ratio in all rats of the five studied groups
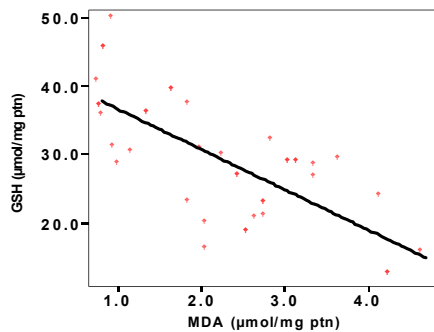
Figure (4): Negative correlation between cardiac malondialdehyde (MDA in µmol/mg protein) and   reduced form of glutathione (GSH in µmol/mg protein) in all rats of the five studied groups
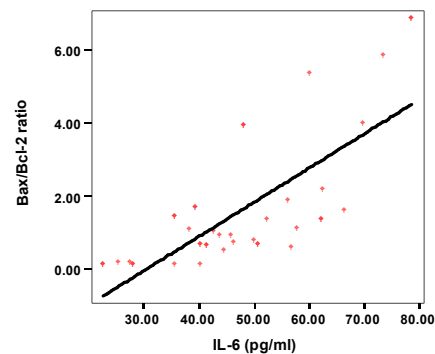
Figure (7): Positive correlation between serum interleukin-6 (IL-6 in pg/ml) and cardiac Bax/Bcl-2 ratio in all rats of the five studied groups

4. Discussion

The results of the present study provide evidence that combined hepatic ischemic preconditioning (IPC); postcoditioning (IPO) and allopurinol preconditioning (Allo) have synergistic protective effect on cardiac tissue oxidative stress and endogenous antioxidant preservation in male albino rats subjected to 30 minutes hepatic ischemia followed by 2 hours reperfusion (I/R). In this experiment, combined IPC+IPO+Allo show statistical significant ($P<0.05$) reduction of cardiac malondialdehyde (MDA) and Bax mRNA and statistical significant increase ($P<0.05$) of cardiac reduced form of glutathione (GSH) and Bcl-2 mRNA compared to IPC+IPO alone. However, that synergism is lost as regards the Bax/Bcl-2 ratio which shows insignificant ($P>0.05$) difference on comparing the three groups IPC + IPO, Allo and IPC+IPO+Allo.

Hepatocytes are important sources of Reactive Oxygen Species (ROS) along with Kupffer cells and neutrophils in hepatic ischemic/reperfusion injury (IRI) **(21)**. **Bhogal *et al.* (22)** suggested that hepatocytes should not be considered bystanders and targets of the injury. They should be seen as active participants in IRI in both the ischemic and reperfusion phases. With ischemia the respiratory cytochromes become redox-reduced, allowing them to directly transfer electrons to oxygen. This will improve the understanding of this complex and multifactorial injury **(23)**.

It is reported that hepatocytes are likely to play at least 2 key roles in hepatic IRI. First, hepatocytes generate significant levels of intracellular ROS during hypoxia and hypoxia/reoxygenation; that augments local tissue damage or affects organs remote from the site of I/R **(24)**. Second, hepatocytes can secrete and release proinflammatory cytokines, chemokines and adhesion molecules that are identified in peripheral tissues including the heart and kidney, and may play an important role in the development of multi-organ failure. **(9,10,25)**. An important function of ROS is the regulation of cytokine gene expression and stimulation of a cellular cascade leading to inflammation **(26)**.

In line with the previous results, the present study shows significant increased level of serum interleukin-6 in rats with hepatic IR, suggesting systemic leakage of proinflammatory cytokines/chemokines released by injured hepatic tissues. Also, there is significant increase of cardiac MDA and significant decrease of cardiac GSH due to hepatic ROS-induced cardiac oxidative stress and due to the systemic inflammatory response. Moreover, positive correlation is found between the serum IL-6 and cardiac MDA.

Accumulating evidences have documented that the cell death observed during the first few hours of myocardial ischemia occurs mainly through apoptosis **(27)**, rather than necrosis, which has long been considered as the predominant form of myocardial damage generated by ischemia **(28)**. Moreover, apoptotic cell death causes considerable cardiomyocyte loss, decreasing contractile function of the heart **(29)**, and eventually acts as a precursor of heart failure **(30)**. Thus, interventions aim at inhibition of mechanisms leading to apoptosis before the process becomes irreversible might therapeutically prevent excessive cell death **(31)**.

Oxidative stress is a major apoptotic stimulus in ischemic heart disease. Reactive oxygen species (ROS) are therefore excessively generated from a likely mitochondria source and then hasten lipid peroxidation, DNA damage, and other direct cellular injuries, consequently initiating apoptosis in cells **(32,33)**. Apoptotic signaling induces apoptosis primarily through three types of complex pathways. They include 1) cytokine/Fas receptor-driven pathway, 2) mitochondrial-driven pathway, and 3) endoplasmic reticulum/Ca2+-driven pathway. Among them, mitochondrial-mediated pathway, including the Bcl-2 family is the best characterized and believed to be critical in regulating apoptosis **(34)**.

An outcome of this study is a significant upregulation of cardiac Bax mRNA expression and a significant decrease in cardiac Bcl-2 mRNA expression with overall significant increase in the Bax/Bcl-2 ratio due to hepatic I/R. Reversal of those results, with lowering the magnitude of increased Bax/Bcl-2 ratio, are observed on inducing hepatic ischemic mechanical preconditioning-postconditioning and/or allopurinol preconditioning.

The Bcl-2 family proteins are key regulators of cell death and survival that can either inhibit or promote apoptosis. Family members include antiapoptotic Bcl-2 and Bcl-xl and proapoptotic Bax, Bad, truncated Bid (tBid), and Bim. Interactions between these antiapoptotic and proapoptotic Bcl-2 proteins exist in a delicate balance at the mitochondrial membrane that determines cell fate. Heterodimerization of antiapoptotic members, such as Bcl-2, with proapoptotic members, such as Bax can inhibit or activate apoptosis depending on the relative levels of each protein **(35)**. The ratio of proapoptotic to antiapoptotic proteins (e.g., Bax/Bcl-2) regulates myonuclei integrity and cell survival by controlling mitochondrial membrane permeability. Decreased mitochondrial membrane stability and pore formation initiates the release of cytochrome c, formation of the apoptosome, and subsequent activation of caspase-9 and caspase-3 leading to mitochondria –mediated apoptosis **(36)**.

In consistent with the present study, the positive correlation between serum IL-6 and cardiac Bax/Bcl-2 ratio due to increased Bax mRNA and decreased Bcl-2 mRNA, Escandell et al. **(37)** reported that Bcl-2 is a negative regulator of interleukin-1b cytokine secretion in murine

macrophages in pharmacological-induced apoptosis. In contrast, **Waxman and Kolliputi (38)** demonstrated that IL-6–mediated protection against hyperoxia is partly mediated by up-regulation of Bcl-2 expression and regulation of Bcl-2 family member interactions. While **Ryazantseva et al. (39)** suggested that the inductive and inhibitory effects of IL-2 on apoptotic process depend on the dose of the cytokine and cell micro-environmental conditions.

Ischemic preconditioning (IPC) is an adaptational response of briefly ischemic tissues which serves to protect against subsequent prolonged ischemic insults and reperfusion injury. Ischemic preconditioning can be mechanical or pharmacological. Direct mechanical preconditioning in which the target organ is exposed to brief ischemia prior to prolonged ischemia has the benefit of reducing ischemia-reperfusion injury (IRI) but its main disadvantage is trauma to major vessels and stress to the target organ. Remote (inter organ) preconditioning is a recent observation in which transient non-lethal ischaemia and reperfusion of one organ confers resistance to a subsequent episode of lethal ischaemia reperfusion injury in a remote organ or tissue without direct stress to the organ **(40)**.

Potential mechanistic pathways underlying remote ischemic preconditioning (RIPC): the actual mechanism through which transient ischemia and reperfusion of an organ or tissue confers cardioprotection is currently unknown although several hypotheses have been proposed to reduce oxidative stresss and preserve mitichondrial function: (1) The neural hypothesis proposes that preconditioning of the organ or tissue remote from the heart generates an endogenous substance such as adenosine, bradykinin or calcitonin gene-related peptide (CGRP), which then activates a local afferent neural pathway stimulating an efferent neural pathway, which terminates at the heart and mediates cardioprotection. (2) The humoral hypothesis proposes that the endogenous substance (such as adenosine, bradykinin, opioids, CGRP, endocannabinoids, Angiotensin I) or some other as yet unidentified humoral factor generated in the remote organ or tissue enters the blood stream and activates its respective receptor in the myocardium thereby recruiting the various intracellular pathways of cardioprotection implicated in ischemic preconditioning. (3) The third hypothesis proposes that transient ischemia and reperfusion of an organ or tissue provokes a systemic protective response, which suppresses inflammation and apoptosis. Recent data suggest that the activation of the mitogen-activated protein kinases (MAPKs) within the remote organ may also contribute to RIPC-induced cardioprotection **(41)**.

Tapuria et al. (40) reported that some studies demonstrate endothelial NO, kinases, opioids, catecholamines and KATP channels (ATP-sensitive potassium channels) as the candidate mechanism in remote preconditioning. Experiments show suppression of proinflammatory genes, expression of antioxidant genes and modulation of gene expression by RIPC as a novel method of IRI injury prevention.

Although preconditioning is a source of scientific inspiration, its clinical applicability is limited by the inability to predict acute ischemic events. Accordingly, postconditioning may confer benefits similar to preconditioning. Postconditioning is a procedure of repetitive brief cycles of reperfusion performed immediately at the onset of reperfusion to induce intracellular protective reactions **(2,42)**.

Both local and remote postconditioning may ultimately prove to be effective in rodent models of acute myocardial infarction by potentially invasive renal manipulation **(43)** and by limb manipulation **(44)**. The previuos studies show that remote postconditioning (rather than conditioning before reperfusion) by ischemic hindlimb manipulation is safe and effective **(43)**. Contradictory result was reported by **Bretz et al. (45)**, that ischemic postconditioning does not attenuate ischemia–reperfusion injury of rabbit small intestine. **Kin et al. (46)** demonstrated that, when the initiation of postconditioning is delayed for greater than 1 minute, the cardiac protection from IRI is lost.

Although it is likely that the precise mediators and their time course of action will vary (depending on the organ rendered ischaemic and the temporal aspects of the ischemia) and the mechanisms underlying remote conditioning (preconditioning or postconditioning) remain elusive, it is likely that some similarities exist between the two **(47)**. It is generally accepted that adenosine release and, hence, activation of the A_{2A} and A_3 receptors have a critical role in the reduction in the infarct size **(48)**. As in the case of humoral factors, a neurogenic arc could be one of the triggers easing the release of adenosine in the myocardium in some forms of preconditioning. Other potential triggers include reaction-elaborated reactive oxygen species, endogenous opioids operating through the κ and δ receptors and nitric oxide. Downstream effectors include protein, kinase C, reperfusion injury-signalling kinases, KATP channels and the mitochondrial permeability transition pore **(47)**.

In the present study, blockage of xanthine oxidase (XO) with allopurinol decreases the production of ROS indicating reduced oxidant stress and is reflected by restoration of GSH levels and reversed MDA content in the heart. Decreased ROS and the subsequent inflammatory response (reduction of serum IL-6), results in attenuation of cardiac tissue apoptosis which is reflected by reduced cardiac Bax mRNA and Bax/Bcl-2 ratio.

The mechanism by which allopurinol exerts a protective effect on liver IR injury is still under debate, but strong evidence exists to support that

allopurinol blocks (XO) and aids in resynthesis of ATP by inhibiting the breakdown of its catabolites, inhibiting the formation of ROS, and preventing mitochondrial membrane damage, therefore decreasing anaerobic respiration **(49)**. Contradictory result is reported by **Rajesh *et al.* (50)** as allopurinol pretreatment failed to upregulate Bcl-2 expression in cardiac I/R model.

Interestingly, in a jejunum-segment model the allopurinol pre-treatment expresses the highest level of apoptotic activity, and thus, the ratio between necrosis and apoptosis significantly altered. Since significant oxidative stress is known to induce necrosis, it is supposed that the effect of allopurinol might direct the dominant cell death type from necrosis to apoptosis by decreasing the level of reactive oxygen species, and rather increasing the number of apoptotic cells. However, the effect of allopurinol is dose-dependent and supposedly the way of administration would have an influence on the results too. The authors suggested that, the effect of administration of allopurinol is contradictory **(51)**.

In consistent with the results of this study, **Foly and Chari (2)** suggested that the additive effects of preconditioning (IPO) and postconditioning (IPC) may be beneficial in human transplantation and needed to be studied. In addition, **Lee and Lee (11)** demonstrated that IPC and Allo act synergistically to protect cells against mitochondrial injury and preserve the hepatic energy metabolism during hepatic I/R.

In conclusion, the results of the present study demonstrate that there is an increase in cardiac tissue apoptosis in the hepatic I/R model, which may be, at least partly, due to enhanced mitochondrial pathway resulting possibly from increased oxidative stress. Remote hepatic mechanical IPC, IPO and Allo preconditioning may act synergistically to protect cardiac tissue against oxidative stress and mitochondrial injury during hepatic I/R.

The underlying mechanisms and pathways need further clarification. Future clinical studies using more than one approach to minimize injury at different time points in the transplant process may be needed to achieve significant clinical benefit.

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The effects of Behavioural Parent Training Program on Families of Children with Attention-Deficit/Hyperactivity Disorder

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Abstract: The present study evaluated the effectiveness of Behavioural Parent Training Program (BPTP) on families of children with ADHD. Using quasi- experimental design, sixty parents of ADHD children from an ADHD centre for children with behavioural and emotional disorders were randomly assigned to experimental and control groups. The program developed by Barkley was administered in nine 90-minutes sessions in nine weeks with a one-month follow-up session. Conners' Parent Rating Scales-Revised and ADHD Rating Scale-IV were employed to measure treatment outcomes. Since data did not meet the assumptions of normality distribution, a series of nonparametric tests using SPSS version-16 were used in the statistical analyses. The results of Friedman Tests showed significant results for all the subscales. Further investigation of the results using Wilcoxon Signed Rank Test also showed a statistically significant reduction in symptoms of ADHD and related problem (Optional behaviour, Cognitive problems/inattention and ADHD Index). The results imply that BPTP can be effective for reducing symptoms of ADHD. The outcome of the study could benefit family counselors, psychologists and specifically for psycho-educational interventions as a single treatment. Theoretical and practical implications of the findings, avenues for future research and limitations of the study are discussed.

Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is one of the most prevalent chronic disorders in childhood psychiatry. it has been estimated, 3–7% of school-aged children are affected by ADHD [1]. ADHD can be detected during childhood; however, its symptoms can continue to adolescence or adulthood [2]. Indeed, in order to make a diagnosis, the DSM-IV-TR mandates an onset of symptoms occurring before the age of seven. People who suffer from the disorder indicate different symptoms that can affect their everyday social interactions or activities which need continuous focus on detail [3].

The prevalence of ADHD in many countries has been reported to be around 8 to 12 % [4]. Conservative estimates indicate that 3 % to 5% and with other estimates as high as 7% to 12% of school-aged children suffer from ADHD [5]. In the prevalence of the disorder in a sample of primary school students (N=2500) in Tehran (Iran), about 3%-5% were estimated to suffer from the disorder [6]. In another study in Iran on 2000 students' parents using ADHD Home-Version, Ghanizadeh [7] found that the rate of prevalence of ADHD was about 10.1%. Its rate among boys and girls was 13.6% and 6.5%, receptivity. This researcher concluded that the rate of probable ADHD in Iran was very similar to that in other countries.

Children with ADHD often exhibit impulsive and disruptive behaviors rendering them hard to control at home and in structured settings like school [8].

These children at school-age begin to demonstrate social deficits, low self-esteem, academic failures, as well as a higher risk for injuries. During adolescence, social and academic problems may persist, and impulsive and risk-taking behaviors (motor vehicle accidents, sexually transmitted diseases, unplanned pregnancy, substance abuse/ smoking) are more prevalent in children with ADHD [9]. Reportedly, stimulant medication has proved to be positively effective on 70% to 80% of ADHD children, but it results in a negative or neutral effect on the rest [10]. Furthermore, research demonstrates that after the termination of medication its effect is not long-lasting, and that 20-30% of children with ADHD does not have a positive response to medication [11].

Although the most common treatment for children with ADHD is medication therapy [12], in 10% to 20% of children who consume the medication no significant improvement has been observed [13]. Attention and emphasis on the role of parents as agents of change in the lives of children is not a new concept. Parent training programs (PTP) have a long-standing record in psychology. The case of Freud's 'Little Hans' study can be mentioned as an example of parent

training programs. Freud trained Hans' father how to deal with his son's Phobia. Parents are regarded as the care-givers, instructors, coaches, leaders, discipliners and the primary factors in their children's change or socialization [14].

Empirical evidence indicates that parental training programs can improve parenting skills, reduce parental stress, and reduce the child's aggressive behavior in families with ADHD children [15]. In Iran, a study was performed by Alizadeh and his colleague on the interaction of parenting styles and ADHD children in Iranian parents showed that parenting style is a pervasive and crucial factor that plays a role in children's psychological development [16]. These researchers note that there is a considerable lack of research about the relationship between parenting styles and child psychopathology in Iran. Behavioural Parent Training explicitly provides parents with instruction in the implementation of behavior modification techniques that are based on social learning principles and behavior modification techniques. The main components of most parent-training programs include providing effective instructions and discipline strategies, building parent-child relationship, as well as using positive reinforcement effectively for a child's compliance and responsible behaviors [17].

However, there is evidence that shows the fading effect of such training programs over time. Some studies have indicated that parental training programs may have no significant effect on the treatment of ADHD [18-21] With regard to high prevalence of ADHD among children and inadequate response to medical treatment; researchers have been focusing on psychosocial treatment for ADHD which has resulted in inconclusive findings. Therefore, further studies are needed to make clear the effectiveness of therapeutic psychosocial interventions for children with ADHD. To best of the researcher's knowledge, there are no published studies that evaluate the effectiveness of Barkley' parent training program on Iranian parents trained in a large group. Therefore, due to the observed inconsistency in the findings of the previous research and in order to investigate the effectiveness of PTP in Iran, the current study is proposed.

Materials and Methods

Using quasi-experimental design, sixty parents of ADHD children who met DSM-IV-TR criteria for ADHD and based on some of exclusive and inclusive criteria from ADHD Center for Children with Behavioural and Emotional Disorders in Kermanshah city, Iran were randomly assigned to experimental and control groups. The program developed by Barkley [22], was administered in nine 90-minutes sessions in nine weeks with a one-month follow-up session, the

content of each session was based on work done by Barkly [22] and is described in Table 1. The sessions typically began with a review of homework tasks, which the parents were asked to, carry out outside of the sessions. Each session concluded with the setting of further homework tasks. Some written materials were also provided and used. Treatment outcomes were evaluated by Conners' Parent Rating Scales-Revised: Short Form CPRS (short form) [23] and ADHD Rating Scale-IV: Home Version [24], the instruments were completed by the parents in four scheduled administrations - pre-intervention, post-intervention 1, post-intervention 2 and follow-up.

Behavioral Parent Training Program manual used in this study was developed by Barkley [22]. This is a structured curriculum consisting of 10 sessions intended to improve parental competence in dealing with child behavior problems, increase parental understanding about the origins of noncompliant and defiant behavior, improve the child's compliance with parental instructions, and decrease family conflict. The core skills include providing positive reinforcement for appropriate behavior, communicating directions effectively, and being consistent with consequences for disruptive behaviors. Parents learn techniques such as positive attending, selective ignoring, token economies, and time-out. Parenting skills are taught through modeling, role-play, and corrective feedback provided by the therapist. Assignments to implement new parenting skills at home were given after each session (See table 1).

Table 1: Barkley's Parent Training Program (1997)

Session 1	Why Children Misbehave
Session 2	Pay Attention
Session 3	Increasing Compliance and Independent Play
Session 4	When Praise Is Not Enough: Poker Chips and Points
Session 5	Time out! And other Disciplinary Methods
Session 6	Extending Time Out to Other Misbehavior
Session 7	Anticipating Problems: Managing Children in Public Places
Session 8	Improving School Behavior from Home: The Daily School Behavior Report Card.
Session 9	Handling Future Behavior Problems
Session 10	Booster Session and Follow-Up Meetings

Data Analysis: Exploratory data analysis showed that data do not follow a normal probability distribution. Therefore, a series of nonparametric tests including Mann-Whitney U test, Friedman and Wilcoxon Signed Rank tests were used in the statistical analyses. The

Statistical Package for Social Science (SPSS) version 16 for windows was used to analyze the data collected.

Results

Results showed that 18 parents (60%) in Experimental group and 14 parents (46.7%) in Control group were more than 35 years old. Five parents (16/7%) in experimental group and 10 parents (33/3%) were between 31-35 years old and 7 parents (23/3%) in experimental group and 6 parents (20%) were 26-30 years old. Results showed that 16 pair of (father and mother) parents (53.3%) in Experimental group and 14 pair of parents (46.7%) in Control group had a good awareness of their children's problem. While, parents' awareness of their children's problem was moderate in 14 pair of (father and mother) parents (46.7%) in Experimental group and 16 pair of parents (53.3%) in Control group. The results of the Mann-Whitney U tests on CPRS-R and ADHD Rating Scale-IV showed no significant difference between the experimental and the control groups at the pre-intervention stage.

To investigate the significance of difference through the four scheduled administrations of the intervention (pre-intervention, Post-intervention1, post-intervention2 and Follow-up session) in Oppositional Behavior, Cognitive problem, Hyperactivity symptoms, Conner's ADHD Index, Inattention (IA), Hyperactivity /Impulsivity (HI), Hyperactivity-Impulsivity (HI) and Inattention (IA) of ADHD children, a series of Friedman tests were conducted. The results have been presented in Table 2. The results from Friedman tests revealed a statistically significant difference in oppositional behaviour ($\chi2=56.46$, p $\leq.001$), Cognitive problem ($\chi2=59.99$, p$\leq.001$), Hyperactivity ($\chi2= 37.53$, p $\leq.001$), Conner's ADHD Index ($\chi2= 45.96$, p $\leq.001$), Inattention ($\chi2 = 52.17$ p $\leq.001$), Hyperactivity /Impulsivity ($\chi2 = 52.75$, p $\leq.001$). Hyperactivity-Impulsivity (HI) and Inattention (IA) ($\chi2 = 64.12$, p $\leq.001$) for experimental group. However, control group did not show any significant difference for the measured variables.

In the last step, a series of Wilcoxon Signed Rank tests were performed to examine the changes in the measured variable over time in the treatment group. Results revealed a statistically significant reduction in Oppositional behavioural significant ($Z = -4.78$, $P \leq .001$, EF=.87), Cognitive problems ($Z = -4.78$, $P \leq .001$, EF=.87), Hyperactivity symptoms ($Z = -4.63$, $P \leq .001$, EF=.84), ADHD Index of ADHD children ($Z = -4.74$, $P \leq .001$, EF=.86), Hyperactivity/ Impulsivity ($Z = -4.001$, $P \leq .001$, EF=.73), Inattention ($Z = -4.45$, $P \leq .001$, EF=.81), Hyperactivity/ Impulsivity and Inattention ($Z = -4.68$, $P \leq.001$, EF=.85) following participation in the training program.

Table 2. Results of Friedman tests for experimental and control group

Variable	Group	Pre intervention M	Mdn	Post intervention 1 M	Mdn	Post intervention 2 M	Mdn	Follow-Up M	Mdn	$\chi2$
Oppositional behavior	Experimental	70.37	70	48.1	48	53.8	53	56.13	56	56.46*
	Control	68.7	68	66.1	63	62.97	61	66.87	66	3.48
Cognitive problem	Experimental	70.43	71	50.5	50	55.57	54	52.23	50	59.99*
	Control	69.33	71	64.47	66.5	66.9	68	64.77	65	5.97
Hyperactivity symptoms	Experimental	73.83	78	57.8	65	66.03	68	61.67	60	37.53*
	Control	77.67	82	76.97	79.5	77.27	80.5	75.2	78	4.05
Conner's ADHD Index	Experimental	69.13	72	56.5	59.5	58.17	60	54.07	54	45.96*
	Control	67.87	68.5	66.1	64	67.97	68	65.47	64	5.13
Inattention (IA)	Experimental	90.57	96	72.67	75	70.8	74	68.37	75	52.17*
	Control	90.57	96	87.2	92	86.17	94	83.9	91	3.47
Hyperactivity /Impulsivity (HI)	Experimental	89.73	96	76.7	87	70.8	75.5	66.23	76	58.75*
	Control	94.13	98	92.13	94.5	88.2	96	88.4	95.5	5.76
HI & IA	Experimental	92.27	95.5	75.03	79.5	60.6	64	61.47	69	64.12*
	Control	93.43	97	93.1	95	90.4	95	85.73	93	5.99

* $p \leq.001$

Discussion

The current study demonstrated the effectiveness of BPTP on some of symptoms of ADHD among children is consistent with findings from other studies. For example, in a study, participants who completed the BPT program relative to waiting-list controls showed parent-reported improvements in the overall severity of their children's ADHD symptoms [25]. Others reported positive effects of BPT programs include the improvement of ADHD symptoms and home behaviours of children [26], reduction of oppositional behaviours[27], and of attention deficit and internalizing symptoms [28]. On the other hand, Weinberg [29] found no behavioural improvement among youngsters at completion of the program [30].

This finding was discussed in the context of a possible ceiling effect from the medications that the youngsters were using [29]. Also, Pisterman et al. [31] found that behavioural parent training of ADHD children was not effective on measures of attention. The study's results suggest that the effectiveness of BPTP is possible that has positive effects on behaviours that are important to parents and in home contexts, For example, BPTP was found to increase parental knowledge of ADHD and decrease parental stress [29], decrease maternal stress [27], increase parenting self-esteem [3], and improve parents' confidence in their child management abilities, knowledge of behavioural principles, and parent-child relationships.

The findings of this study are supported by Social Learning Theory [32-34]. According to this theory, all behaviours are learned through a combination of positive and negative reinforcement and modeling. Within this theory learning takes place indirectly by receiving information, observing others or modelling. Bandura's theory also declares that people can learn behaviour without direct experiencing and in absence of any rewards. However, in social learning the social interaction between learners and role models is crucial. Additionally, our finding can be supported by Behaviour Modification Principles (BMP) [35]. Since ADHD is a development delay in the self-regulation of behaviour by internal means of representing information and motivating goal –directed behaviour, then intervention that directly alters the nature of the stimuli controlling behaviour as well as the pattern, timing, or salience of such a consequence by socially arranged means would be useful, at least for symptomatic reduction in some settings and tasks.

In sum, the results of this study support the notion that parent training programs can benefit for families in a number of ways such as reconstructing and creating a new bridge for communication and interaction with their children and elimination most of related problem such as parental stress and changing their strategists toward them. Additionally, present study provides preliminary evidence that BPTP can be effective for decreasing ADHD symptoms and other related problems to ADHD among samples of children with ADHD as a single treatment.

Limitations and implications of the study: This study has several limitations that may suggest some interesting avenues for future research. The first limitation that should be acknowledged is that this study involved only the parents of the children not the children themselves. The second limitation that should be noted   is that the effect of the parent training program on children's behaviors was evaluated only at home not at school. The last limitation that should be addressed here is about medical concerns, co-morbid diagnoses, and utilization of psychotropic medications that may influence the outcome of the research, were not considered in this study.

Despite the above-mentioned limitations, some theoretical and practical implications of the present study can be suggested. The findings indicating significant effects of Barkley's program on the Iranian parents of children with ADHD imply that Barkley's program can be used as a therapeutics solution to reduce some of the symptoms in ADHD children. Therefore, it is suggested that mental health professionals, social workers, and psychologists working with ADHA children use Barkley's program in their therapeutic interventions.   In terms of theoretical implication, the current empirical research supported Barkley's program as a useful short-term treatment approach in reducing of ADHD symptoms.

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8/24/2011

Effect of Testosterone on Hind Limb Regeneration in Tadpoles of the Egyptian Toad, *Bufo Regularis* Reuss

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Abstract: The present study investigated the role of Testosterone on the regenerative capacity in two metamorphic stages of the tadpoles of the Egyptian toad, Bufo regularis Reuss, after amputation of the hind limb at the mid-shank level. It indicated an enhancing effect of Testosterone treatment on limb regeneration in the prometamorphic (stage 56), where 90% of the cases regenerated toes ranging from five to one compared with 77.3% in the control group, also the differential effect of testosterone on the number of toes was obvious in the treated animals, where 30% and 35% of the cases regenerated five and four toes respectively compared with 27.3% and 31.8% in the control group. In the metamorphic stage (stage58), the effect of testosterone was also obvious, where 38.6% of the treated cases restored toes compared with 13.3% of the cases in the control group. 45.5% of the treated cases restored part of the foot compared with 20 % of the cases in the control group. Histological observations of the treated limbs revealed that the formation of thick epithelial covering and complete skin is faster than that of the control animals. This may indicate that the enhancing effect of testosterone on limb regeneration, this may be due to the acceleration of wound healing either by its action upon the proliferative phase of healing which involves immune processes such as reepithelialization and angiogenesis or by the production of IGF-1 or by its stimulatory effect through Wnt/β-catenin signaling resulting in the initiation of the early phases of limb regeneration.

Key Words: Limb regeneration, Amphibia, Testosterone.

List of Abbreviations:
As Astragalus **BL** Blastema **C** Cartilaginous collar **CD** Cellular debris **CF** Cartilage or procartilage formation **CP** Cartilaginous cap **E** Epiphysis **FC** Fibrocytes or fibrocellular accumulation **M** Muscle fibres or Muscle Group **MG** Multicellular glands **ML** Melanophores **MS** Mesenchymal cells **PH** Phalanges **SC** Scar of fibrocellular tissue **TF** Tibio-fibula **WE** Wound epithelium

Introduction

Limb regeneration is one of the best examples of organ/appendage regeneration in vertebrates and has been called 'epimorphosis' since it requires blastema formation and proliferation (**Brockes, 1997; Suzuki *et al.*, 2006**) though there has been criticism of the classical definition of epimorphosis and morphallaxis (**Agata *et al.*, 2007**). Among tetrapods, the cellular and molecular mechanisms involved in limb development are highly conserved, where fully developed limbs share a common skeletal pattern (**Muneoka and Sassoon, 1992**). On the other hand, the regenerative responses of limbs after amputation differ from animal to animal among tetrapods. Birds cannot regenerate limbs at any stage of development and, surprisingly, mammals have slightly better limb regenerative capacity than that of birds. Embryonic and neonatal mice can regenerate their digit tips if they are amputated through the distal phalanx (**Borgens 1982; Reginelli *et al.*, 1995**), and similar digit tip regeneration occurs in humans (**Douglas, 1972; Illingworth, 1974**). After amputation at a more proximal level, a neonatal mouse cannot regenerate lost parts, and hypertrophy of amputated bones occurs (**Masaki and Ide, 2007**). In contrast, amphibians have exceptionally high regenerative capacity for limb regeneration. Urodele amphibians such as newts and salamanders can regenerate their limbs following amputation any time during their life cycles, although there is a non-regenerative mutant in axolotls (**Sato and Chernoff, 2007**). Anuran amphibians such as *Xenopus* are intermediate between urodele amphibians and other vertebrates in terms of their regenerative capacity, in that they can completely regenerate developing hind limb buds prior to the onset of metamorphosis, but regenerative capacity declines gradually as metamorphosis proceeds (**Dent, 1962; Muneoka *et al.*, 1986; Suzuki *et al.*, 2006**).

Many investigations have dealt with the factors affecting either retardation or enhancement of the regenerative capacity among urodeles and anurans, by using several experimental means such as mechanical, electrical, chemical, and hormonal means.

Hormones as well as hormone-like growth factors are well known to promote cellular

differentiation and regeneration (**Leon *et al.,* 1998; De luca *et al.*, 1999**).

Dyson and Joseph (1968) concluded that the treatment of rabbit females with testosterone stimulates their regenerative growth in the ear.

Beran *et al.* (1982) concluded that testosterone and some of the synthetic analogs tested exert their hemopoietic effect, at least partly, by affecting the maintenance of erythroid and granulocytic stem cells, directly by increasing their survival or proliferation or indirectly by increasing the input from multipotent stem cell pool, or by both mechanisms.

Kinderman and Jones (1993) proved that testosterone propionate administration during facial nerve injury results in an increase in ribosomal levels in hamster facial motoneuron system (FMN).

Bardin, 1996; Katznelson *et al*., 1996; Bhasin *et al.*, 1997&2000; Swerdloff and Wang, 2003 stated that treatment with testosterone improves muscle mass and strength, bone density, and reduces visceral fat in a variety of subjects.

Brown *et al*. (2001) suggested that testosterone enhances the rate of regeneration by increasing the neuronal cytoskeletal response after axonal injury. And suggested a common mechanism for gonadal steroid action on regenerating motoneurons across species.

Ustünel *et al.* (2003) determined that testosterone can induce protein synthesis in gastrocnemius muscle fibres, and induces changes in shape and size, and also can change the appearance and the number of fibres.

Sinha-Hikim *et al*. (2003) Concluded that testosterone -induced muscle fiber hypertrophy is associated with an increase in satellite cell number, a proportionate increase in myonuclear number, and changes in satellite cell ultrastructure.

Prokai-Tatrai *et al.*(2007) stated that testosterone can activate synthesis of bcl-2 protein, which prevents cell apoptosis in the injured regions.

Cayan *et al.* (2008) showed that testosterone has a significant role to increase bladder smooth muscle, leading to improvement in bladder functions in postmenopausal women with urogenital system dysfunction.

Little *et al*. (2009) suggested that testosterone has neuroprotective effects on morphology and function in both highly androgen-sensitive as well as more typical motoneuron populations, further supporting a role for testosterone as a neurotherapeutic agent in the injured nervous system.

Wilson *et al.*(2009) suggested that Testosterone has neuroprotective effects on morphology in both males and females.

Fu *et al.*(2011) suggested that testosterone promotes cell proliferation and differentiation via G protein-coupled receptors and different downstream pathways in the L6 cell line, although the related

molecular mechanisms need to be elucidated in future studies.

The present study was deemed necessary in view of elucidating further the effect of testosterone on hind-limb regeneration in a prometamorphic stage (stage 56) and a metamorphic stage (stage 58) of the Egyptian toad, *Bufo regularis* Reuss after amputation at the mid-shank level.

2 Material and Methods:

Early tadpoles of *Bufo regularis* Reuss were collected from the ponds of Abou Rawash, Giza Governorate, Egypt. The tadpoles were reared in glass aquaria (60 x 30 x 30cm) in the laboratory at room temperature 27°C ± 2°C, in Department of Zoology, Faculty of Science, Cairo University.

Two stages were selected for this study. Staging of the individuals before the operation was carried out according to the normal table of **Sedra and Michael (1961)**. The selected stages were numbered 56 and 58. The most distinctive external criteria of these stages are as follows:

Stage 56 (A prometamorphic stage)

Age 28 days, length 25mm and tail is 14.5 mm long. Hind limb is about 1.9 mm in Length. Landmarks between thigh, shank and foot are more distinct. Melanophores are scattered on all toes and are especially dense on the last three toes.

Stage 58 (A metamorphic stage)

Age 39 days, length 30 mm and tail is 17 mm long. Elbow of right fore-limb is piercing or has already pierced the overlying skin. The left fore-limb has passed out through the wide, spout-like opening of the branchial chamber; thus this limb is now completely exposed. About 4-5 tubercles on the palm are prominent. The fourth toe is the longest. Web clearly developed between 2-3 and 3-4 toes.

Tadpoles of each stage were randomly divided into two groups: a control group and a treated one. Chloretone (Sigma) was used as an anaesthetic medium in tap water with the concentration 1:2000. Each operated case was then transferred to a Petri dish containing half concentration of the anaesthetic medium, then shortly to another Petri dish containing tap water in which the operated case recovered and became motile within few minutes.

For each group, amputation was carried out on the left hind-limb at the level of the mid-shank (Fig. 1). The right limb was kept intact. Amputation was carried out by using iridectomy scissors and fine watch-maker's forceps. Experimental tadpoles were injected intraperitoneally after amputation with Testosterone propionate (Testone-E, Misr Co. for Pharm Ind., Cairo, Egypt) at a single dose of 5µl / individual.

To clarify the early post-operative histological changes within the stump tissues, individuals were

selected and fixed at regular intervals, in a time series of 1, 3, 5, 7, 10 and 15 post-operative days, while the remaining tadpoles were left to reach two weeks after complete metamorphosis. A total of 250 cases were operated. Out of these, 80 cases were serially sectioned and studied microscopically.

The amputated limbs were then dehydrated through ascending grades of alcohol, cleared in cedar wood oil and finally embedded in parablast. Limbs were serially sectioned longitudinally at a thickness of 7 microns. The sections were stained with haematoxylin and eosin for general histological structures. A total of 160 cases (metamorphosed toadlets) were fixed, and morphologically examined.

To investigate the pattern of skeletal elements, transparencies of the operated limbs by using Victoria blue stain (**Bryant and Iten, 1974**) and Alizarin Red S stain (**Sedra, 1950**) were made.

Photomicrographs of representative sections and limbs of both control and treated animals were prepared at a known magnification.

3 Results
STAGE 56Group: 1/56 (Control group)A) Histogenesis of time-series•

By the first post-amputation day, the wound surface was covered with two or three layers of epithelial cells. Epithelial covering was thicker at the most distal tip of the regenerate. Nuclei of the epithelial cells were large and rounded. The basement membrane was indistinct. The activity of macrophages in removing cellular debris was noticed at the stump surface (Fig. 4).• By the third post-amputation day, the main bulk of the blastema was fibro-cellular in nature. Some blastemal cells began to redifferentiate into cartilage cells added to the stump skeleton .• By the fifth post-amputation day, the upper layer of the epidermis was cornified. Procartilaginous streaks were evident in the distal regions as an early indication of autopodial elements on the way to redifferentiate, with indentation at the most distal part of the regenerate as a first sign of toes formation. Muscles were surrounding the skeleton of the regenerate (Fig. 5).• By the seventh post-amputation day,the epidermis was stratified and the dermis was thin and multi-cellular glands were embedded within the dermis. The shank region was restored completely with its skeletal support. Distally, a chondrifying centre representing the foot skeleton was observed. Muscles were surrounding the skeleton of the regenerate. Lymph spaces were seen beneath the skin.• By the tenth post-amputation day, the shank was completely restored. Distally, long skeletal elements were redifferentiated representing the autopodial skeleton. Muscles were well-redifferentiated surrounding the skeletal elements. The skeletal elements were normally articulating with each other .• By the fifteenth post-amputation

day, redifferentiation progressed distally resulting in the restoration of toes with their skeletal support (Fig. 6).

B) Final cases
i) General morphological characteristics (Table 1 & Fig.2):
- 44 cases were operated. Out of these:
• 12 cases had regenerated five toes**.** One of them, that was demonstrated with Victoria blue stain, weak chondrification of phalanges of the 1st toe and the terminal phalanges of 2nd toe was observed (Fig. 7).
• 14 cases had regenerated four toes each. One of them, showed that all the regenerated limb segments were normal while the fourth toe was short (Fig. 8).
• Four cases had regenerated three toes each. One of them, that was demonstrated with Alizarin red preparation, astragalus and calcaneum were short and completely fused (Fig. 9).
• Two cases had regenerated two toes each.
• Two cases had regenerated one toe each.
• Two cases had regenerated part of foot.
• Four cases had regenerated part of the shank region with a tapering end.
• Four cases had regenerated part of the shank region with a blunt end.

ii) Histological observations

Two cases were studied microscopically. Both of them showed advanced histogenesis, restored normal skeletal elements with normal configuration and articulation between phalanges. Muscles were well-restored surrounding the skeletal elements (Fig. 10).

Group: 2/56 (Testosterone -treated group):
A) Histogenesis of time-series:
• By the first post-treatment day, a thick epithelial covering closed the wound surface. Basement membrane was indistinct; the wound cover was dermis free. Activity of macrophages in removing cellular debris was noticed. Dedifferentiation of muscles began around the stump skeleton (Fig. 11).
• By the third post-treatment day, the epidermal covering was two or three cells thick. Basement membrane was discontinuous. Unicellular glands were observed. The dedifferentiated mesenchymal cells formed a blastema. The whole regenerate was in the form of a cone (Fig.12).
• By the fifth post-treatment day, melanophores and multicellular glands were spread beneath the epidermis. Mitotic activities of the blastemal cells resulted in more elongation of the regenerate with its pointed distal tip.
• By the seventh post-treatment day, the tibio-fibula was completely restored. Distally, a chondrifying centre representing the autopodial skeleton was

observed. Muscles were surrounding the skeleton of the regenerate (Fig. 13).
• By the tenth post-treatment day, more redifferentiation was observed. redifferentiation of muscles around the skeletal elements was observed
• By the fifteenth post-treatment day, further redifferentiation progressed distally resulting in the restoration of toes with its skeletal support. Skeletal elements were normally articulating with each other (Fig. 14)**.**

B) Final cases:
   i) General morphological characteristics (Table 1 & Figs. 2):
- 40 cases were operated:
• 12 cases had regenerated five toes each. One of them, that was demonstrated with Alizarin red preparation, weak ossification of terminal phalanges of 1st , 2nd ,3rd and 5th toes were noticed (Fig. 15).
• 14 cases had regenerated four toes each. In one of them, that was demonstrated with Victoria blue stain, most skeletal elements were strongly chondrified (Fig. 16).
• Five cases had regenerated three toes each. One of them, that was demonstrated with Victoria blue stain**,** it showed partial fusion between the basal phalanges of the 1st and 3rd toes with skeletal elements of foot region was noticed (Fig. 17).
• Two cases had regenerated two toes each.
• Three cases had regenerated one toe. In one of them, that was demonstrated with Victoria blue stain, chondrifying phalanges supporting the toe were obvious (Fig. 18).
• Two cases had regenerated part of foot.
• Two cases had regenerated part of the shank region with a tapering end.

ii) Histological observations:
 Two cases were studied microscopically. In the first case, the one that regenerated three toes, restoration of most skeletal elements and soft tissues was well observed**.** In the second case; that regenerated two toes, most of the skeletal elements of the foot and toes were restored (Fig.19).

STAGE 58
Group: 1/58 (Control group)
 A) Histogenesis of time-series
• By the first post-amputation day, the wound surface was covered with stratified epithelium. The epithelial cells were having large and rounded nuclei. Some activity of macrophages in removing cellular debris was noticed.
• By the third post-amputation day, the epithelial covering was two or three cells thick. Basement membrane was seen discontinuous. Dermis was still hardly seen. Mitotic activities of the blastemal mesenchyme cells were noticed (Fig. 20).

• By the fifth post-amputation day, few melanophores and multicellular glands were noticed. Cartilage redifferentiation was noticed on both sides of stump skeleton. The distal part of the blastema was still fibrocellular in nature.
• By the seventh post-amputation day, the upper layer of the epidermis was cornified.. Cartilage redifferentiation progressed at both sides of the tibio-fibula shaft. Mesenchymal cells of the blastema were still noticed at the distal end of the regenerate (Fig. 21).
• By the tenth post-amputation day, the upper layer of the epidermis was cornified. Melanophores and multicellular glands were noticed. cartilage redifferentiation progressed to form a cap above the collar, while blastema cells were still fibrocellular in nature.
• By the fifteenth post-amputation day, melanophores and multi-cellular glands were highly spread beneath the skin. A cartilaginous collar was formed around the distal end of the shaft of the tibio-fibula. The collar was extending apically to form a cap. A fibrous scar was surrounding the distal part of the skeleton (Fig. 22).

B) Final casesi) General morphological characteristics (Table 1 & Fig. 3)
-30 cases were operated. Out of these:
• Four cases had restored one toe each. In one of them, the shank region was straight with a thin foot ending with a toe-like protrusion. Upon demonstration with Victoria blue preparation, chondrifying phalanges supporting the toe were obvious (Fig. 23).
• Six cases had restored part of the foot. In one of them, that was demonstrated with Victoria blue stain the regenerated part of foot was small,chondrification was obvious at the base of foot (Fig. 24).
• Eight cases had restored the shank region with a tapering end. One of them, that was demonstrated with Alizarin red preparation, there was incomplete restoration of skeletal elements (Fig. 25).
• Eight cases regenerated part of the shank region with a blunt end.
• Four cases were negative.

ii) Histological observations
     The examined case regenerated nearly the whole shank with a blunt end; it had a toe-like protrusion laterally, in which, multicellular glands and melanophores were well-spread within the skin. A large cartilaginous condylar cap was formed around the stump skeleton. Muscle fibres were surrounding the skeletal tissue, but merging distally into fibrous scar (Fig. 26).

Group: 2/58 (Testosterone -treated group):
A) Histogenesis of time-series:

• By the first post-treatment day, the wound surface was covered with a thin layer of epithelial cells that were condensed at the distal margin of the stump. These cells were cuboidal with rounded nuclei while the most outer cells were squamous with flattened nuclei-basement membrane and dermis was not seen. Activity of macrophages in removing cellular debris was noticed (Fig. 27).

• By the third post-treatment day, the epithelial covering was two or three cells thick. Basement membrane was discontinuous. Some blastema cells were redifferentiated into procartilage cells above the stump skeleton, while other blastema cells were still undifferentiated having fibro-cellular nature. The regenerate ended with ablunt end (Fig. 28).

• By the fifth post-treatment day, epidermal covering was formed of thick stratified squamous epithelium with underlying thin dermis. Melanophores and multicellular glands were observed. Most of the blastema cells were redifferentiated into cartilage cells and were added to the stump skeleton, while fibrocellular tissue was still observed beneath the skin. Some muscle fibres were redifferentiated at the stump edges, surrounding the skeletal elements (Fig. 29).

• By the seventh post-treatment day, the upper layer of the epidermis was cornified, melanophores and multicellular glands were seen. The addition of cartilage cells to the stump skeleton resulted in the formation of thick collar around the tibio-fibula shaft. mesenchymal cells of the blastema were intermingled with fibres above the stump skeleton (Fig. 30).

• By the tenth post-treatment day, melanophores and multicellular glands were noticed. A chondrifying centre was formed distally and articulating with the cartilaginous cap. Muscle fibres were redifferentiated around the skeletal tissues. Loose fibrocellular connective tissue was obviously seen beneath the skin. The distal end of the regenerate was protruding outwards (Fig. 31).

• By the fifteenth post-treatment day, epidermis was cornified. The cartilaginous collar formed around the distal part of the shaft of tibio-fibula was extending apically to form a cap. Fibrous bundles were forming a scar underneath the skin (Fig. 32).

B) Final cases:
i) General morphological characteristics (Table 1& Fig.3):
- 44 cases were operated:
•Two cases had restored two toes, One of them, that was demonstrated with Victoria blue stain, the shank region was short and the foot was considerably ending with toes appeared as two fused small protuberances (Fig.33).

• 15 cases had restored one toe, in one of them, that was demonstrated with Victoria blue preparations, the lateral toe -like protrusion was supported by chondrifying phalanges (Fig.34)

• 20 cases had restored part of the foot. In one of them, that was demonstrated with Alizarin red preparations, complete restoration of tibio- fibula and no skeletal support at the restored part of foot was shown (Fig. 35).

• Six cases had restored the part of the shank region with a tapering end.

• One case had restored part of the shank region with a blunt end.

ii) Histological observations:

The examined case had regenerated part of the foot. A cartilaginous collar was formed representing the distal epiphysis of tibio-fibula and extending distally into a cartilaginous element which is the skeletal support of the foot part (Fig. 36).

4. Discussion

The present study aimed to investigate the effect of testosterone on the restoration of the regenerative capacity in a prometamorphic stage (stage 56) and a metamorphic stage (stage 58) of the Egyptian toad, *Bufo regularis* Reuss, after amputation at the mid-shank level. Selection of the experimental stages was based on the following: Stage 56 represents the prometamorphic stage, during which the regenerative capacity starts to drop down. Stage 58 represents the metamorphic stage, where the regenerative capacity is reduced or completely lost.

Scadding (1979) stated that neither gonadectomy, nor injections of testosterone or 17-beta estradiol, had apparent effect on the rate of regeneration or histological appearance of limb regenerates in the newt *Notophthalmus viridescens*. Neither promotion nor inhibition of limb regeneration was observed.

Tarsoly *et al*., (1979) concluded that testosterone exerts a direct peripheral effect on the callus cells, presumably on their enzyme system.

Vita et al.(1983) tested the effect of testosterone on the reinnervation of the anterior tibialis sciatic nerve following crush in rabbits. And showed that there is accelerative effect of Testosterone on the regeneration process.

Sassoon et al. (1986) concluded that testosterone induces both chondrogenesis and myogenesis in juvenile larynx and that this process may contribute to the pronounced sexual dimorphism of the adult vocal organ.

Testosterone has a documented ability to modulate the activity of immune, fibroblast, and myogenic precursor cells, which are all components of regeneration (**Grounds, 1987; Zhang et al., 1998; Friedl et al., 2000; Horiguchi et al., 2002 and Schneider et al., 2003**).

Jones et al.(2001) showed that exogenous administration of testosterone immediately after

nerve injury impacts positively on the functional recovery through actions mediated by the androgen

receptor. The mechanism by which steroidal enhancement of the regenerative properties of
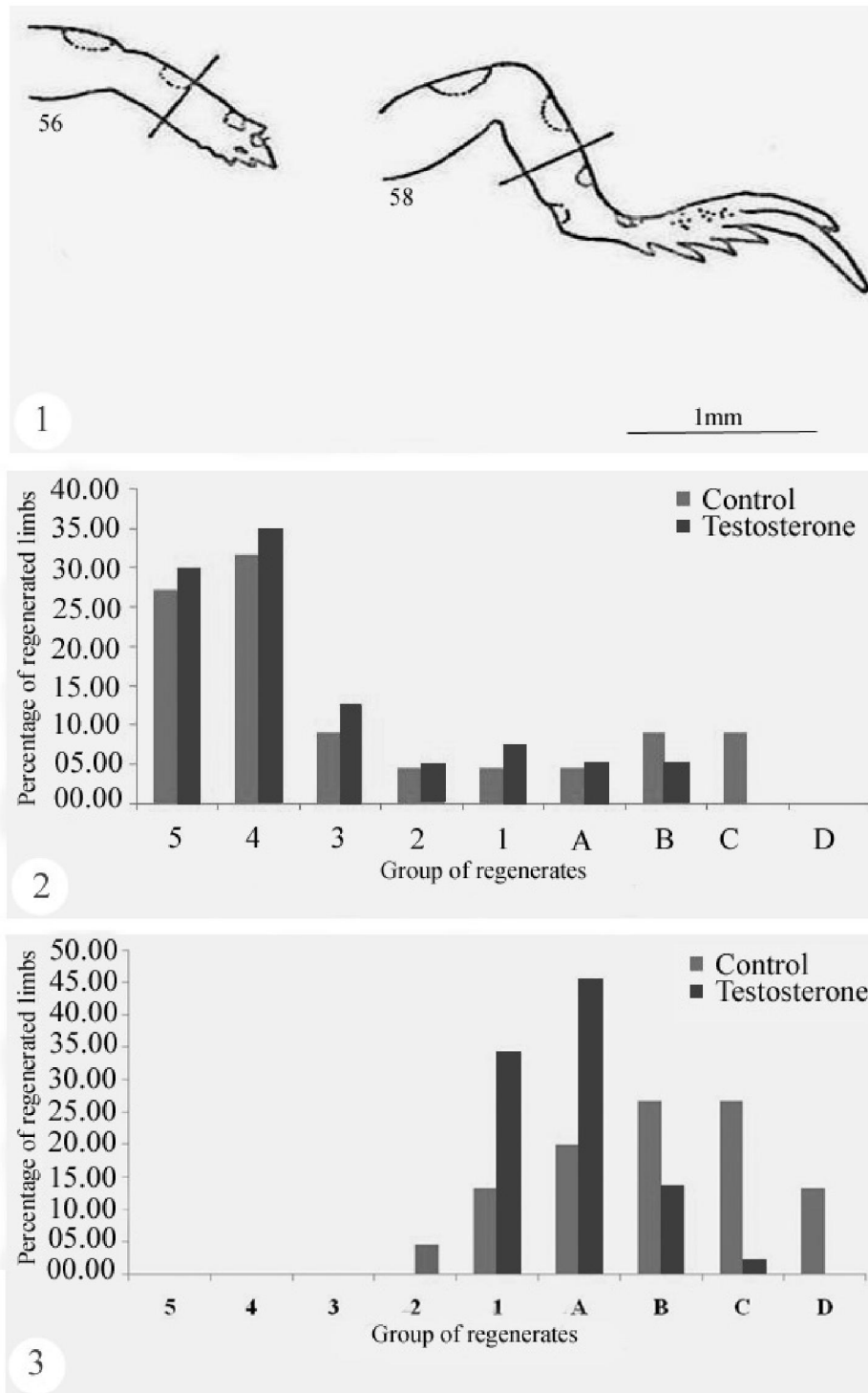
Fig. (1): Diagrammatic drawing of the left hind-limbs of stages 56 and 58 of the tadpoles of *Bufo regularis* Reuss, shown in antero-lateral view. The level of amputation is represented by a line transecting the mid-shank level.

Fig. (2): A histogram showing comparison between the regenerates of control and experimental tadpoles treated with Testosterone after amputation at the mid-shank of stage (56) of *Bufo regularis*.

Fig. (3): A histogram showing comparison between the regenerates of control and experimental tadpoles treated with Testosterone after amputation at the mid-shank of stage (58) of *Bufo regularis*.
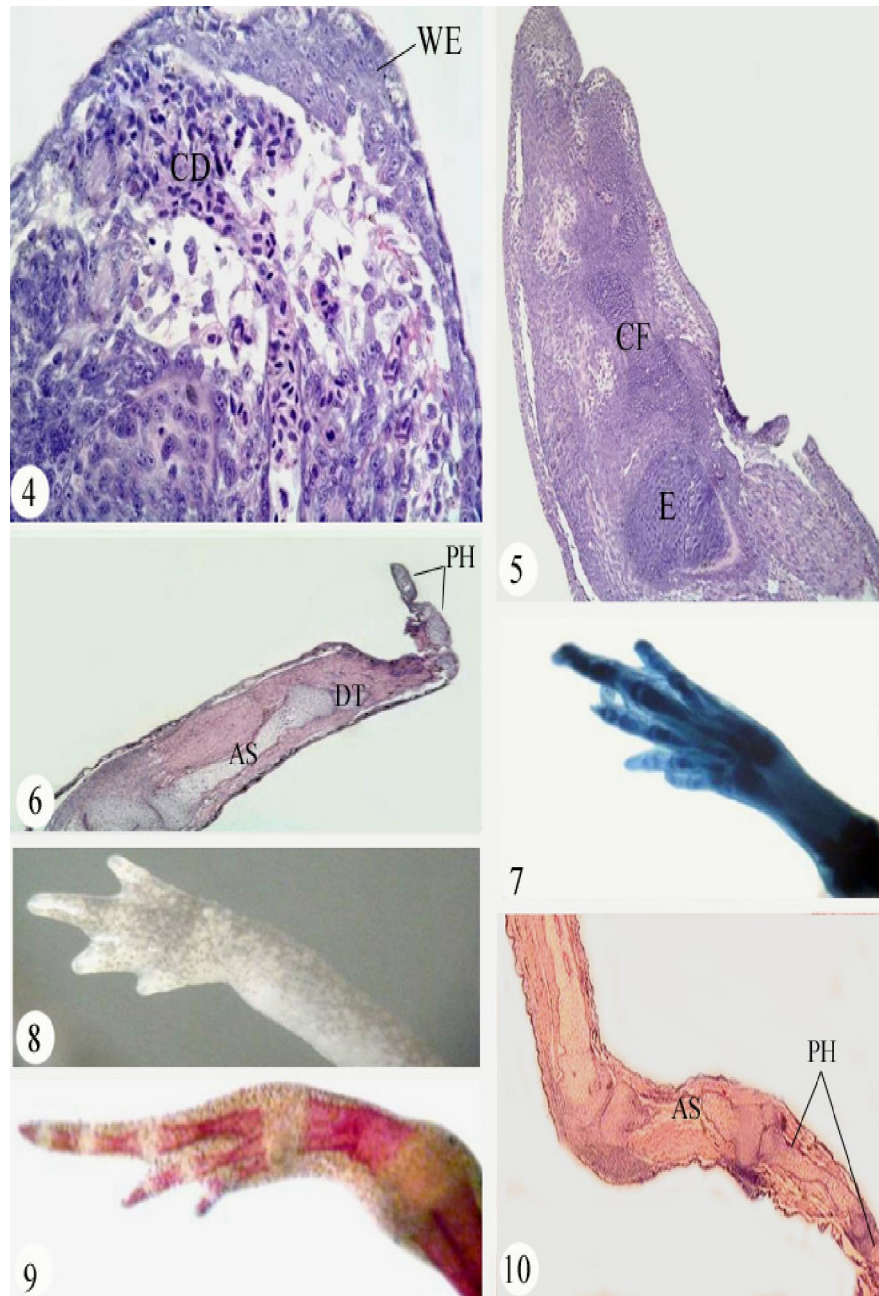
Fig. (4): A photomicrograph of a longitudinal section of a regenerate of left hind limb, fixed one day after amputation. H&E stain (X 200).
Fig. (5): A photomicrograph of a longitudinal section of a regenerate of left hind limb, fixed five days after amputation. H&E stain (X 100).
Fig. (6): A photomicrograph of a longitudinal section of a regenerate of left hind limb, fixed fifteen days after amputation. H&E stain (X 100).
Fig. (7): A photomicrograph of a regenerate of left hind limb, fixed two weeks after metamorphosis and demonstrated with Victoria blue stain (X 25).
Fig. (8): A photomicrograph of a regenerate of left hind limb, fixed two weeks after metamorphosis(X 25).
Fig. (9): A photomicrograph of a regenerate of left hind limb, fixed two weeks after metamorphosis and demonstrated with Alizarin red stain (X 25).

Fig. (10): A photomicrograph of a longitudinal section of a regenerate of left hind limb, fixed two weeks after metamorphosis. H&E stain (X 40).

Fig. (11): A photomicrograph of alongitudinal section of a regenerate of left hind limb, fixed one day after amputation. H&E stain (X 200).

Fig.(12): A photomicrograph of alongitudinal section of a regenerate of left hind limb, fixed three days after amputation. H&E stain (X 200).

Fig.(13): A photomicrograph of a longitudinal section of a regenerate of left hind limb, fixed seven days after amputation. H&E stain (X 40).

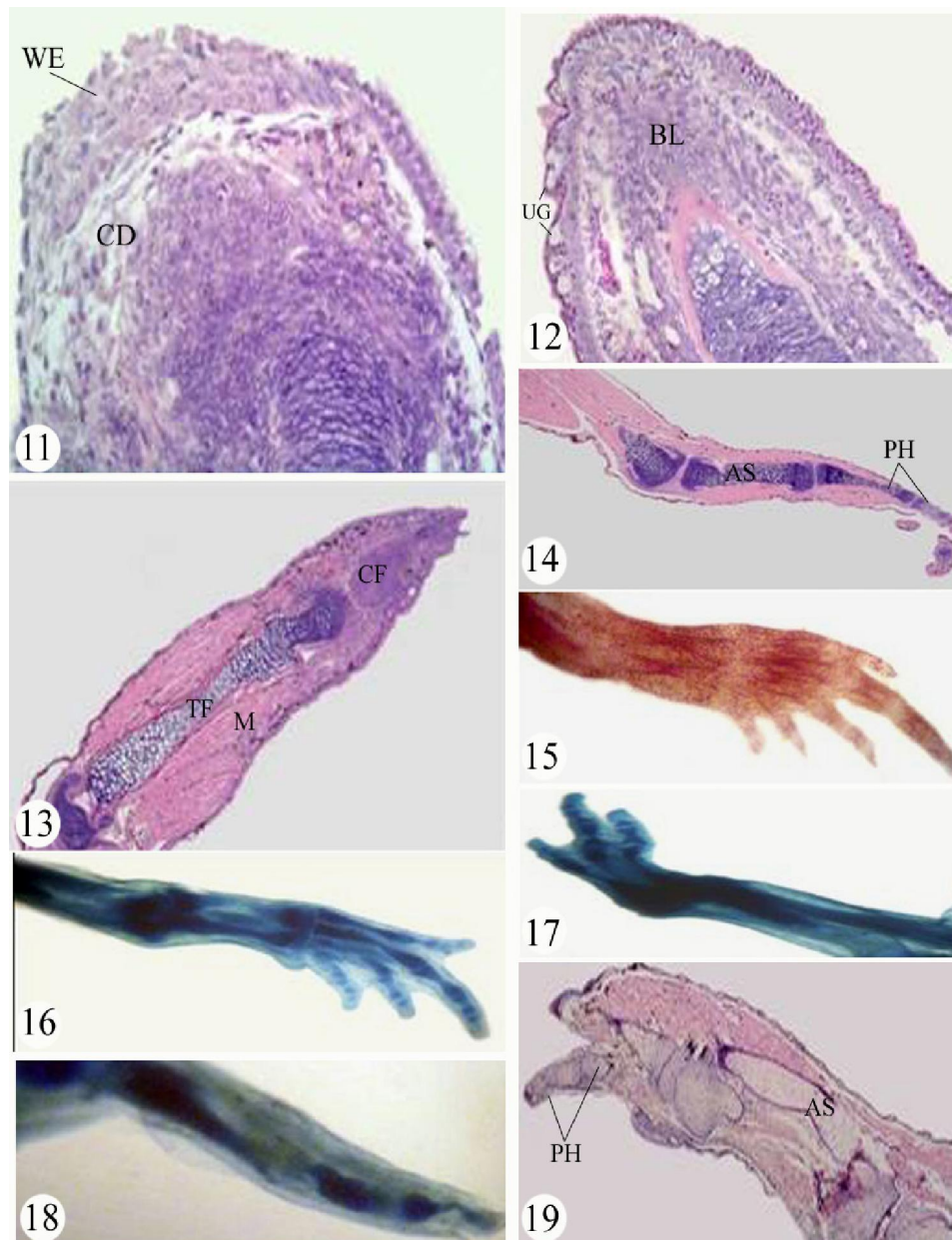**Fig. (14):** A photomicrograph of aLongitudinal section of a regenerate of left hind limb, fixed fifteen days after amputation. H&E stain (X 40).

Fig. (15): A photomicrograph of a regenerate of left hind limb, fixed two weeks after metamorphosis and demonstrated with Alizarin red stain (X 25).

Fig. (16): A photomicrograph of a regenerate of left hind limb, fixed two weeks after metamorphosis and demonstrated with Victoria blue stain (X 25).

Fig. (17): A photomicrograph of a regenerate of left hind limb, fixed two weeks after metamorphosis and demonstrated with Victoria blue stain (X 25).

Fig. (18)**:** A photomicrograph of a regenerate of left hind limb, fixed two weeks after metamorphosis and demonstrated with Victoria blue stain (X 25).

Fig. (19)**:** A photomicrograph of a longitudinal section of a regenerate of left hind limb, fixed two weeks after metamorphosis. H&E stain (X 40).

Fig. (20): A photomicrograph of a longitudinal section of a regenerate of left hind limb, fixed three days after amputation. H&E stain (X 100).

Fig. (21)**:** A photomicrograph of a longitudinal section of a regenerate of left hind limb, fixed seven days after amputation. H&E stain (X 40).

Fig. (22)**:** A photomicrograph of a longitudinal section of a regenerate of left hind limb, fixed fifteen days after amputation. H&E stain (X 100).
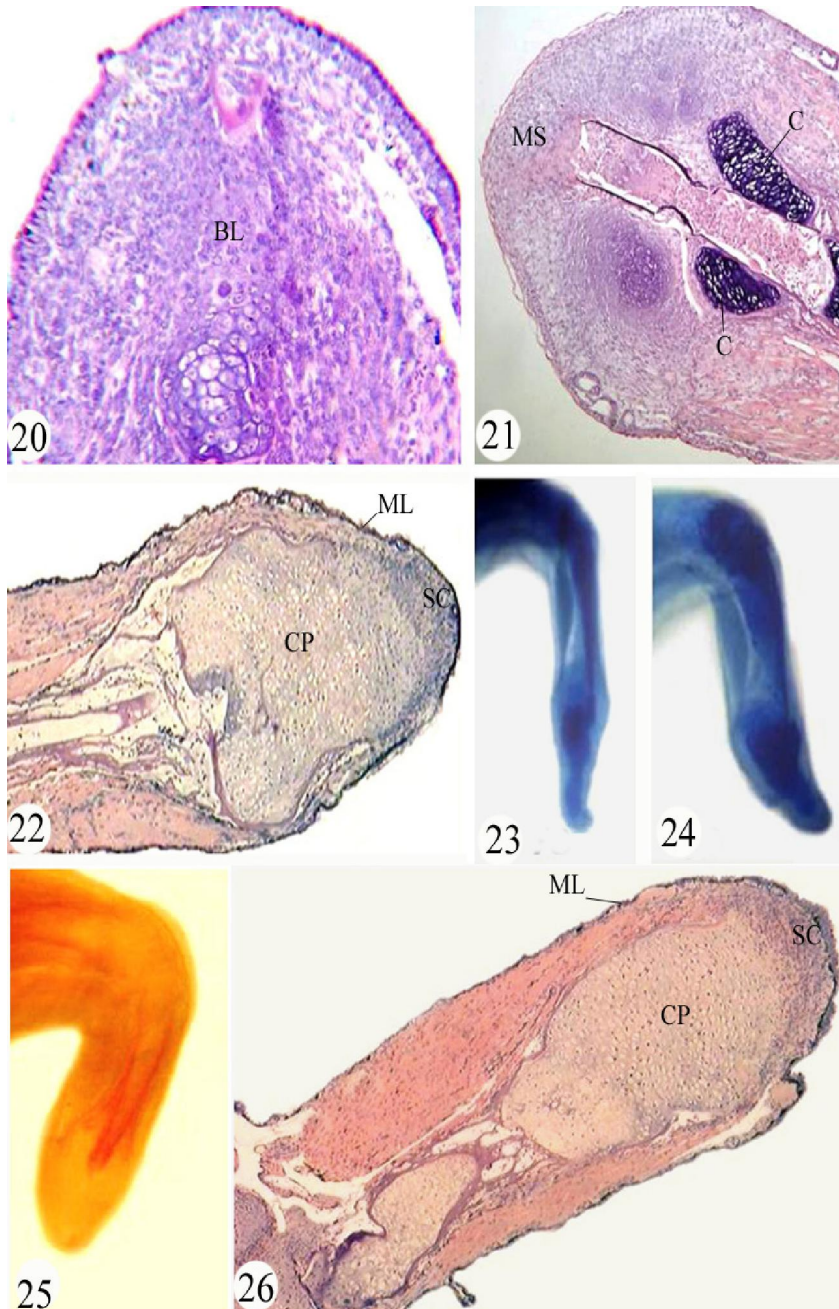
Fig. (23)**:** A photomicrograph of a regenerate of left hind limb, fixed two weeks after metamorphosis and demonstrated with Victoria blue stain (X 25).

Fig. (24)**:** A photomicrograph of a regenerate of left hind limb, fixed two weeks after metamorphosis and demonstrated with Victoria blue stain (X 25).

Fig. (25)**:** A photomicrograph of a regenerate of left hind limb, fixed two weeks after metamorphosis and demonstrated with Alizarin red stain (X 25).

Fig. (26)**:** A photomicrograph of a longitudinal section of a regenerate of left hind limb, fixed two weeks after metamorphosis. H&E stain (X 40).

Fig. (27)**:** A photomicrograph of alongitudinal section of a regenerate of left hind limb, fixed one day after amputation. H&E stain (X 200).

Fig.(28)**:** A photomicrograph of alongitudinal section of a regenerate of left hind limb, fixed three days after amputation. H&E stain (X 200).
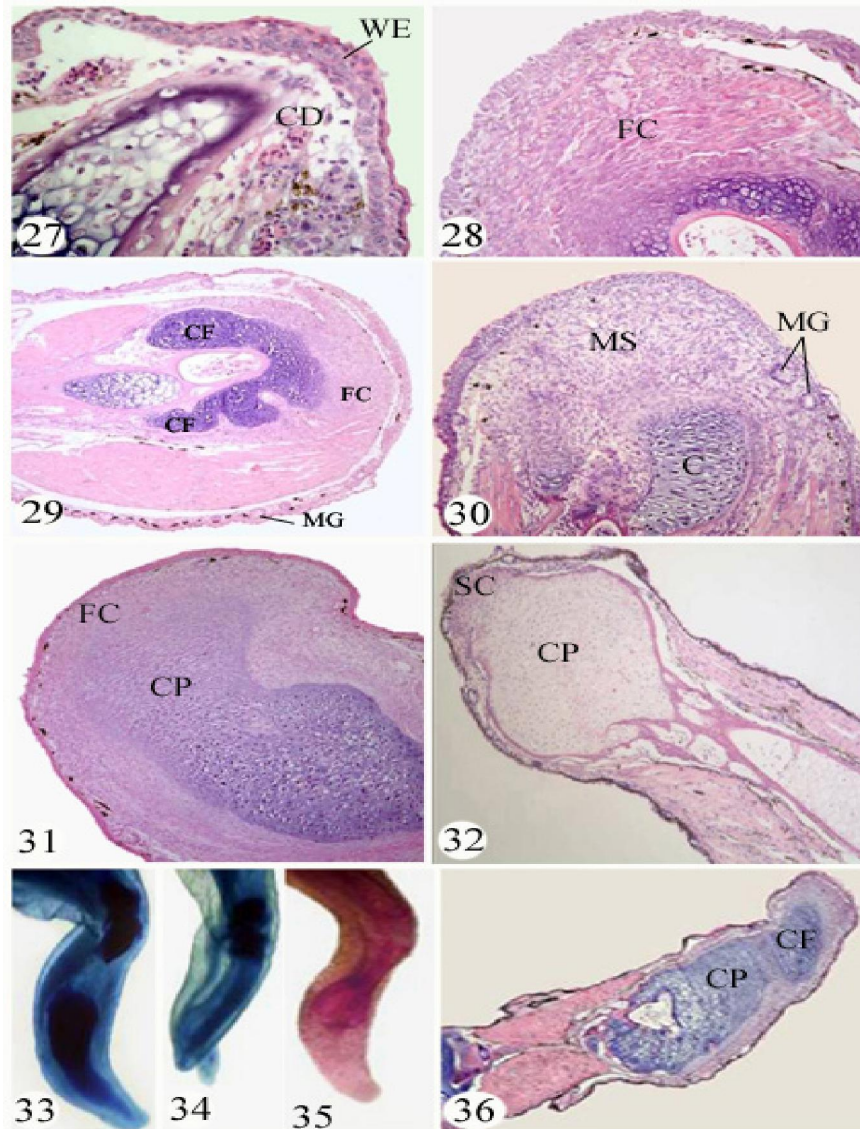
Fig.(29)**:** A photomicrograph of aLongitudinal section of a regenerate of left hind limb, fixed five days after amputation. H&E stain (X 100).

Fig.(30)**:** A photomicrograph of a longitudinal section of a regenerate of left hind limb, fixed seven days after amputation. H&E stain (X 100).

Fig.(31)**:** A photomicrograph of alongitudinal section of a regenerate of left hind limb, fixed ten days after amputation. H&E stain (X 100).

Fig.(32)**:** A photomicrograph of aLongitudinal section of a regenerate of left hind limb, fixed fifteen days after amputation. H&E stain (X 40).

Fig. (33)**:** A photomicrograph of a regenerate of left hind limb, fixed two weeks after metamorphosis and demonstrated with Victoria blue stain (X 25).

Fig. (34)**:** A photomicrograph of a regenerate of left hind limb, fixed two weeks after metamorphosis and demonstrated with Victoria blue stain (X 25).

Fig. (35)**:** A photomicrograph of a regenerate of left hind limb, fixed two weeks after metamorphosis and demonstrated with Alizarin red stain (X 25).

Fig. (36)**:** A photomicrograph of a longitudinal section of a regenerate of left hind limb, fixed two weeks after metamorphosis. H&E stain (X 40).

injured motoneurons occurs may involve pre-existing androgen receptors, heat shock proteins, and modulation of the cellular stress response.

Testosterone generally has immunosuppressive and anti-inflammatory properties (**McCruden and Stimson, 1991; Giglio** *et al.,* **1994; Wichmann** *et al.,* **1997; Savita and Rai, 1998**), although there is evidence that Testosterone promotes inflammation in dermal wound healing (**Ashcroft and Mills, 2002; Ashcroft** *et al.***, 2003**).

Testosterone increases expression of the nerve growth factor (**Tirassa** *et al.***, 1997**) and mediates promotion of neurite growth and interneural communication through branching and arborization (**Kujawa** *et al.***, 1991**).

Phillip *et al.***(2001)** conclude that testosterone has a direct, local, GH-independent effect on growth of the tibial epiphyseal growth plate and IGF-1 receptor abundance in hypophysectomized and castrated rats.

White *et al.* **(2009)** showed that Nandrolone decanoate (ND) (exogenous testosterone) administration can enhance castrated mouse muscle regeneration during the recovery from bupivacaine-induced injury. ND had a main effect for increasing muscle MyoD and cyclin D1 mRNA expression at 14 days.

The present results indicated an enhancing effect of testosterone treatment on limb regeneration in stage 56, where 90% of the cases regenerated toes ranging from five to one compared with 77.3% in the control group, also the differential effect of testosterone on the number of toes was obvious in the treated animals, where 30% and 35% of the cases regenerated five and four toes respectively compared with 27.3% and 31.8% in the control group.

In the metamorphic stage (stage58), the effect of testosterone was also obvious, where 38.6% of the treated cases restored toes compared with 13.3% of the cases in the control group. And 45.5% of the treated cases restored part of the foot compared with 20 % of the cases in the control group.

The present results agree with and support the results of (**Dyson and Joseph 1968; Vita** *et al.***, 1983;Grounds 1987; Zhang** *et al.***, 1998; Friedl** *et al.***, 2000; Horiguchi** *et al.***, 2002 and Schneider** *et al.***, 2003**). Who showed the accelerative effect of testosterone on the regeneration process.

Histological observations of the treated limbs revealed that the formation of thick epithelial covering and complete skin is faster than that of the control animals.

Demling (1999) found that anabolic agents, human growth hormone, HGH, and the testosterone analogue, oxandrolone, after severe burn injury,

significantly decreased weight and nitrogen loss and increased healing with nearly identical benefits.

Testosterone is needed for the wound healing process since decreased levels impede healing (**Stanford** *et al.***, 1999; Demling, 2000; Demling and Orgill 2000**).

Karim *et al.* **(1973)**; **Janssens and Vanderscheuren (2000)** demonstrated a significant increase in net protein synthesis, especially in muscle and skin, with high doses of Testosterone delivered parenterally.

Previous studies have shown the importance of testosterone on dermal wound healing (**Ashcroft and Mills, 2002; Ashcroft** *et al.,* **2003**) and the modulatory effects of this hormone on immune responses (**Cutolo** *et al.,* **2002; Palaszynski** *et al.,* **2004**).

Robert and Demling (2005) showed that exogenous administration of anabolic agents ,human growth hormone, insulin-like growth factor-1, insulin, testosterone and its analogs maintained or increased lean body mass as well as directly stimulate the healing process through their anabolic and anticatabolic actions.

The anabolic properties of testosterone were defined in the 1930s. These include an increase in muscle size, synthesis, and strength. Increased skin thickness has also been noted with administration of testosterone to hypogonadal men. The importance of testosterone is evident by the complications seen with low Testosterone levels, which include sarcopenia or lost lean mass, increased rate of development of osteoporosis, anemia, thinning of skin , weakness, and impaired wound healing (**Carson-Jurica** *et al.***, 1990; Kuhn, 2002 and Matsumoto, 2002**)

Engeland *et al.* **(2009)** suggested that human mucosal healing rates are modulated by testosterone levels. Based upon when between-group differences were observed, testosterone may impact upon the proliferative phase of healing which involves immune processes such as reepithelialization and angiogenesis.

Hobbs *et al.* **(1993)** indicated that 6 weeks treatment of normal men with testosterone leads to an increase in serum IGF-I levels.

IGF-1 is considered to be a wound healing stimulant, increasing cell proliferation and collagen synthesis (**Lieberman** *et al.,* **1994; Lin** *et al.,* **1998*; Coerper** *et al.,* **2001; Blumenfield** *et al.,* **2002).**

From the conclusions of **Stanford** *et al.* **(1999); Demling (2000); Demling and Orgill (2000);Engeland** *et al.* **(2009)** it may be suggested that the enhancing effect of Testosterone on limb regeneration may be due to its acceleration of wound healing either by its action upon the proliferative phase of healing which involves immune processes such as reepithelialization and angiogenesis or by the production of IGF-1.

Bhasin *et al.* (2006) proposed that testosterone could promote the differentiation of mesenchymal multipotent cells into the myogenic lineage while inhibiting adipogenic differentiation by modulating nuclear translocation of β-catenin.

Singh *et al.*(2009) indicated that testosterone promotes the nuclear translocation of β-catenin through an AR-mediated mechanism in C3H 10T1/2 cells.

Hong *et al.*(2011) Concluded that testosterone regulates β-catenin protein level and proliferation rate in mesenchymal tumour(desmoid tumour).

Zhao *et al.*(2011) provided that Testosterone increases cellular β-catenin content which promotes the expression of β-catenin-targeted genes and myogenesis in the muscle-derived stem cells of cattle.

B-catenin is essential for adult skeletal muscle growth and regeneration *in vivo* (**Polesskaya *et al.*, 2003; Reya and Clevers, 2005; Armstrong *et al.*, 2006**)

Yokoyama *et al*. (2007) demonstrated that Wnt/β-catenin signaling plays an essential role during the early phases of limb regeneration and is important, but not absolutely required, during the subsequent phases of limb regeneration in *Xenopus*.

From the conclusions of **Bhasin *et al.* (2006) ; Yokoyama *et al*. (2007); Singh *et al.* (2009); Zhao *et al.* (2011)**.   It is suggested that Testosterone may enhance the limb regeneration by its stimulatory effect through Wnt/β-catenin signaling resulting in the initiation of the early phases of limb regeneration.

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10/25/2011

Efficacy of Behavioural Parent Training Program in Reducing Parental Stress among Iranian Parents of Children with ADHD

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Abstract: The present quasi-experimental study was performed to evaluate the efficacy of Behavioural Parent Training Program (BPTP) in reducing parental stress. The sample for this study consists of 60 parents of children with ADHD was randomly assigned to experimental and control groups. The experimental group received the Barkley's parent training program. All participants completed the Parental Stress Index /Short Form (PSI/SF) at four different time points. A Mixed Model ANOVA using the SPSS 19.0 (SPSS Inc., Chicago, IL, USA) was used in data analysis. The results from mixed model ANOVA reflected that mean changes in parental stress were significantly different between two groups. In addition, Post hoc analysis revealed a statistically significant decrease in parental stress only for the experimental group. The present study in line with some previous studies provides some preliminary evidence that supports the effectiveness of Barkley's parent training program to reduce parental stress for Iranian parents with ADHD children. The implications of the study findings and limitations of the research method along with recommendations for future studies are discussed.

Keywords: AHDA children; Barkley's parent training program; parental stress, parents

1-Introduction

Attention deficit hyperactivity disorder (ADHD) is one of the most common psychiatric disorders diagnosed in children. Research shows that ADHD not only bedevils children, but also negatively influences their parents and siblings [1-3]. Previous studies show that parents of ADHD children face many challenges and experience very high levels of stress [1, 4-8]. Studies reporting parents of children with ADHD use higher levels of alcohol consumption in response to their increased stress [9] and use corporal punishment [10] reflect that parents of children with ADHD don't use appropriate techniques to reduce stress and should be trained. Other studies also show that parents with ADHD children need more services in their community [11]. In light of the above mentioned discussion and high prevalence of ADHD among Iranian school-age children [12] the present study was conducted to evaluate the ability of the parent training program developed by Barkley to reduce parents stress.

2. Material and Methods

This quasi-experimental study was carried in Kermanshah, Iran. The study involved 60 Iranian parents (fathers=30 and mothers=30) of children with ADHD which randomly assigned to experimental and control groups. The required sample size was determined using Snedecor and Cochran`s [13] formula, with the test power=90% and $\alpha = .05$. The required information to calculate sample size was obtained from a previous study conducted in Iran [2].

$$n = 1 + 2c\left(\frac{s}{d}\right)^2$$

$$SD = \sqrt{\frac{s_{x1}^2 + s_{x2}^2}{2}} = \sqrt{\frac{26.73^2 + 42.8^2}{2}}$$

$$s = 19.55$$
$$MD = 43 - 28.1 = 14.9$$

$$n = 1 + 2 \times 7.5 \times \left(\frac{19.55}{14.9}\right)^2$$

$$n = 1 + 2 \times 7.5 \times 1.72 = 26.8 = 27$$

$$27 \times 10\% = 2.7 \;\square\; 3$$

$$27 + 3 = 30$$

Note: SD= Standard Deviation, D= Mean Difference, C=Constant (depends on level α .05, power selected 95%)

Finally, the sample size for each group was considered 30 subjects.

Intervention: Barkley's Parent Training Program

The treatment used in this study to train parents with ADHD is called "Defiant Children". According to Barkley [14, 15], this parent training program can be applied to manage and reduce ADHD symptoms among children. The following is a description of Barkley's 10 session program. The participants met once a week for one and half hours over a period of 10 weeks.

Session one: Why Children Misbehave: Session One has two primary objectives. First, the participants were informed of the cause and maintenance factors of defiant behaviors in children. A discussion was facilitated relating to the reciprocal interfamilial and interpersonal interactions that contributed to childhood misbehaviour [14]. The second objective of the session was to commence constructive group formulation and cohesion of the participants [14].

Session two: Pay Attention: The objective of the second session was to educate the participants about the way in which their parent-child interaction styles could motivate the children to show positive behaviour. The participants were trained on how to manage their ADHD children's behaviours and how to avoid attending to negative behaviours in order to elicit positive behaviours from the children [14].

Session three: Increasing Compliance and Independent Play: The primary objective for Session Three was to enable participants to generalize the effects from the previously learned attending skills into settings outside of special time. The researcher/therapist trained the participants to effectively utilize the attending skills to increase immediate child compliance with the parents' or caregivers' commands [15].

Session four: When Praise Is Not Enough: Poker Chips and Points: The main aim of the fourth session was to set a formal system of positive reinforcement at home that would make privileges depending on the child's compliance. Participants were instructed to produce a developmentally suitable token economy for their children at home. The children would be consistently and generously be reinforced by tokens for their desirable behaviour and obeying their parents' demands [15].

Session five: Time out! And other Disciplinary Methods: The fifth session had two objectives. The first objective was to enable the participants to differentiate between and the effective use of the cost response and the time out from reinforcement behavioural procedures. The second objective was to train the participants on how to implement time-out as well as other methods of disciplining children (Barkley, 1991).

Session six: Extending Time Out to Other Misbehaviour: The primary objective o f session six was to assist the participants in resolving any problems encountered when using the time out procedure during the past week. A discussion was conducted on the application of the time out from reinforcement procedure for one or two additional non-compliant behaviours observed in the home [16].

Session seven: Anticipating Problems: Managing Children in Public Places: The seventh session mainly focused on training the participants to use the child behaviour management methods, which they had learned previously, in public settings. They were taught a four-session procedure of think aloud-think ahead to predict and decrease the children's public misbehaviour. The value of predicting problematic behaviour was emphasized as a pivotal tool in successful management of children's misbehaviour in public [16].

Session eight: Improving School Behaviour from Home: The Daily School Behaviour Report Card: At this session, the nature of children's problematic behaviours displayed at school was reviewed. The participants were taught to implement a daily report card for their children's behaviour at school. The handout entitled 'Using a Daily School Behaviour Report Card' were distributed and presented. This was followed by a discussion on incorporating the report card with the daily journal of communications between the parent and the teacher that is currently in use in these children's schools (Barkley, 1991).

Session nine: Handling Future Behaviour Problems: The objective for session nine was to encourage the participants to think about the possible future child behavior problems and how they could utilize the previously taught methods to address these problems [15].

Session ten: Booster Session and Follow-Up Meetings: This session was conducted one month after completion of the treatment. This session included an overall review of the programme. There was a discussion on the participants' use of the procedures that they had learned during the programme. Any necessary support was provided by the therapist to correct the parents' home token system [15].

The program was administered in 90 minute sessions in nine weeks and a one-month follow up session. Treatment outcome was evaluated by Parent Stress Index/Short Form (PSI/SF) [17]. The parents completed the scale at four data points (pre-intervention, post-intervention-1, post-intervention-2 and a follow up) of the research instruments.

Data were analyzed using the SPSS 19.0 software package (SPSS Inc., Chicago, IL, USA). Frequency distributions, percentages, means and standard deviations were used to describe data. In order to determine the homogeneity of the two groups in terms of age, sex, employment status and educational

attainment a series of bivariate analyses including independent t- test and Chi-Square were performed to compare the experimental and control groups. Mixed Model was used to evaluate changes in parents stress. Experimental group vs. Control group served as a between-subjects variable and time (pre-intervention, post-intervention-1, post-intervention-2 and a follow up) was a within-subjects variable.

3-Results

Of the 60 parents in the study, 60% parents in the experimental group and 46.7% parents in the control group were more than 35 years old. Five parents (16.7%) in the experimental group and 10 parents (33.3%) in the control group were between 31-35 years old and 7 parents (23.3%) in the experimental group and 6 parents (20%) in the control group were between 26-30 years old. The results showed that 16 pairs of (father and mother) parents (53.3%) in the experimental group and 14 pairs of parents (46.7%) in the control group were aware of their children's problem. The parents' awareness of their children's problem was moderate in 14 pairs of (father and mother) parents (46.7%) in the experimental group and 16 pairs of parents (53.3%) in the control group. In order to determine homogeneity in parent's age across the two groups, an independent t-test was conducted. Result of the independent t-test revealed no significant difference between two groups (t $_{(58)}$ =-.8, p=.935). In order to compare the homogeneity of the two groups in terms of sex, employment status and educational attainment, chi-square analyses were conducted (Table 4.8). Result revealed no significant difference between

the two groups in terms of sex ($x^2_{(1)}$=.067, p= .796), employment status ($x^2_{(4)}$=5.09, p=.278), and educational attainment ($x^2_{(1)}$=.073, p= .787).

The test results revealed no statistically significant difference between the experimental group (M = 108.33, SD = 16.87) and control group (M = 113.26, SD = 16.20) at the pre-intervention stage (t $_{(60)}$ = -1.15, p = 0.25).

A Mixed Model ANOVA was conducted to examine mean changes in parental stress between groups and across time points. Since assumption of multivariate homogeneity of variances was violated (Mauchly's W = .718, p ≤ .01), the statistics from the Greenhouse-Geisser correction for the test of the main effect and the test of the interaction effect were utilized. Results of mixed-model ANOVA revealed that the main effect for group was significant (F $_{(1, 58)}$ = 57.67, p ≤.001, η^2_p = .50 (See Table 1).

Table 1. Tests of Between-Subjects Effects

Source	df	F	η^2_p
Intercept	1	2964.61***	0.98
Group	1	57.67***	0.50
Error	58		

*** p≤.001

A significant main effect for time was also obtained, (F $_{(2.49, 144.85)}$ =76.29, p≤.001, Eta-squared = .57). A significant time × group was also obtained (F $_{(2.49, 144.85)}$ =68.76, p≤.001, η^2_p = .54) (See Table 2).

Table 2. Tests of Within-Subjects Effects

Source		df	F	η^2_p
Time	Sphericity Assumed	3	76.30***	0.57
	Greenhouse-Geisser	2.4975	76.30***	0.57
	Huynh-Feldt	2.663844	76.30***	0.57
	Lower-bound	1	76.30***	0.57
Time × Group	Sphericity Assumed	3	68.76***	0.54
	Greenhouse-Geisser	2.4975	68.76***	0.54
	Huynh-Feldt	2.663844	68.76***	0.54
	Lower-bound	1	68.76***	0.54
Error(Time)	Sphericity Assumed	174		
	Greenhouse-Geisser	144.855		
	Huynh-Feldt	154.5029		
	Lower-bound	58		

*** p≤.001
Mauchly's W=0.72 , x^2 (5)= 18.82, p=0.002

Therefore, the results from mixed model ANOVA reflects that mean changes in parental stress are significantly different between two groups.

In the next step of analysis, post hoc analysis revealed a statistically significant decrease in

parental stress only for the experimental group. Table

3 and Figure 1 depict the within-group change in parental stress of the two groups. There were trends towards larger decreases on mean parental stress

scores for the experimental group ($F_{(1, 29)}$=353.42, $P \leq 0.001$). No significant mean changes scores were found on parental stress for control group.

Table 3. Changes in parental stress in control and experimental group

| Time points | Group | | | |
| | Experiment | | Control | |
	Mean	SD	Mean	SD
Pre-intervention	108.3	16.88	113.3	16.2
Post-intervention-1	85.2	14.3	105.8	18.21
Post-intervention-2	85.6	15.00	111.4	19.91
Follow up	54.4	9.52	111.3	18.58
F	353.42***		0	
Eta Squared	0.92		0	

***p≤.001

Figure 1 accompanied by Table 3, illustrates that only parents in the experimental group reported a statistically significant decrease in parental stress.

Figure 1. Changes in parental stress in control and experimental group
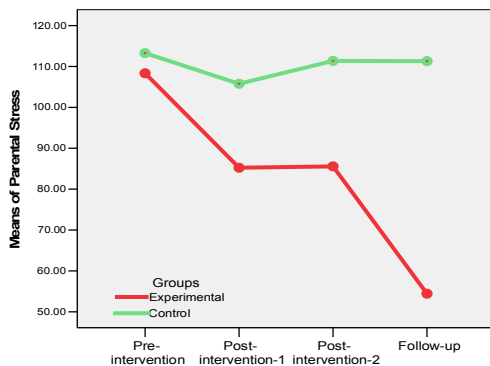
4-Discussions

A growing body of literature shows that parents of children with ADHD suffer from high levels of distress, depressed affect, and substance use [18]. Similarly, there is emerging evidence that parents of children with ADHD experience increased levels of parenting stress. Consequently, increased levels of parenting stress are associated with disruptions to the parent-child relationship and parenting practices and disruptions in parent psychological functioning [19-21].

The present study was conducted on sample of Iranian parents with ADHD children. The findings from Mixed Model ANOVA revealed statistically significant decreases in parents' reported stress in comparison to the control group. These results along with some previous studies [22-27] acknowledge the effectiveness of Barkley's 10-session parent training program to reduce parent stress among parents with ADHD children. However, a few studies didn't find statistically significant decrease in parent stress in comparison to the control group [3].

The effectiveness of this program can be garnered by the fact that parent training help parents to deal successfully with the many challenges that produce new attitudes in order to reach behavioural changes towards their children. This is supported by the notion that a change in the parenting style can lead to a change in the interactions between the parent and the child. As Johnston and Mash [28] reported, when parenting style is constructive, it can improve parental stress and self-esteem. In addition, our results is also supported by this notion that improving communication and relationships between parent and children is related with less stress, enhanced parental monitoring, and improved positive behaviours in children [29]. According to Social Learning Theory [30, 31] all behaviours are learned through a combination of positive and negative reinforcement and modelling. Thus, one of the objectives of this study was to establish formal positive reinforcement system in a home that privileges were contingent on child compliance. This discipline strategy was successful in reducing ADHD symptoms and parental stress. To encourage the participants think about the possible future child behaviour problems and utilize the taught methods in BPTP to address these problems is effective in

reducing ADHD symptoms and parental stress. Consistent with self-efficacy theory, success in behavioural accomplishments (through the acquisition of effective parenting skills) can raise mastery expectations and reduce parental stress.

In addition, social learning theory and behaviour modification techniques supported behavioural training for parents in analyzing their own problems with their children and as it was indicated using of the theories were effective in reducing ADHD symptoms, behavioural problems in children and managing parental stress.

The results from this study lend support to the contention that BPTP can have therapeutic benefits not only for targeted children with ADHD, but also for their parents. This finding, hopefully can serve as an impetus for investigating other ways in which BPTP may indirectly positively affect parents and family functioning within the ADHD population. The results of this study support the notion that parent training programmes can benefit families in a number of ways such as reconstructing and creating a new bridge for communication and interaction with their children and elimination most of related problem such as parental stress and changing their strategists toward them. There are a few limitations of the present study that are worth mentioning. First limitation that should be acknowledged is that participants were not screened for their own psychopathology. Thus, in the future studies participants should be screened for other psychological problems prior to acceptance into the research program. Second limitation of the present study is the lack of objective measures of parents' treatment outcome. Therefore, additional studies that include objective measures for measuring of treatment outcome are needed. The last limitation that should be addressed is that experimental and control groups were not matched for the severity of the child's ADHD. Previous studies show that the severity of the child's ADHD is significantly contributed with parental stress [32]. In light of this limitation, it is suggested that future studies should consider the severity of the child's ADHD in research design.

Despite the above-mentioned limitations, some practical and theoretical implications of the present study can be suggested. Theoretically, this study supported the Barkley's 10-session parent training program as an effective program to reduce parent stress in parents with ADHD children. In terms of practical implication, it is suggested that counsellors working with families of ADHD children use this training program.

Acknowledgement

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9/18/2011

Stresses in the sphere rests on a rigid plane horizontal surface

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Abstract: In this paper, Analytical stresses are obtained for the sphere rests on a rigid plane horizontal surface. It is assumed that the support reaction consists of a concentrated vertical force equal to the weight of the sphere. We are started with the solution for a point force acting on the surface of a half-space and determine the tractions on an imaginary spherical surface passing through the point of application of the force, then complete the solution by superposing appropriate spherical harmonics. The results differ significantly from the classical elasticity solutions that are based on the assumption that the body is fully formed before the loading is applied. The self-equilibrated tractions due to self-weight and the concentrated force alone and with the approximations obtained using $n = 2$ and $n = 4$. The process is clearly convergent and as with Fourier series approximations, the error exhibits more zero crossings as the number of terms increases.
[Reza Masoudi Nejad. **Stresses in the sphere rests on a rigid plane horizontal surface.** Life Science Journal. 2011;8(4):290-295] (ISSN:1097-8135). http://www.lifesciencesite.com.

Keywords: Stress; Rigid plane; Vertical force; Spherical harmonics; Elasticity; Fourier series

1. Introduction

The majority of contacts fall into one of two classes; those where at least one of the contacting bodies is convex, so that the contact size depends on the normal load – an incomplete contact, and those where the contact-defining body has a surface profile with distinct discontinuities in surface slope which define the contact size – a complete contact. Partial slip contact problems are of great practical interest because the damage caused by slip within notionally stationary contacts encourages the nucleation of fatigue cracks. Most solutions to partial slip problems within the literature are based on half-plane theory, and employ the usual Amontons–Coulomb frictional law, which remains the most appropriate model for most metallic contacts, and is also used here. The pioneering solution describes the response of a Hertzian contact subject to a constant normal force and oscillatory shear (Cattaneo, 1938). Mindlin revisited the problem ten years later, and extended the solution to consider more complex sequences of loading (Mindlin, 1949; Mindlin & Dereciewicz, 1953). The most important development in this family of solutions since then has been the simultaneous discovery by Jager (1998) and by Ciavarella (1998) that the 'corrective' shear traction distribution within the stick zone is similar in form to the contact pressure sustained by the contact under a lighter load-this is true for any half-plane contact. As half-plane theory can model only incomplete contacts the contact pressure always falls smoothly to zero at the contact edge, so that there is always a region of slip present under a monotonically increasing load, and which starts at the edge. Recently, the authors examined the case of two similar elastic cylinders pressed end-on-end and twisted, and investigated the development of slip. That problem incorporates an unusual type of contact-where the size is simultaneously defined by both bodies-and where the shearing traction is anti-plane with respect to a diametral plane (Kartal, Hills, Nowell, & Barber, 2010).

2. Preliminaries
2.1. The Boussinesq solution

We shall now apply similar arguments to solve the Boussinesq problem, in which a point force F in the $z-direction$ is applied at the origin $R = 0$ on the surface $z = 0$ of the half-space $z \succ 0$.

We note that the force is normal to the surface, so that there is no tangential traction at any point on the surface — i.e.

$$\sigma_{zx} = \sigma_{zy} = 0 \; ; \; all \; x,y, \; z = 0 \qquad (1)$$

We therefore seek a suitable partial integral of $1/R$ to be dimensionless in R and singular at the origin, but otherwise to be continuous and harmonic in $z \succ 0$.

It is easily verified that the function

$$\varphi = \int_{-\infty}^{0} \frac{d\varsigma}{\sqrt{x^2 + y^2 + (z-\varsigma)^2}} = \ln(R + z) \qquad (2)$$

The force applied at the origin is

$$F = -2\pi \int_0^\infty r \sigma_{zz}(r,h) dr$$

$$= -6\pi h^3 \int_0^\infty \frac{rdr}{(r^2+h^2)^{5/2}} = -2\pi \qquad (3)$$

and hence the stress field due to a force F in the $z-direction$ applied at the origin is obtained from the potential

$$\varphi = -\frac{F}{2\pi}\ln(R+z) \qquad (4)$$

2.2 Other singular solutions

We have already shown how the singular solution in $(R+z)$ can be obtained from $1/R$ by partial integration, which of course is a form of superposition. A whole sequence of axially symmetric solutions can be obtained in the same way. Defining

$$\phi_0 = \frac{1}{R} \qquad (5)$$

We obtain

$$\phi_{-1} = \ln(R+z) \ ; \ \phi_{-2} = z\ln(R+z) - R$$

$$\phi_{-3} = \frac{1}{4}\{(2z^2-r^2)\ln(R+z) - 3Rz + r^2\}$$

$$\phi_{-4} = \frac{1}{36}\{3(2z^3-3zr^2)\ln(R+z) + 9zr^2$$
$$-11z^2R + 4r^2R\} \ ; \qquad (6)$$

By the operation

$$\phi_{n-1}(r,z) = \int_{-\infty}^0 \phi_n(r,(z-\varsigma))d\varsigma \ . \qquad (7)$$

The inverse operation is one of differentiation, so that

$$\phi_n = \frac{\partial \phi_{n-1}}{\partial z} \ . \qquad (8)$$

We can therefore also extend the sequence to functions with stronger singularities such as

$$\phi_1 = -\frac{z}{R^3}; \phi_2 = \frac{3z^2}{R^5} - \frac{1}{R^3}; \phi_3 = -\frac{15z^3}{R^7} + \frac{9z}{R^5}. \qquad (9)$$

If the half-space is indented by a frictionless punch, so that the surface $z = 0$ is subjected to normal tractions only, a simple formulation can be obtained by combining solution and defining a relationship between ϕ and ω in order to satisfy identically the condition $\sigma_{zx} = \sigma_{zy} = 0$ on $z = 0$. We write

$$\phi = (1-2\nu)\varphi \ ; \ \omega = \frac{\partial \varphi}{\partial z} \qquad (10)$$

Obtaining

$$\sigma_{zx} = z\frac{\partial^3\varphi}{\partial x \partial z^2} \ ; \ \sigma_{zy} = z\frac{\partial^3\varphi}{\partial y \partial z^2} \ , \qquad (11)$$

Therefore

$$\sigma_{RR} = \frac{\partial^2\phi}{\partial R^2} + R\cos\beta\frac{\partial^2\omega}{\partial R^2} - 2(1-\nu)\frac{\partial\omega}{\partial R}\cos\beta \qquad (12)$$

$$\sigma_{\theta\theta} = \frac{1}{R}\frac{\partial\phi}{\partial R} + \frac{\cot\beta}{R^2}\frac{\partial\phi}{\partial\beta} + \frac{1}{R^2\sin^2\beta}\frac{\partial^2\phi}{\partial\theta^2}$$

$$-(1-2\nu)\frac{\partial\omega}{\partial R}\cos\beta + \frac{2\nu}{R}\frac{\partial\omega}{\partial\beta}\sin\beta \qquad (13)$$

$$+\frac{\cos^2\beta}{R\sin\beta}\frac{\partial\omega}{\partial\beta} + \frac{\cot\beta}{R\sin\beta}\frac{\partial^2\omega}{\partial\theta^2}$$

$$\sigma_{\beta\beta} = \frac{1}{R}\frac{\partial\phi}{\partial R} + \frac{1}{R^2}\frac{\partial^2\phi}{\partial\beta^2} + (1-2\nu)\frac{\partial\omega}{\partial R}\cos\beta$$

$$+\frac{2(1-\nu)}{R}\frac{\partial\omega}{\partial\beta}\sin\beta + \frac{\cos\beta}{R}\frac{\partial^2\omega}{\partial\beta^2} \qquad (14)$$

$$\sigma_{\beta R} = \frac{1}{R}\frac{\partial^2\phi}{\partial\beta\partial R} - \frac{1}{R^2}\frac{\partial\phi}{\partial\beta} + (1-2\nu)\frac{\partial\omega}{\partial R}\sin\beta$$

$$-\frac{2(1-\nu)}{R}\frac{\partial\omega}{\partial\beta}\cos\beta + \cos\beta\frac{\partial^2\omega}{\partial\beta\partial R} \qquad (15)$$

$$\sigma_{\theta R} = \sigma_{\theta\beta} = 0 \qquad (16)$$

3. Stress field due to rests on a rigid plane
3.1. Governing equations

The general solution for a solid sphere with prescribed surface tractions can be obtained using the spherical harmonics. The addition of the singular harmonics permits a general solution to the axisymmetric problem of the hollow sphere, but the corresponding non-axisymmetric. Using equations (4)-(10), we obtain

$$\varphi_0 = (1-2\nu)\varphi = -\frac{F(1-2\nu)}{2\pi}\ln(R+z) \qquad (17)$$

$$\omega_0 = \frac{\partial\varphi}{\partial z} = -\frac{F}{2\pi R} \qquad (18)$$

These can be written in spherical polar coordinates centred on the point of application of the force as

$$\varphi_0 = -\frac{F(1-2\nu)}{2\pi}\ln(R+R\cos\beta) \qquad (19)$$

$$\omega_0 = -\frac{F}{2\pi R} \qquad (20)$$

and the corresponding non-zero stress components are obtained by substitution into equations (12)-(16) as

$$\sigma_{RR} = \frac{F[1-2\nu-2(2-\nu)\cos\beta]}{2\pi R^2} \qquad (21)$$

$$\sigma_{\theta\theta} = -\frac{F(1-2\nu)(1-\cos\beta-\cos^2\beta)}{2\pi R^2(1+\cos\beta)} \tag{22}$$

$$\sigma_{\beta\beta} = -\frac{F(1-2\nu)\cos^2\beta}{2\pi R^2(1+\cos\beta)} \tag{23}$$

$$\sigma_{\beta R} = -\frac{F(1-2\nu)\sin\beta\cos\beta}{2\pi R^2(1+\cos\beta)} \tag{24}$$

$$\sigma_{\theta R} = \sigma_{\theta\beta} = 0 \tag{25}$$

Points on the surface of the sphere are defined by the equation
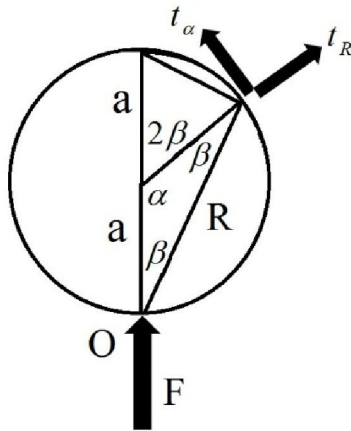
$$R = 2\alpha\cos\beta \tag{26}$$

Figure 1. Configuration, loading and geometry.

As shown in figure 1. At this point we have

$$\sigma_{RR} = \frac{F[1-2\nu-2(2-\nu)\cos\beta]}{8\pi a^2\cos^2\beta} \tag{27}$$

$$\sigma_{\theta\theta} = -\frac{F(1-2\nu)(1-\cos\beta-\cos^2\beta)}{8\pi a^2\cos^2\beta(1+\cos\beta)} \tag{28}$$

$$\sigma_{\beta\beta} = -\frac{F(1-2\nu)}{8\pi a^2(1+\cos\beta)} \tag{29}$$

$$\sigma_{\beta R} = -\frac{F(1-2\nu)\sin\beta}{8\pi a^2\cos\beta(1+\cos\beta)} \tag{30}$$

$$\sigma_{\theta R} = \sigma_{\theta\beta} = 0 \tag{31}$$

However, to find the implied tractions on the spherical surface, we need to rotate the local coordinate system clockwise through an angle β, obtaining the radial and tangential tractions

$$t_R = \sigma_{RR}\cos^2\beta + \sigma_{\beta\beta}\sin^2\beta$$
$$+2(-\sigma_{\beta R})(-\sin\beta)\cos\beta \tag{32}$$

$$t_\alpha = (\sigma_{\beta\beta}-\sigma_{RR})(-\sin\beta)\cos\beta$$
$$+(-\sigma_{\beta R})(\cos^2\beta-\sin^2\beta) \tag{33}$$

In these equations, notice that the sign convention for a Cartesian coordinate system (x,y)

aligned with (R,β) at the surface would involve a negative shear stress $(\sigma_{xy}=-\sigma_{\beta R})$ and the clockwise rotation introduces a negative sign into the terms involving $\sin\beta$. The direction of the shear traction t_α is chosen so as to be consistent with the angle α subtended at the centre of the sphere, defined such that $\alpha=0$ corresponds to the point of application of the force. From figure 1, we then have

$$2\beta = \pi - \alpha \tag{34}$$

Substituting from (27)-(31) into (32), (33) and simplifying, we obtain

$$t_R = \frac{F[4(1-2\nu)-(7-8\nu)\cos\beta]}{8\pi a^2} \tag{35}$$

$$t_\alpha = \frac{F[2(1-2\nu)-3\cos\beta-(7-8\nu)\cos^2\beta]\sin\beta}{8\pi a^2(1+\cos\beta)\cos\beta} \tag{36}$$

Or in terms of α,

$$t_R = \frac{F[4(1-2\nu)-(7-8\nu)\sin(\alpha/2)]}{8\pi a^2} \tag{37}$$

$$t_\alpha = -\frac{F[3+6\sin(\alpha/2)-(7-8\nu)\cos\alpha]\cos(\alpha/2)}{16\pi a^2(1+\sin(\alpha/2))\sin(\alpha/2)} \tag{38}$$

These expression are not of Fourier form in the angle α and hence the process of adding terms to satisfy the traction boundary conditions on the sphere will be more complex than in the two-dimension case. More seriously, the traction t_α is singular as $\alpha\to0$ — i.e. at the point of application of the force. This result was first remarked by Sternberg and Rosenthal. It might still be possible to satisfy the boundary condition using an infinite series of spherical harmonics, but the series would probably be only very slowly convergent because of the singularity.

To prevent this problem and improve the convergence of the series, we need to superpose additional potentials chosen so as to cancel this singularity. As a preliminary to this process, we can expand (38) near $\alpha=0$ by writing

$$\sin\alpha \approx \alpha; \sin(\alpha/2) \approx \frac{\alpha}{2};$$
$$\cos\alpha \approx 1-\frac{\alpha^2}{2}; \cos(\alpha/2) \approx \frac{1-\alpha^2}{8}, \tag{39}$$

Obtaining

$$t_\alpha \approx \frac{F(1-2\nu)}{2\pi a^2\alpha} - \frac{F(5-4\nu)}{8\pi a^2} + O(\alpha) \tag{40}$$

As $\alpha\to0$.

To choose a suitable potential to cancel the first term, notice that the surface is very nearly plane when $\alpha\to0$, so we can look for potentials giving shear tractions on the surface of the half plane with this singular form. This may seem curious, since the original Boussinesq solution gave identically zero

tractions on this plane, but we note that the singular tractions are one order lower than the singularity associated with the point force, which is order R^{-2}. We therefore choose additional potentials from equation (6) that are one order less singular than those in (19), (20). Notice that these will introduce singular tractions both t_R and t_α, the former is undesirable, so we use the same combination make the dominant singular term in t_R be zero.

We therefore choose

$$\phi_1 = 2(1-\nu)CR[\cos\beta\ln(R+R\cos\beta)-1] \quad (41)$$

$$\omega_1 = C\ln(R+R\cos\beta) \quad (42)$$

We find the tractions due to this potential as $\alpha \to 0$ to be

$$t_R \approx -\frac{C}{2a} + O(\alpha) \quad (43)$$

$$t_a \approx -\frac{C}{a\alpha} + \frac{C(1-2\nu)}{2a} + O(\alpha) \quad (44)$$

Superposing this on the original stress function, it is clear that we can cancel the unwanted singularity by choosing

$$\frac{F(1-2\nu)}{2\pi a^2} - \frac{C}{a} = 0 \quad, \quad (45)$$

Or

$$C = \frac{F(1-2\nu)}{2\pi a} \quad (46)$$

With this choice, the tractions everywhere on the surface of the sphere are bounded, but we notice from equation (44) that the traction t_α will be non-zero at $\alpha = 0$. Now it is easily verified that all the axisymmetric spherical harmonics give zero values of shear traction $\sigma_{R\beta}$ on the axis $\beta = 0$. In other words, although the magnitude of the traction is continuous, it's direction changes discontinuously at the origin. This is itself a kind of singularity.

This additional singularity can also be removed by superposing the next higher order potentials, once again chosen form (6) so as to satisfy – i.e.

$$\phi_2 = \frac{(1-\nu)AR^2}{2}[(2\cos^2\beta - \sin^2\beta) \quad (47)$$
$$\times\cos\beta\ln(R+R\cos\beta)-3\cos\beta+\sin^2\beta]$$

$$\omega_2 = AR[\cos\beta\ln(R+R\cos\beta)-1] \quad (48)$$

We find the traction due to the superposition of all the above potentials at $\alpha \to 0$ to be

$$t_R \approx -\frac{F(1-2\nu)}{2\pi a^2} + O(\alpha) \quad (49)$$

$$t_\alpha \approx -\frac{F(1+12\nu-16\nu^2)}{8\pi a^2} + A + O(\alpha). \quad (50)$$

And hence we can eliminate the cowlick by choosing

$$A = \frac{F(1+12\nu-16\nu^2)}{8\pi a^2}. \quad (51)$$

Thus, a suitably smooth form of the point force solution for the sphere is provided by the potentials

$$\phi = \phi_0 + \phi_1 + \phi_2$$
$$= -\frac{F(1-2\nu)}{2\pi}\ln(R+R\cos\beta)$$
$$+\frac{2F(1-2\nu)(1-\nu)R[\cos\beta\ln(R+R\cos\beta)-1]}{2\pi a}$$
$$+\frac{F(1+12\nu-16\nu^2)(1-\nu)R^2}{16\pi a^2}$$
$$\times[(2\cos^2\beta-\sin^2\beta)\cos\beta\ln(R+R\cos\beta)$$
$$-3\cos\beta+\sin^2\beta] \quad (52)$$

$$\omega = \omega_0 + \omega_1 + \omega_2$$
$$= -\frac{F}{2\pi R} + \frac{F(1-2\nu)\ln(R+R\cos\beta)}{2\pi a}$$
$$+\frac{F(1+12\nu-16\nu^2)R}{8\pi a^2}$$
$$\times[\cos\beta\ln(R+R\cos\beta)-1] \quad (53)$$

To confirm that the tractions now remaining to be removed are smooth, we plot them in figure 2 as functions of α.
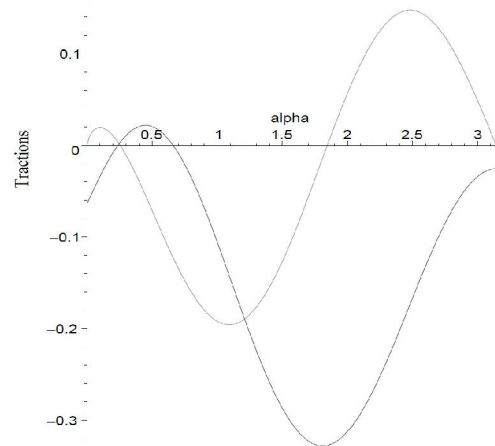

Figure 2. Tractions on the spherical surface associated with the stress functions of equation (52, 53). The curve passing through the origin is the shear traction t_α.

3.2. Gravitational

The body force due to self-weight is conveniently introduced as a hydrostatic stress

$$\sigma_{RR} = \sigma_{\theta\theta} = \sigma_{\alpha\alpha} = -\rho g\tilde{R}\cos\alpha \quad, \quad (54)$$

Where $\tilde{R}$ is here measured from the centre of the sphere. This adds an additional term $-\rho g a \cos\alpha$. Into the traction component t_R, whilst leaving t_α unchanged. Also, we note that the force F must support the weight of the sphere, so

$$F = \frac{4\pi \rho g a^3}{3} \ . \qquad (55)$$

3.3 Spherical harmonics

To complete the solution, we superpose a series of spherical harmonics. Thus, we add the new potentials

$$\phi_3 = \sum_{i=1}^{n} A_i \tilde{R}^{i+1} P_{i+1}(\cos\alpha)\omega = \sum_{i=1}^{n} B_i \tilde{R}^i P_i(\cos\alpha), \quad (56)$$

evaluate the additional tractions on the surface $R = a$. since we can only use a finite number of terms in the series, we can satisfy the traction-free boundary condition might be chosen, but the must convergent is to use a Galerkin or 'weighted residual' method. For example, if we write the approximate tractions in the form

$$\tilde{t}_R = t_R^P + \sum_{i=1}^{n} A_i t_{Ri}^A(\alpha) + \sum_{i=1}^{n} B_i t_{Ri}^B(\alpha) \qquad (57)$$

$$\tilde{t}_\alpha = t_\alpha^P + \sum_{i=1}^{n} A_i t_{\alpha i}^A(\alpha) + \sum_{i=1}^{n} B_i t_{\alpha i}^B(\alpha) \ , \qquad (58)$$

we can define an error measure

$$E = \int_0^\alpha (\tilde{t}_R^2 + \tilde{t}_\alpha^2)d\alpha \ . \qquad (59)$$

An optimal choice of the constants A_i, B_i can then be made by requiring

$$\frac{\partial E}{\partial A_i} = 0 \ ; \quad \frac{\partial E}{\partial B_i} = 0 \ , \qquad (60)$$

for $i = 1, n$. Notice that the error measure has been weighted uniformly in $0 \prec \alpha \prec \pi$. An alternative choice here would be to weight according to the volume of surface of the sphere, which would introduce a factor of $\sin\alpha$ into the integral. This would probably give better accuracy near the equator (where there is more surface area) and less near the poles.

The effect of this process is to weight the traction-free condition according to the practicable in mathematica. In Figure 3 we present the self-equilibrated tractions t_R due to self weight and the concentrated force alone and with the approximations obtained using $n = 2$ and $n = 4$ respectively. The process is clearly convergent and as with Fourier series approximations, the error exhibits more zero crossings as the number of terms increases. Oscillations near $\alpha = 0$ (Gibb's phenomenon) are to

be expected with large numbers of terms, but this effect has been to some extent mitigated by the removal of the stronger discontinuous effects in the above analysis.
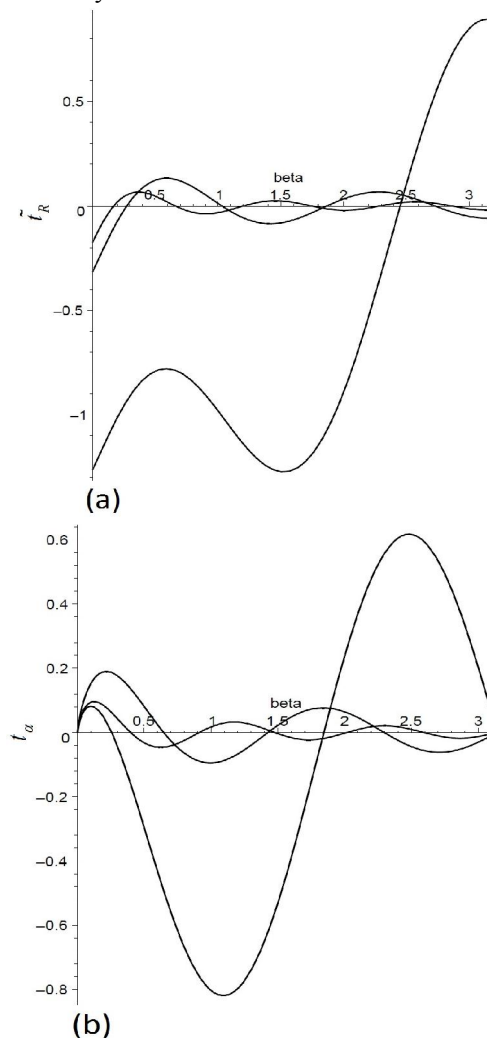

Figure 3. Tractions in the approximate solution (a) $\tilde{t}_R$ and (b) $\tilde{t}_\alpha$, normalized by $\rho g a$ for the case $\nu = 0.3$.

4. Conclusion

To obtain the Stresses in the sphere rests on a rigid plane horizontal surface, equations are solved utilizing the Boussinesq solution method and determined the tractions on an imaginary spherical surface passing through the point of application of the force, then complete the solution by superposing appropriate spherical harmonics.

Alternative choice here would be to weight according to the volume of surface of the sphere, which would introduce a factor of $\sin\alpha$ into the integral. This would probably give better

accuracy near the equator (where there is more surface area) and less near the poles.

The effect of this process is to weight the traction-free condition according to the practicable in mathematica. In Figure 3 we present the self-equilibrated tractions t_R due to self-weight and the concentrated force alone and with the approximations obtained using $n = 2$ and $n = 4$. The process is clearly convergent and as with Fourier series approximations, the error exhibits more zero crossings as the number of terms increases

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3/20/2011

Surgical Management of Post-Discectomy Spondylodiscitis with Transforaminal Lumbar Interbody Fusion (TLIF) and Posterior Instrumentation

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Abstract: Background: Post operative lumbar disc space infection is relatively uncommon. The anterior approach has been the traditional surgical approach for treatment of this complication. Posterior approach was sometimes added for instrumentation only. Purpose: To present the results and clinical outcome, at a minimum of twelve months, following transforaminal lumbar interbody fusion (TLIF) and posterior instrumentation for post-discectomy spondylodiscitis. Study design: A case series Materials and Methods: Nine patients (age 38– 68 years; mean: 47.8 years) with post-lumbar discectomy spondylodiscitis, were treated surgically by TLIF and posterior spinal instrumentation. All patients had significant back pain despite a full conservative treatment regimen by broad spectrum antibiotics and brace. The follow-up ranged from 12 to 36 months with an average of 22 months. All patients were available for follow up which included physical examination, scoring of function and radiographs. Outcome measures: To assess the invasiveness of the operation, we evaluated operative time, blood loss, and complications. Visual pain analogue scale (VPAS), activities of daily living (ADL) (Barthel index), CRP, and ESR in the preoperative, postoperative and final follow-up periods were used to evaluate the surgical outcome. Results: Although we encountered some postoperative complications including wound infection; at the final follow-up visit, VPAS and Barthel index improved in all patients. Changes in CRP and ESR revealed suppression of infection in all cases. Conclusion: Surgical treatment for postoperative spondylodiscitis with TLIF and posterior spinal instrumentation provides patients with satisfactory final outcomes.

1. Introduction

The relatively uncommon complication of postoperative spondylodiscitis was first described as a clinical entity by **Turnbull in 1953** [1]. Postoperative spondylodiscitis represents 30.1% of all cases of pyogenic spondylodiscitis [2] and has been reported to occur after almost every open and minimally invasive spinal procedure, including laminectomies [3,4], discectomies [5–18], and fusions with or without instrumentation [5,13,19–22]. It has also been documented to occur following less invasive procedures, such as discography [23–25], chemonucleolysis [26, 27], myelography [28], paravertebral injections and lumbar puncture [29].

The optimal management of postoperative infections of the spine is controversial. Infections after discectomy or laminectomy are usually treated non operatively with long-term antibiotics [30-33]. Surgical debridement is usually reserved for patients in whom medical management of the disease has failed, those with neurological compromise, unstable mechanical deformity, an epidural abscess, or intractable pain [34- 38].

Due to the fact that instrumentation placed for fusion operations in otherwise normal patients has been shown to increase postoperative infection rates, many authors have expressed understandable concern about the placement of instrumentation in an infected patient. Historically, many have preferred to recommend bed rest and prolonged spinal bracing rather than placing internal implants. Others have advocated a staged operation with a period of antibiotic therapy bridging the debridement and instrumentation procedures [39- 42].

In recent series, excellent outcomes have been demonstrated for single-stage procedures in which hardware placement is performed within and adjacent to debrided areas, and these studies have not shown significantly increased rates of infection recurrence [38, 43,44].

In addition, even, it has been shown that transforaminal lumbar interbody fusion (TLIF) yields satisfactory results, offers excellent exposure with minimal risk; particularly in cases of repeat spine surgery, in which the presence of scar tissue makes traditional posterior lumbar interbody fusion techniques difficult or impossible [45]. Also, TLIF seems to be a viable alternative to anteroposterior

circumferential fusion or anterior lumbar interbody fusion [45].

Thus it was hypothesized that, in cases of spondylodiscitis not responding to appropriate treatment; or those with either neurological compromise or with severe intractable back pain; these patients may benefit from a TLIF and posterior instrumentation.

2. Patients and Methods

A prospective study of 9 patients with post-discectomy spondylodiscitis was conducted in the period from January 2008 to January 2010. The follow up continued till February 2011. 6 patients were male and 3 were females. The age range was 38-68 years with a mean age of 47.8 years.

All our patients had undergone open discectomies as a method of treatment for symptomatic prolapsed lumbar discs, which was complicated by infection in the operated disc spaces. Conservative treatment with broad spectrum antibiotics and bracing failed in all cases. The antibiotic regimen was chosen empirically to cover gram positive, gram negative and anaerobic organisms. Initially and for the first 2 weeks, Ampicillin/ sulbactam and metronidazole were administered intravenously. This was followed by oral ciprofloxacin and clindamycin until normalisation of the CRP. The mean duration of the conservative treatment was 3.3 months (range: 1.5–5.5). Despite adequate and prolonged conservative treatment, the nine patients studied continued to suffer from significant low back pain, the average severity of which, assessed by the visual pain analogue scale (VPAS), was 8.1 (range: 6-10). Plain radiographs revealed disc space narrowing with erosion and sclerosis of the adjacent end-plates in all cases. Accordingly, those patients were treated by one stage surgical debridement, TLIF and posterior instrumentation.

Preoperative evaluation included full examination of the patients and their radiological data, including plain radiographs and magnetic resonance imaging (MRI). In addition, laboratory tests were performed in the form of white blood cell count (WBC; count/mm3), C-reactive protein (CRP; mg/dl), and erythrocyte sedimentation rate (ESR; mm/h). Patients were evaluated by Barthel Index [46], which has been used since the 1960s because of its high reliability and validity [47], as regards the activities of daily living (ADL), and the VPAS as regards the severity of back pain.

The invasiveness of surgery was evaluated by calculating the operative time and blood loss and recoding the complications. Patients were mobilized within the first few postoperative days, wearing a semi flexible lumbosacral brace. All patients received a six-week antibiotic regimen (3 weeks intravenous and 3 weeks oral), according to the result of culture and sensitivity. If no organism was identified, the empirical preoperative antibiotic regimen was continued.

During the first 6 weeks (the antibiotics period), ESR and CRP were done on weekly basis, then they were done again during each follow up visit. Plain radiography, VPAS and Barthel Index were checked at intervals of 6 weeks then 3, 6, 12, 24 and 36 months postoperatively. All patients were available for follow-up. The mean follow-up period was 22.2 months (range: 12–36).

Surgical technique:

The patient is placed in the prone position. To prevent cross contamination, autologous posterior iliac cancellous bone graft is first harvested and its incision closed. Posterior spinal elements are exposed through a midline longitudinal incision. A subperiosteal dissection of the paraspinous muscles is completed to the transverse processes. Pedicle screws are sized and inserted, under C-arm x-ray guidance, before decompression to minimize blood loss and achieve distraction. The spinal canal is entered through a unilateral laminectomy and inferior facetectomy. The interspinous ligament as well as the ligamentum flavum on the opposite side are left intact. The exiting nerve root is identified and protected. The thecal sac is gently retracted medially if necessary. Discectomy is performed through this unilateral approach. Radical debridement with resection of all infected and necrotic disc and bony tissue is performed and samples are sent for culture and sensitivity. After the initial discectomy, gradual distraction is applied to the pedicle screws on the opposite side. An osteotome is used to achieve flat endplate surfaces, until bleeding bone is reached. Bone graft is packed inside the interbody space, and then distraction is released. The construct is compressed to establish an optimum graft-bone interface and to re-establish lumbar lordosis. The rod-screw system is tightened. Bone graft is also laid over the transverse processes after adequate decortication to establish a circumferential fusion.

Statistical Methods

Preoperative, intraoperative, and postoperative data were collected and maintained in a single computer database. Data were statistically described in terms of range, mean and frequencies. Comparison of the pre and postoperative means to calculate the significance was done by the paired "t" test. All statistical calculations were done using SPSS

(Statistical Package for the Social Science version 15; SPSS Inc., Chicago, IL, USA).

3. Results

VPAS and Banthel index showed significant improvement. ESR and CRP returned to normal or near normal at latest follow up (Table 1). The average blood loss was 0.74 Litre (range: 0.5-1.2). The average operative time was 165.5 minutes (range: 120-240).

Cultures of the pus samples obtained during surgery showed no growth in two cases, *Staphylococcus Aureus* in 5 cases, Klebsiella in one case and *Escherichia Coli* in one case. There has been no residual infection, recurrence of infection or metal work failure to date. Adequate radiological fusion was achieved in all cases.

Postoperative Complications:

Transient L5 nerve root palsy in one patient, which resolved spontaneously over approximately 4 months. One other patient had wound infection which was cured in 3 weeks, by repeated dressings in addition to the routinely administered antibiotics. There were no other notable complications related to the procedure.

Table (1): Comparison of the preoperative and postoperative means of the evaluation parameters:

	Preoperative		Postoperative		Significance
	Mean	*Range*	*Mean*	*Range*	
VPAS	*8.1*	6-10	*1.3*	0-3	
Barthel index	*42.2*	30-60	*94.4*	80-100	P < 0.001
ESR (1st hour)	*95.75*	64-120	*17.3*	10-30	
CRP	*51.4*	38-66	*< 6*		
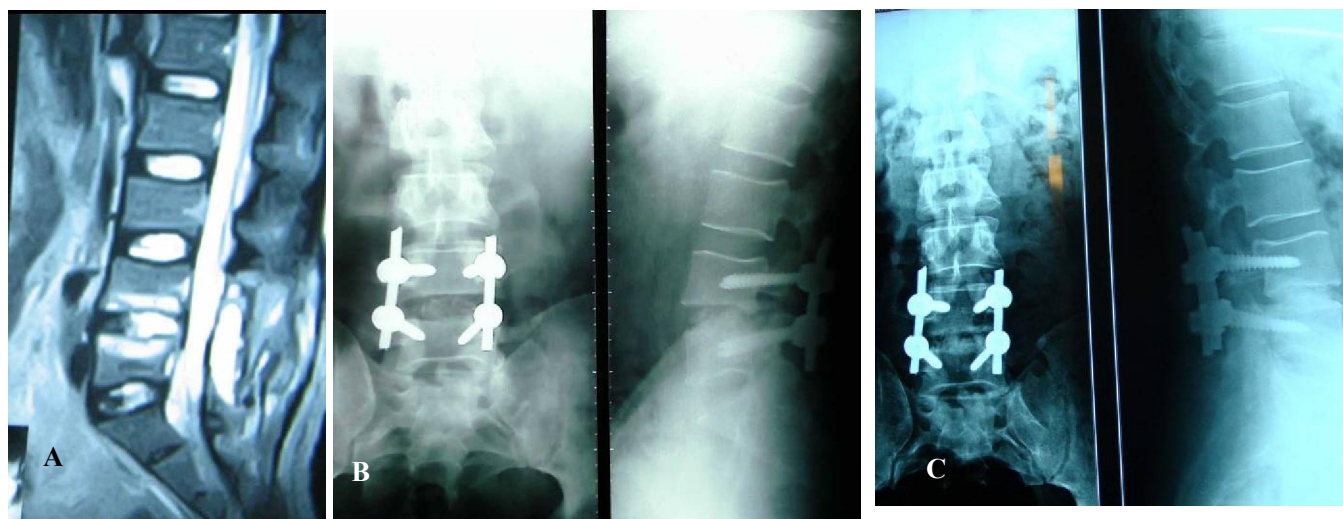
Fig. (1): A: Preoperative MRI showing L4/5 spondylodiscitis. B: postoperative plain radiographs following TLIF with autologous iliac bone graft and posterior instrumentation. C: 2.5 years follow up plain radiographs showing satisfactory fusion.

4. Discussion

Postoperative spondylodiscitis represents almost 30.1% of all cases of pyogenic spondylodiscitis [2] and has been reported to occur after almost every open and minimally invasive spinal procedure. In most patients, the infection is often mild, self-limited and will resolve spontaneously without any treatment intervention. In many cases, there may be a delay in diagnosis because of the frequent occurrence of back pain after spinal surgery. In fact, some reports have shown misdiagnoses in this patient population because of the lack of suspicion of infection as a causative factor [12,13]. In addition, the patient may tend to seek advice elsewhere due to increasing back pain, and thus the exact follow up and incidence of the reported cases may be misleading. Inspite of this, it has been reported that the incidence of postoperative spondylodiscitis after any type of spinal procedure ranges from 0.26 to 20% [3,4,7,8,12,14,23,48]. This incidence and severity generally increases with the complexity of the procedure [49], ranging from 0.6% to 3.7% after discectomy [14, 50] to 3.7% to 20% after posterior instrumented fusion [51,52]. This explains the small number of cases recruited in this study, being only nine cases over two years.

The exact cause of postoperative spondylodiscitis is controversial, the majority of

investigators think that it results from the direct inoculation of an offending pathogen into the avascular disc space [17,23] . Some authors believe that there are two types of spondylodiscitis, a septic form caused by an infectious agent and an aseptic form resulting from an inflammatory reaction [10,26] . Others believe that there is no such thing as an aseptic spondylodiscitis and that this form is actually the result of a less virulent, low grade infection [23, 53]. Once inoculated, the process of infection and discitis begins. More than often, the main causative organism is not identified. When an organism is identified, the most common infectious etiologic agent is *Staphylococcus aureus* followed by other Staphylococcus species [8,13,16,17,19,21,24,54-56] and anaerobic organisms [2]. Other less common organisms include *Streptococcus viridans* and other Streptococcus species [55], *Escherichia coli*, *Pseudomonas aeruginosa* [4], fungus and others [53,56]. Because all the patients in the current study are from the low socio-economic class and because of the difficulty to identify the causative organism, we elected not to perform CT guided biopsy and give the patients empirical broad spectrum antibiotics covering both aerobic and anerobic pathogens.

It has been reported that the WBC is elevated in only 42.6% of spondylodiscitis cases [56]. That is why we did not rely on WBC as an outcome measure in the current study although it was done as a part of the routine blood investigations. The laboratory studies most sensitive and indicative of the presence of an inflammatory process are the ESR and the CRP. However, it should be noted that in adults, ESR trends are confused by associated medical conditions and the nonspecific elevation in the rate that often occurs with increasing age. Nevertheless, the ESR is a useful tool in the management of adult pyogenic spondylodiscitis, and the authors of most studies on this disease, view a 50 to 66% reduction in the ESR as compatible with eradication of infection [57-59]. The current study showed 82% reduction in the ESR.

Plain radiographic signs of spondylodiscitis are not sensitive and tend to lag behind physical examination findings and laboratory markers. The first plain radiographic sign often noted between the fourth and sixth postoperative week is a loss of intervertebral disc space height. This can be accompanied with blurring or clouding of the vertebral end plates above and below the infected disc space [13]. CT scanning, MRI with gadolinium and also, radionuclide studies are more sensitive. MRI is the radiographic imaging modality of choice in diagnosing postoperative spondylodiscitis [5] with a reported sensitivity and specificity of 93% and 97%, respectively [60]. It has been shown that MRI is superior to both gallium 67 and technetium 99 bone

scanning in diagnosing postoperative discitis and will demonstrate disc changes sooner than CT [60].

Complete eradication of infection should be verified by postoperative normalization of ESR and CRP levels. Trends in these values are greatly affected by concomitant medical conditions and the inflammatory response to surgery. Follow-up magnetic resonance imaging may be useful as well, but interpretation of these images is made difficult by the presence of enhancing non infected granulation tissue and artifacts from the hardware.

In the treatment of spondylodiscitis, numerous authors have preferred to recommend bed rest and prolonged spinal bracing rather than surgical intervention. Others have advocated a staged operation with a period of antibiotic therapy bridging the debridement and instrumentation procedures [39-42].Open surgical drainage for spondylodiscitis was historically reserved for patients with an epidural abscess [61]. The prognosis is stated to be better when treatment is instituted early during the infection [62,63].

There are no obvious advantages to avoidance of hardware placement into debridement cavities. Indeed, the reported sporadic cases of extrusion of anteriorly placed grafts indicate that fixation should be used if possible [64].

The use of interbody grafts in patients with spinal osteomyelitis is accepted [65-67]. Autologous interbody bone grafting in the setting of an active infection was first reported for chronic vertebral osteomyelitis by **Wiltberger** [68] in 1952, and has been used safely ever since [69,70].

In most articles in which single-stage procedures for spinal infections have been described, anterior debridement with placement of allograft or autograft has been used, combined with placement of a posterior stabilizing construct. This approach is based on the principle that instrumentation placed posteriorly involves a second operating field that is not (at least directly) contaminated. The first report in which this strategy was used was published by **Fountain** [71] . **Fountain** presented a mixed series of patients, and the treatment for infection was anterior corpectomy and fusion as well as posterior stabilization with Harrington rods. The first series describing the consistent placement of posterior instrumentation at the time of debridement was published in 1988 by **Redfern et al.**, [72]. In 1996, **Rath et al.,** [73] reported on a series of 43 patients with thoracic or lumbar spondylodiscitis who were treated entirely via a posterior approach, however, the transforaminal approach was not used. In 2003, **Liljenqvist et al.,** [44] reported on a series of 20 patients with thoracic or lumbar spondylodiscitis who all underwent single-stage operations consisting of anterior debridement and reconstruction in which

an expandable titanium cage was used, along with posterior fixation in which a pedicle screw/rod construct was used.

Reconstruction of the anterior column for the treatment of spondylodiscitis has received great interest, because it shares 80% of the lumbar spine load, and such reconstruction places the interbody graft under compression and increases the fusion rate [74-76]. Anterior lumbar interbody fusion may frequently require involvement of an access surgeon and may be a separate approach for the posterior instrumentation [77]. Posterior Lumbar Interbody Fusion (PLIF) is also commonly used but requires bilateral exposure with loss of the posterior tension band at the level of fusion. It decreases the bony surface for posterior fusion, requires significant retraction of the neural elements, and more importantly, particularly in spondylodiscitis cases, cannot be performed safely in a revision case secondary to scar tissue formation [45].

In 1982, with the rationale of offering a secure fusion in a single stage operation, **Harms and Rolinger** [78] pioneered a modified PLIF technique called transforaminal lumbar interbody fusion (TLIF). Compared with the more traditional techniques, it provided several advantages by accessing the spinal canal and disc via a path that runs through the far-lateral portion of the vertebral foramen. Also due to the fact that minimal retraction on the nerve roots and dural sac is required, the surgical risk for neurological deficit is significantly lower. In addition, TLIF achieves a single-stage circumferential fusion through only a posterior approach.

The use of TLIF technique in the management of spondylodiscitis cases has not yet, to the best of our knowledge, been described in literature. It seems a logical pathway to achieve debridement, access the disc space, bypass the scared zone, and simultaneously achieve solid circumferential fusion, avoiding the more complicated anterior approach. Although it may be considered technically demanding, the mean operative time, mean blood loss and complication rate have all shown to be reasonable in the current study. Furthermore, the good results achieved make this technique ideal for managing postoperative spondylodiscitis. One limitation of this study is the inability to recruit a larger number of patients because it highly focused on a certain population.

Conclusion

These results demonstrate that TLIF, is a useful therapeutic tool in dealing with cases of postoperative spondylodiscitis as it offers adequate debridement, good postoperative stability, and allows a single-stage circumferential fusion through only a single posterior approach, with minimal complication rates. A possible follow up to this study would be to use the described technique for all cases of spondylodiscitis.

Case presentation:

A 38 years old male farmer developed L4/5 spondylodiscitis following open discectomy. The diagnosis was confirmed by MRI (Fig. 1A). Following full dose broad spectrum antibiotics and bracing for 8 weeks, the CRP was back to normal but he continued to complain of back pain of increasing severity with night exacerbation and inability to perform his routine activities of daily living. Repeated inflammatory markers showed evidence of reactivation of infection. His final CRP check was 38 and ESR was 64 at the first hour. Disc space debridement, TILF with autologous iliac bone graft and posterior instrumentation with titanium pedicle screw system, were performed (Fig. 1B). The patient achieved satisfactory fusion, his back pain improved dramatically and there has been no recurrence of infection, at 2.5 years follow up (Fig. 1C).

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