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Effects of Designed Ultrasonic Field in Different Frequency Sonophoresis Using the Carrier of Liposomes

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Abstract: The objective of the study was to investigate the skin permeability of designed diffuse ultrasonic field and the application of different frequency exposed on liposome transdermal delivery. The specimens were exposed to ultrasound by frequencies of 20, 60 kHz and 1 MHz with the intensities 0.43 W/cm^2. In the exposed experiments, the diffuse ultrasonic field was performed using an inclined incident transducer and a designed wedge in the 20 and 60 kHz. The frequency of 1 MHz transducer was operated directly in the skin sample. These exposure methods have been compared to the unexposed samples by recording the permeated depth of the rhodamine in the skin. Experimentally, the results revealed that the ultrasonic frequency of 60 kHz has a better permeated depth about 168 nm under the skin surface. In general, applied higher intensity of ultrasound gave greater permeated depth than lower intensity. However, safe application of higher intensity ultrasound should be practiced by careful selection of exposure parameters. It is the principle reason for the lower intensity applied in the study.
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Keywords: liposome, diffuse ultrasonic field, acoustic radiation force, Sonophoresis

1. Introduction

Transdermal delivery system is a simple and convenient drug delivery system. It offers several advantages than traditional drug delivery such as oral delivery and injections including elimination of the first-pass metabolism, lower the pain, and possible maintained release of drugs. However, human skin is an efficient barrier. The outer layer of this barrier called the stratum corneum, is the main structure to cause the low skin permeability of transdermal delivery of drugs. Therefore, it is difficult to deliver the higher molecular weight drugs across the skin. Several physical and chemical methods have been reported in the literature, which have successfully increased the level of drugs delivery across and into the skin. These methods, for example, include the ultrasound, chemical enhancers and electric fields [1-3].

The use of ultrasonic technology in the biological or medical application has been a convenient clinical tools and development for many years [4-5]. Ultrasound under suitable conditions has been shown to enhance the transdermal transport. This phenomenon is referred to as sonophoresis. Sonophoresis is a physical technique to enhance the transdermal delivery of drugs using ultrasound energy [6]. In the recent researches, low frequency ultrasound has been shown to be more enhancement in the transdermal delivery than the high frequency ultrasound [7]. This high efficiency of low frequency ultrasound creates from cavitation effects, which is important reason for skin permeabilization [8]. Cavitation is the gaseous nuclei growing in liquid under the ultrasound exposure. It involves either the rapid growth and collapse of a bubble (called the transient cavitation), or vibration motion of a bubble (called the stable cavitation) in the ultrasound field. Cavitation is affected by numerous parameters including the presence of cavitation nuclei. The cavitation nuclei may exist in many forms including microbubbles that are already existence in the liquid or made by artificial way. Many methods to enhance the cavitation have been reported in the literature. For example, researchers have used microspheres, silica particles, and ultrasonic contrast agents to enhance cavitation [9]. Ultrasonic contrast agents are typically gas-encapsulated microbubbles with diameters of the order of 1-10 μm. Contrast agents are filled with a gas that may be air or substance of higher molecular weight, such as perfluoropropane. The shell can be stiff or more flexible, and the shell thickness can vary from 10-200 nm. Liposome has a similar structure as the contrast agents. It has composite structures made of phospholipids and may contain small amounts of other molecules. The size of the liposomes can vary from low micrometer range to tens of micrometers. Liposomes are artificially prepared vesicles made of lipid bilayer. It can be filled with drugs, and used to deliver drugs for cancer and other diseases. Physical methods such as iontophoresis, ultrasound, and tape-stripping can further assist the delivery of drugs encapsulated in liposomes. Dahlan *et al.* have considered the effects of the low frequency ultrasound and liposomes on skin [10]. It has to notice that the liposomes can repair skin damage, which could limit the drug permeation. They find that the influence of liposome was evident within 5 min of its application, and the smaller liposomes were more

effective at repairing skin disruption caused by sonication. In addition, they think the skin repair by liposomes seems to depend on the extent of the disruption caused by ultrasound application. Though the ultrasound can assist the transdermal delivery of drugs in liposomes, it still exist some questions. Such as the exposure of high intensity ultrasound will increase the temperature in the liquid. The thermal effect induced by high temperature will damage the liposome and render the drug inside ineffective. Thus, liposomes solution in and not in an ultrasonic field will be discussed in this study, and the permeation depth of the entrapped material within liposomes (rhodamine B) was compared. The diffuse ultrasonic field was performed using the combination of an inclined incident transducer and a designed wedge. To prevent the thermal effects appeared in the exposure experiment, the lower ultrasonic intensity was applied to drive the transducer. Three driving frequencies of the ultrasonic field are selected and the distribution conditions of skin permeation depth examined.

2. Theory

In an ultrasonic field, the force act on the particle is determined by a balance among the diffusion force, the gravitational force and the acoustic radiation force. When the acoustic standing wave field is produced in a dilute suspension of particles, the acting force is known as the primary acoustic radiation force. For a spherical particle with a radius R dispersed in an inviscid fluid, and an acoustic force due to a one-dimensional standing plane wave field this is described by

$$F_{ac} = 4\pi R^3 \kappa E_{ac} F \sin(2\kappa x) \qquad (1)$$

where x is the position of the particle relative to the nearest pressure antinode of the wave field; κ is the acoustic wave number; E_{ac} is the acoustic energy density, and F is the constant acoustic factor. The constant F is given by

$$F = \frac{1}{3}\left[\frac{5\Lambda - 2}{1 + 2\Lambda} - \frac{\gamma_p}{\gamma_f}\right] \qquad (2)$$

In Eq. (2), Λ is the ratio of particle density to fluid density and γ_p and γ_f are the compressibility of the particle and the fluid, respectively. Eq. (1) yields the acoustic radiation force and is reasonable for any particle that is much smaller than the acoustic wavelength. If the above condition is satisfied, then the acoustic constant factor F is independent of the size and shape of the particle [11]. Eq. (1) indicates that the primary acoustic radiation force can drive the particles to the pressure nodes or the antinodes of the acoustic field. When the constant acoustic factor F is positive, then the particles move toward the pressure nodes, if F is negative, then the particles are driven to the pressure antinodes.

3. Materials and methods

3.1 Diffuse ultrasonic field

To produce a wide and uniform exposure surface, the suitable design was needed. The acoustic field is about using the diffuse field theory of Sabine to create a uniform sound field for the radiation experiment [12-13]. With this theory, the ultrasonic beam had to be oblique incident to the finite boundary. After repeatedly reflection of the sound wave, a uniform sound field would be obtained in the surfaces of the space. The cuboid acrylic wedge, shown schematically in Fig. 1(a), with the bottom area of 62×65 mm and the height of 120 mm was used to create the uniform irradiation field. The top corner of the exposure wedge was made an oblique and triangle plane with the length of 75 mm to mount the ultrasonic transducer. Ultrasonic beam of the transducer was incident with the angle 45° from the oblique plane toward the boundary of the wedge at the far end. The transducers with the frequencies of 20 and 60 kHz were used to fix on the wedge. In Fig. 1(b), the acrylic case was applied as a boundary to fix the transducer of 1 MHz. The exposure area was determined as the boundary of the case. Furthermore, the exposure area indicated in the figure was used to contact the skin samples. All sampling positions of the exposure area were shown in the Fig. 2. Each permeated depth of six randomly selected regions of each sampling position was taken. The permeated depth was measured by Nikon C1 plus confocal microscopy. The mean values of permeated depth in the six regions was indicated the depth of one sampling position in the exposure area. An ultrasonic transducer was positioned above the sampling position A1 of the exposure area. Two custom built transducers with operating frequencies of 20 and 60 kHz (Broadsound Corporation, Taiwan) were used for application ultrasound. The exposure experiment of 1 MHz was operated by ultrasonic diathermy system (ZMI, ULS-1000). The exposure and measurement system with a diffusion field comprised an ultrasonic transducer that could produce a diffuse sound field was devised, and is presented in Fig. 3. The transducer was driven by a continuous sine wave from a function generator (GW instek, SFG-830). The intensity of the sound field was measured using a miniature PVDF ultrasonic hydrophone probe (Force Institute, MH28-10). In this experiment, the output intensities were set as 0.19 and 0.45 W/cm2. The signal obtained from the hydrophone was analyzed using a LeCroy WaveSurfer 422 digitizing oscilloscope. Ultrasound was exposed to the skin samples for 5 minutes to prevent the increasing temperature on the skin. All experiments were performed at room temperature. When the skin samples were exposed or sham-exposed to ultrasonic irradiation, the permeated depth distribution of liposomes, affected or unaffected by the ultrasonic waves, was visible.

3.2 Material and skin preparation

Skin exposure experiments were carried out in vitro

with full thickness pig skin of the ear (Yorkshire). Superfluous tissues such as fat and muscle were removed. Skin was cut into square pieces (10×10 cm), and was stored in a freezer until the experiments were performed. Egg yolk phosphatidylcoline (EPC) and cholesterol (Sigma Chemical Co., St. Louis, MO) in the molar ratio of 4:1 were mixed in a round-bottomed flask. The fluorescence materials (rhodamine) were dissolved in the suspension. Then the suspension was prepared by dissolving in chloroform. Subsequently, the organic solvent was evaporated under the vacuum, and the lipid film formed was then left under a stream of nitrogen to remove traces of the organic solvents. The resulting dried lipid film was dispersed with a buffer solution (Hepes: 0.1 M, pH 5). The solution was vortex mixed above the phase-transition temperature (room temperature) and yielded the lipid suspensions. Lipid suspensions were operated with the mechanical shaking for 30min. After that, the ultrasonic processor was used to crushing the lipid membrane and obtained liposomes with the diameter of 200 nm.

4. Results and discussions

Table 1 presents the permeated depth of liposomes at each sampling position for exposed or sham-exposed to ultrasonic irradiation with three different frequencies. In this table, the permeated depth of liposomes, are presented in a unit of micrometer. Sham irradiation experiments are used to compare the influence of the ultrasonic irradiation in the liposomes. In addition, the sham irradiation experiments were measured the permeated depth after maintained the liposome solution about 30 min in the skin. Figure 4 shows the permeated depth distribution of the exposure area of the skin samples without exposure to ultrasound, based on the color plot. The sampling position A1 to A9 indicate the relative position in the exposure area. The color scale is given by MATLAB package, and expanded from 130 to 200. The average value of permeated depth of liposomes in the skin sample is about 138 μm, as indicated in Table 1. Based on the value of permeated depth, the distribution of the liposomes was about 130 to 145μm in the Z-axis. In this condition, the attraction of molecule and the absorption of the skin afford the liposomes to permeate the skin sample.

Figures 5(a)-(c) plot the distribution of permeated depth with ultrasound exposure obtained from the data in Table 1. In the 20 and 60 kHz exposure experiment, the sound beam is incident into the cuboid acrylic wedge to produce a diffuse ultrasonic field and expose the skin sample. Figures 5(a) plot the results of exposure to the ultrasonic frequency of 20 kHz in the intensity of 0.45 W/cm^2. In this image, the distribution of permeated depth is from 148.3 to 173.3 μm, and the average permeated depth of liposomes is 159 μm, as shown in Table 1. It must be notice that the thermal effects induced by ultrasound will be avoided in this research. Thus the ultrasonic transducer does not contact the skin sample directly in the exposure experiments and the shorter exposure time can reduce the temperature rise. Comparing to the sham irradiation results, the average permeated depth under the ultrasonic exposure is increased about 20 μm. The greatest depth was 173.3 μm and appeared in the sampling position A5. Figures 5 (b) plots the distribution of permeated depth exposed to the ultrasonic frequency of 60 kHz with the intensity of 0.45 W/cm^2. In this image, the distribution of permeated depth is from 151.7 to 185 μm. The average permeated depth of liposomes is 168 μm, as shown in Table 1. The average permeated depth is exceeded about 30 μm to the sham-exposed result. It is also better than the result of 20 kHz about 10 μm. As can be seen in the Table 1, all sampling positions appeared more than 165 μm permeated depth except A7 and A9. The greatest depth was 185 μm and appeared in the sampling position A2. Figures 5 (c) plots the distribution of permeated depth exposed to the ultrasonic frequency of 1 MHz. Comparing to the wedge exposure, this experiment is used the traditional sonophoresis apparatus. The ultrasonic transducer will contact the skin sample directly. It must be notice that the exposure area is about 30×30 mm. The average permeated depth of liposomes is 159.5 μm and the greatest depth is 167.7 μm appeared in the sampling position A2. The average permeated depth is exceeded about 20 μm to the sham-exposed result.

Figs. 6(a)-(c) show the effects of the ultrasound exposure and thus clarify the change in the permeated depth of the sampling position between the exposed or sham-exposed to ultrasonic irradiation. These figures plot the average values of permeated depth as a function of sampling position at frequency of 20, 60 kHz and 1 MHz, respectively. One sampling position represents an arithmetic mean over six sampling points. As can be seen in these figures, the permeated depth of treated samples are greater than the sham-exposed skin. In the sampling position A1, the permeated depth of the exposed samples are over 170 μm than the control samples in the frequencies of 20 kHz and 60 kHz in Figs. 6(a)-(b). Based on the corresponding dimensions of the wedge presented in Fig. 2, the sound beam is incident with the angle 45° . When ultrasound is applied, the sound wave is initially reflected from the boundary of the wedge and the reflected beam points to the sampling position A1, A2, A4 and A5. The first reflected sound wave penetrate through the wedge and produce greater acoustic radiation force. Thus, the acoustic radiation force affects the liposomes and pushes them down to the skin. It can be seen that the two figures has greater permeated depth in the sampling positions in the A2 and A5. Fig. 6(c) is the average values of permeated depth as a function of sampling position for the skin sample with exposure

frequencies 1 MHz. In Figs. 6(c), the distribution of the permeated depth is from 148.7 to 167.7 μm. Notably, the exposure area in the 1 MHz irradiation experiments is smaller than the wedge experiments. Thus, it can be seen that under the 60 kHz irradiation, the average depth result and the distribution of the permeated depth is greater than the other frequencies.

Table 1. The permeated depth of the different sampling positions are exposed to ultrasound at two output intensities by using the 20, 60 kHz and 1 MHz frequencies. The unit of the recorded values are micrometer. In this table, the (AVG) is the average permeated depth in the series of sampling positions.

Frequency / sampling position	Sham exposed	20 kHz	60 kHz	1 MHz
A1	130	171.7	173.3	160
A2	130	158.3	185	167.7
A3	133.3	150	165	157.3
A4	136.7	161.7	165	164.7
A5	145	173.3	176.7	163
A6	141.7	158.3	168.3	154.7
A7	145	153.3	158.3	148.7
A8	145	153.3	171.7	157.3
A9	138.3	148.3	151.7	162
AVG	138	159	168	159.5

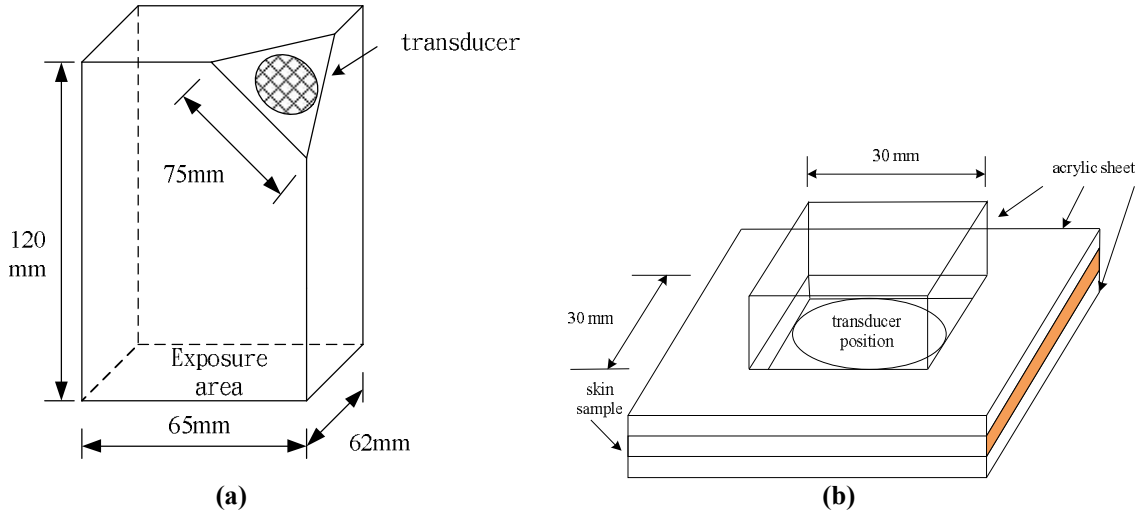

Figure 1. (a)The dimensions of the exposure wedge. The orientation of the transducer is fixed in the corner of the wedge. (b)The exposure chamber used in the 1 MHz experiments.

5. Conclusions

This study examined various subjects. First, the design wedge with inclined incidence of sound wave were applied to investigate the permeated effects of ultrasound. Second, three ultrasonic frequencies of 20, 60 kHz and 1 MHz were applied. Third, the average permeated depth of liposomes in each experiment were described and the permeated depth distribution of the sampling position in the skin samples were compared. An ultrasonic intensity of 0.45 W/cm^2 and the frequency of 60 kHz permeated the liposomes more effectively than other setup. An appropriate ultrasonic frequency inclined incident into the designed wedge could induce the permeability of liposomes and increased the permeated depth of particles in skin samples.

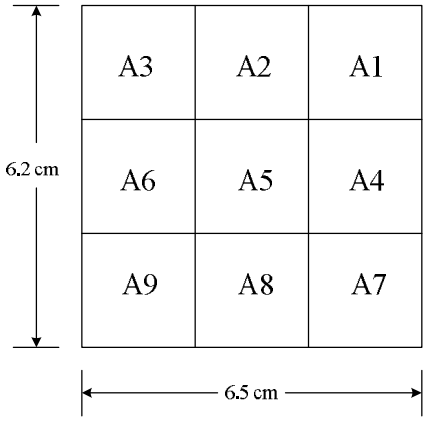

Figure 2. The sampling positions of the exposure area applied in the experiments. The diameter is about 62×65 mm in the wedge bottom. The sampling positions of ultrasonic diathermy system is the same as this figure, only the exposure area of ultrasonic diathermy system is about 30×30 mm.

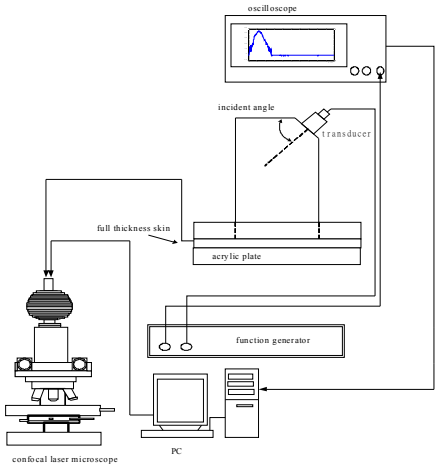

Figure 3. Schematic diagram of the isonation and measurement apparatus used in the exposure experiments.

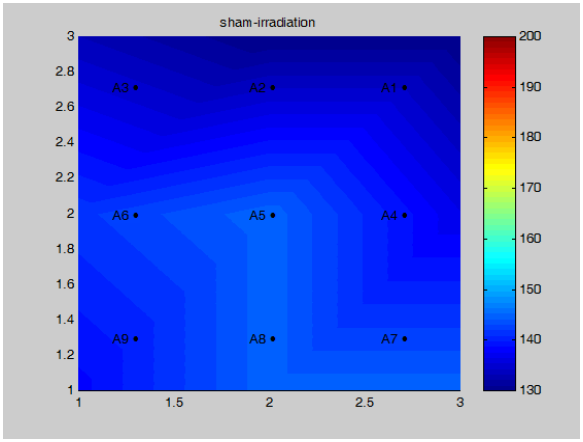

Figure 4. Color mapping of the permeated depth distribution for the skin sample with no sound field applied. Color plot corresponds to the magnitude of depth value. A1 to A9 is the sampling position with respect to the skin sample.

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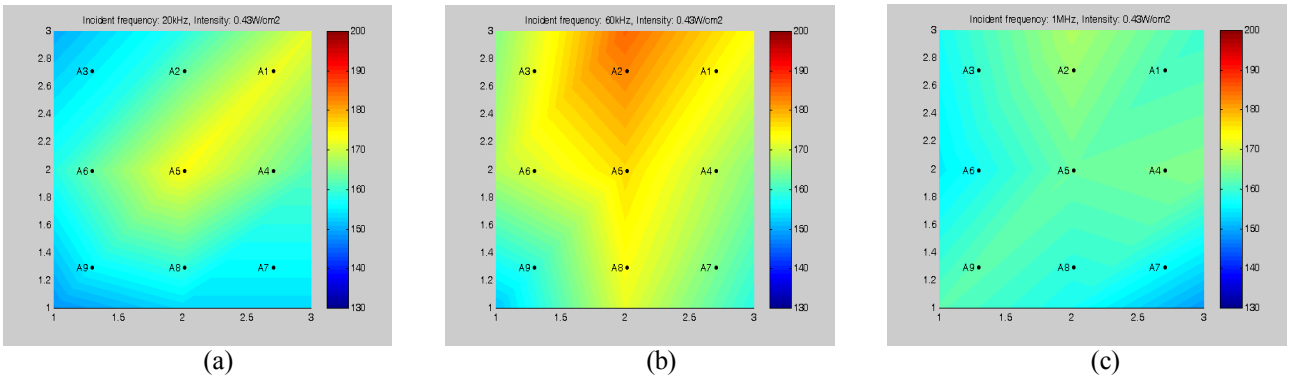

(a) (b) (c)

Figure 5. Color mapping of the permeated depth distribution for the skin sample with exposure frequencies of 20, 60 kHz and 1MHz: (a) demonstrate the frequency of 20 kHz with intensities 0.45 W/cm^2, (b)(c) demonstrate the frequency of 60 kHz and 1 MHz.

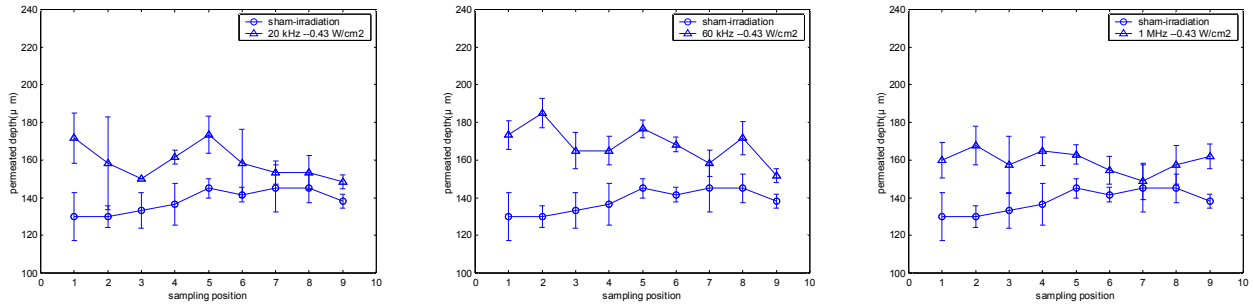

Figure 6. The average values of permeated depth as a function of sampling position for the skin sample with exposure frequencies of 20, 60 kHz and 1 MHz: (a) demonstrate the frequency of 20 kHz with intensities 0.45 W/cm^2, (b)(c) demonstrate the frequency of 60 kHz and 1 MHz.

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Theoretical and Numerical Analyses on Multi-Layered Ceramic Scaffold due to High Pressure for Tissue Engineering

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Abstract: Tissue engineering scaffolds provide temporary mechanical support for tissue regeneration and transfer global mechanical load to mechanical stimuli to cells through its architecture. The manufacturing process may cause the deformation and internal defects in multi-layered ceramic scaffold (MLCS) that results in the malfunction for tissue engineering applications. This work aims to investigate the deformation of MLCS that composed of nearly a hundred of ceramic and metal powder films interleaved and stacked due to high pressure at constant elevated temperature. On theoretical analysis, classical laminated plate theory, linear elastic assumptions and equilibrium equations were adopted. Associated with the practical process three types of boundary conditions (BCs) were used, such as all edges simple-supported, two edges simple-supported and the other two free, and four edges free. Also, two more conditions need be added, including four fixed points at corners and the elastic foundation at bottom. As for the numerical simulation the finite element method (FEM) incorporated with software ANSYS was used to obtain the displacement field of MLCS to validate the analytical prediction. Compared with the numerical results the analytical solutions were found satisfactorily acceptable, i.e., the errors were about 0.1%- 6.2% for the BCs of four edges free and four corners fixed. The errors about 0.13%- 6.15% were also acceptable for the BCs of two edges simple-supported and the others free. However, the analytical solution for the case of all the edges simple-supported did not agree with the numerical results. Finally, the proposed theoretical methodology alternatively provides an analytical method, instead of FEM and ANSYS, to analyze a nearly and over hundred layered MLCS for tissue engineering scaffolds.

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Keywords: Theoretical derivation, Finite element analysis, Multi-layered ceramic scaffold, Deformation, Tissue engineering

1. Introduction

The physical characteristics required of scaffolds for tissue engineering necessitate the application of novel processing techniques for its design and fabrication. Scaffolds have been studied and fabricated using conventional techniques such as fiber bonding, solvent casting, particulate leaching, membrane lamination and melt molding [1]. In scaffold-based tissue engineering (TE) strategies, the successful regeneration of tissues and organs from matrix-producing connective tissue cells or anchorage dependent cells relies on the application of suitable substrates or scaffolds [2]. The scaffolds, built from synthetic or natural materials, serve as temporary surrogates for the native extracellular matrix (ECM). The challenge in scaffold-based TE is to construct biologic replicas in vitro such that the engineered composite becomes integrated for transplantation in vivo for the recovery of loss or malfunctioned tissues or organ. The composite should subsequently function coordinately with the rest of the body without risk of rejection or complications [3, 4].

Numerous studies have been conducted on forming particular porous microarchitectures inside scaffolds and foam structures to obtain acceptable mechanical properties [5-7]. In addition, several groups have developed porous bone substitutes using various biomaterials including polymers, ceramics, metals and composites in an attempt to obtain biomechanical properties matching natural bone [8-10]. Bioceramics, especially calcium phosphates (CaP), are known as bone-resembling materials with excellent biocompatibility but limited mechanical strength [10–12]. Although porous ceramic materials are remarkably stiff and strong compared to polymers and composites, they are typically too brittle to resist significant cyclic loading [10, 13].

Computer-aided tissue engineering (CATE) is a newly emerging field that can be classified within three major categories: computer-aided tissue modeling, computer- aided tissue informatics and computer-aided tissue scaffold design and manufacturing [14, 15]. Application of CATE allows us to explore many novel ideas in modelling, design and fabrication of tissue scaffolds with enhanced functionality and improved interactions with cells. This is particularly useful in modeling and design of

complex bone tissue scaffolds and replacement structures that require us to simultaneously consider many biological and biophysical design requirements [16–18]. Available methods for characterization of mechanical properties of porous scaffolds and heterogeneous tissues were primarily based on using experimental approaches [19, 20], finite element numerical calculation [21-23], or effective property modeling. However, although the asymptotic homogenization theory has been well developed [24–27], the application of the theory requires a finite element implementation and the associated computational algorithm for its numerical solution.

In theoretical derivation by force method we assume that the thin plate resting on an elastic foundation [28]. For elastic foundation problem, Adewale [29] studied that the singularity function method to solve the semi-infinite orthotropic rectangular plates on a Winker-type elastic foundation. Jayachandran and Vaidyanathan [30] investigated the postbuckling response of the isotropic square thin plate subjected to biaxial compression resting on elastic foundations by the finite element method. Shen [31] presented the performances of perfect and imperfect, antisymmetrically angle-ply and symmetrically cross-ply laminated plates under combined loading and resting on Pasternak-type or softening nonlinear elastic foundations from which the results for Winkler elastic foundations were obtained as a limiting case. Horibe and Asano [32] reported the method for calculating the large deflection of a rectangular plate on an elastic foundation by the boundary integral equation method. Finally, our work is to determine the deflection of MLCS due to high pressure at constant elevated temperature with the assumption of laminated plate resting on an elastic foundation theoretically and validated numerically. The numerical simulation by FEM with software ANSYS was used to obtain the displacement field of MLCS for verification.

2 Theoretical Formulation

For simplicity, assume a homogeneous, isotropic and linearly elastic multi-layered thin plate of uniform thickness h, dimensions a, b, modulus of elasticity E and Poisson's ratio v. The plate rests on an elastic foundation and is subjected to a biaxial inplane loading N_x, N_y and a transverse distributed load q as shown in Fig. 1. Also, the intensity of the reaction p at every point of the bottom plate is proportional to the deflection w at that point, as $p=kw$ with k being the modulus of foundation. The deflection due to vertical pressure should be balanced by the reactive deformation of elastic foundation from the force method. Accordingly, the differential equation for deflection in rectangular coordinates is

$$\frac{\partial^4 w}{\partial x^4}+2\frac{\partial^4 w}{\partial x^2\partial y^2}+\frac{\partial^4 w}{\partial y^4}=\frac{1}{D}\left(q+N_x\frac{\partial^2 w}{\partial x^2}+N_y\frac{\partial^2 w}{\partial y^2}+2N_{xy}\frac{\partial^2 w}{\partial x\partial y}-kw\right) \quad (1)$$

where $D=Eh^3/[12(1-v^2)]$ is the flexural rigidity of the plate, E is Young's modulus, and v Poisson's ratio.

Associated with the texts [28] and practical manufacturing process, three types of BCs were discussed, such as all edges simple-supported (S-S-S-S), two opposite edges simple-supported and the other two free (S-F-S-F), and four edges free (F-F-F-F). Also, two more conditions need be necessarily added, including four corners fixed and the bottom plate as elastic foundation.

For the case of BCs S-S-S-S as shown in Fig. 1(c), the load distributed over the surface is

$$q=\frac{16q_0}{\pi^2}\sum_{m=1,3,5...}^{\infty}\sum_{n=1,3,5...}^{\infty}\frac{1}{mn}\sin\frac{m\pi x}{a}\sin\frac{n\pi y}{b} \quad (2)$$

where q_0 is the intensity of the load at the center of the plate. The BCs for simple-supported edges are

$$w=0,\ \frac{\partial^2 w}{\partial x^2}=0 \quad \text{for} \quad x=0,a \quad (3a)$$

$$w=0,\ \frac{\partial^2 w}{\partial y^2}=0 \quad \text{for} \quad y=0,b \quad (3b)$$

To satisfy with all BCs the deflection can be expressed as

$$w=\sum_{m=1,3,5...}^{\infty}\sum_{n=1,3,5...}^{\infty}a_{mn}\sin\frac{m\pi x}{a}\sin\frac{n\pi y}{b} \quad (4)$$

Substituting Eqs. (2) - (4) into Eq. (1) with rearrangements [33] and neglecting the details, we receive

$$w=\frac{16q_0}{\pi^6 D}\sum_{m=1,3,5...}^{\infty}\sum_{n=1,3,5...}^{\infty}\frac{\sin\frac{m\pi x}{a}\sin\frac{n\pi y}{b}}{mn[(\frac{m^2}{a^2}+\frac{n^2}{b^2})-\frac{N_x m^2}{D\pi^2 a^2}-\frac{N_y n^2}{D\pi^2 b^2}+\frac{k}{\pi^4 D}]} \quad (5)$$

Next, consider the case of S-F-S-F as illustrated in Fig. 1(d) where $0\le x\le a$ and $-b/2\le y\le b/2$. The BCs are

$$w=0,\ \frac{\partial^2 w}{\partial x^2}=0 \quad \text{for} \quad x=0,a \quad (6a)$$

$$\left.\begin{aligned}&(\frac{\partial^2 w}{\partial y^2}+v\frac{\partial^2 w}{\partial x^2})=0\\ &D[\frac{\partial^3 w}{\partial y^3}+(2-v)\frac{\partial^3 w}{\partial x^2\partial y}]=(EI\frac{\partial^4 w}{\partial x^4})\end{aligned}\right\} \quad \text{for} \quad y=\pm\frac{b}{2} \quad (6b)$$

where EI is the flexural rigidity of plate. The deflection is in the form as

$w=w_1+w_2$

where $$w_1=\frac{4a^4(q-N_x-N_y)}{10^3\pi^5 kD}\sum_{m=1,3,5...}^{\infty}\frac{1}{m^5}\sin\frac{m\pi x}{a} \quad (7)$$

and

$$w_2=\sum_{m=1,3,5...}^{\infty}\frac{a^4(q-N_x-N_y)}{10^3 kD}(A_m\cosh\frac{m\pi y}{a}+$$

$$B_m \frac{m\pi y}{a}\sinh\frac{m\pi y}{a} + C_m \sinh\frac{m\pi y}{a} + D_m \frac{m\pi y}{a}\cosh\frac{m\pi y}{a}) \quad (8)$$

Eqs. (7) and (8) are satisfied with the BCs. The four constants in Eq. (8) can be determined by satisfying the BCs and the symmetry. Similarly, the deflection of plate is found [33] and expressed as

$$w = \sum_{m=1,3,5...}^{\infty} \frac{a^4(q-N_x-N_y)}{10^3 kD} \sum_{m=1,3,5...}^{\infty} (\frac{4}{m^5\pi^5} A_m \cosh\frac{m\pi y}{a} + B_m \frac{m\pi y}{a}\sinh\frac{m\pi y}{a})\sin\frac{m\pi x}{a} \quad (9)$$

$$A_m = \frac{4}{m^5\pi^5} \frac{\nu(1+\nu)\sinh\alpha_m - \nu(1-\nu)\alpha_m\cosh\alpha_m}{(3+\nu)(1-\nu)\sinh\alpha_m\cosh\alpha_m - (1-\nu)^2\alpha_m} \quad (10a)$$

$$B_m = \frac{4}{m^5\pi^5} \frac{\nu(1-\nu)\sinh\alpha_m}{(3+\nu)(1-\nu)\sinh\alpha_m\cosh\alpha_m - (1-\nu)^2\alpha_m} \quad (10b)$$

where $\alpha_m = m\pi b/2a$ and $\lambda = EI/aD$

Substituting $\lambda = 0$ into Eq. (10), we obtain the constants in Eq. (9) for the case of S-F-S-F.

Finally, for the case of F-F-F-F as illustrated in Fig. 1(e) where $-a/2 \le x \le a/2$ and $-b/2 \le y \le b/2$.

In such case the BCs are

$$(\frac{\partial^2 w}{\partial x^2} + \nu\frac{\partial^2 w}{\partial y^2}) = 0$$
$$D[\frac{\partial^3 w}{\partial x^3} + (2-\nu)\frac{\partial^3 w}{\partial y^2\partial x}] = (EI\frac{\partial^4 w}{\partial y^4}) \quad \text{for } x = \pm\frac{a}{2} \quad (11a)$$

$$(\frac{\partial^2 w}{\partial y^2} + \nu\frac{\partial^2 w}{\partial x^2}) = 0$$
$$D[\frac{\partial^3 w}{\partial y^3} + (2-\nu)\frac{\partial^3 w}{\partial x^2\partial y}] = (EI\frac{\partial^4 w}{\partial x^4}) \quad \text{for } y = \pm\frac{b}{2} \quad (11b)$$

The deflection of w can be expressed as

$$w = \{\frac{q}{384D(\gamma+\delta)}[\lambda(16x^4 - 24a^2x^2 + 5a^4) + \delta(16y^4 - 24b^2y^2 + 5b^4)]$$

$$+ \sum A_n \cosh\frac{n\pi y}{a}\cos\frac{n\pi x}{a} + \sum B_n \cosh\frac{n\pi x}{b}\cos\frac{n\pi y}{b}$$
$$+ \sum C_n y\sinh\frac{n\pi y}{a}\cos\frac{n\pi x}{a} + \sum D_n \sinh\frac{n\pi x}{b}\cos\frac{n\pi y}{b}\}\frac{D(q-N_x-N_y)}{10qk} \quad (12)$$

where δ/λ and A_n ,…, D_n are some constants and n=1,3,5,…. In a particular case of EI=0, n=1, δ/λ =1, $A_1 = B_1$, $C_1 = D_1$, and $\lambda = EI/aD$, we have a square plate carrying a biaxial inplane loading and a uniform pressure and supported only at the corners. After calculations the constants, $B_1 = 5479.3868$ and $D_1 = -33354.3362$, are obtained [33].

From the above-mentioned investigation, the deflection w associated with three BCs of MLCS can be accomplished.

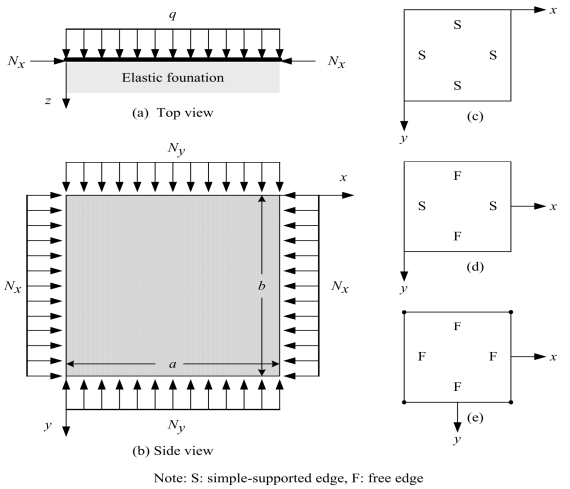

Figure 1. Scheme of MLCS under vertical and lateral pressures on elastic foundation at various boundary conditions

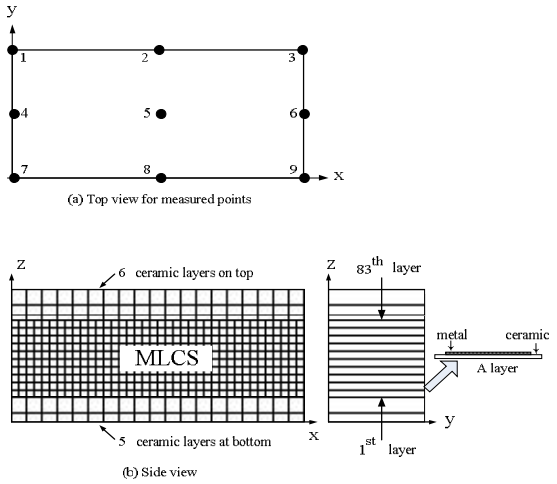

Figure 2. The geometry of 83-layered MLCS

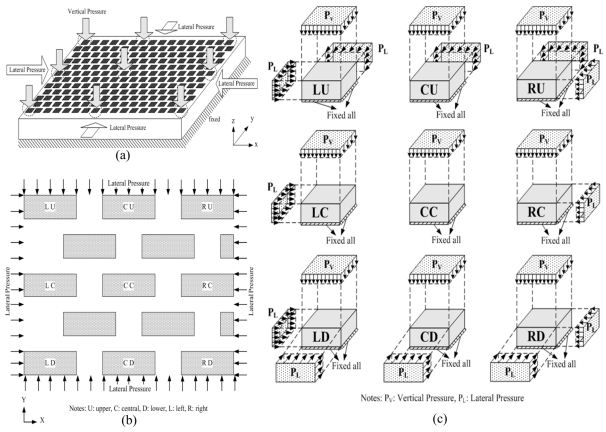

Figure 3. Scheme of MLCS under vertical and lateral pressures (a) side view (b) top view (c) loading positions for nine locations in MLCS

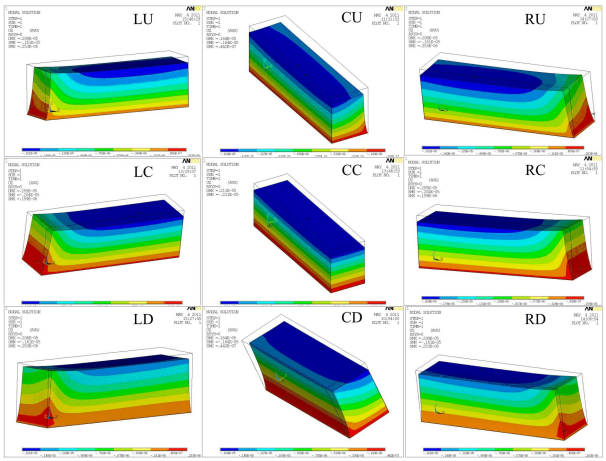

Figure 4. The displacements of u, v and w subjected to uniformly vertical pressure by loading positions for nine locations in MLCS

3 Numerical Simulation

Figure 2 shows the geometry of MLCS, and the dimensions of a ceramic film are $1.368mm \times 0.345mm \times 2.25\mu m$ (L × W × T), and a metal powder film 1.188 mm × 0.175 mm × $1.6\mu m$, respectively. The Young's moduli of both films, such as E_{BaTiO3}=3.53GPa and E_{Ni}=4.09GPa, were obtained by nanoindentation testing [33], however, the Poisson's ratio and coefficient of thermal expansion were adopted from [35], 0.33 for ceramic and 0.26 for metal powder films, and the coefficients of thermal expansion are $9.8ppm/^0K$ and $13.3ppm/^0K$, respectively. The SEM micrographs of ceramic film and printed metal powder film were also obtained [33]. It can be observed that the grain boundary becomes more and more clear with the increase of temperature and time. That means almost no phase changes occur in the manufacturing process of MLCS.

The MLCS were subjected to high hydrostatic pressure at elevated temperature in processing. Two types of vertical loading were investigated, such as the uniform pressure and slant pressure of 1° inclination. The vertical pressures include 8000, 10000, 12000, 16000 and 20000psi. Also two lateral pressures were added, such as the uniform and linearly distributed pressures. The temperature was kept at constant of 85^0C. The BCs include fixed at bottom and other sides free as shown in Figure 3.

For Simplicity, the sample is reasonably assumed elastic, isotropic and homogeneous. The friction and gap between layers can be ignored. A pre-study was performed by using software, ANSYS. Eight-node solid element (Solid 45) and twenty-node solid element (Solid 95) were used [34, 35]. In order to assure the preciseness of simulation the convergence analysis was reasonably done in advance. By the results of convergence analysis, the errors at special area obtained from both coarse and refined meshes of the metal electrode were less than 0.002%. In order to observe and focus on the special zone that the mapped mesh method was properly adopted.

4 Results

In nanoindentation two test points were so close that an error of Young's modulus was found. After a series of tests of the point distances of 10, 50 and 100μm, it is suggested that two test points should be at least 100μm apart. Only two load-displacement curves were deviated away, then these two unloading curves couldn't be adopted for Young's modulus calculation [33].

The numerical results of deformations, i.e., u_x, u_y, u_z of metal electrode at the first layer(bottom), 20^{th} ,40^{th} ,60^{th} , and 80^{th} (top)layer, subjected to uniformly vertical pressure, 69MPa($\approx$10000psi) of practical use, were showed in Fig. 4, as an example. The results due to other pressures, such as 8000-20000psi were omitted. Also, it is found that the deformations and stresses do not change significantly due to a 1° inclination of vertical pressure. Hence, the results of slightly inclined pressure were neglected. It needs be mentioned that the locations of maximal deformation were also marked according to Fig. 3(b).

The theoretical analysis for S-S-S-S, S-F-S-F and F-F-F-F plates subjected to uniformly vertical pressure combined with biaxial compression and resting on elastic foundations. For all cases the material properties, dimensions and uniform pressure are E=3.810GPa, v=0.3, h=429.55μm, a=b=0.15m and q=69MPa, respectively. Young's modulus E and Poisson's ratio v are obtained by the rule of mixtures of two parts. The theoretical results of deformations, u_z, of metal electrode at the top layer, subjected to uniformly vertical pressure, 69MPa and a biaxial inplane loading for various BCs were selected and listed in Table 1. The errors of the numerical results compared with the analytical solutions were listed in Table 2.

Table 1. The deformation, u_z, of analytical solutions of the top layer subjected to uniformly vertical pressure, 69*MPa* by various boundary conditions

location	B. C.	Deflection of measured point (μm)								
		1	2	3	4	5	6	7	8	9
LU	S-S-S-S	0	0	0	0	0.03	0.06	0	0.05	0.09
	S-F-S-F	0	1.36	1.32	0	1.53	1.55	0	1.53	1.55
	F-F-F-F	0	1.08	1.27	0.57	1.4	1.45	0.86	1.56	1.53
LC	S-S-S-S	0	4.72	9.66	0	4.72	9.66	0	4.72	9.66
	S-F-S-F	0	1.92	1.9	0	1.92	1.9	0	1.92	1.9
	F-F-F-F	1.26	1.96	1.97	1.26	1.96	1.97	1.26	1.96	1.97
LD	S-S-S-S	0	0.05	0.11	0	0.03	0.05	0	0	0
	S-F-S-F	0	1.53	1.55	0	1.53	1.55	0	1.36	1.32
	F-F-F-F	0.99	1.63	1.57	0.49	1.36	1.42	0	1.08	1.27
CU	S-S-S-S	0	0	0	1.38	1.38	1.38	2.07	2.07	2.07
	S-F-S-F	1.37	1.37	1.37	1.56	1.56	1.56	1.56	1.56	1.56
	F-F-F-F	1.36	1.36	1.36	1.52	1.52	1.52	1.61	1.61	1.61
CC	S-S-S-S	251	251	251	251	251	251	251	251	251
	S-F-S-F	2.01	2.01	2.01	2.01	2.01	2.01	2.01	2.01	2.01
	F-F-F-F	2.01	2.01	2.01	2.01	2.01	2.01	2.01	2.01	2.01
CD	S-S-S-S	2.38	2.38	2.38	1.19	1.19	1.19	0	0	0
	S-F-S-F	1.56	1.56	1.56	1.56	1.56	1.56	1.37	1.37	1.37
	F-F-F-F	1.61	1.61	1.61	1.52	1.52	1.52	1.36	1.36	1.36
RU	S-S-S-S	0	0	0	0.06	0.03	0	0.09	0.05	0
	S-F-S-F	1.32	1.39	0	1.55	1.56	0	1.55	1.56	0
	F-F-F-F	1.27	1.11	0	1.45	1.42	0.57	1.53	1.58	0.86
RC	S-S-S-S	9.66	4.83	0	9.66	4.83	0	9.66	4.83	0
	S-F-S-F	1.9	1.96	0	1.9	1.96	0	1.9	1.96	0
	F-F-F-F	1.97	1.96	1.26	1.97	1.96	1.26	1.97	1.96	1.26
RD	S-S-S-S	0.11	0.05	0	0.05	0.03	0	0	0	0
	S-F-S-F	1.56	1.56	0	1.55	1.56	0	1.32	1.39	0
	F-F-F-F	1.57	1.65	0.99	1.42	1.38	0.49	1.27	1.11	0

Notes:
- please refer to Fig. 4(b) for positions (U: upper, C: central, D: lower, L: left, R: right)
- please refer to Fig. 3 for measured points

Table 2. The errors of compared with numerical results and analytical solutions of the top layer at various locations on S-F-S-F and F-F-F-F

location	B. C.	Error of deflection of measured point (%)								
		1	2	3	4	5	6	7	8	9
LU	S-F-S-F	--	0.48	0.57	--	1.04	2.65	--	3.83	0.51
	F-F-F-F	--	20	3	28	9.3	4.1	2.4	1.9	1.3
LC	S-F-S-F	--	1.58	2.37	--	5.08	2.81	--	1.73	2.37
	F-F-F-F	33.4	0.5	1.1	32.6	3.1	0.6	33.4	0.3	1.1
LD	S-F-S-F	--	3.8	0.53		4.07	6.15	--	1.55	0.57
	F-F-F-F	12.3	2.6	1.3	41	14.8	2.5	--	21.7	3
CU	S-F-S-F	4.28	0.13	4.28	5.51	0.95	1.95	1.29	5.14	1.29
	F-F-F-F	3.4	0.7	3.4	3.4	2.9	0.1	2	2	2
CC	S-F-S-F	0.74	2.5	1.74	0.3	4.62	0.3	0.74	2.63	1.74
	F-F-F-F	0.6	2.6	0.6	0.2	4.7	0.2	0.6	2.7	0.6
CD	S-F-S-F	1.31	5.13	1.31	1.97	4.22	5.53	4.28	2.24	4.28
	F-F-F-F	2	1.9	2	0.1	6.2	3.4	3.4	3.1	3.4
RU	S-F-S-F	0.6	2.5	--	2.7	2	--	0.5	2.4	--
	F-F-F-F	3	18.4	--	4.1	7	31.6	1.3	1.2	2.4
RC	S-F-S-F	2.4	0.5	--	2.8	1.7	--	2.4	0.7	--
	F-F-F-F	1.1	0.8	33.4	0.6	1.9	32.6	1.1	0.9	33.4
RD	S-F-S-F	0.5	2.4	--	6.1	1.1	--	0.6	0.3	--
	F-F-F-F	1.3	3.2	12.3	2.5	12.6	37.9	3	20.1	--

Notes:
- " -- "denotes the data of the fixed edge can neglected.
- please refer to Fig. 4(b) for positions (U: upper, C: central, D: lower, L: left, R: right)
- please refer to Fig. 3 for measured points

5 Discussion

From Figure 3 it is obvious to see only the first quadrant of MLCS needs be analyzed due to the geometric symmetry, and also six locations, such as LU, CU, RU, RC, RD and CC, are considered. After simulation the largest deformation of u_x occurs at locations RC and LC, and u_y at locations CU and CD similarly, since both largest u_x and u_y look the same after a rotation 90°, i.e., they are compressed by vertical pressure of 69*MPa* and subjected to lateral pressure simultaneously. However, the largest u_z occurs at location CC with both-side lateral pressure balanced. From Figure 4 it is easily found that the deformation, u_z, of electrode films decrease as the lateral pressure number increasing, i.e., more pressure cumulatively acting on the top layer. Also, the deformations become larger at the corners of electrode because of the well-known free-edge effect.

For practical applications, both vertical pressure (69*MPa*) and lateral pressure are applied simultaneously. All the deformations, u_x, u_y and u_z, are increasing from the bottom to the top, especially the maximal values occur at the top layer.

The trends of stress and deformation fields due to other pressures, i.e., 8000, 12000, 20000*psi*, are similar to those results as above mentioned for 69*MPa*, since our priory assumptions for both films are linear and elastic. Nevertheless, the pressure over 20000*psi* will crush the films, i.e., it is too high to fabricate MLCS.

The MLCS green sheets were divided into nine measure points per one region with suitably different boundary conditions. Compared with the numerical results and the analytical solutions of nine measure points were found satisfactorily acceptable. As shown in Table 2, the errors were about 0.1%- 6.2% for the boundary conditions of four edges free and four corners fixed. The errors about 0.13%- 6.15% were also acceptable for the boundary conditions of two opposite edges simple-supported and the others free. However, the analytical solutions did not agree with the numerical results for the case of all the boundary conditions simple-supported.

6 Conclusion

The deformation field in MLCS subjected to vertical and lateral pressures at high temperature were obtained by theoretical analysis and numerical simulation incorporated with FEM and ANSYS. The duration of high temperature tests were performed to assure no phase changes in both ceramic and electrode films. Also, nanoidentation tests were done to obtain the Young's modulus of both films as the input data for simulation. Finally, the concluding remarks can be summarized as follows:

- The material properties of both ceramic and metal powder films, as the input data, were obtained by nanoindentation.
- The assurance of no phase changes of both films was confirmed by high temperature tests in advance.
- The analytical prediction of deflection and stress fields was validated by the numerical simulation of FEM and software ANSYS with very small errors.

The achieved analytical methodology can be used to multi-layered plate instead of conventional numerical methods with commercial softwares for applications of tissue engineering scaffolds.

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Finite Element Simulations of the Contact Stress between Rotary Sinus Lift Kit and Sinus Membrane during Lifting Process

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Abstract: In this study, a three-dimensional elastic-plastic finite element model is used to simulate the lifting process of the sinus membrane using a rotary sinus lift kit. The effects of the edge radius of sinus lift kit and the feeding rate on the contact stress of sinus membrane are explored. Three different edge radii, i.e. 0.4, 0.6 and 1.0 mm, and three different feeding rates, 1, 3 and 5 mm/s, are discussed. The results show that a rotary sinus lift kit with a smaller edge radius has a lower contact stress distribution on the sinus membrane. The results also indicate that a higher feeding rate results in a larger plastic zone on the sinus membrane.
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Keywords: Sinus Lifting, Contact Stress, Feeding Rate, Finite Element Analysis

1. Introduction

When the height of alveolar bone is not enough, the sinus lift approach must be applied to add the bone height such that the success rate of dental implantation can be enhanced. To do this, in the past few years, the rotary sinus lift approach was developed to substitute the traditional osteotome technique. Sinus lift surgery can be done safely and accurately by using the rotary sinus lift approach. Two maxillary sinuses are respectively placed on upper sides of roots of molars in the form of cavities surrounded by bones. When a molar on maxilla is extracted, the insufficient bone mass of maxilla gives a difficulty for the placement of dental implants. The method of maxillary sinus lifting procedure for the placement of dental implants was firstly introduced by Tatum [1] in 1976. The procedure was intended to increase the height of alveolar bone in posterior maxilla. Traditionally, when the remaining height is less than 5mm, open window technique should be taken since longer time is required for the treatment. However, if the remaining height is no less than 5mm, the osteotome technique is used to increase the height of alveolar bone [2~5]. The investigation by Fugazzatto and Vlasssis [6] indicated that no differences were observed in implant survival rates as the osteotome or the open window technique was used. Due to less surgery trauma and less postoperative reaction, the osteotome technique has been used clinically in recent years [7]. The experimental study of John and Steen [8] indicated that the damage of the maxillary sinus membrane was the main complication of maxillary sinus lifting. Vernamonte et al. [9] showed that shaking during knocking process may lead to complications of brain or ear cavity damages and make the patient feel uncomfortable. At present, the rotary sinus lift approach has been developed to replace the traditional knocking approach so that sinus lifting can be carried out in a safer and more precise way [10~15]. Huang et al. [16] and Tu et al. [17] used a three-dimensional finite element model to simulate the bone temperature rise during drilling process.

This research aims to investigate the effects of the edge radius design of rotary sinus lift kit on the contact stress of the sinus membrane during sinus lifting. The contact stress distributions on the sinus membrane for various feeding rates are also explored. Accordingly, in this study, a three-dimensional finite element model is proposed to simulate the contact behavior between the sinus membrane and the sinus lift kit. The results can provide a reference for the design of rotary sinus lift kit.

2. Finite Element Model

The rotary lifting process of sinus membrane is explored in this study. The effect of feeding rate on the deformation of sinus membrane during rotary lifting process is also studied. A three-dimensional elastic-plastic finite element model is used to simulate the contact behavior between the sinus membrane and the sinus lift kit during the lifting process. Because the shape of sinus membrane is changed over time during the lifting process, dynamic simulations are performed using the commercial ABAQUS/Explict package. It is known that shape of sinus membrane is complicated. For simplicity, the sinus membrane is modeled as a thin disk with a diameter of 40 mm and a thickness of 0.5 mm [18]. The simplified model is established

according to the maximum width in a single tooth loss area in alveolar bone and the size of the standard of human alveolar bone. In this study, a CAS rotary sinus lift kit (Korea) is employed. The model of the CAS sinus lift kit is constructed by using the software Solidworks, as shown in Fig. 1. The CAS CAD model is later input to ABAQUS/CAE and assembled with sinus membrane model. In performing the simulations, the contact behavior between the sinus membrane and the sinus lift kit is modeled using surface to surface contact discretisation.

Figure 2 shows the configuration of the contact model. Finite element models of the sinus membrane and the sinus lift kit are shown in Fig. 3. The nodes on the circumference surface of the sinus membrane are fixed during the lifting process. In this model, the element types selected for the sinus membrane and the sinus lift kit are 8-node brick elements C3D8 and 4-node tetrahedron elements C3D4, respectively. The friction coefficient between lift kit and sinus membrane is set as 0.3. There are 6005 nodes and 4736 elements in sinus membrane, 1056 nodes and 4450 elements in sinus lift kit. The mechanical properties of the sinus membrane used in finite element simulations are listed in Table 1 [18~19]. The material of the lift kit is stainless steel SUS420, the same as those used in normal drills for oral implant [20].

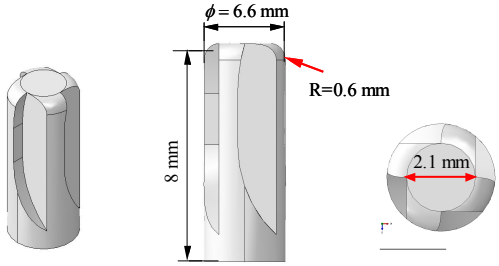

Fig. 1 Appearance of the CAS rotary sinus lift kit.

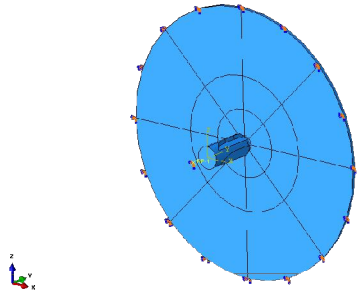

Fig. 2 The configuration of the contact model.

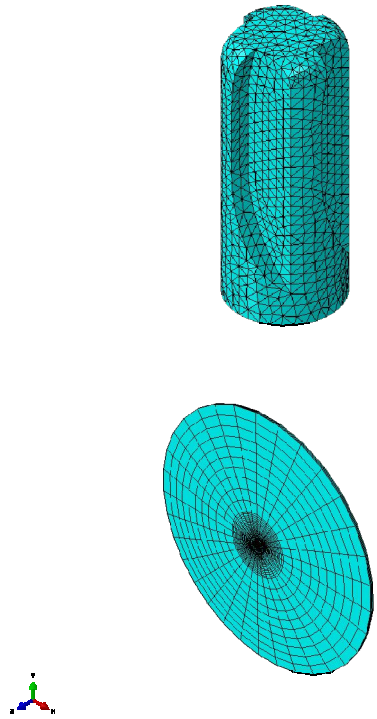

Fig. 3 The finite element models of sinus membrane and sinus lift kit

Table 1. Mechanical properties of sinus membrane and lift kit used in finite element model [18-20].

Properties of materials	sinus lift kit	Sinus membrane
Density (Kg/m^3)	7840	1000
Young's modulus(MPa)	210000	70.3
Yielding strength (MPa)	585	3.91
Tensile strength (MPa)	760	8.6
Poisson's ratio	0.24	0.45

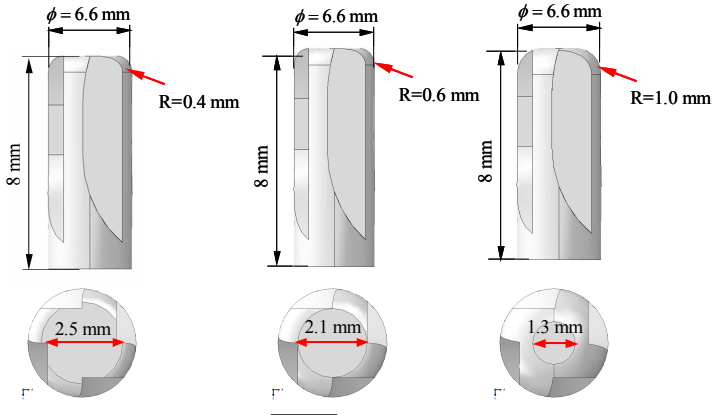

Fig. 4 Appearances of sinus lift kit with different edge radius designs

3. Results and Discussion

As shown in Fig. 1, the edge radius R of the original KIT lift kit is 0.6 mm. In this study, the edge radius is changed and its effect on the contact stress is explored. Three different radii R and three different feeding rates are investigated.

3.1 Lift kit with different radius designs

The sinus lift kits with different radii R, i.e. 0.4, 0.6 and 1.0 mm, are shown in Fig. 4. The rotation speed of each lift kit is fixed as 800 rpm in simulations and the total lifting time is 1 second. Figure 5 shows the von-Mises stress distributions on the sinus membrane for sinus lift kit with different radius designs at lifting displacement 1 mm. The maximum von-Mises occurs on the membrane are 0.58, 0.6 and 0.75 MPa for the lift kit with radius of R=0.4, 0.6 and 1.0 mm, respectively. The results indicate that the maximum von-Mises increases with increasing corner radius. This can be attributed to the variation of contact area between the siuns membrane and the lift kit. It can be seen from Fig. 4 the contact area decreases as the edge radius increases. A smaller contact area results in a larger contact stress. All the maximum von-Mises stresses stated above are less than the yield strength 3.91 MPa of the sinus membrane. It means that the sinus membrane can sustain 1 mm lifting displacement for the lift approach with all the three different radius designs and no structure damage will occur.

The contact von-Mises stress distributions on the sinus membrane for a lifting displacement of 3 mm is shown in Fig. 6. The maximum von-Mises occurs on the membrane are 2.9, 3.25 and 4.2 MPa for the lift kit with a radius of R=0.4, 0.6 and 1.0 mm, respectively. Compared with Fig. 5, it can be observed that contact stresses increase obviously as the lifting displacement increases from 1 mm to 3 mm. In the case of lifting displacement 3 mm, the membrane stress caused by the lift kit with a radius of R=1.0 mm exceeds the yielding strength of the sinus membrane. Figure 7 presents the contact von-Mises stress distribution on the sinus membrane for a lifting displacement of 5 mm. In Fig. 7, the maximum von-Mises occurs on the membrane are 3.4, 4.3 and 4.9 MPa for the lift kit with radius of R=0.4, 0.6 and 1.0 mm, respectively. With this lifting displacement, all the maximum stress exceeds the yield strength of sinus membrane but is still less than its tensile strength. It should be noted that a stress larger than the yield stress will result in plastic deformation in sinus membrane even though the load is released after surgery. From the results shown above, it can be concluded that the sinus lift approach with a smaller edge radius, in this study R=0.4 mm, has a lower contact stress distribution on the sinus membrane than those with other radius designs.

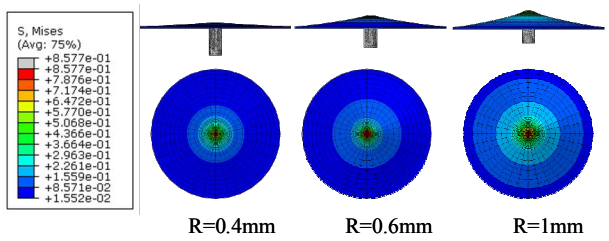

Fig. 5 The von-Mises stress distributions on the sinus membrane for lifting displacement 1 mm

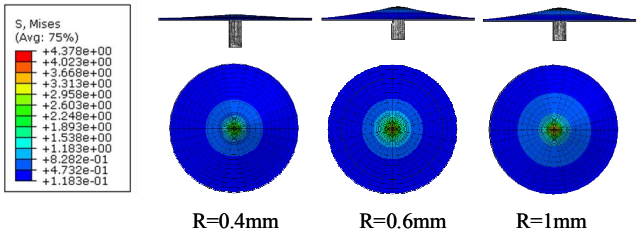

Fig. 6 The von-Mises stress distributions on the sinus membrane for lifting displacement 3 mm

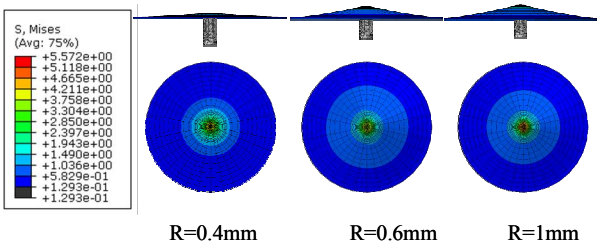

Fig. 7 The von-Mises stress distributions on the sinus membrane for lifting displacement 5 mm

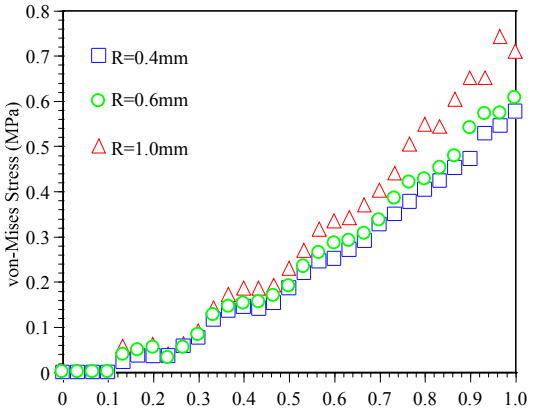

Fig. 8 Variations in von-Mises stress with lifting time at lifting displacement 1 mm for various corner radii

Figures 8~10 plot the variations of the von-Mises stress with the lifting time for different corner radii and lifting displacements. In these figures, the stresses are taken at the center point of the sinus membrane. The results of Figs. 8~10 confirm that the von-Mises stress accumulated in the membrane increases as the lifting time increases.

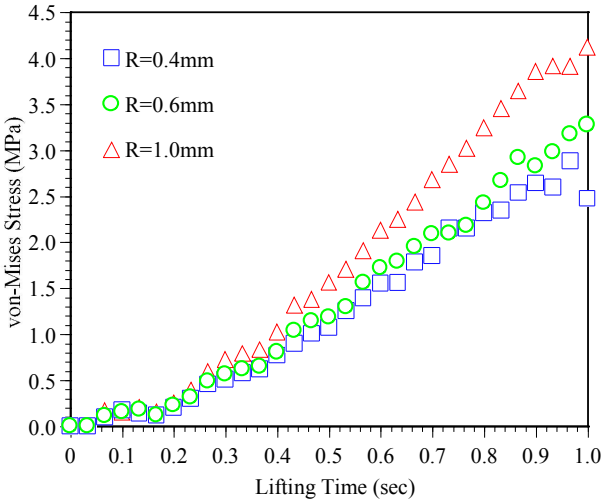

Fig. 9 Variations in von-Mises stress with lifting time at lifting displacement 3 mm for various corner radii

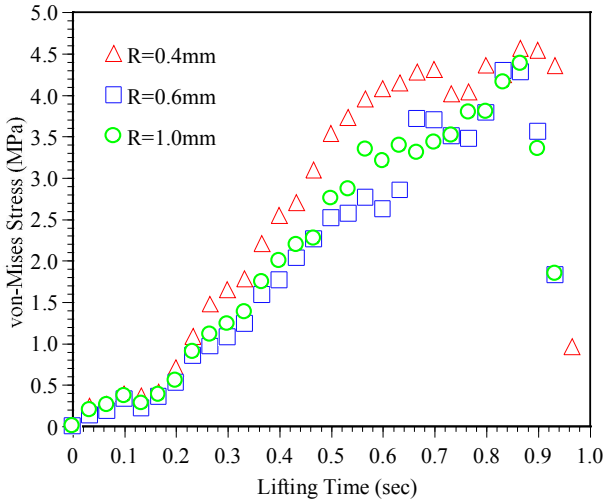

Fig. 10 Variations in von-Mises stress with lifting time at lifting displacement 5 mm for various corner radii

3.2 Lift approach with different feeding rate

In the following discussions, a sinus lift kit with an edge radius of R=0.6 mm is used. The rotation speed of the lift kit is fixed as 800 rpm in simulations and the total lifting time is 1 second. The forces applied to the sinus membrane are explored during the lifting process. Figure 11 shows the variation in applied force with lifting time for various feeding rates. Three different feeding rates, i.e. 1, 3, 5 mm/s, are discussed. From the simulation results, it can be observed that the feeding rate has a significant influence on the applied force. A larger feeding rate results in a larger applied force on the siuns membrane. This can induce a larger contact stress on the sinus membrane. Figure 12 presents the von-Mises stress distributions on the sinus membrane for various feeding rates. In order to understand the effect of feeding rate on the plastic deformation region in sinus membrane, the stress exceeds yielding strength 3.91 MPa is presented in gray color, as shown in Fig. 12. It is easy to see that the plastic zone size increases with increasing feeding rate. The plastic zone size can be obtained as 0, 0 and 3.04 mm for feeding rate of 1, 3 and 5 mm/s, respectively.

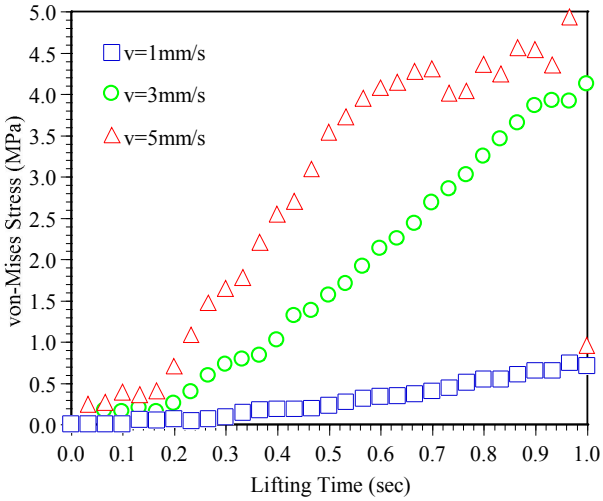

Fig. 11 Variation in applied force with lifting time as function of feeding rate

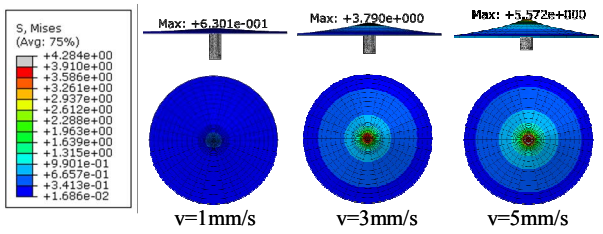

Fig. 12 The von-Mises stress distributions on the sinus membrane for various feeding rate

4. Conclusion

This study proposes a three-dimensional elastic-plastic finite element model to simulate the contact behavior between the KIT rotary sinus lift kit and the sinus membrane during lifting process. The effects of the edge radius of the sinus lift kit and the feeding rate on the contact stress of sinus membrane are explored. Based on the numerical results, the following conclusions can be drawn:

1. A rotary sinus lift kit with a smaller edge radius design can has a lower contact stress distribution on the sinus membrane.
2. A higher feeding rate results in a larger plastic zone on the sinus membrane.
3. This result can provide a reference for manufacturers or relevant organizations in developing more advanced rotary lift kits.

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Young's Moduli of Human Tooth Measured using Micro-Indentation Tests

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Abstract: Micro-indentation test results were used to curve fit the Young's moduli of human tooth enamel and dentine in this work. The applied load and unloaded curve portion effects on the measured Young's moduli were investigated. The variation in measured Young's modulus for different applied loads, from 10 *mN* to 500 *mN* , was studied. The experimental results indicate that the measured Young's moduli are very sensitive to the applied load if the load is greater than 100 *mN*. The measured results also reveal that the measured Young's moduli were dependent upon the unloaded curve data portion to be curve fitted. The large portion of the unloaded curve data could be used, i.e. 90% of unloaded curve data, to curve fit a smaller Young's modulus value.
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Keywords: Human tooth; Young's modulus; Micro-indentation test

1. Introduction

The mechanical properties of materials, i.e. Young's modulus, hardness, are very important for biomechanical engineering research. The knowledge of tooth mechanical properties plays a key role in predicting the mechanical behavior and helps clinicians to understand the tooth stress distribution under different bite loads. However, because of the small size of human teeth, it is difficult to measure tooth material properties using traditional methods. In the last decade, micro-depth sensing indentation tests have been used to obtain the Young's modulus of teeth[1-9]. It is assumed that the variation in the load and unloading curve in the micro-scale indentation test is dominated by the material properties[10, 11]. Linear elastic behavior occurs particularly during the unloading period. The portion of the unloaded part of the load-indentation depth curve data is used to derive the Young's modulus of the tested specimen. The slope at the initial unloading point in load-indentation depth curve is derived to approximate the Young's modulus of the tested specimen[11, 12]. However, different portions of the unloaded curve may affect the derived slope in this method. Finding a proper portion of the unloaded curve to derive a reliable Young's modulus in the micro-indentation test has always been discussed. Besides this problem, the applied load and the loading position may also have significant effects on the measured data. As noted, based on the hardness and density, the tooth structure is considered as two distinct portions, enamel and dentine. In these two parts, the tooth structures are porous and inhomogeneous. This porous structure may introduce significant variation in the measured load and penetration depth data. In other words, a wide variation in the measured Young's modulus values is expected when using this method.

This work examines the maximum applied load and load position effects on the variation in measured Young's moduli in the enamel and dentine portions of human teeth.

2. Load-indentation depth curves

The micro-indentation test technique has been widely used to measure the surface properties for a long time. The penetration deformation and its spring back behaviors during the loading and unloading processes in the indentation test have been used to extract the corresponding material elastic and plastic properties. The material behavior at initial unload is considered as an elastic recovery in essence. The load-indentation depth curve slope at the initial unloading can be derived the Young's modulus of the tested specimen[11]. Referring to the load-indentation depth curve, as shown in Figure 1, a stiffness at the initial unload can be expressed as,

$$S = \left.\frac{dP}{dh}\right|_{h=h_{\max}} \qquad (1)$$

where S, P, and h denote stiffness, load, and indentation depth, respectively.

Based upon Sneddon's analysis[13], the load (P) -indentation depth (h) relationship for a rigid cylinder indenter can be approximated as,

$$P = \left(\frac{4GR}{1-\nu}\right)h \qquad (2)$$

where R, G, and ν are the cylinder radius, shear

modulus and Poisson's ratio, respectively. The circular contact area is πR^2, and elastic modulus E can be in terms of the shear modulus and Poisson's ratio as $E=2G(1+\nu)$, differentiating P with respect to h, it leads to

$$S = \frac{dP}{dh} = \frac{2}{\sqrt{\pi}} \frac{E}{(1-\nu^2)} \sqrt{A} \qquad (3)$$

In a real case the indenter cannot be perfectly rigid. The deformation of the indenter therefore contributes to the measured displacement. It is convenient to define a reduced modulus E_r[14],

$$\frac{1}{E_r} = \frac{(1-\nu^2)}{E} + \frac{(1-\nu_i^2)}{E_i} \qquad (4)$$

where E and ν are the tested specimen's elastic modulus and Poisson's ratio, respectively, E_i and ν_i are the indenter's elastic modulus and Poisson's ratio, respectively. Equation (3) can be rewritten as

$$S = \frac{dP}{dh} = \frac{2}{\sqrt{\pi}} \sqrt{A} E_r \qquad (5)$$

Therefore, providing a reasonable estimate of the Poisson's ratio and contact area, the specimen's modulus can be computed from the initial unloading slope.

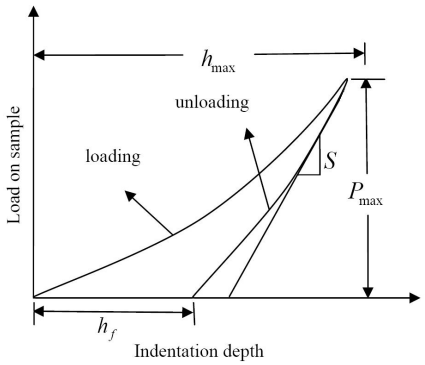


Figure 1. Load-indentation depth curve of the micro-indentation test.

3. Micro-indentation tests

3.1 System of micro-indentation tests

A Nano Indenter XP, produced by the MTS System Corporation, was used in this work. The fundamental characteristics of the system are listed as below,

1. Resolution of indenter displacement: 0.1 nm
2. Range of indentation depth: 25 nm ~ 500 μm
3. Maximum applied load: 0.5 N
4. Resolution of load: less than 500 mN
5. Linear loading controlling system

3.2 Specimen preparation

A human molar tooth aged 60 years old was chosen to make the specimen. The specimen was embedded with a cold-curing epoxy resin and its interior sectional surface was chosen to perform the indentation tests. The specimen's surface, as shown in Figure 2, was hand-ground polished using wet silicon carbide papers of #80, #800, #1200, and #2500 grit sizes progressively. Fine polishing was achieved on a rotary polishing machine using 0.1 μm-size aluminum oxide suspensions. The specimen was stored in dry conditions at room temperature before testing.

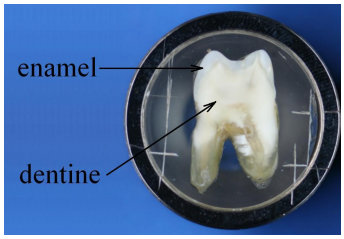


Figure 2. Specimen for micro-indentation tests.

3.3 Experiment setup

The micro-indentation tests in this study were performed using a calibrated Berkovich indenter. To investigate the applied load effect on the variation in molar tooth Young's modulus measured using the micro-indentation tests, six groups of indentation tests were carried out with maximum applied loads of 10, 30, 50, 100, 200, and 500 *mN*, respectively. A total of 12 groups were studied for measuring both the enamel and dentine moduli. In order to observe the variation in measured data, an 8 x 8 indent matrix, as shown in Figure 3, was measured for each test group. The loading, holding and unloading time periods for each indention test were controlled at 15, 30, and 15 seconds, respectively. The allowable drift rate was less than 0.5 nm/s.

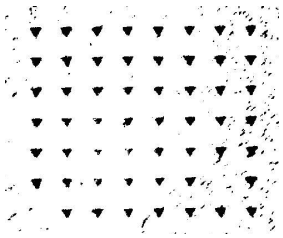


Figure 3. Residual indents of one group micro-indentation tests.

To study the possible adopted portion of the unloaded curve effect on the measured Young's modulus, different percentages of unloaded data of the load-indentation depth curve were used to derive the slope. Five groups of test data, i.e. 20%, 30%, 50%, 70%, and 90% of the unloaded data, were chosen to generate the corresponding Young's moduli.

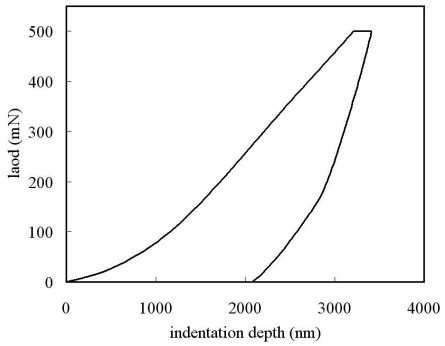


Figure 4. The measured load-indentation depth cycle on enamel part.

4. Results and discussions

Figure 4 shows the variation in load and indentation depth on the enamel portion in a loading cycle. As mentioned in the previous section, due to the porosity of teeth, some measured data are unreasonable. Eliminating these unavailable data, there are more than 50 acceptable results in the 8 x 8 test data collected. These available load-indentation depth curves were used to derive the corresponding Young's modulus values. Figures 5 and 6 show the variation in the obtained moduli with different maximum loads for the enamel and dentine parts of the tested tooth. The modulus values were derived using the top 50% unloaded curve data. Some data were distributed unlike a Gaussian distribution. The key reason for this wide spread distribution may come from the inhomogeneous and porous tooth structures. In the micro-indentation test, the indent size was just a few micro-meters. It is possibly affected by the local porosity, leading to a non-Gaussian distribution data.

The average value and the corresponding standard deviation of the derived Young's modulus are listed in Table 1. Some distribution cases show no obvious central peak at the average modulus, however, the standard deviation is small and most deviation to average modulus ratios listed in Table 1 are less than 10%. Nevertheless, the results in this study present extracting enamel and dentine Young's modulus using micro-indentation tests can still obtain quite narrow-band distributed data.

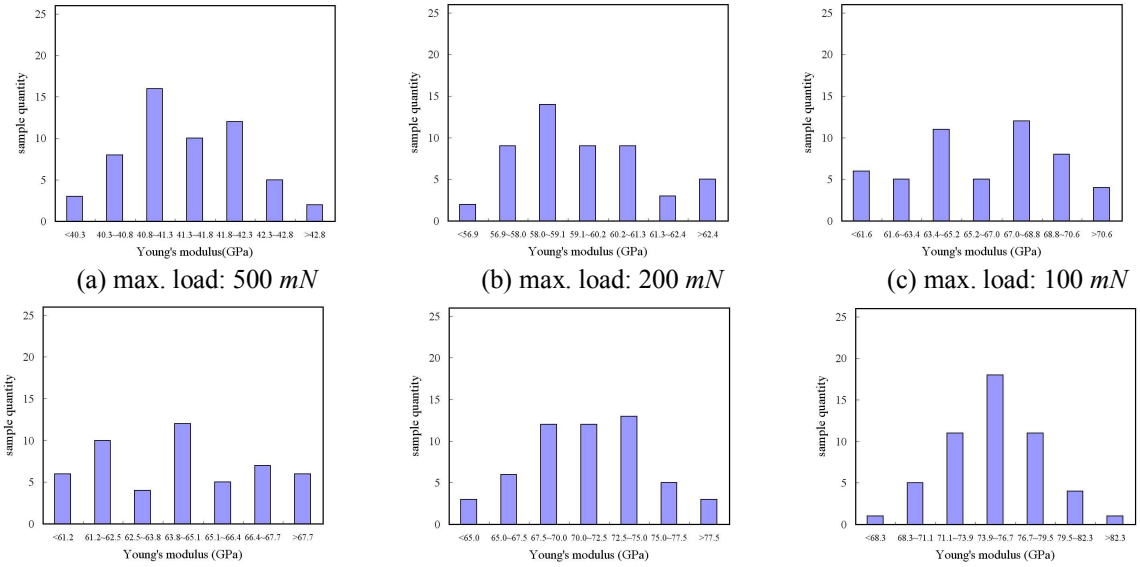
Figure 7 presents the enamel and dentine average moduli which vary with different percentages i.e. 20%, 30%, 50%, 70%, and 90% of the adopted unloaded data. The results reveal that the derived modulus decreases with the increase in adopted unloaded curve data in most cases. The largest modulus value is derived with 20% of the adopted unloaded data. In general, the percentage of adopted unloaded data is not sensitive to the derived results. Similar results were observed for the dentine cases. A greater applied load may derive a smaller Young's modulus value. The ratio of the modulus at 20% to the one at 90% is between 103.8% ~ 106.2% for the dentine cases. Based on the measured results, the unloaded data in the 20% to 50% range from the initial unloaded data is suggested for deriving the Young's moduli values.

The variation in average Young's modulus with applied load is shown in Figure 8. For dentine, the mean value of the derived Young's modulus decreases from 20.7 GPa to 16.2 GPa as the applied load is increased from 30 mN to 500 mN. A difference of 21% is introduced. Similarly, the mean value of the derived Young's modulus for enamel decreases from 75.3 GPa to 41.5 GPa as the applied load is increased from 10 mN to 500 mN. The difference is extended to 45% in this case. The measured results reveal that the derived Young's modulus is very sensitive to the applied load in the micro-indentation test of tooth specimen.

A comparison between the published values for enamel and dentine Young's moduli[2, 3, 5-7, 15] and the results derived in this study is listed in Table 2. Figure 9 shows the variation in published moduli and results derived in this work. All of the results indicate that the derived Young' modulus values relate significantly to the applied load. To eliminate the local porosity effect, a lower applied load 10 *mN* is suggested to measure the tooth Young's modulus using the micro-indentation method. The derived mean values for enamel and dentine are then 75.3 and 19.6 *GPa*, respectively.

Table 1. The average moduli and its standard deviation for the enamel and dentine portions.

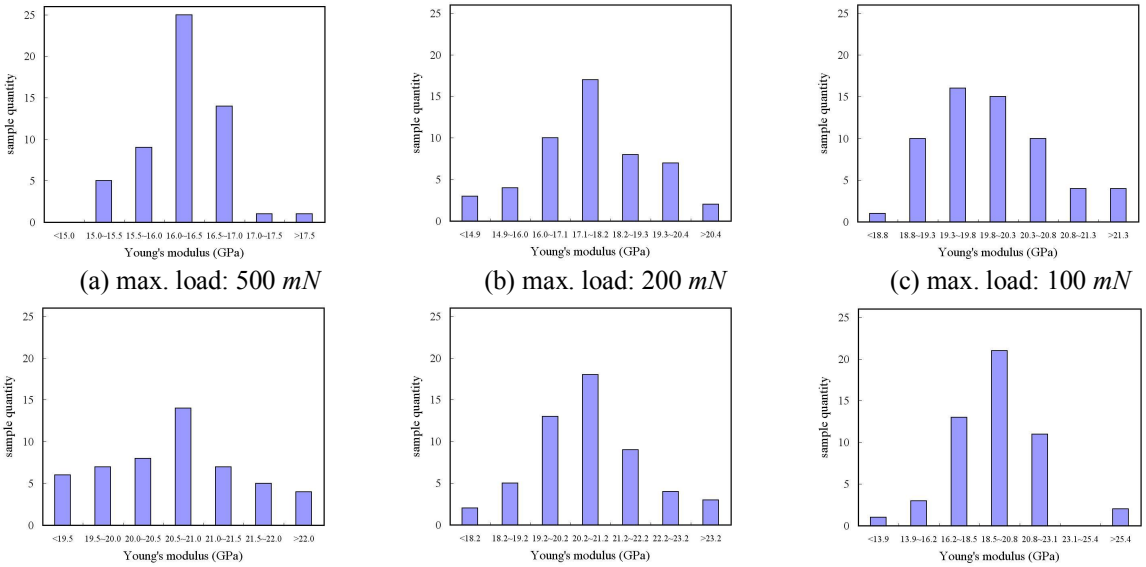
max. load (*mN*)	enamel			dentine		
	Young's moulus E (*GPa*)	standard deviation D (*GPa*)	ratio D/E	Young's moulus E (*GPa*)	standard deviation D (*GPa*)	ratio D/E
10	75.3	3.6	4.8%	19.6	2.7	13.8%
30	71.2	3.9	5.4%	20.7	1.4	6.6%
50	64.3	2.7	4.1%	20.7	0.9	4.4%
100	65.4	3.3	5.0%	20.0	0.7	3.6%
200	59.6	1.8	3.0%	17.6	1.6	9.3%
500	41.5	0.8	1.8%	16.2	0.5	3.1%



(a) max. load: 500 *mN* (b) max. load: 200 *mN* (c) max. load: 100 *mN*

(d) max. load: 50 *mN* (e) max. load: 30 *mN* (f) max. load: 10 *mN*

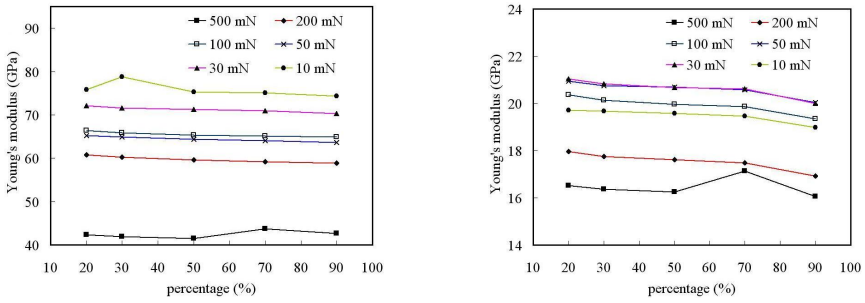
Figure 5. The variation in Young's moduli in the tooth enamel portion with different loads.



(a) max. load: 500 *mN* (b) max. load: 200 *mN* (c) max. load: 100 *mN*

(d) max. load: 50 *mN* (e) max. load: 30 *mN* (f) max. load: 10 *mN*

Figure 6. The variation in Young's moduli in the tooth dentine portion with different loads.



(a) enamel (b) dentine

Figure 7. The derived Young's modulus values with different percentages adopted using unloaded curve data.

Table 2. Literature values for enamel and dentine moduli compared with the results in this work.

enamel			dentine		
	applied load (*mN*)	Young's modulus (*GPa*)		applied load (*mN*)	Young's modulus (*GPa*)
Mahoney *et al.* [2]	50	74.5 ~ 99.9	Mahoney *et al.* [2]	50	18.2 ~ 24.0
Mahoney *et al.* [2]	150	73.3 ~ 95.2	Mahoney *et al.* [2]	150	16.4 ~ 22.5
Marshall *et al.* [3]	30	62.1 ~ 65.0	Marshall *et al.* [3]	30	18.6 ~ 20.7
Balloch *et al.* [5]	0.0025	60.0 ~ 70.0	Balloch *et al.* [5]	0.0025	17.0 ~ 23.0
He *et al.* [15]	10	115*	Hosoya *et al.* [6]	10	21.0 ~ 27.7
He *et al.* [15]	250	95*	Hosoya *et al.* [7]	3	22.7 ~ 29.2
This work (50 indents)	10	64.9 ~ 84.5	This work (50 indents)	10	13.3 ~ 29.3
This work (50 indents)	500	39.5 ~ 43.3	This work (50 indents)	500	15.1 ~ 18.3

* Literature providing the mean values only.

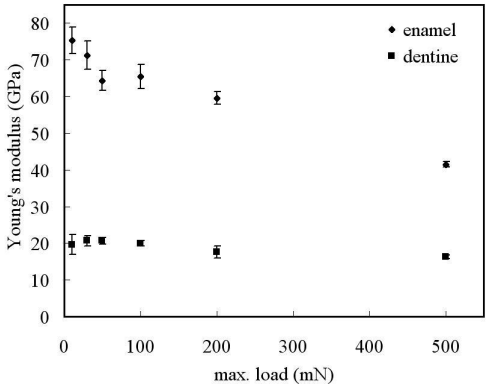
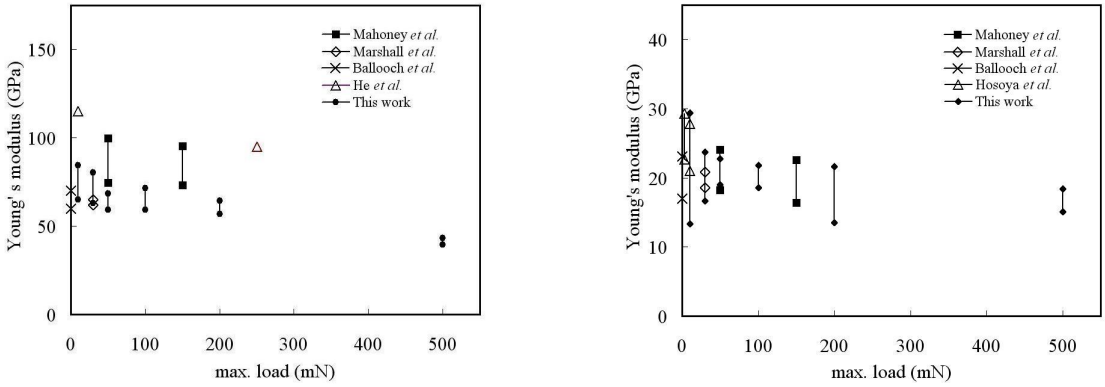


Figure 8. The variation in derived Young's modulus for a tooth with different micro-indentation test applied loads.



(a) enamel (b) dentine

Figure 9. The variation in published Young's modulus with the applied loads.

5. Conclusions

This study presented the adopted unloaded data percentage and applied load effects on the derived Young's modulus values for a studied tooth. The results reveal that the percentage of adopted unloaded data is not sensitive to the derived Young's modulus for the tooth. The unloaded data in the 20% to 50% range from the initial unloaded data is suggested for deriving the Young's moduli values. However, the measured results also indicated that the derived Young's modulus is very sensitive to the applied load in the micro-indentation test. To eliminate the local porosity effect a lower applied load, 10 *mN*, is suggested. Because of the local porosity and inhomogeneous tooth structure, a wide variation in measured Young's moduli was found for enamel and dentine in this study. For safety considerations, a conservative estimate of the Young's modulus value in tooth mechanics analysis is suggested.

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Fatigue Response of Ti/APC-2 Nanocomposite Laminate Prosthesis

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Abstract: The post mechanical behavior of Ti/APC-2 hybrid nanocomposite laminate prosthesis after low velocity impact was investigated. The three layered Ti/APC-2/Ti cross-ply nanocomposite laminates were fabricated according to the modified the diaphragm curing process based on the previous experience. Each sample, L×W×t=240mm×25mm×1.55mm, was first subjected to free drop of a rigid steel ball (diameter=12.7mm) of 1m and 2m high. Then, the samples were due to static tensile tests at room temperature to measure their residual ultimate strength and longitudinal stiffness as the base-line data for constant stress amplitude tension-tension cyclic tests. The corresponding S-N curve, fatigue strength and life were obtained. Also, the mechanism of damage by impact and failure of separation were observed. The mechanical properties do not reduce significantly due to low-velocity impact, even if the damage area is obviously large for 2m high free drop. Similarly, the fatigue resistance of impacted samples does not lose much. It is mainly attributed to the damage only occurs on the surface of impacted samples with little influence inside the laminates by low-velocity impact. It can be concluded that the enhancement by nanoparticles and superior bonding capability of matrix PEEK with Ti sheets take the responsibility of improvement of mechanical responses.

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Keywords: Ti alloy; Gr/PEEK(APC-2); Nano; Composite; Impact; Tension; Fatigue

1. Introduction

Osteo-arthritis affects joints between bones due to the growth of small lumps of bone on the contacting surfaces of the joints. This prevents the joints from sliding in the usual manner, causing excruciating pain during movement[1]. Replacing a natural joint with an artificial joint offers good potential to alleviate the problem. In hip joint replacement operations, the head of the femur is sectioned-off, and the soft marrow is removed to create a hollow intra-medullar cavity through the centre of the femur shaft. An artificial implant (mainly comprising of a long stem and a head) is then glued into the femoral cavity. The implant head fits into the acetabular socket of the hip bone. The critical mechanical property requirements of the implant material include (but are not limited to) high specific bending stiffness, high bending stiffness comparable to that of the surrounding cortical bone, biocompatibility, corrosion resistance and high endurance limit. It is noteworthy that the loads on a typical joint fluctuate approximately a million times a year[2].

Titanium (Ti) and its alloys have been utilized in medical practice, for they have high-strength/density ratios, excellent corrosion resistance to biological environments, and strong adhesion to bone tissue[3].

A lower modulus implant material would result in the construction of a more biomechanically compatible prosthesis. In this respect, carbon and aramid fiber reinforced polymer matrix (such as polyether ether ketone – PEEK) composite materials are gaining importance, because they offer the potential for implants with tailor-made stiffness in contrast to metals. They possess superior biomechanical properties, such as better fatigue strength, chemical resistance, environmental stability, and resistance to sterilization by c-radiation and biocompatibility[4-6].

Owing to the brittleness of thermosetting matrix resulted by cross-linking, the epoxy resins and epoxy-based fiber composites are susceptible to impact damage. Thermoplastics, having greater toughness, are considered to have the potential for alleviating this problem[7].

For example, during the last ten years, the Defence Research Establishment (DREV) has been studying the application of composite materials for generic anti-tank recoilless gun[8]. To achieve the desired weight reduction, a graphite-epoxy composite material was used to manufacture the gun tube and venturi. Although the ballistic viability of such a design was well proven during tests and evaluations, one major concern that remains is sensitivity of the thermoset resin (epoxy) to the low-level damage accumulation due to a mixture of normal loading and rough handling in the field. Such damage is generally

barely visible, and it is therefore important to develop an adequate NDT inspection technique to permit testing as close as possible to an operational environment.

Although damage inflicted by low-velocity impact appears quite complicated, the major failure modes include only matrix cracking, delamination, and fiber breakage[9]. As pointed out by Sun and Rechak[10], the delamination mode of failure is induced by matrix cracks which occur prior to other failure modes. Thus, suppression of matrix cracking will suppress delamination. It is conceivable that the use of tougher matrices will yield composites that are more resistant to impact damage.

Except for the degree of damage, the plate specimens did not differ from beam specimens in failure modes or impact tolerance properties[11], i.e., no plate size effect. The postimpact load-carrying capability of a composite laminate is of prime concern to the design engineer. After a tool-drop type accident where no damage is visible from the surface, the structure is still expected to carry the full spectrum of loading. However, it may be wrong of overestimation. In all cases the residual strength decreased as the impact velocity increased. From the results[10] the tough matrix composites may provide excellent impact resistance properties at low-impact velocities. However, beyond a certain threshold velocity, the use of tough matrix materials may result in more laminate tensile and flexural strength reduction than that of brittle matrix materials.

Additionally, the PEEK composites have significantly lower contact rigidity, i.e., for a given contact force the resulting indentation in the PEEK composites would be larger, yielding a larger contact area, and, therefore, a low contact pressure. A larger contact area with lower pressure will reduce the transverse shear stress concentration and thus minimize local matrix cracking.

From the above-mentioned it is clear that little reduction in strength in the tough PEEK composite laminates prior to the low velocity, i.e., $\leq$ 25m/s. Beyond this point the matrix damage occurred and substantial fiber breakage followed. That explains the sudden drop of strength in PEEK laminates when impacted at velocities higher than 25m/s.

Based on the knowledge of merits and disadvantages of PEEK composites within the scope of low velocity we fabricated the Ti/APC-2 hybrid nanocomposite laminates to investigate their resistance and mechanical properties by tensile and fatigue tests after free drop of a steel ball at 1m and 2m high. Nejad[12] investigated the stresses in the sphere rests on a rigid plane horizontal surface. The related surface treatment and adding nanoparticles are stated as follows.

Anodic method is a commonly used surface treatment, however, the bonding capability of polymer composites to titanium thin plates is still a problem. In order to improve the interfacial bonding capability, Ramani et al.[13] found the chromic acid anodic method was excellent. Chromic acid anodic oxidation produced an oxide layer of thickness 40~80 nm for the 5 V and 10V treatments[14]. Ibrahim et. al.[15] investigated the influence of resin-tags on shear-bond strength of butanol-based adhesives.

In recent years, inorganic nanoparticles filled polymer composites have attracted attention because the filler/matrix interface in these composites might constitute a great area and influence the properties of composites at rather low filler concentration[16]. Herein, our concern is focused on an engineering application, i.e., dispersing SiO_2 nanoparticles 1wt% optimally on the interfaces of APC-2 composite laminates to improve the mechanical properties of samples due to static and cyclic loadings as an extension of previous work[17].

From the bonding of Ti with APC-2 by the modified diaphragm curing process, Ti/APC-2 hybrid nanocomposite laminates were successfully fabricated. The mechanical properties of samples subjected to tensile and cyclic tests at room temperature after low velocity impact were obtained. The failure mechanisms were observed.

2. Experimental

The twelve-inch wide prepregs of Carbon/PEEK (Cytec Industries Inc., USA) unidirectional plies were cut and stacked into cross-ply $[0/90]_s$ laminates. The nanoparticles SiO_2 (Nanostructured & Amorphous Materials, Inc. USA) possessed the average diameter 15±5 nm, specific surface area 160±20 m^2/g, spherical crystallographic and amorphous powder. The optimal amount of SiO_2 was found 1% by wt. of laminates. The grade 1 (H: 0.015%, O: 0.18%, N: 0.03%, Fe: 0.2%, C: 0.08%) Ti sheets, supplied by Kobe Steel Ltd (Japan), were 0.5mm thick after rolled, heated and flattened with scratch brushing. The ultimate tensile strength of Ti is 353MPa, and modulus of elasticity 109GPa.

Prior to lamination, the slimmed Ti alloy sheets were subjected to pretreatment in order to create the tough bonding with APC-2 prepregs. After a series of tests, the surface treatment by chromic acid anodic method of electro-plating was found better as demonstrated by the results of tensile tests. After anodic processing, the thickness of oxide coating film was about 40~80nm. The anodic oxide coating was observed uniform by SEM, and the composition of coating consisting of TiO_2 by EDS.

The APC-2 prepregs were sandwiched with the

Ti alloy sheets to produce Ti/APC-2 hybrid three-layered laminated composites. The modified diaphragm curing process was adopted[9]. The hybrid composite specimen was a plate of 240mm(L)×25mm(W)×1.55mm(t) as shown in Figure 1.

The samples were divided into three groups, such as virgin, due to 1m high free drop, and 2m high free drop. The steel ball was 12.7mm in diameter and 8.3g of weight. The velocities at the contact of sample surface was 4.41m/s for 1m high, and 6.28m/s for 2m high, respectively.

An MTS-810 servohydraulic computer-controlled dynamic material testing machine was used to conduct the tensile test and constant stress amplitude T-T cyclic test with stress ratio=0.1, frequency=5HZ, sinusoidal wave form under load-controlled mode at room temperature after the free drop of a steel ball.

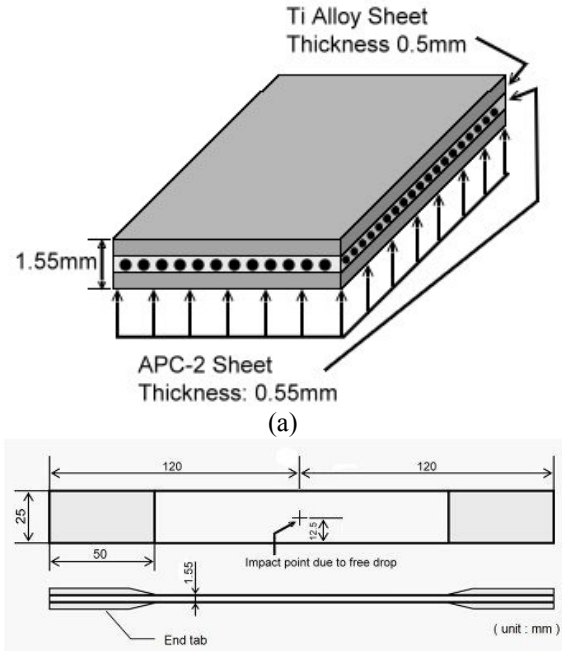

Figure 1. The (a) geometry, dimensions and supported conditions of Ti/APC-2 cross-ply nanocomposite laminate, (b) due to impact of free drop by steel ball.

3. Results

The mechanical properties of Ti/APC-2 cross-ply laminates were listed in Table 1(a) for virgin samples, (b) for samples due to 1m high free drop, and (c) for samples due to 2m high free drop. Also, the mechanical properties of Ti/APC-2 cross-ply nanocomposite laminates were in Table 2(a) for virgin specimens, (b) for specimens after a free drop at 1m high, and (c) for specimens after a free drop at 2m high, respectively. The stress-strain curves of Ti/APC-2 nanocomposite laminates after a 1m high free drop impact was shown in Figure 2. for example. It should be paid attention that there is a kink angle, i.e., knee, in stress-strain curves of hybrid laminates, such as Al/APC-2 and Ti/APC-2 W/WO nanoparticles.

Table 1 The mechanical properties of Ti/APC-2 cross-ply laminates (a) virgin samples, (b) samples after a free drop impact at 1m high, and (c) samples after a free drop impact at 2m high.

(a)

Sample No.	Ultimate Load (KN)	Ultimate Strength (MPa)	Maximum Strain	Longitudinal Stiffness	
				Initial tangent E_{11i} (GPa)	Secant E_{11s} (GPa)
1	24.71	637.68	0.0170	102.80	30.38
2	24.05	620.65	0.0171	101.56	29.23
3	25.21	650.58	0.0175	101.24	29.39
Average	24.66±0.58	636.3±15.01	0.017±0	101.86±0.82	29.66±0.63
Notes:	E_{11i}(Tangent Modulus)：0.0005≤ε≤0.00165 E_{11s}(Secant Modulus)：0.007≤ε≤0.013				

(b)

Sample No.	Ultimate Load (KN)	Ultimate Strength (MPa)	Maximum Strain	Longitudinal Stiffness	
				Initial tangent E_{11i} (GPa)	Secant E_{11s} (GPa)
1	22.57	582.45	0.0171	101.81	26.83
2	23.24	599.74	0.0178	94.70	26.98
3	23.34	602.32	0.0182	93.98	25.42
Average	23.05±0.42	594.84±10.8	0.018±0.001	96.83±4.33	26.41±0.86
Notes:	E_{11i}(Tangent Modulus)：0.0005≤ε≤0.00165 E_{11s}(Secant Modulus)：0.007≤ε≤0.013				

(c)

Sample No..	Ultimate Load (KN)	Ultimate Strength (MPa)	Maximum Strain	Longitudinal Stiffness	
				Initial tangent E_{11i} (GPa)	Secant E_{11s} (GPa)
1	22.84	589.42	0.0153	105.03	30.06
2	22.38	577.55	0.0151	103.64	29.73
3	23.06	595.10	0.0159	105.37	29.56
Average	22.76±0.35	587.36±8.96	0.015±0	104.68±0.92	29.78±0.26
Notes:	E_{11i}(Tangent Modulus)：0.0005≤ε≤0.00165 E_{11s}(Secant Modulus)：0.007≤ε≤0.013				

Table 2 The mechanical properties of Ti/APC-2 cross-ply nanocomposite laminates (a) virgin samples, (b) samples after a free drop impact at 1m high, and (c) samples after a free drop impact at 2m high.

(a)

Sample No.	Ultimate Load (KN)	Ultimate Strength (MPa)	Maximum Strain	Longitudinal Stiffness	
				Initial tangent E_{11i} (GPa)	Secant E_{11s} (GPa)
1	24.96	644.13	0.0160	95.28	31.89
2	25.37	654.71	0.0174	98.95	29.50
3	25.68	662.71	0.0175	99.39	30.60
Average	25.34±0.36	653.85±9.32	0.017±0.001	97.87±2.26	30.66±1.2
Notes:	E_{11i}(Tangent Modulus)：0.0005≤ε≤0.00165 E_{11s}(Secant Modulus)：0.007≤ε≤0.013				

(b)

Sample No.	Ultimate Load (KN)	Ultimate Strength (MPa)	Maximum Strain	Longitudinal Stiffness	
				Initial tangent E_{11i} (GPa)	Secant E_{11s} (GPa)
1	26.26	674.98	0.0185	94.39	30.30
2	25.52	655.96	0.0187	94.14	28.25
3	25.41	655.74	0.0184	100.65	29.15
Average	25.73±0.46	662.23±11.05	0.019±0.0002	96.39±3.69	29.23±1.03
Notes:	E_{11i}(Tangent Modulus)：0.0005≤ε≤0.00165 E_{11s}(Secant Modulus)：0.007≤ε≤0.013				

(c)

Sample No..	Ultimate Load (KN)	Ultimate Strength (MPa)	Maximum Strain	Longitudinal Stiffness	
				Initial tangent E_{11i} (GPa)	Secant E_{11s} (GPa)
1	22.03	568.52	0.0162	99.19	26.54
2	22.17	572.13	0.0157	99.49	27.58
3	23.05	594.84	0.0163	102.50	28.30
Average	22.42±0.55	578.5±14.27	0.016±0.0003	100.39±1.83	27.47±0.88
Notes:	E_{11i}(Tangent Modulus)：0.0005≤ε≤0.00165 E_{11s}(Secant Modulus)：0.007≤ε≤0.013				

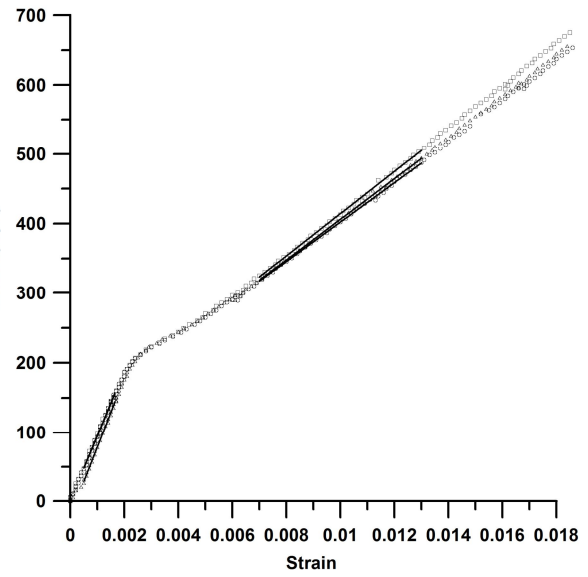

Figure 2. The stress-stain curves for Ti/APC-2 cross-ply nanocomposite laminates after a 1m high free drop impact

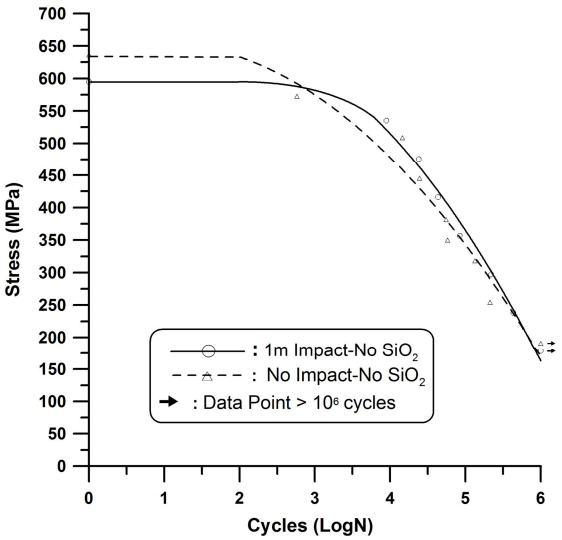

Figure 3. The S-N curves for Ti/APC-2 hybrid composite laminates with and without the impact of 1m high

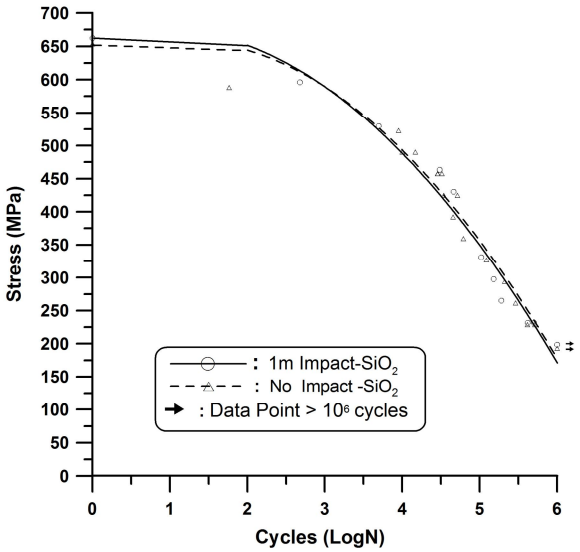

Figure 4. The S-N curves for Ti/APC-2 hybrid nanocomposite laminates with and without the impact of 1m high

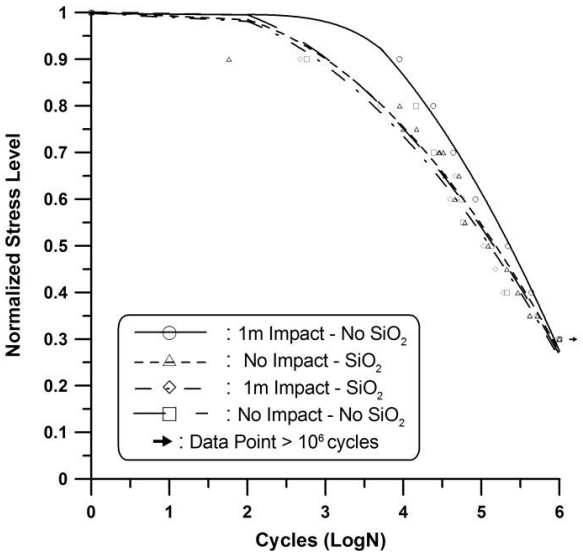

Figure 5. The normalized S-N curves for Ti/APC-2 composite laminates W/WO nanoparticles due to the impact of 1m high or not.

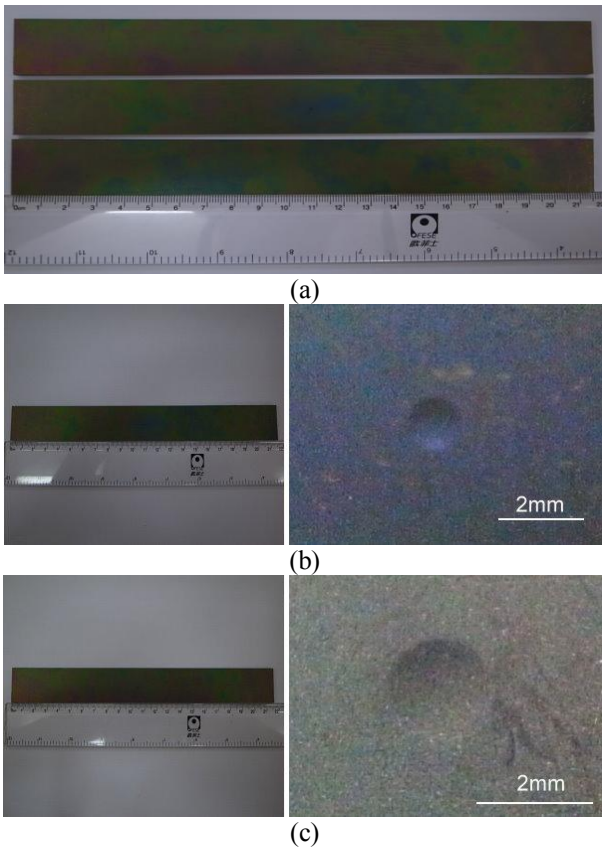

Figure 6. The (a) virgin samples, (b) impacted sample due to 1m high free drop, and (c) impacted sample due to 2m high free drop by a steel ball.

The stress vs. cycles (S-N) curves for Ti/APC-2 cross-ply composite laminates with and without impact of 1m high free drop were illustrated in Figure 3. The S-N curves for Ti/APC-2 cross-ply nanocomposite laminates with and without impact of 1m high free drop were also shown in Figure 4. in contrast. The combined S-N curves, normalized by their corresponding ultimate strength, were presented in Figure 5. The photos of impact indentation were shown in Figure 6.

4. Discussion

It is obvious to see the enhancement of mechanical properties by adding the SiO_2 nanoparticles in Ti/APC-2 nanocomposite laminates in comparison with the virgin laminates, especially a slight increase of ultimate strength with the stiffness almost unchanged, as depicted in Tables 1(a) and 2(a). However, after the impact of 1m high free drop, the retention of ultimate strength was significantly well, i.e., about 10% more due to the spreading of

nanoparticles in APC-2 laminae in the hybrid laminates when compared with Tables 1(b) and 2(b). Similarly, the longitudinal and secant stiffnesses were also kept unchanged. Referring to the statement in[4] that PEEK composites can't resist the damage due to impact at high velocity, i.e., over 25m/s, nevertheless, the innovative hybrid laminates fabricated herein may have the capability to sustain the impact of high velocity with the improvement of mechanical properties by nanoparticles and metallic sheets. It is the urgent topics for further research.

Generally, it is reasonable that the S-N curves for Ti/APC-2 composite laminates due to impact is lower than that of the same virgin laminates without any impact. However, the S-N curves for Ti/APC-2 nanocomposite laminates with and without impact were very close, i.e., no significant difference. That strongly confirmed the above-mentioned concepts of enhancement and improvement of mechanical and cyclic properties by adding nanoparticles in APC-2 laminae of Ti/APC-2 nanocomposite laminates.

Although the area of impacted surface due to 2m high free drop is greater than that of 1m high free drop, the reduction of mechanical properties of samples impacted by 2m high free drop is not significant as shown in Figure 7. The main reason is that the damage occurred mostly on the surface area with good retention of properties inside the hybrid laminates, i.e., both 1m and 2m high free drop are belonged to low-velocity impact. The velocity of 6.28m/s is near a quarter of high velocity, i.e., the high-velocity impact should be over 25m/s. Additionally, good fatigue resistance of impacted samples due to 2m high free drop can also demonstrate the slight damage occurred in the hybrid laminates. Thus, the problem of high-velocity impact is a worthwhile topics for further research.

5. Conclusion

Ti/APC-2 nanocomposite hybrid laminates were fabricated. The mechanical properties, such as ultimate strength and stiffness, were obtained for virgin samples and impacted samples due to 1m and 2m high free drop. Based on the received properties the cyclic tests were conducted to obtain the S-N curves for three types of laminates.

The mechanical properties do not reduce significantly due to low-velocity impact, even if the damage area is obviously large for 2m high free drop. Similarly, the fatigue resistance of impacted samples does not lose much. It is mainly attributed to the damage only occurs on the surface of impacted samples with little influence inside the laminates by low-velocity impact. It can be concluded that the enhancement by nanoparticles and superior bonding capability of matrix PEEK with Ti sheets take the responsibility of improvement of mechanical responses.

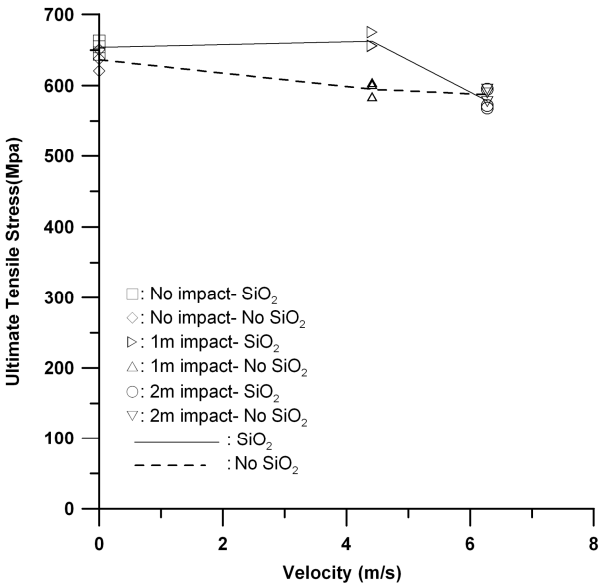

Figure 7. The ultimate strength vs. impact velocity curves for Ti/APC-2 W/WO nanoparticles composite laminates with and without the impact of 1m and 2m high.

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Computer-Assisted Navigation System Helps Experienced Surgeon Improve Outcome in Total Knee Arthroplasty

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Abstract: Navigation-assisted total knee arthroplasty (TKA) reportedly improves component alignment. Also, experience on the knee surgery is complementary to the success of total knee arthroplasty even with the use of navigation system. One hundred and twenty-five consecutive patients who underwent navigation-assisted TKA and 125 patients who underwent conventional TKA by an experienced surgeon were evaluated for mechanical alignment, perioperative hemodynamic status, and early complications. Patients with navigation-assisted TKA showed better mechanical axis, coronal and sagittal axis of the femoral component and coronal axis of the tibia components. Patients in the navigation-assisted TKA group experienced less blood loss, needed fewer transfusions, and required fewer hospitalization days and fewer early complications. Navigation-assisted TKA improved mechanical and component alignment, perioperative hemodynamic status.

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Keywords: Computer-assisted navigation system, total knee arthroplasty

1. Introduction

Navigation-assisted systems reportedly improves coronal and sagittal alignment of total knee arthroplasty (TKA)[1-7]. Previous reports demonstrated that navigation by sole reference to epicondylar axis along femur and tuberosity of tibia failed to improve rotational alignment of the femoral component [8]. Experienced knee surgeons usually determine the rotational alignment with several reference landmarks and complement the pitfall of computer system. Assistance with the navigation technology, femoral medullary violation and excessive soft tissue release can be avoided. Less invasive procedure in TKA is a prerequisite for a stable perioperative hemodynamic status which may contribute to fewer complications [9]. We compared the component alignment and perioperative medical status of patients who underwent navigation-assisted and conventional TKA. We hypotheses that an experienced knee surgeon can improve outcome of total knee arthroplasty with the usage of computer-assisted navigation system.

2. Material and Methods

This study protocol was approved by the institutional review board to review the charts and medical data of the patients. Between March 2005 and October 2007, one hundred and twenty-five consecutive patients who underwent navigation-assisted TKA for osteoarthritis, rheumatoid arthritis and traumatic osteoarthritis disorders were enrolled. One hundred and twenty-five patients were replaced with conventional TKA between February 2004 and September 2006. All patients received surgery in a single institution and performed by a single surgeon who had been performing knee arthroplasty for more than 15years. Within the overlap period (March 2005 to September 2006), 84 patients were separated for navigation-assisted and conventional TKA according to patients' wishes. Preoperatively, age, gender, laterality, body mass index (BMI), and the medical conditions of all patients were recorded. Pre-operative anesthetic status of the patients was graded according to the American Society of Anesthesiology classification system (ASA grade). The post-operative alignment of the components and related complications were documented.

Operative technique

Surgical site preparation

Both groups had received TKA in the same standard operation room. Aseptic dragging and skin preparation was applied as standard operative protocol. The operations were carried out using a pneumatic tourniquet at a pressure of 300 mmHg, with a single injection of antibiotics (cefamezin 1 g) before surgery. A mid-vastus approach was used through a midline skin incision.

Conventional total knee arthroplasty

In the conventional implantation group, femoral intramedullary and tibial intramedullary alignment guides were used. With IM guidance system, we used the standard knee arthroplasty instrumentation to perform the distal femoral cut, chamber cut, and proximal tibia cut. Proper implant sizing and position was adjusted with the surgeon's experience. The femoral and tibial components were implanted with cementing. The wound was closed over a suction drain. A compression bandage was applied from the ankle to the proximal portion of the thigh.

Navigation-assisted total knee arthroplasty

In the navigation-assisted procedures, both femur and tibia alignment cutting were measured extramedullary using a navigation system (BrainLab®; Heimstetten, Germany). This infrared-base system, by anatomically mapping the knee and kinematic registration, sets up a working model on patients' knee (Figure 1). Two fixed reference array with marker spheres which were fixed to the distal femur (4mm) and proximal tibial (3mm) by bi-cortical screws were tracked using an infra-red camera. The center of femoral head, mapping of distal femur, upper tibia, and bony landmarks of the ankle were registered. The femoral component was referenced parallel to the anterior cortex of distal femur. The marked epicondylar line was further checked using Whiteside's line and a posterior condylar line as used in the conventional manner to determine rotation of the femoral component. Then the size and positioning of the prosthesis was checked with the aid of the navigation system.

The distal femur cut and chamber cut were performed less than one degree deviation under navigation system guidance. An extramedullary guide connected to a reference array was employed to check the tibial cutting level, varus-valgus angle, and tibial slope angle. The rotation of the tibial component was adjusted parallel to the axis between the medial third of the tuberosity and the center of the tibial plateau. For double checking confirmation, an additional extramedullary guiding rod was used as a reference for the midpoint of the anterior ankle joint in determining the rotation of the tibial component. The mechanical axis of within 1 degree deviation was created by sequential soft tissue release under real time computer imagine guidance. The femoral and tibial components were implanted with cementing technique. The wound was subsequently closed over a suction drain and a compression bandage was applied. There were no patella re-surfacing in both groups.

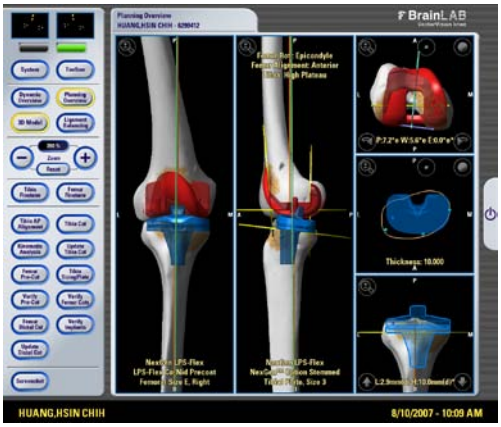


Fig. 1 The photograph shows a working model for TKA after anatomic mapping and kinematic registration

Postoperative management

Both groups received the same postoperative rehabilitation protocol. Antibiotic (cefamezin 1g per 8 hours) was administered for 1 day postoperatively following the guide of infection department. The amount of blood in the drains was recorded. For post operation pain control, passive continuous movement of the knee started after removal of the drainage, alone with intramuscular and oral analgesics. Active knee motion was encouraged 2 days after surgery. With the exception of non-steroid anti-inflammatory drugs for at least 3 weeks, no additional thromboprophylaxis was used in this study series.

Blood transfusion was given if the hemoglobin level was less than 8 mg/dl or if the hemoglobin level was more than 8mg/dl with an unstable hemodynamic status during or after surgery. The hemoglobin levels of all patients were examined on the next morning postoperatively. Patients were discharged after their knee motion approached 95 degrees flexion. All complications within 90 days after surgery were recorded.

Radiographic measurements

At the 6-weeks follow-up, radiography of the knee and long-leg standing radiograph were taken and the weight-bearing femorotibial angle and the mechanical axis were calculated [10]. All measurements were performed by a single colleague. The coronal femoral component angle (ideal angle: 90°) was measured from the medial angle between the mechanical load axis of the femur and the horizontal axis of the 2 prosthetic condyles on an anterior-posterior radiograph. The coronal tibial component angle (ideal angle: 90°) was measured from the medial angle between the anatomic axis of

the tibia and the horizontal axis of the tibial tray on the radiograph. Sagittal plane alignment was measured on a lateral radiograph. The sagittal femoral component angle (ideal angle: 90°) was measured from the angle between the anterior cortex of the distal femur and a line drawn perpendicular to the distal part of the femoral component on a lateral radiograph. The sagittal tibial component angle (ideal angle: 83–90°) was measured from the posterior angle between the midline axis of the tibia and a line drawn across the tibial tray.

Statistical analysis

Patient demographics were analyzed using Pearson's chi-square test, whereas the component alignment and the data of medical status were analyzed using an independent sample t-test. A *p*-value of less than 0.05 was considered to be significant.

3. Results

There were no significant differences in the age, gender, laterality, BMI, causes of operation, and ASA grade between patients who underwent navigation-assisted TKA and conventional TKA (Table 1).

Implant alignment

Table 2 showed that the mechanical axis in the navigation-assisted TKA group was closer to the normal axis than those of conventional TKA group (0.39 ± 2.15° versus 2.01 ± 2.76°, $p < 0.01$). The mean coronal angle of the femoral component was 89.58 ± 1.69° in the navigation-assisted TKA group and 88.95 ± 2.15° in the conventional TKA group ($p = 0.22$). The mean coronal angle of the tibia component was 90.01 ± 1.26° in the navigation-assisted TKA group and 89.34 ± 1.73° in the conventional TKA group ($p < 0.01$). There was a significant difference between the 2 groups with respect to the coronal femoral component position and the coronal tibial component position within 3° deviation ($p = 0.04$ for the femoral components, $p < 0.01$ for the tibial components) and within 1° deviation ($p = 0.05$ for the femoral components, $p < 0.01$ for the tibial components). There were suggestive of more precision of coronal component alignment in the navigation-assisted group than in the conventional group.

The sagittal alignment of the femoral component reflects the anatomical alignment with respect to the anterior femoral cortical bone. The mean sagittal angle of the femoral component was 90.5 ± 2.4° in the navigation-assisted TKA group and 93.3 ± 3.7° in the conventional TKA group ($p < 0.01$). Greater precision was noted for the femoral component sagittal angle within both a 3° and a 1° deviation of the ideal 90° in the navigation-assisted TKA group ($p < 0.01$ and $p < 0.01$, respectively). In terms of the tibial component sagittal alignment of 83–90°, the sagittal angle of the tibial component did not differ between the 2 groups ($p = 0.396$). The posterior slope of the tibial component was 2.26 ± 2.25° in the navigation-assisted TKA group and 2.3 ± 2.63° in the conventional TKA group ($p = 0.986$).

During of the learning period of navigation technique, the component alignments were compared between the conventional TKA group and navigation group in the overlap period. The femoral and tibial coronal alignments were closer to the ideal degree in the navigation group (p=0.019 and 0.007 respectively) (Table 3). Interestingly, the femoral component coronal alignment in conventional group TKA was also improved during the period of usage of navigation system (p=0.008), but not for the tibial component coronal angle (p=0.899) (Table 4).

Medical status and early complications

There was no significant difference in either the tourniquet time or the decrease in hemoglobin levels on the first postoperative day. However, when compared with the conventional group, the patients who underwent navigation-assisted TKA experienced less blood loss ($p = 0.032$), needed fewer blood transfusions ($p < 0.01$) and required fewer hospitalization days ($p < 0.01$) (Table 5). The complications that arose within 90 days after surgery are shown (Figure 2). There were 2 cases of delayed wound healing and 1 case of upper gastrointestinal bleeding in the navigation-assisted group. In the conventional TKA group, there were 3 cases of superficial infection, 2 cases of cerebral infarction, 2 cases of periprosthetic fracture, 2 cases of upper gastrointestinal bleeding, and 1 case each of angina pectoralis and deep infection.

Table 1 Clinical Summary of Patients in Both Groups

	Conventional group	Navigation group	*p*-value
Number	125	125	1.00
Age (years)	67.25 ± 8.95	66.3 ± 10.88	0.449
Gender Male:Female	25:100	25:100	1.00
Laterality (left:right)	61:64	60:65	0.90
Body mass index (kg/M^2)	28.33 ± 4.85	27.97 ± 4.63	0.559
Cause of operation OA: RA:other	113:9:3	110:12:3	0.842
ASA grade 1:2:3	2:85:38	0:75:50	0.141

Table 2. Comparative data of Component Alignment in Both Groups

		Conventional group (n = 125)	Navigation group (n = 125)	*p*-value
Mechanical axis (degree)		2.01 ± 2.76	0.39 ± 2.15	<0.01
Femoral component				
Coronal angle (degree)		88.95 ± 2.15	89.58 ± 1.69	0.22
	within 3° deviation	109 (85.8%)	118 (93.7%)	0.04
	within 1° deviation	50 (39.4%)	65 (51.6%)	0.05
Sagittal angle	(degree)	93.3 ± 3.7	90.5 ± 2.4	<0.01
	within 3° deviation	73 (57.5%)	103 (81.7%)	<0.01
	within 1° deviation	28 (22%)	59 (46.8%)	<0.01
Tibia component				
Coronal angle (degree)		89.34 ± 1.73	90.01 ± 1.26	<0.01
	within 3° deviation	112 (88.2%)	125 (100%)	<0.01
	within 1° deviation	55 (43.3%)	84 (66.7%)	<0.01
Sagittal angle	(degree)			
	83–90°	116 (91.3%)	111(88.1%)	0.396

Table 3 Comparative Data of Component Alignment within the Overlap Period

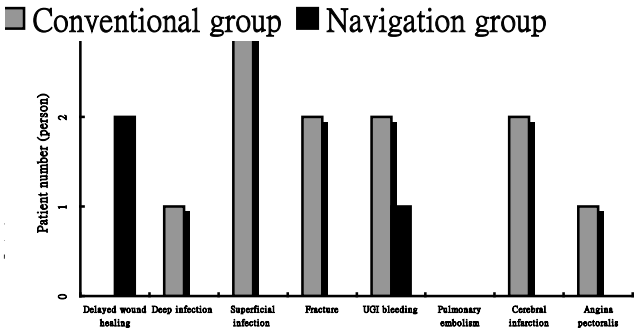
	Conventional group (n = 50)	Navigation group (n=34)	*p*-value
Femoral component			
Coronal angle (degree)	89.07 ± 2.55	89.92 ± 1.84	0.019
Tibia component			
Coronal angle (degree)	89.16 ± 1.73	90.19 ± 1.06	0.007

Table 4 Comparative Data of Conventional Group Before and After the Usage of Navigation System

	Before (n = 75)	After (n = 50)	*p*-value
Femoral component			
Coronal angle (degree)	88.87 ± 1.84	89.07 ± 2.55	0.008
Tibia component			
Coronal angle (degree)	88.94 ± 1.73	89.16 ± 1.73	0.899

Table 5. Hemodynamic Status and Hospitalization Days in Both Groups

	Conventional group	Navigation group	*p*-value
Tourniquet time (minutes)	121.9 ± 21.5	118.1 ± 22.6	0.182
Hemoglobin level decrease on the first postoperative day (mg/dl)	2.13 ± 0.98	1.97 ± 0.98	0.193
Blood loss in drainage bottle (cc)	525.6 ± 323.1	277.1 ± 205.6	0.032
Blood transfusion number (%)	29 (23.2%)	9 (7.2%)	<0.01
Hospitalization(days)	7.42 ± 3.67	5.77 ± 2.18	<0.01



Most investigators have found that navigation-assisted TKA achieves more accurate component alignment than that obtained using conventional implantation [11-13]. In addition to better alignment, we demonstrate a more stable perioperative hemodynamic status and fewer complications when using navigation-assisted TKA. Improvement of the component alignment by a single experienced surgeon was also observed in this study.

No definite association between implant alignment and early postoperative range of motion or knee function has previously been demonstrated. However, a misaligned component promotes early loosening through increased wear caused by suboptimal implant loading [14-18]. Using an improved computed tomography protocol, higher accuracy of implant alignment could be obtained through the use of a navigation system [19]. Surgeons need to define the rotation of the femoral component through a compromise between different landmarks [20]. With collaboration of navigation systems and surgeon's experience on the rotation determination, bony cut, and soft tissue balancing outcome of TKA can be improved even the optimum rotational alignment of the femoral component has not been clearly defined [8,21,22]. We observed that after usage of navigation system, surgeons can improve the bone cutting and placement of femoral component even in the conventional procedure. Computer navigation system can be a tool in modifying the surgical technique and surgical outcome in an experienced surgeon.

In contrast to the conventional surgical procedure, the navigation system presents a real-time intraoperative mechanical axis and soft tissue gap evaluation. With precise bone cutting, soft tissue release can be minimized. Our study demonstrates that the avoidance of intramedullary violation and limited soft tissue release during navigation-guided balancing of the prosthesis produces a less invasive surgical environment. This less invasive environment might be a factor contributing to the lower blood loss, fewer

blood transfusions, hospitalization days, and complications. The increased blood loss in conventional TKA may be due to intramedullary jigging of the femur and more soft tissue dissection during balancing of the prosthesis[23,24]. The navigation-assisted operation saves blood, lessens the risks of transfusion, and may be useful in patients for whom blood products are not acceptable.

Systemic emboli phenomena during preparation of the femur and tibia are well-recognized complications associated with TKA [25]. They are widely believed to be the cause of intraoperative hypotension and reduced cardiac output, which may lead to circulatory collapse, change of mental status, or cerebral infarction [26]. Navigation-assisted TKA significantly reduced systemic emboli as detected by transcranial Doppler ultrasonography [27]. Computer-assisted TKA resulted in the release of significantly fewer systemic emboli than the conventional procedure using intramedullary alignment[19]. In conventional group, there were 2 cases of cerebral infarction and 1 case of angina pectoralis. One cerebral infarction occurred during the operation and the other developed 2 days after the surgery. However, no infarction or cardiac attack incident was noted in our navigation group.

In addition to systemic emboli, there were 2 cases of upper gastrointestinal bleeding, 1 case of periprosthetic fracture, and 1 case of deep infection in the conventional TKA group. By comparison, there was just a single case of upper gastrointestinal bleeding in the navigation-assisted TKA. No previous reports, however, have suggested possible explanations to account for these differences. Less bone marrow and soft tissue damage in the navigation-assisted TKA and pain management [28,29] might reduce the need for anti-inflammatory medication. This might explain the less frequent occurrence of upper gastrointestinal bleeding. The undistorted metaphyseal intramedullary canal may contribute to decreased prosthesis infection during sepsis[30,31] or decreased incidence of periprosthesis fracture.

In conclusion, using navigation-assisted system by an experienced knee surgeon also contributes to the better outcome of TKA. Better alignment, less invasion and preservation of the microarchitecture of the distal femur through the avoidance of intramedullary violation contribute to the better hemodynamic status and fewer early complications.

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Navigation-Assisted Total Knee Arthroplasty with Normal Pressure Drainage Reduces Blood Loss – A Prospective Comparative Study of Three Modalities

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Abstract: Several modalities have been developed to reduce perioperative blood loss during total knee arthroplasty (TKA) and a navigation system has been successfully introduced in TKA. This study compared the blood loss of navigation-assisted TKA and conventional TKA in the presence of negative or normal pressure drainage. Patients were separated into 3 groups. We enrolled 60 patients undergoing conventional TKA with negative pressure drainage in Group A, and those undergoing navigation-assisted TKA with negative or normal pressure drainage were enrolled in Group B (64 patients) and C (66 patients), respectively. Haemovac drainage volume, reduction of haemoglobin, estimated total blood loss, range of motion at 3 months after surgery, number of blood transfusions and hospitalisation days were all recorded. There were no differences in the demographic data of these 3 groups. Patients in Group B had significantly decreased total drainage volume, estimated total blood loss and blood transfusion rate than those in Group A. The significant reduction of total drainage volume, estimated total blood loss and blood transfusion rate were also noted in Group C when compared with Group B. Patients in Group C had a significantly reduction in haemoglobin, heaemovac drainage volume, estimated total blood loss, blood transfusion rate and hospitalisation days when compared with Group A. Navigation-assisted TKA with normal pressure drainage is a potential modality for the reduction of the haemovac drainage volume, perioperative blood loss and transfusion rate without compromising range of motion at 3 months after surgery.
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Keywords: Navigation, total knee arthroplasty, blood loss

1. Introduction

Total knee arthroplasty (TKA) is a well-developed technique with a highly satisfactory success rate for treating knee disorders. Post-TKA complications are significant concerns to knee surgeons. Excessive perioperative blood loss leading to unstable hemodynamic status is a concern. Haemolytic and allergic reactions and viral infection are the concerns following blood transfusion. Several modalities have been developed to reduce perioperative blood loss, including hypotension anesthesia [1], drain clamp [2-4], fibrin tissue adhesive [5], compression bandage, cryotherapy [6], tranexamic acid injection [7, 8] and sealing of the femoral canal using an autologous bone block.

A navigation system has been successfully introduced to assist in TKA. This system is reportedly beneficial in improvement of alignment and accuracy, in complex cases, and in sparing the intramedullary canal [9, 10]. Reduced blood loss during navigation-assisted TKA was reported by Kalairajah et al. [11]. However, details for the combination of drainage modification and the navigation system have not been reported. A prospective study was carried out to compare the blood loss among patients after conventional TKA with negative pressure drainage, navigation-assisted TKA with negative pressure drainage, and navigation-assisted TKA with normal pressure drainage. We hypothesized that navigation-assisted TKA with normal pressure drainage would further improve the perioperative blood loss.

2. Material and Methods

We conducted a prospective, comparative study between January 2005 and September 2009 to evaluate the perioperative blood loss and postoperative range of motion among patients by conventional TKA with negative pressure drainage (Group A), by navigation-assisted TKA with negative pressure drainage (Group B) and by navigation-assisted TKA with normal pressure drainage (Group C). Indications for TKA included advanced osteoarthritis, rheumatoid arthritis and traumatic arthrosis. Contraindications included knee sepsis with previous osteomyelitis, a remote source of ongoing infection, extensor mechanism dysfunction, severe vascular disease and recurvatum deformity secondary to muscular weakness. This study received approval from the institutional review board of the hospital and informed consent was obtained from all patients.

We enrolled 60,64 and 66 patients into Group A, B and C, respectively. The patients were separated into 3 groups according to their own wishes and financial concerns when the surgery was scheduled. The use of acetylsalicylic acid was stopped 1 week before the surgery and continued on the next day after the surgery. Other nonsteroidal anti-inflammatory drugs were not restricted before or after surgery. No antithrombus medications were given perioperatively. All surgeries were conducted under general anesthesia and were performed by or under the direct supervision of the senior orthopedic surgeon. Conventional TKA was performed in a bloodless field using a pneumatic tourniquet at a pressure of 300 mm Hg after a single injection of antibiotic (cefazolin sodium 1 g). A midvastus approach was used through a midline skin incision of 10-12cm. The femur and tibia bone cuts were adjusted via an intramedullary guide. The prosthesis (Advantim Knee System, posterior stabilizing type; Wright Medical Technology, Arlington, TN, USA) was implanted with cement fixation. A 1/8 inch haemovac(Zimmer Haemovac; Zimmer, Warsaw, IN, USA) was inserted as a closed drainage system and was maintained at a negative pressure (700 mm Hg). The haemovac was removed 72 hours after TKA or when the drainage volume was less than 50 ml in the preceding 8 hours.

A navigation-assisted TKA was performed on the Group B patients with a similar midvastus approach through a midline skin incision. The synovium was partially removed to enable precise registration. An infrared camera was equipped to track two fixed references with marker spheres that were fixed to the distal femur (4 mm pins) and the proximal tibia (3 mm pins) using two bi-cortical half-pins inserted via separate 0.5cm incision. A CT-free Navigation System (Vector Vision; Brain LAB, Heimstetten, Germany) with anatomical mapping of the knee and kinematics analysis was employed to generate a working model of the patient's knee. The centre of the femoral head, ankle landmarks and mapping of the distal femur and proximal tibia were registered. The femoral component was referenced parallel to the anterior cortex of the distal femur. A multiple referencing method using the epicondylar line, Whiteside line and posterior condylar line was adapted for the rotation of the femoral component. The rotation of the tibial component was adjusted to that of the femoral component and was parallel to the axis between the medial third of the tibial tuberosity and the center of the tibial plateau. An additional extramedullary guiding rod was used as a reference for the midpoint of the anterior ankle joint to assist with the determination of the rotation of tibial component. Osseous cut was achieved without intramedullary violation and the prosthesis (LPS-Flex system, Nexgen; Zimmer, Warsaw, IN, USA) was implanted with cement fixation. The ideal mechanical axis of within a 1.0˚deviation was obtained after soft tissue balancing with the aid of a real-time computer screen. The tourniquet was released when the wound was closed. A 1/8 inch haemovac was also inserted as a closed drainage system and was maintained at a negative pressure (700 mm Hg). The haemovac was removed 72 hours after TKA or when the drainage volume was less than 50 ml in the preceding 8 hours.

Patients in Group C were operated on using the same navigation-assisted procedure as Group B. However, the tourniquet was released when the joint was closed and a 1/8 inch haemovac was inserted as a close drainage system and maintained at a normal pressure (760 mmHg) without compressing the haemovac to a negative pressure. The drainage was removed 24 hours after surgery.

Intraoperative blood loss for all patients was less then 30 ml due to the application of the tourniquet. Body fluid supplements were administrated with 0.9% normal saline or 0.9% NaCl with 5% glucose to stabilize the vital signs and urine output perioperatively. Blood transfusion with packed red blood cells was indicated when the haemoglobin (Hb) concentration was less than 8 mg/dl or 8-9 mg/dl with unstable vital signs. All patients started continuous passive motion after the removal of the drain. Bedside exercise for active flexion-extension, quadriceps training and walker-aided ambulation were conducted under the assistance of a physical therapist. All patients were discharged when the range of motion of the knee was greater than 90˚, the wound was clean and dry and the patient's general condition was stable. We recorded the perioperative complications and range of motion 3 months after surgery to compare the effect of the navigation technique or drainage management on the knee motion.

Estimation of total blood loss

Patient blood volume (PBV) was calculated according to the formula [12]:

PBV (in litres) = k1 × height $(m)^3$ + k2 × weight (kg) + k3

Where: k1 = 0.3669, k2 = 0.03219, k3 = 0.6041 for males;

k1 = 0.3561, k2 = 0.03308, k3 = 0.1833 for females.

The loss of Hb (Hbloss) was calculated according to the formula [8]:

Hbloss = PBV × (Hbi - Hbe)

Where: Hbloss (in g) is the amount of Hb loss, Hbi (in g/l) is the Hb concentration before surgery and Hbe is the Hb concentration at 24 h after the surgery. Finally, the estimated blood loss of the first postoperative day (in ml) was calculated by using the following formula [1]:

Estimated blood loss = Hbloss/Hbi

The estimated total blood loss was the sum of the haemovac drainage volume of the following days and the estimated blood loss.

Statistical Analysis:

Haemovac drainage volume, Hb level on the first postoperative day, reduction in Hb level, estimated total blood loss, range of motion 3 months after surgery, and days of hospitalisation were all recorded and statistically analyzed using ANOVA and Scheffé's method. The blood transfusion rate was compared using Pearson's chi-square test. A p value of less than 0.05 was regarded as statistically significant.

3. Results

There were no statistical differences in the demographic data of the patients in the 3 groups (Table 1). Patients in Group B had significantly lower haemovac drainage volume ($p < 0.01$), blood transfusion rate ($p < 0.01$), estimated total blood loss ($p < 0.01$) and better range of motion 3 months after surgery ($p < 0.01$) than the patients in Group A, although Hb loss (g) or Hb reduction did not significantly differ between the two groups. Patients in Group C had also significant reduction in the haemovac drainage volume ($p < 0.01$), blood transfusion rate ($p < 0.01$) and estimated total blood loss ($p < 0.001$) than the patients in Group B (Table 2).

In the comparison between Groups A and C, we observed that patients in Group C showed significant reductions in haemovac drainage volume ($p < 0.01$), Hb reduction($p = 0.03$), estimated total blood loss ($p<0.01$), blood transfusion rate ($p < 0.01$), hospitalisation days ($p < 0.01$) and better range of motion 3 months after surgery ($p < 0.01$) when compared to Group A (Table 2).

Major complications, such as symptomatic deep venous thrombosis, thromboembolic disorders, major organ failure and deep infections were all reviewed. One patient in Group A was diagnosed perioperatively with an acute infarction of the right corona radiata. A significant reduction of Hb (14.5 mg/dl drop to 8.8 mg/dl) and haemovac drainage volume (2270 ml) was found at 24 hours after surgery. The estimated total blood loss was 2745 ml and this may have contributed to the cerebrovascular accident. Otherwise, no other major complications were encountered within the 3 groups.

The navigation groups (B and C) had better range of motion 3 months after surgery when compared with Group A and there was no significant difference between Group B and C ($119.5 \pm 9.3°$ vs $115.8 \pm 12.4°$, respectively, $p = 0.213$). Therefore, the improvement in the range of motion was significant in the navigation groups compared to conventional TKA, and there was no difference between the use of negative pressure or normal pressure drainage in patients who underwent navigation-assisted TKA.

4. Discussion

In this prospective comparative study, reduced blood loss was achieved after navigation-assisted TKA with normal pressure drainage compared to navigation-assisted TKA with negative pressure drainage and conventional TKA with negative pressure drainage. The blood transfusion rate was also lower than observed in previous reports [13, 14] (Table 3).

Some modalities have been proposed to reduce blood loss during TKA and hence to decrease blood transfusion and associated complications. These include a modified drainage system [2-4], tranexamic acid administration [7,8], minimal incision surgery [14], and computer-assisted surgery [9, 11]. It is conceded that skillful surgery is an important strategy for reducing blood loss. Minimal -incision TKA has been suggested to reduce blood loss and decrease the transfusion rate from 24.5% to 10.1% [14]. A navigation-system has been introduced to TKA with the advantages of improved alignment and accuracy and sparing the intramedullary canal [10]. Previous reports also demonstrated that computer-assisted TKA is associated with lesser blood loss than conventional techniques [11,15]. In our study, Hb reduction was significant in Group A compared to Group C, but not in the comparison between Groups A and B or Groups B and C (Table 2). The Hb level reflects the concentration of Hb in the PBV and it was obviously affected by body size. Due to the limited number of cases, the reduction and loss of Hb was not statistically significant although the mean value was less in Group B when compared with Group A. The reduction in Hb does not precisely represent the actual blood loss during the procedure. Therefore we identified the actual blood loss and analyzed the differences among each group by calculating the estimated total blood loss. When we compared Group A with Group C, the Hb reduction, haemovac drainage volume and estimated total blood loss were significantly less in Group C. These results imply that normal pressure drainage would further enhance the reduction in perioperative blood loss in addition to avoidance of medullary violation through the navigation technique and neither wound complications nor ROM deficit were encountered postoperatively. In addition, different brands of posterior stabilizing prosthesis would not change the amount of blood loss because of the same approach and osseous resection. To our knowledge, we provide the first evidence indicating that navigation-assisted TKA with normal

pressure drainage is beneficial for the improvement of the perioperative blood loss, and blood transfusion rate when compared with the conventional procedures. The mean estimated total blood loss was 639.3 ± 212.8ml in Group C, which was the lowest compared with previous studies [13, 16](Table 3).

A prospective, comparative study in 90 patients who underwent TKA, observed no benefits using the postoperative drainage systems [17]. Another prospective study reported no advantages for the postoperative drainage systems, including the incidences of swelling or persistent drainage, and range of motion [18-20]. However, excessive haematoma is a concern in patients without drainage and may become the source of infection. Therefore drainage is still preferred by most orthopaedic surgeons in TKA. The efficacy of a drainage clamp for reducing blood loss is still controversial in patients with TKA. Drain clamping for 30 min with an intra-articular injection of saline and adrenaline has been reported to be an effective method for reducing blood loss during TKA [21]; however, patients with drain clamping for 24 hours after TKA reportedly have more complications than those with clamping for 1 hour [4]. No significant differences in blood loss or postoperative Hb levels were reported between a 2-hour clamp group and a control group [2].

A negative pressure produced by the drainage system can evacuate a haematoma from the joint, but it can also breakdown the intra-articular pressure caused by the haematoma and joint capsule tension, and leads to more perioperative blood loss. Therefore, we suggested a closed system with normal pressure drainage. The intra-articular blood can be drained out under balanced pressure without inducing further blood loss. In this study, the mean drainage volume was 130.5 ± 87.2 ml, which was lower than observed for the conventional TKA, the navigation-assisted group with negative pressure drainage and with previous reports (Table 3). Also, the retained blood clot within the knee joint did not compromise the range of motion after the surgery (Table 2) or resulted in major complications.

The use of tranexamic acid is an alternative approach for the reduction of blood loss. Plasmin binds to fibrinogen or fibrin structures to induce fibrinolysis. Tranexamic acid blocks the lysine-binding site of plasminogen that inhibits the conversion of plasmin. Tranexamic acid reportedly reduces the drainage volume by 50% [7, 8], but it does not significantly reduce the hidden blood loss caused by the extravasation of red blood cells just after tourniquet release [13].

The limitation of this study was the randomization of the patients. Due to the extra-cost of the navigation system, patients` decision to undergo conventional or navigation TKA was based on their own wishes that would make blind randomization incapable. However, the preoperative demographic data showed no statistical differences among these 3 groups.

Table 1 Demographic data for the Groups A, B and C

Variables	Group A (n = 60)	Group B (n = 64)	Group C (n = 66)	p
Age (years)	66.0 ± 7.5 (48 - 77)	64.1 ± 11.8 (27 - 86)	64.9 ± 5.6 (50 - 77)	0.488
Male/female	11/49	8/56	8/58	-
OA/RA /trauma	53/6/1	56 / 7 / 1	62 / 4 / 0	-
Body weight (kg)	66.8 ± 12.8 (42 - 107.2)	68.0 ± 13.9 (47 – 109)	68.4± 9.6 (52 – 111)	0.745
Body height (m)	1.53 ± 0.07 (1.43-1.73)	1.54 ± 0.08 (1.4 –1.75)	1.54 ± 0.05 (1.44 – 1.66)	0.750
Patient blood volume(ml)	3752.6± 583.5 (2614.0-5083.0)	3780.7 ± 671.3 (2869.2-5658.4)	3799.6 ± 424.9 (2989.1-5181.3)	0.897
Preoperative Hb (mg/dl)	13.12±1.31 (9.8 - 15.6)	12.87 ± 1.46 (9.2 - 6.7)	13.09 ± 1.28 (9.2 - 16.7)	0.538

Group A: Conventional TKA with negative pressure drainage
Group B: Computer-assisted TKA with negative pressure drainage
Group C: Computer-assisted TKA with normal pressure drainage
OA: Osteoarthritis; RA: Rheumatoid arthritis;
Hb: Haemoglobin
ANOVA $p < 0.05$ is considered significant difference

Table 2 Comparative perioperative data of Group A,B and C

Variables	Group A (n = 60)	Group B (n = 64)	Group C (n = 66)	ANOVA p	Scheffe p Group A vs B	Scheffe p Group B vs C	Scheffe p Group A vs C
Hb loss (gm)	8.07 ± 4.25 (0.32 – 23.93)	7.53 ± 3.09 (1.87 – 16.98)	6.64 ± 2.57 (0.69 – 12.21)	0.056	-	-	-
Hb reduction (mg/dl)	2.14 ± 1.05 (0.1 – 5.7)	1.98 ± 0.70 (0.5 – 3.6)	1.75 ± 0.66 (0.2 – 3.2)	0.03	0.56	0.29	0.03
Haemovac drainage amount (ml)	582.7 ± 393.2 (125–2745)	336.6 ± 222.7 (5 – 1090)	130.5 ± 87.2 (0 – 460)	<0.01	<0.01	<0.01	<0.01
Estimated total blood loss (ml	1190.4 ± 562.0 (329.1– 4395.0)	918.4 ± 338.5 (146.0-2106.5)	639.3 ± 212.8 (89.8 – 1090.4)	<0.01	<0.01	<0.01	<0.01
Hospitalisation days	8.7 ± 2 (5 – 20)	7.9 ± 2.2 (5–17)	6.9 ± 2.2 (3 – 14)	< 0.01	0.119	0.072	<0.01
ROM (degree)	105.4 ± 22.3 (80-130)	119.5 ± 9.3 (90- 135)	115.8 ± 12.4 (70 - 150)	<0.01	<0.01	0.21	<0.01
Blood transfusion (%)ǂ	28.3	9.4	0	< 0.01			

Group A: Conventional TKA with negative pressure drainage
Group B: Computer-assisted TKA with negative pressure drainage
Group C: Computer-assisted TKA with normal pressure drainage
ROM : Range of motion 3 months after surgery ;
Hb: Haemoglobin
p value of less than 0.05 was regarded as statistically significant.
ǂ Blood transfusion rate was compared using Pearson's chi-square test

Table 3 Series of perioperative blood loss, Hb reduction and transfusion rate

Authors	Numbers of TKA	Drain volume (ml)	Hb reduction (mg/dl)	Estimated blood loss (ml)	Transfusion rate (%)
Dae et al. [15]	50 (CATKA)	464.9	2.59	–	–
Good et al. [13]	27 (TCTKA) 24 (placebo)	385 845	2.8 2.8	1045 1426	11.1 58.3
Kalairajah et al.[11]	30 (CTKA) 30 (CATKA)	1747 1351	5.3 3.6	– –	– –
Sehat et al. [16]	101 (CTKA)	733	3.0	1498	–
Tenholder et al. [14]	69 (MIS) 49 (CTKA)	– –	3.2 3.8	– –	10.1 24.5
Tsumara et al.[19]	106 (CTKA)	662	2.4	–	–
Present series	60(CTKAN) 64 (CATKAN) 66 (CATKAP)	582.7 ± 393.2 336.6 ± 222.7 130.5 ± 87.2	2.14± 1.05 1.98± 0.7 1.75± 0.66	1190.4 ± 562.0 918.4 ± 338.5 639.3 ± 212.8	26.6 9.4 0

(CTKA) : Conventional TKA
(CTKAN) : Conventional TKA with negative pressure drainage
(CATKAN): Computer-Assisted TKA with negative pressure drainage
(CATKAP) : Computer-Assisted TKA with normal pressure drainage
(TCTKA): Tranexamic acid administration following conventional TKA
(MIS): Minimal incision TKA; Hb: Haemoglobin

In this study, navigation-assisted TKA with normal pressure drainage was proven to be the most effective at reducing perioperative blood loss, transfusion rate and hospitalisation days. Navigation-assisted TKA affords a technical advance in reducing blood loss by the avoidance of an intramedullary violation. Normal pressure drainage avoids excessive blood loss while evacuating intra-articular haematomas. This drainage strategy is simple and easy and no additional equipment or nursing care is required.

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The Critical Dimension Definition of Femoral for Custom-made Total Knee Arthroplasty by the Application of Geometric Modeling

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Abstract: The main purpose for the design of knee prosthesis is to construct patient's individual anatomical, geometric model and satisfy his/her real geometric and physiology characteristic. The reconstruction of 3D knee geometric model is the basis of knee prosthesis related design. In this study, Mimics software was used to obtain CT image to construct the complete knee actual 3D geometric model, to analyze and define the critical dimension needed for the most complicated femur resection in custom-made total knee arthroplasty (TKA), including critical dimensions of anterior femur resection, distal femur resection and posterior condylar resection. Based on the obtained information, one can setup the critical dimensions and perform the location analysis that are suitable for knee arthroplasty for individual patient's knee characteristic, shape and damage conditions. Then, based on these data to design and manufacture custom-made knee prosthesis to fulfill press-fit design purpose.
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Keywords: Mimics, femoral, 3D geometric modeling, total knee arthroplasty (TKA), custom-made, knee prosthesis

1. Introduction

In Total Knee Arthroplasty(TKA), the prosthesis under different resection and implantation parameters affect knee interior stress distribution is the subject that can hardly be solved by traditional anatomical research[1]. However, this is the unavoidable critical factor when performing TKA. Therefore, the establishment of the real human 3D knee model has become significant. According to the related research[2][3], better joint prosthesis and medullar cavity press-fit produced better surgical results, longer durability, less pain at the initial stage of implantation, and reduce loosening problems in the future.

2. Literature review

The study of computer image aided in medical care and knee arthroplasty has been going on for many years [4][5][6][7]. Among them, the use of CT (Computed Tomography) image reconstruction for knee 3D model to assist medical professionals performing surgery planning and guiding has the longest history and is the most mature one. The main reason is its advantage on bone identification.

Since the image format (DICOM) of CT/MRI cannot be read by CAD software, conversion software is needed to convert DICOM format into CAD readable format. Mimics, a commercial software, was chosen to do the format conversion. One can define various custom-made knee critical dimensions using 3D knee model. An integrated approach of CAD/CAM was presented for the concurrent development of custom-made femoral stem [8].

Mimics is a highly integrated and user friendly 3D image software and has performed well in [9][10][11]. Gray threshold is used in Mimics as the basis to build 3D geometric model from CT/MRI image. Human body can be categorized into 2000~4000 density levels. Gray threshold, base on human density, is used to represent human bone, ligament, muscle, blood vessel, etc [12]. Through the selection of the Gray threshold in CT/MRI medical image data, Mimics is able to generate 3D geometric model. Geometric model can accurately identify the required critical points and dimensions by observing this model from different view angle and direction, such as locating CT layer that contains lateral epicondyle and medial epicondyle. A design system combining clinical experience and engineering knowledge was developed for the manufacture for femoral component of knee prosthesis [13].

In the study of knee mechanics, dynamics model, and anatomical characteristics, Lewis etc. [14] suggested that the optimal fixed axis of rotation for the human knee joint should passes the centers of medial and lateral epicondyle. Mensch etc. [15] concluded that line connects center of medial and lateral epicondyle is very closed to transepicondylar axis (TEA). Therefore, TEA is considered the most reliable anatomical reference index. In total knee arthroplastry prosthesis, to reconstruct precise knee mechanical axis is very important at improving surgery field and increasing the life of prosthesis [16]. One of the critical factors for a successful surgery is to locate the precise femoral anatomic axis for

femoral prosthesis rotation to ensure the proper distal femoral resection surgery and the installation of prosthesis.

Transepicondylar axis connects femoral tips of lateral epicondyle and medial epicondyle. It is used to describe maximum flexion movement and define the alignment of femoral rotational axis for total knee arthroplasty prosthesis. If femoral prosthesis can be placed parallel to the transepicondylar axis, it is possible to obtain normal patellar track, decrease the stress between patellar and femur, and the friction between tibia and femur [17]. This is probably the only bone index in arthroplasty. However, it is not possible to visualize this axis during the surgery. The only way to locate this axis is by touching. With the assistance of CT image, one can locate the axis on the image in advance and use Mimics to construct 3D geometric model surgical procedure simulation. Furthermore, base on the patients' knee characteristic and damage condition, one can proceed on the defining of the critical dimension and resection alignment of arthroplasty and complete the definition of the necessary dimension and information of custom-made knee prosthesis.

3. Research method and results

To construct the knee geometry model, and to define and analyze the critical dimensions, one should obtains patient's CT image files; use Mimics to reconstruct knee 3D model; measure the location of the required TEA, femoral anatomic axis, mechanical axis and tibia anatomic axis for total knee prosthesis replacement surgery; refer to alignment angle of the total knee prosthesis replacement surgery tools; simulate surgery resection procedure; conduct the definition of the characteristic dimension and resection alignment for the knee arthroplasty and complete the definition of the necessary dimension and analysis required for custom- made knee prosthesis surgery.

3.1 Femur 3D geometry construction

By using the better bone structure recognition capability feature of CT, CT image was imported to Mimics. Fig. 1 is the flow chart regarding the construction of knee geometry model.

The built in gray threshold was used to select femur to form different mask layers; use "image edit" feature to "add" and "erase" image boundary to increase the precision of the reconstructed image. Also, region growth feature was used to break up the image to obtain more precise femur voxel. Finally, Mimics 3D calculation feature was used to build up the femur. Fig. 2 shows the finalized femur 3D model structure.

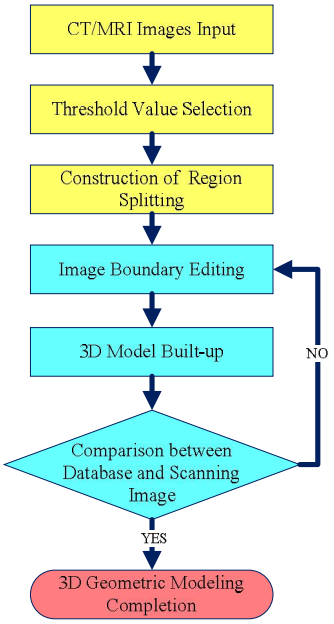

Fig. 1 Knee geometry model construction flow chart

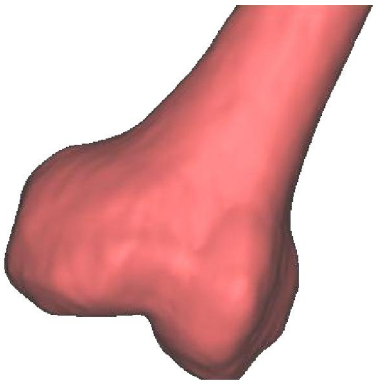

Fig. 2 Femur 3D geometry model

3.2 Femur critical dimension definition

The accuracy of the axes of knee prosthesis is very important. To define the knee critical dimensions, there base lines have to be located first; they are femoral anatomic axis, TEA and mechanical axis. By using the relationship among these three axes, anterior femur critical dimensions a, distal femur critical dimensions b, and posterior condylar critical dimensions c are defined. Fig. 3 shows the relationship between theses axis and is discussed in the following paragraph.

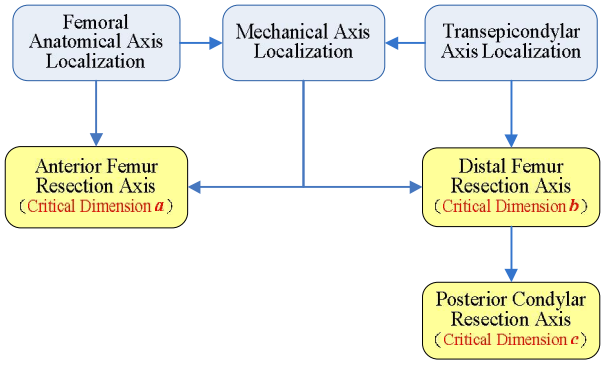

Fig. 3 Flow chart of the custom-made knee prosthesis critical dimension definition

3.2.1 Femoral anatomical axis

On CT image, two cross sections, located at 1/3 distal end, were selected at the proper location of femur medullary cavity, cancellous bone was identified and center was chosen at each cross section using Mimics. The axis connecting these center points is called femoral anatomical axis.

3.2.2 Transepicondylar axis and mechanical axis

By connecting the tips of lateral epicondyle and medial epicondyle, one can locate transepicondylar axis (TEA), also called the rotation axis. Mechanical axis can be located by using medial tip of distal femur as the reference point to obtain the locating axis that is parallel to TEA, then use the intersect of locating axis and anatomical axis as the rotation axis, rotate anatomical axis in the direction of medial side of knee, 7° for male and 5° for female to get mechanical axis, as shown in Fig. 4.

3.3 Femur critical dimension and resection axis

The purpose of knee truncation is to remove the excess worn or lesion affected knee surface and portion for the implantation of knee prosthesis. Therefore, the design dimensions of knee prosthesis have to closely match the shape and contour of the truncated knee bone; the knee resection is the most critical step in knee arthroplasty prosthesis. The precise location of each resection axis (surface) greatly affects the results of surgery.

Usually, the femoral resection has three steps: (1) anterior femur resection (2) distal femur resection (3) posterior condylar resection. These three resection axes were used to designate the critical dimensions a, b and c respectively, as shown in Fig. 5.

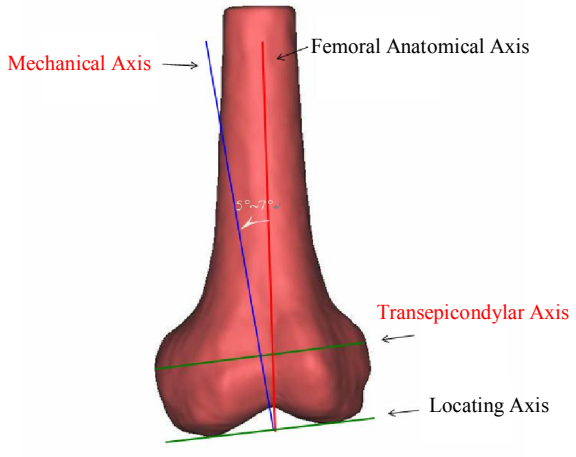

Fig. 4 Definition of femoral mechanical axis

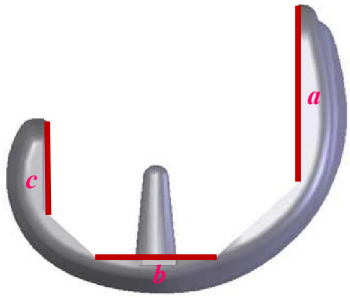

Fig. 5 Femur critical dimensions

3.3.1 Anterior femur critical dimension *a*

The first step of knee femoral resection is anterior femur resection. There are three methods to determine anterior femur resection axis: 1. Parallel to transepicondy line; 2. Posterior condylar connecting line 3-degree turning outward; 3. Vertical line of whiteside line (trochlear axis). These three methods are equally good, however, the first method was used in this study. The key is to make sure the resection axis and TEA are parallel, as shown in Fig. 6. It is critical that the resection surface and bony cortex surface of femoral shaft are even to avoid damaging femur bony cortex. The resection length ***a*** is one of the prosthesis critical dimensions, as shown in Fig. 7.

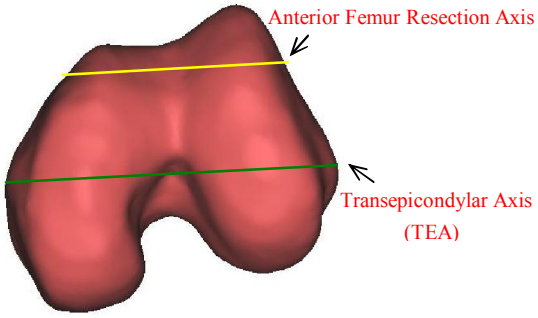

Fig. 6 Schematic of anterior femur resection axis

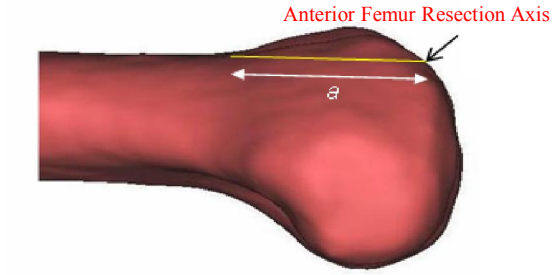

Fig. 7 Schematic of anterior femur critical dimension ***a***

3.3.2 Distal femur critical dimension *b*

Transepicondylar axis (TEA) is the index used to define distal femur critical dimension since distal femur resection axis has to parallel to TEA. Because it is very difficult to locate TEA during knee arthroplasty prosthesis, one can use medial tip of distal femur as a reference point to setup the locating axis that is perpendicular to mechanical axis as shown in Fig. 8. Base on the characteristics of distal femur and the damage conditions, one can moves the locating axis parallel upward about 8mm~10mm to obtain distal femur resection axis, as shown in Fig. 9 where ***b*** is the second critical dimension.

3.3.3 Posterior condylar critical dimension *c*

Posterior condylar resection is the last step to perform at knee arthroplasty prosthesis. The resection surface must be parallel to anterior femoral resection axis as shown in Fig. 10.

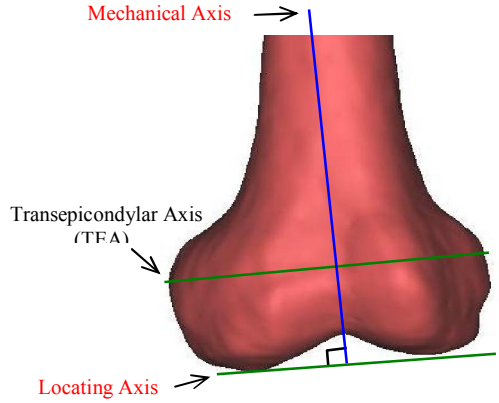

Fig. 8 Schematic of the locating axis for the distal femur critical dimension

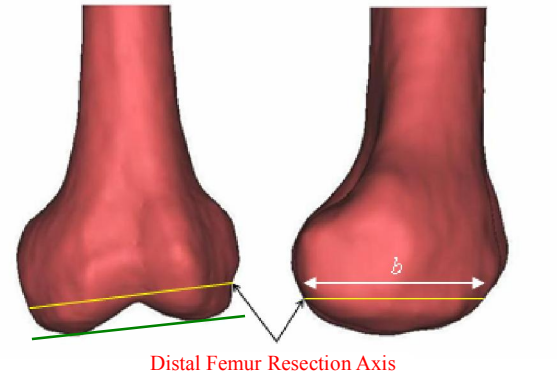

Fig. 9 Schematic of distal femur critical dimension ***b***

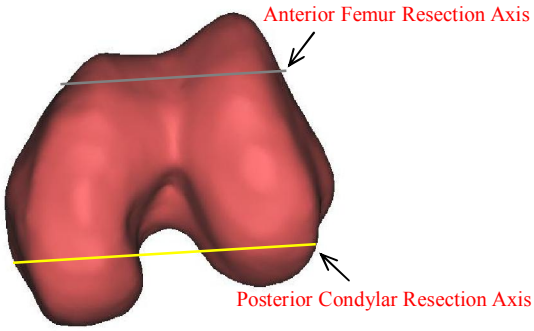

Fig. 10 Schematic of posterior condylar resection axis

The resection location is related to patients' condyle characteristic (including damage conditions) and knee prosthesis. The resection length c is the third critical dimension as shown in Fig. 11. In general, posterior condylar resection axis is perpendicular to distal femur resection axis. The ideal situation is knee joint space of distal femur and posterior condylar after resection is the same at flexion and extension.

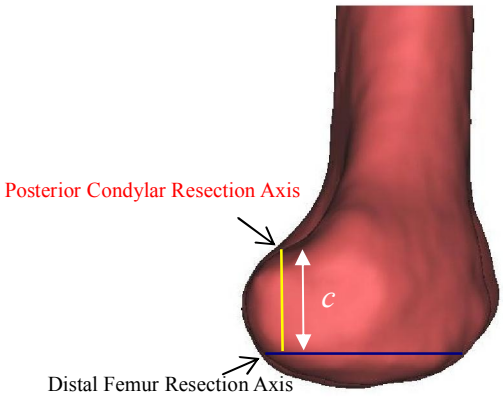

Fig. 11 Schematic of posterior condylar critical dimension ***c***

4. Conclusion

(1) Mimics is used to construct 3D model according to CT and MRI image. Its section method is based on the proper choice of the gray threshold to obtain the precise knee structure contour figure. Because the highly complexity of the knee structure, manual figure section is necessary. It is even difficult to quickly section MRI image. Since manual section requires more experience and technique, the reconstruction efficiency is low.

(2) CT has a better recognition capability of identifying the bone structure. Bone contour can be shown more clearly. MRI has better recognition capability on ligament, muscle and blood vessel. The proper use of the advantage of these two images can construct a much better knee 3D model.

(3) The gray threshold range of Mimics to identify the bone structure is from 226 to 1729. The 3D model prototype is constructed base on this threshold. After the refinement of 3D prototype, the reconstructed model is more close to the actual condition. The refinement includes the comparison/repair of the prototype with CT image from different view angles, directional characteristics etc. The precision of the 3D model built by Mimics is related to the CT/MRI scanning layer thickness. Thinner layer produces better resolution.

(4) To define the knee critical dimensions, three base lines have to be located; they are femoral anatomic axis, transepicondylar axis and mechanics axis. By using the relationship between these three axes, anterior femur critical dimension ***a***, distal femur critical dimension ***b***, and posterior condylar critical dimension ***c*** are defined. These dimensions need to be adjusted according to knee size, bone contour characteristics and damage conditions to fulfill the requirement of the individual custom-made knee prosthesis design.

(5) It is easier to measure the axes, which difficult to be located in actual surgery, by using 3D geometry model defined dimensions, such as transepicondylar axis, etc. In this study, femur resection procedures are setup based on the actual surgical experience simulation. By observing characteristics of the 3D model from different view angles, direction, etc., it is possible to greatly improve the precision of the critical dimension alignment to ensure the knee prosthesis design to fulfill the press-fit purpose.

Acknowledgement

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The Metallographic Analysis Optimization of Aluminum Alloy for Medical Equipment Parts

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Abstract: The purpose of this research is to study the metallographic analysis optimization of the T6-treated forging Al alloys, 7075, 6061, and casting Al alloy A356 for medical equipment parts. The influences of the metallographic image under several control factors, types of polishing solution, etching time, polishing speed, etc., are investigated. Taguchi method is employed to evaluate the optimization of aluminum alloy metallographic analysis. By using Matrox Inspector 4.0 image analysis software, the metallographic images are converted to gray scale. According to the gray scale values, the major factor of the metallographic image optimization for Al alloy 7075 and 6061 is the etching time, while the polishing speed is the least control factor. For Al alloy A356, the type of polishing solution is the main factor for the optimization of metallographic image, while the least influential factor is the etching time. The optimized metallographic factors combination can be used on the development and design of the medical equipment parts in the future.

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Keywords: Al alloy 7075, 6061, A356, metallographic analysis, Taguchi method, gray scale

1. Introduction

In recent year, owing to the shortage of the energy supply, energy saving awareness has risen and the demand of light-weighted with high strength and high stiffness materials has increased. It has been recognized that alumina strengthened aluminum alloy metallic base composite material has good strength/weight ratio, especially its wear-resistant property is much better than that of strengthen base material. Since aluminum has very good anti-abrasive, mechanical and reusable properties, this type of materials have been widely used in medical equipment parts, general household, chemistry, aeronautics, aerospace, automobile and motorcycle. Due to the advanced of the technology and new materials development, metallographic research area has been broadened. Initially, metallography in coordinate with physical metallurgy focused on materials microstructure and metallurgical phenomena discussion and is hardly used in engineering applications. However, the progress of the metallographic technology has made this technology widely used at materials related production, machining manufacturing, medical equipment parts and material-breakage analysis [1][2][3][4][5]. Normally, chemical composition analysis and mechanical properties test are performed to understand the characteristics of an unknown material. However, simple mechanical property test (such as tensile, impact test) cannot show all material characteristic or defect. Metallographic analysis has become an important and direct test method to verify material characteristics.

2. Main content

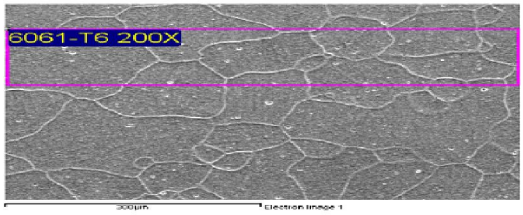
This project studies the optimization of the aluminum alloy metallographic analysis and discusses the factors that affect the metallographic image the most. Firstly, introduce three most effective factors (types of polishing solution, etching time, and polishing speed) into Taguchi method to perform the optimized metallographic analysis, and then use Matrox Inspector 4.0 image analysis software to analyze each group of metallographic images. After gray scale numerical statistic analyses, 0~ 255 gray scale peak values are chosen as Taguchi analysis data.

2.1 The choice of aluminum specimen

This study chose commonly used forged aluminum alloy 7075, 6061 and casting aluminum alloy A356 as test specimens to perform aluminum alloy metallographic optimization analyses. [5][6]

2.2 The design of Taguchi method

This study used Taguchi method $L_9(3^4)$ array structure to design metallographic analysis factors [7][8][9][10][11]. The control factors are: types of polishing solution (A), material etching time (B) and polishing speed (C). Each factor was set to three different levels. Table 1 and 2 show each factor, variation level and $L_9(3^4)$ array table. AxB is the interaction factor between polishing solution and materials etching time.



Element	Weight%	Atomic%
Al	87.33	87.76
Si	12.67	12.24
Totals	100.00	

Fig. 1 EDS composition analysis for Al alloy 7075

Table 1. Metallographic processing control factors

f	Parameter		Level 1	Level 2	Level 3
A	Polishing solution		Alumina 0.3 micro	Alumina 0.05 micro	SiO_2 0.06 micro
B	Etching time	7075	10s	20s	30s
		A356	28s	35s	42s
		6061	30s	40s	50s
C	Polishing speed		200 rpm	300 rpm	400 rpm

Table 2. $L_9(3^4)$ Array

EXP	A	B	AxB	C
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

2.3 The making of polishing solution

Reference Keller’s etching solutions were adjusted properly and used as the aluminum alloy chemical etching solution in this study. Composition for both reference and adjusted solutions are shown in Table 3：

Table 3 Reference etching solution and adjusted etching solution

Keller’s etching solution (reference)	*HF(1ml)+HCL(1.5ml)+HNO_3(2.5ml)+H_2O(95ml)* [1]
	HF(1ml)+HCL(1ml)+HNO_3(66ml) [6]
	HF(1ml)+HCL(1.5ml)+HNO_3(2.5ml)+H_2O(95ml) [11]
	HF(2ml)+HCL(3ml)+HNO_3(5ml)+H_2O(190ml) [12]
Adjusted Keller’s etching solution	***HF(3ml)+HCL(1.5 ml)+HNO_3(2.5ml)+H_2O(93 ml)*** This is used as the aluminum alloy chemical etching solution in this study.

2.4 Aluminum alloy composition analysis

EDS of SEM was used to analyze the composition and element ratio of 7075, 6061 and A456 aluminum alloy to confirm that all the materials are qualified for the experiment.

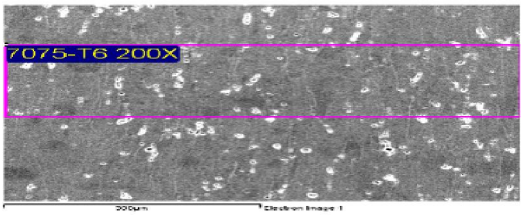
2.5 HV surface hardness analysis

Vickers hardness test was used to confirm the materials used in this experiment are T6-treated forging and casting aluminum alloy. 12 points were chosen on each series of the material. Each point was pressed for 10 seconds with 300g loading. Average hardness was calculated for comparison.

3. Experimental results

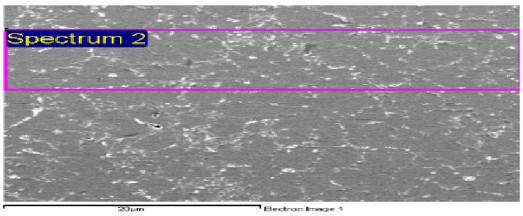
3.1 Aluminum alloy composition analysis results

Fig. 1 shows the result of Al alloy 7075 composition analysis; Fig. 2 shows the Al alloy A356 composition analysis result; Fig. 3 gives the composition analysis result for Al alloy 6061. All the results meet the industrial requirements.



Element	Weight%	Atomic%
Mg	2.42	2.81
Al	89.64	93.69
Cr	0.46	0.25
Cu	1.84	0.82
Zn	5.64	2.43
Totals	100.00	

Fig. 2 EDS composition analysis for Al alloy A356



Element	Weight%	Atomic%
Al	87.33	87.76
Si	12.67	12.24
Totals	100.00	

Fig. 3 EDS composition analysis for Al alloy 6061

3.2 HV surface hardness analysis results

Vickers hardness test was performed on aluminum alloy 7075, A356 and 6061. The results are shown in Fig. 4.

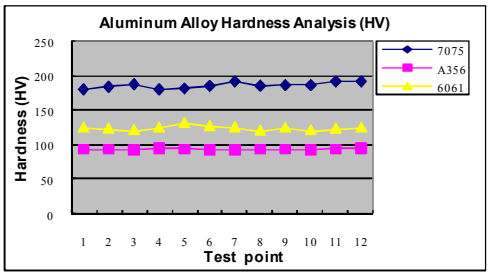


Fig. 4 Aluminum alloy HV hardness test results

3.3 Metallographic image gray scale value statistic analysis

Matrox Inspector 4.0 image analysis software analyzes each group of metallographic image. After gray scale numerical statistic analysis, 0~ 255 gray scale distribution peak values were chosen as Taguchi analysis data. (Gray scale with wider distribution and lower peak value has clear crystalline grain on its metallographic structure). Fig. 5 ~ Fig. 10 are the results of gray scale conversion of experimental data for three different aluminum alloy undergo Taguchi method test:

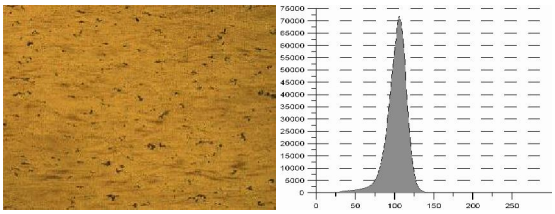


Fig. 5 Al alloy 7075 metallographic image (200X) and gray scale distribution curve (for Exp. No. 1)

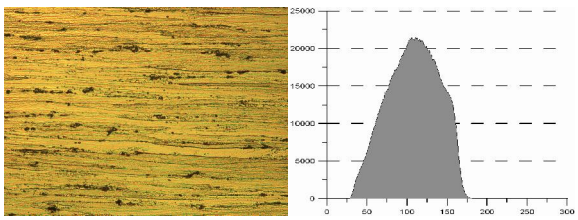


Fig. 6 Al alloy 7075 metallographic image (200X) and gray scale distribution curve (for Exp. No. 9)

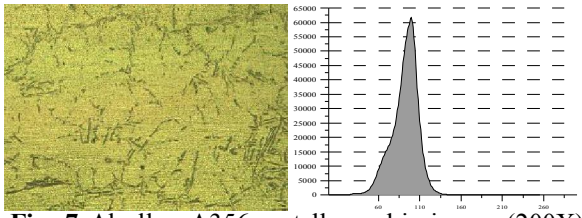


Fig. 7 Al alloy A356 metallographic image (200X) and gray scale distribution curve (for Exp. No.3)

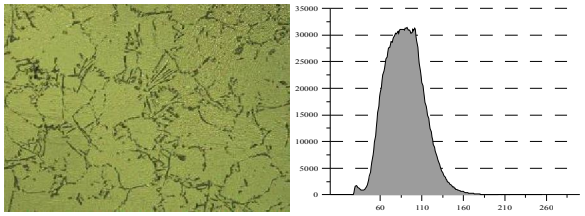


Fig. 8 Al alloy A356 metallographic image (200X) and gray scale distribution curve (for Exp. No.8)

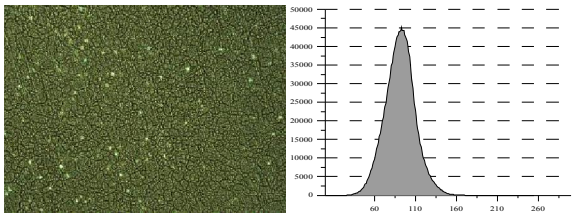


Fig. 9 Al alloy 6061 metallographic image (200X) and gray scale distribution curve (for Exp. No.1)

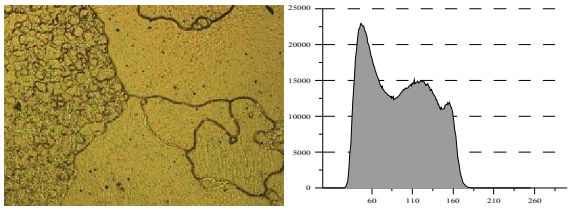


Fig. 10 Al alloy 6061 metallographic image (50X) and gray scale distribution curve (for Exp. No.10)

3.4 Metallography optimization analysis

Metallographic analyses were done based on factors level shown in Table 1; Matrox Inspector 4.0 software was used to perform image analysis. Gray scale with wider distribution and lower peak value has clear crystalline grain on its metallographic structure. This characteristic agrees with STB (the smaller the better). Fig. 11~13 show the S/N ratio results. The optimized factors for Al alloy 7075 metallographic analysis are A2, B3 and C1; For Al alloy A356 metallographic analysis, the optimized factors are A2, B3 and C1. The optimized factors for Al alloy 6061 metallographic analysis are A3, B3 and C3.

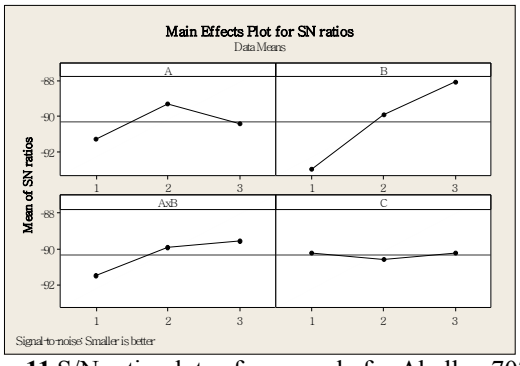


Fig. 11 S/N ratio plots of gray scale for Al alloy 7075

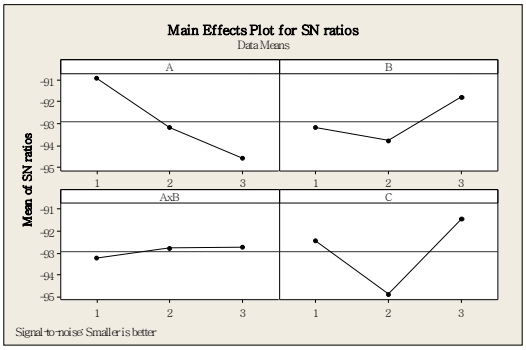


Fig. 12 S/N ratio plots of gray scale for Al alloy A356

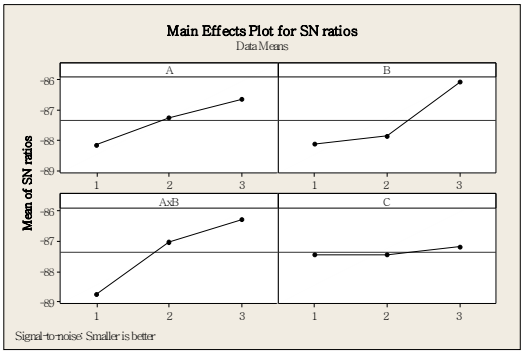


Fig. 13 S/N ratio plots of gray scale for Al alloy 6061

3.5 Metallographic gray scale variance analysis

Substitute S/N ratio into variance analysis calculation formula to perform the gray scale variance analysis to understand the optimized degree of contribution of each control factor on metallographic analysis. Analysis results are shown in Table 4 ~ Table 6. In Table 4, the major factor that affects Al alloy 7075 metallographic optimization is factor B (material etching time); In Table 5, the major factor that affects the metallographic optimization of Al alloy A356 is factor A (polishing solution); In Table 6, the major factor for the metallographic optimization of Al alloy 6061 is factor B (material etching time) if the factor AxB was not considered.

Table 4 Gray scale value variance analysis of Al alloy 7075

Factors	Degree of freedom *df*	Variation *S*	Variance *V*	Degree of contribution ρ
A	2	6.18	3.09	12.24
B	2	37.51	18.76	74.30
AxB	2	6.55	3.27	12.97
C	2	0.25	0.12	0.49
Total	8	50.49		100.00%

Table 5 Gray scale value variance analysis of Al alloy A356

Factors	Degree of freedom *df*	Variation *S*	Variance *V*	Degree of contribution ρ
A	2	20.55	10.28	44.35
B	2	6.13	3.06	13.22
AxB	2	0.43	0.21	0.92
C	2	19.24	9.62	41.51
Total	8	46.35		100%

Table 6 Gray scale value variance analysis of Al alloy 6061

Factors	Degree of freedom *df*	Variation *S*	Variance *V*	Degree of contribution ρ
A	2	3.45	1.72	16.68
B	2	7.48	3.74	36.21
AxB	2	9.59	4.79	46.41
C	2	0.14	0.07	0.69
Total	8	20.65		100%

3.6 Optimized metallographic analysis factors combination

The S/N ratios can determine the optimized metallographic analysis factors combination. The optimized combination factors are summarized in Table 7.

Table 7 Optimized metallographic analysis factors combination

Factor / Optimized comb. / Type	Polishing solution	Etching time	Polishing speed
7075	A2：Alumina 0.05micro	B3：30 s	C_1：200 rpm
A356	A1：Alumina 0.3 micro	B3：42 s	C_3：400 rpm
6061	A3：SiO_2 0.06 micro	B3：50 s	C3：400 rpm

4. Conclusion

(1)Matrox Inspector 4.0 software was used to perform image analysis. Gray scale with wider distribution and lower peak value has clear crystalline grain on its metallographic structure.

(2)The optimized factors for Al alloy 7075 metallographic analysis are A2(Alumina 0.05micro), B3(etching time 30 seconds) and C1(polishing speed 200rpm).

(3)The optimized factors for Al alloy A356 metallographic analysis are A1(Alumina 0.3micro), B3(etching time 42 seconds) and C3(polishing speed 400 rpm).

(4)The optimized factors for Al alloy 6061 metallographic analysis are A3 (SiO2 0.06micro),

B3 (etching time 50 seconds) and C3 (polishing speed 400 rpm).

(5)The optimized metallographic factors combination can be used on the development and design of the medical equipment parts in the future.

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3/3/2012

Comparison between Antioxidant Activities of Phenolic Extracts from Different Parts of Peanut

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Abstract: Peanut hull and skin are waste products of the food industry. Adding value to these products was the aim of this work. This goal was achieved by studying the phenolic content of the skin, hull and defatted flour of both roasted and unroasted peanuts. The antioxidant activity of the phenolic extracts was determined. The roasted peanut skin extract was then chosen and tested for its power of inhibition of flaxseed oil oxidation. The anticarcinogenic activity of the roasted peanut skin extract on different cell line carcinomas was examined. Both the extractable polyphenols (EPP) and the non extractable polyphenols (NEPP) were determined in the examined parts of the peanuts. Results revealed that NEPP was always higher than EPP and that highest phenolic content was found to be present in the skin. Roasted hull, unroasted hull, roasted skin, unroasted skin, roasted defatted flour, and unroasted defatted flour contained EPP 4.33, 3.38, 41.5, 56.2, 7.33 and 7.23 mg/g, respectively; and contained NEPP 7.4, 5.3, 102, 113.38, 10.3, and 13.4 mg/g, respectively; they also contain total polyphenol extract (TPE) 11.69, 8.73, 144.37, 169.19, 17.48, and 21.31 mg/g, respectively. Free radical scavenging activity (FRSA%) at 100µl conc. reached 87.0 % for roasted and unroasted peanut skin , ca. 79% for roasted and unroasted peanut hull, and least between 45.51-60.45% for unroasted and roasted defatted peanut flour, respectively. FRSA of BHT (0.1%) was 77.81%. Antioxidant activity (AOA) as measured by β-carotene/ linoleate method revealed AOA for roasted skin > unroasted skin > roasted hull > BHT > unroasted hull > unroasted defatted flour > roasted defatted flour, with values 89.13 > 86.65 > 80.33 > 76.33 > 75.27 > 39.34 > 30.37%, respectively. Roasted peanut skin extract (PSE) was chosen for further investigation. Percent reduction of oxidation in flaxseed oil when compared to control oil reached 27.10% and 22% for oil + PSE, and oil + BHT, respectively, as measured by p-anisidine value; and 41.99% and 38.88%, respectively, as measured by peroxide value. PSE exhibited potential as an anticarcinogenic agent, but needs further investigations.

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Key words: peanut hull, peanut skin, peanut flour, extractable polyphenol, non extractable polyphenol, antioxidant activity, anticarcinogenic activity.

1. Introduction

Peanut seed hulls and skins are considered as waste products of the food industry. Recently many seed hull extracts have been proven to exhibit antioxidant activities (Shahidi et al., 2006; Rao et al., 2010; Taha et al., 2011;Singer and Wagdy, 2011; Win et al., 2011). The antioxidant activities of these extracts are mainly due to presence of phenolic compounds. Phenolic compounds or polyphenols constitute one of the most numerous and widely distributed groups of substances in the plant kingdom and are an integral part of both human and animal diets (Panickar and Anderson, 2011). They are a structural class of organic chemicals characterized by the presence of one or more of phenol units (Quideau et al., 2011). The number and characteristics of these phenol structures underlie the unique physical, chemical and biological properties of particular members of the polyphenol class. Phenolic compounds can be divided into several groups including: phenolic acids, flavonoids, tannins, stilbenes and lignans (Han et al., 2007). The interest in phenolic compounds came from the discovery that they exhibit antioxidant properties (free radical scavenging and metal chelating activities) thus their possible beneficial implications in human health (Carrasco-Pozo et al., 2011). Flavonoids have applications as antibiotics, antiulcer, and anti-inflammatory agents (Oueslati et al., 2012), as well as in the treatment of diseases such as hypertension, vascular fragility, allergies, hypercholesterolemia (Bravo, 1998; Arts and Hollman, 2005). Epidemiological studies support the hypothesis that consumption of diets rich in fruits and vegetables decrease the risk of cardiovascular disease, diabetes, and cancer (Panickar and Anderson, 2011).

Peanut (Arachis hypogea L.) is an important oilseed crop. It is not only important for the production of oil, but also for direct consumption. They are consumed raw, roasted, pureed, or mixed with other foods or in different processed forms of which peanut butter is the most important. Recently, peanuts have gained much attention as functional food (Fransisco and Resurrection, 2008). Peanut

shells and skins are usually removed before processing or even when eaten as condiment. Shells and skins are sometimes burned or used in animal feed or as fertilizers. Peanut skin, shell, and kernel extracts were reported to exhibit different levels of antioxidant activity (Duh and Yen, 1997; Yu et al., 2005; Talcott et al., 2005 and Win et al., 2011). Roasting was reported to increase the antioxidant capacity of intact peanuts due to the formation of Maillard reaction products (Talcott et al., 2005). Fransisco and Resurrection (2009a) reported peanut skin to contain a complex series of procyanidin oligomers. While Win et al. (2011) studying peanut skins, hulls raw kernels and roasted kernel flour found that they contain p-hydroxy benzoic acid, chlorogenic acid (not detected in hull), p-coumaric acid (not detected in hull), Ferulic acid and epicatechin (present only in skin), resveratrol, quercetin (not detected in hull), luteolin (present only in hull), kaempferol (detected only in raw and roasted kernel flour).

Peanut is an international edible crop utilized in all countries. It would be an achievement if the huge amounts of hulls and skins resulting after its utilization could be upgraded to a valuable product by producing phenolic extracts from them. Peanuts are usually roasted before eating and before being added to many food recipes. The aim of the present research was to study the effect of roasting on the phenolic content and antioxidant activity of peanut hulls, skins, and defatted kernels (flour). Emphasis was made to extract the condensed tannins or nonhydrolysable polyphenols as they are usually an important but neglected part of the polyphenols. The antioxidant activity of the most effective peanut extract will be examined for their power to inhibit lipid oxidation.

2. Materials and Methods

Materials:

Peanut (*Arachis hypogea L.*) was brought from the local market. Peanuts were hulled and skinned manually, then ground using a pulverizor and sieved to pass through 60 mesh screen. The hulled skinned kernels were ground then defatted in a soxhlet apparatus using n-hexane. The defatted kernel (flour) was spread to dry, then reground in a Ball Mill and sieved to pass through 60 mesh screen. Peanuts with shells were roasted at 150°C in an air draft oven for 30 minutes.

Cell line Carcinomas:

Liver Carcinoma Cell Line (HEPG2), Colon Carcinoma Cell Line (HCT_{116}), Cervical Carcinoma Cell Line (HELA), Breast Carcinoma Cell Line (MCF7), were supplied and used in The National Cancer Institute, Biology Department, Cairo, Egypt.

Analytical methods:

Moisture, oil, protein, ash, crude fibre contents were determined in peanut hulls, skins and defatted flour according to A.O.A.C. (2005). Different crude phenolic extracts of the same samples were determined according to Hung et al. (2002).

Extractable polyphenols (EPP):

Powdered samples (500 mg) were extracted sequentially with 40 ml of methanol : water (50:50 v:v) and 40 ml of acetone: water (70:30 v:v) at room temperature for 60 min in each case, centrifuged at 2500 xg for 15 min. Combined extracts were made up to 100 ml with distilled water. EPP were determined by Folin Ciocalteu according to (Hung *et al.,* 2002) using Gallic acid as standard.

Condensed tannins or non hydrolysable polyphenols (NEPP):

The residue after centrifuge were treated with 40 ml conc. HCl in 1- butanol (50 ml/L) in a water bath at 100°C for 3 hrs, with occasional shaking, then centrifuged at 2000 xg, the supernatant was made up to 50 ml with the same solvent; and absorbance measured at 555 nm using tannin as standard.

The combined supernatants (EPP + NEPP) were designated total phenolic extract (TPE), and concentrated in a rotary evaporated at 50°C for the determination of the antioxidant activity, radical scavenging activity, and anticarcinogenic activity.

Antioxidant activity was determined by two methods: Free radical scavenging activity according to (Kuda*et al*., 2005). The second method used is the coupled oxidation of β- carotene/ linoleic acid method described by (Al-Shaikhan *et al.,* 1995).

Anticarcinogenic activity of the phenolic extract of roasted peanut skin was determined in the National Cancer Institute Cairo, Egypt (Biology Department) on several cell line carcinomas. This was determined by measurement of potential cytotoxicity of the phenolic extracts which was carried out by the Sulfo-Rhodamine-B stain (SRB) assay, according to the method of (Skehan *et al*., 1990).

Effect of phenolic extract on the inhibition of lipid oxidation

The oxidative stability of flaxseed oil with and without the addition of the phenolic extract resulting from roasted peanut skin was determined as follows: One hundred g flaxseed oil samples were stored in 200ml open beakers in a draught air oven at 60°C in the dark for 18 days. Combined phenolic extract (TPE) at 0.5% as well as 0.01% BHT were added to the oil samples. The oil samples were analysed at 2 days intervals to determine the progress in the formation of peroxide value (PV) and p-anisidine

value (p-AnV) of the oil. PV and p-AnV were determined according to (A.O.C.S, 1998).

Statistical analysis:

The results are presented as average ± standard deviation (SD). All results were evaluated statistically using analysis of variance according to McClave & Benson (1991).

Results and Discussion

Table 1 shows the chemical composition of roasted and unroasted peanut hull, skin and defatted flour. Roasting process decreased the crude fibre content in the hull and skin. On the other hand the protein content in the hull, skin and flour increased upon roasting. Oil and ash contents were hardly affected by roasting. Protein content of hull, skin, and flour were 3.9, 11.8, and 52.4%, respectively, upon roasting they reached 4.9, 13.0, and 56.0%, respectively. Statistical analysis indicated a significant difference at $p<0.05$ between roasted and unroasted peanut hull and skin for all the examined constituents. There was no significant difference in ash content of both roasted and unroasted hulls. On the other hand, roasting caused a significant difference between the moisture and protein contents of the roasted meal and the unroasted meal.

Kerr *et al.* (1986) reported that peanut hull contains 8.2% protein. While Hegazy *et al.* (1991) analysed peanut hull flour and found it to comprise 7.92 % moisture, 6.90% protein, 1.30% oil, 4.23% ash and 49.2% crude fibre. No chemical composition has been reported in the literature for peanut skin. Batal *et al.* (2005) studying the nutrient composition of 17 peanut meal samples, they reported that crude protein ranged between 40.1-50.9 with a mean of 45.6% and mean values of oil, fibre and ash were 2.5, 8.3 and 5.0%, respectively. While studying the physicochemical characterisation of heat (HPF) and cold (CPF) pressed peanut meal flours, Juliana and Zhengxing (2008) found that HPF contained 5.9% moisture, 49.8% protein, 0.9% fat, 8.6% ash and 8.0% crude fibre; and CPF contained 7.1% moisture, 52.1% protein, 1.6% fat, 7.6% ash and 9.7% fibre.

Table 1: Chemical Composition of Peanut Hull, Skin and Defatted flour.

Sample	Moisture %	Protein %	Oil %	Ash %	Fiber %	NFE %
Skin	8.01±0.1^{b}	11.8±0.20^{b}	7.1±0.45^{a}	2.1±0.11^{b}	55.0±0.17^{a}	15.9±0.29^{b}
Roasted skin	8.5±0.29^{a}	13.0±0.31^{a}	6.3±0.26^{b}	2.7±0.21^{a}	50.0±0.69^{b}	19.4±0.45^{a}
L.S.D.	0.0740	0.4532	0.2325	0.2222	0.4038	0.2772
Hull	8.5±0.040^{a}	3.90±0.12^{b}	0.13±0.02^{a}	3.10±0.22	60.0±0.40^{a}	23.47±0.28^{b}
Roasted hull	6.41±0.40^{b}	4.90±0.10^{a}	0.1±0.02^{b}	3.20±0.12	58.0±0.11^{b}	27.39±0.30^{a}
L.S.D.	0.0535	0.2267	0.0227	0.000	0.3322	0.5074
Defatted flour	7.3±0.33^{b}	52.40±0.53^{b}	0.50±0.02	5.10±0.22	8.3±0.18	26.40±0.05^{a}
Roasted Defatted flour	7.82±0.13^{a}	56.00±0.15^{a}	0.50±0.03	5.37±0.03	8.00±0.10	22.41±0.29^{b}
L.S.D.	0.0520	0.2240	0.000	0.000	0.000	0.5563

Different letters in each column (between roasted and unroasted) indicates significant differences at P<0.05, ± = Standard deviation, NFE= Nitrogen Free Extract

Phenolic compounds in different parts of roasted and unroasted peanuts

Phenolic compounds have attracted much interest recently because *in vitro* and *in vivo* studies suggest that they have a variety of beneficial biological properties which may play an important role in the maintenance of human health. Phenolic compounds exhibit a wide range of physiological properties, such as antioxidant, anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardioprotective and vasodilatory effects (Benavente-Garcia *et al.,* 1997; Samman *et al.,* 1998; Puupponen-Pimia *et al.,* 2001; Manach, *et al.,* 2005).

According to the solubility criterion, polyphenols may be classified into extractable polyphenols (EPP) and nonextractable polyphenols (NEPP) (Saura-Calixto *et al.,* 2007). EPP are low and intermediate molecular mass phenolics that can be solubilized in organic or aqueous organic solvents. NEPP are high molecular mass compounds (proanthocyanidins or condensed tannins, and hydrolysable tannins) or polyphenols bound to other food matrix components such as dietary fibre and protein that can be found in the residues of aqueous organic extracts (Sayago-Ayerdi *et al.,*2007). Most studies on food polyphenols and dietary intake address exclusively EPP. In fact most studies ignore NEPP which remain in the residue, although these compounds possess high bioactivities. Arranz *et al.* (2009; 2010) in their study of EPP and NEPP in fruits reported that the amount of NEPP (112-126 mg/100g of fresh fruit) was much higher than the EPP (18.8-28mg/100g of fresh fruit). Most peanuts are eaten roasted. The determination of EPP and NEPP in the hull, skin and defatted meal of roasted and unroasted peanuts seemed important.

Table 2: Effect of roasting peanuts on EPP, NEPP and TPP extracts from different parts.

Sample	EPP(mg/g)	NEPP(mg/g)	TPE(mg/g)
Skin	56.2±0.53 [a]	113.38±0.83. [a]	169.19±0.18 [a]
Skin roasted	41.5±0.3 [b]	102±1.07 [b]	144.37±0.57 [b]
LSD (5%)	2.1716	0.9737	0.950
Hull	3.38±0.14 [b]	5.3±0.38 [b]	8.73±0.44 [b]
Hull roasted	4.33±0.13 [a]	7.4±0.46 [a]	11.69±0.40 [a]
L.S.D. (5%)	0.3117	0.952	0.9534
Defatted flour	7.23±0.41 [b]	13.4±0.46 [a]	21.31±0.47 [a]
Roasted defatted flour	7.33±0.12 [a]	10.3±0.31 [b]	17.48±0.50 [b]
L.S.D. (5%)	0.504	0.8827	1.104

EPP= Extractable polyphenol, NEPP=Nonextractable polyphenol, TPE = Total polyphenol extract (EPP+NEPP).
Different letters in each column (between roasted and unroasted) indicates significant differences at $P<0.05$, ± = Standard deviation

Table 2 gives the values for EPP, NEPP and TPE (EPP + NEPP) for roasted and unroasted hull, skin, and defatted flour of peanuts. Results in table reveal that the NEPP was always higher than the EPP as stated by (Sáyago- Ayerdi *et al.,* 2007; Arranz *et al.,* 2009; 2010). Thus it is important to determine this part of the polyphenols that has been usually ignored. It is noteworthy to mention than NEPP have bioactive properties (Arranz *et al.*, 2009). Hydrolysable tannins are hydrolysed by week acids or weak bases to produce carbohydrate and phenolic acids. Almost all phenolic acids possess antioxidant properties as well as other bioactivities. The condensed tannins or proanthocyanidins also possess biological activities (Hongxiang *et al.,* 2004; Park *et al.,* 2011). Statistical analysis indicated a significant difference between roasted and unroasted samples at $p<0.05$ for EPP, NEPP and TPE. Highest phenolic content was found to be present in the skin part. Several authors reported peanut skin to be very rich in phytochemicals (Yu *et al.,* 2007; Win *et al.,* 2011). Roasting of the hull increased EPP, NEPP, and naturally TPE. On the contrary, roasting decreased PP compounds in the skin and defatted flour. Win *et al.* (2011) reported that roasting increased total phenolic compounds, radical scavenging activity and inhibition % of linoleic acid peroxidation in peanut kernel flour. Yu *et al.* (2005) found that among several processing methods and extraction solvents, the combination of roasting and ethanol extraction were the most efficient method in extracting phenolics with high antioxidant activity from peanut skins.

Antioxidant activity of extracts from different parts of roasted and unroasted peanuts:

The antioxidant activity (AOA) of phenolic compounds may result from the neutralization of free radicals initiating oxidation processes or from the termination of radical chain reactions. Also AOA of phenolic compounds is due to their high tendency to chelate metals. In this investigation two different methods have been used for the determination of the AOA of the extracts: the first method is the DPPH free radical scavenging activity (FRSA) and second method, is the inhibition of β-carotene co-oxidation in a linoleate model system. In the first method DPPH* is used, it is one of the free radicals widely used for testing preliminary radical scavenging activity of a compound or plant extract. The principle involved in this method is that the antioxidants (phenolic extracts) act with the stable free radical on DPPH* (having a deep violet color) and convert it to DPPH with discoloration.

Table 3: Radical Scavenging Activity of Roasted and Unroasted Different Peanut Extracts.

Sample	25µl	50µl	100µl	L.S.D.
Skin	81.53±0.16 [a/3]	85.33±0.30 [a/2]	87.33±0.51 [1]	0.3982
Roasted skin	80.36±0.21 [b/3]	84.76±0.15 [b/2]	87.10±0.27 [1]	0.4014
L.S.D. (5%)	0.4190	0.5441	0.00	-------
Hull	73.9±0.36 [a/2]	78.63±0.15 [a/1]	78.77±0.15 [a/1]	0.3059
Roasted hull	73.7±0.46 [b/3]	75.23±0.13 [b/2]	79.20±0.20 [b/1]	0.3143
L.S.D. (5%)	0.9316	0.3246	0.4006	------
Defatted flour	34.39±0.34 [b/3]	41.27±0.35 [b/2]	45.51±0.16 [b/1]	0.3467
Roasted defatted flour	36.67±0.32 [a/3]	56.47±0.46 [a/2]	60.45±0.17 [a/1]	0.3997
L.S.D. (5%)	0.7451	0.9167	0.3680	------
BHT (0.1%)	54.6 ± 0.365	72.61±0.247	77.81±0.624	

Different letters in each columns (between roasted and unroasted) indicates significant difference at ($p<0.05$), different numbers in rows (between concentrations) indicates significant difference at $p<0.05$, ± = standard deviation.

Table 3 indicates the antioxidant activity (AOA) of EPP, NEPP and TPE for skin, hull and meal of peanuts as determined by the FRSA method. Peanut skin extracts exhibited the highest FRSA followed by hull extracts then the meal extracts with values of87.33, 78.77, and45.51%, respectively, compared to 77.81% for BHT which means that the TPE of peanut skin is superior to BHT(0.1%), and rather close to hull extract but much higher than the defatted flour extract. The FRSA of the different parts of peanut were well correlated with the amount of TPE for the same parts. Statistical analysis revealed a significant difference between roasted and unroasted skin, hull and meal except for roasted and unroasted peanut skin at 100µl extract where there was no significant difference. As for the statistical analysis between different concentrations of the same extract, there was a significant difference at $p<0.05$ between the same extract at all three concentrations. Unroasted hull extract at both 50 and 100µl showed no significant difference. Roasting caused increase in FRSA of roasted defatted flour (60.45) over unroasted defatted flour (45.51). This is in agreement with Win *et al.* (2011) who reported that roasting increased total phenolic compounds, radical scavenging activity and % inhibition of linoleic acid peroxidation in peanut kernel flour.

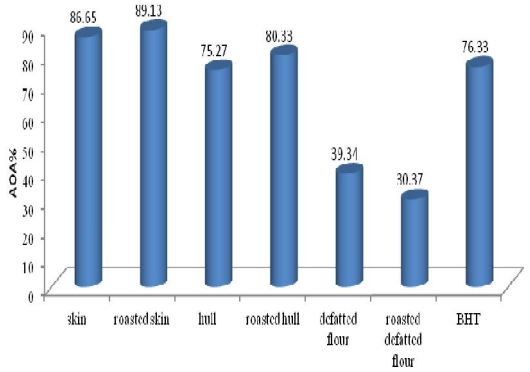

Figure 1: Antioxidant activity (AOA %) of roasted and unroasted parts of peanuts as measured by β-carotene/linoleate method

Antioxidant activity as measured by β-carotene/linoleate method is given in Figure1. It is clear from the figure that peanut skin TPE both roasted and unroasted demonstrate higher AOA than the hull and defatted flour extracts, and even higher than BHT(0.1%). This is in accordance with the results of the phenolic content and FRSA. AOA for roasted skin > skin > roasted hull > BHT >hull > defatted flour > roasted defatted flour, with values 89.13 > 86.65 > 80.33 > 76.33 > 75.27 > 39.34 > 30.37% AOA. Statistical analysis between roasted and unroasted skin, hull, and meal displayed a significant difference at $p<0.05$. Roasting resulted in an increase in AOA of the skin and hull but not the meal. Win *et al.* (2011) reported on the antioxidant activity of peanut skin, hull, roasted kernel flour phenolic extracts as indicated by FRSA% and % inhibition of linoleic acid peroxidation, and that the skin was the highest in phenolic compounds and AOA. Several authors displayed the high AOA of peanut skins (Nepote *et al.,* 2002; Yu *et al.,* 2005; Hoang *et al.,* 2008; Chukwumah *et al.,* 2009). The antioxidant activity of different peanut parts is due to the presence of a group of phenolic compounds (Yen and Duh 1995; Nepote *et al.,* 2002; Ali and Abdedaiem, 2010; Win *et al.,* 2011). Peanut phenolic compounds reported in the literature include: resveratrol in the methanolic extract of the skin (Ballard *et al.,*2009), total catechinsprocyanidin dimers, trimmers and tetramers identified in directly peeled peanut skins (Yu *et al.,* 2007), and A-type proanthocyanidins in roasted peanut skins (Monagas *et al.,* 2009). A reversed phase high performance liquid chromatography was developed for the simultaneous determination of five phenolic compounds, two stilbenes and eight flavonoidsin peanut skins extract (Francisco and resurrection, 2009b). They reported the presence of gallic, protocatechuic, epigallactocatechin, catechin, β-resorcyclic (internal standard), caffeic, procyanidin B_2, epicatechin, epigallocatechingallate, p-coumaric, ferulic, piceid, epicatechingallate, catechingallate, resveratrol and quercetin. While (Win *et al.,* 2011) found that p-hydroxybenzoic acid and resveratrol was present in skin, hull, raw kernel and roasted kernel flour of peanuts chlorogenic acid, and p-coumaric acid were present in the skin, raw and roasted kernel flour. Ferulic acid and epicatechin were present only in the skin of peanuts. Luteolin was detected only in the hull and kaempferol detected only in the raw and roasted kernel flour. This diversity in phenolic compounds would act in synergism with one another ending up with quite a strong antioxidant activity of peanuts, especially the skin.

Effect of peanut skin phenolic extract (PSE) on the oxidative stability of Flaxseed oil

The oxidative stability of oils and fats is one of the most important parameters for their quality assessment. A number of methods for such assessment have been developed. Here flaxseed oil has been subjected to accelerated oxidation at 60^{O}C for 18 days. The control sample was flaxseed oil without any addition, and flaxseed oil with BHT added at 0.01%, and the investigated sample was flaxseed oil with added 0.5% roasted peanut skin

extract (PSE). PV and p-AnV were measured every two days and their values were taken as an indication of the oxidative stability of the oil.

Table 4: Storage Stability of Flaxseed Oil (control), Flaxseed Oil + Peanut Skin Extract, and Flaxseed Oil + BHT as Measured by Increase in Peroxide Value (meq/Kg)

Storage (days)	control	Peanut skin extract	BHT
Zero	0.95 ± 0.612	0.95 ± 0.469	0.95 ± 0.425
3	15.25 ± 0.382	10.15 ± 0.672	7.25 ± 0.359
6	28.15 ± 0.536	19.09 ± 0.736	16.95 ± 0.561
9	39.95 ± 712	28.15 ± 0.355	29.95 ± 0.681
12	51.09 ± 0.666	33.75 ± 0.711	33.15 ± 0.712
15	69.09 ± 0.358	41.05 ± 0.582	43.05 ± 0.483
18	86.8 ± 0.539	50.35 ± 0.638	53.05 ± 0.569

BHT= Butylated hydroxyl toluene (0.01%)
Value is the average of four replicates ± standard deviation.

Table 5: Storage Stability of Flaxseed Oil (control), Flaxseed Oil +Peanut Skin Extract, and Flaxseed Oil + BHT as Measured by Increase in p-Anisidine value

Storage (days)	Control	Peanut skin extract	BHT
Zero	4.09 ± 0.621	4.09 ± 0.621	4.09 ± 0.621
3	10.80 ± 0.392	6.80 ± 0.298	7.60 ± 0.456
6	18.10 ± 0.0	10.30 ± 0.512	11.10 ± 0.551
9	26.70 ± 0.0	19.20 ± 0.617	15.40 ± 0.716
12	35.40 ± 0.0	20.40 ± 0.432	20.80 ± 0.501
15	45.70 ± 0.0	24.80 ± 0.339	26.10 ± 0.723
18	55.60 ± 0.0	28.50 ± 0.531	33.60 ± 0.653

BHT= Butylated hydroxyl toluene (0.01%)
Value is the average of four replicates samples ± standard deviation.

Tables 4 and 5 represent the PV and p-AnV, respectively, for the control oil, oil + PSE, and oil + BHT. It is evident from the tables that PSE and BHT both inhibited oxidation of flaxseed oil to almost close levels during the first 12 days. At day 15 and day 18, 0.5% PSE delayed oxidation of the oil more than 0.01% BHT. At zero day the three oil samples had a PV 0.95meq/Kg then the PV developed to 86.8, 50.35, 53.05 meq/Kg, at day 18 of heating for control oil, oil + PSE, oil + BHT, respectively. This result indicates 41.99% reduction in oil oxidation due to PSE addition and 38.88% reduction due to BHT addition compared to control. On the other hand, at zero day the three oils showed 4.09 p-AnV which increased during the period of the experiment to reach at end of the 18 days 55.60 for control oil, 28.50 for oil + PSE, and 33.60 for oil + BHT p-AnV. Reduction in oil oxidation when compared to control oil reached 27.10% and 22% due to oil + PSE, and oil + BHT, respectively.

The antioxidant activity of methanolic extracts of peanut hulls was tested in soybean and peanut oils that were subjected to accelerated oxidation (Duh and Yen, 1997). They reported similar results that hull extracts at all tested concentrations displayed an AOA and that at 0.48and 1.20% hull extracts delayed oil oxidation more than 0.02% BHA. Hoang *et al.* (2008) found that ethyl acetate phenolic skin extract from both conventional and high oleic acid peanuts exhibited moderate antioxidant activity in lard or rapeseed oil. This was indicated by the Schaal Oven Test. While the reducing power, FRSA, inactivation of hydroxylic and superoxide free radicals were medium, comparable to synthetic antioxidants. O'Keefe and Wang (2006) studied the effect of peanut skin extracts on quality and storage stability of beef products (ground and ground with added salt, phosphate and nitrite/erythorbate).The reduction in oxidation was pronounced in ground beef and ground beef with salts as indicated by color and TBARS. Contrary to our findings (Nepote *et al.,* 2002) tested the AOA of peanut skin on sunflower oil by applying accelerated oxidation. Their results show the peanut skin extracts did not reach the activity level of BHT.

Anticarcinogenic activity of roasted peanut skin extract (PSE)

This evaluation was carried out in the National Cancer Institute, Biology Department, Cairo. The

experiment was done by the Sulfo-Rhodamine-B stain (SRB) assay. Roasted peanut skin phenolic extract has been chosen and evaluated as a chemopreventive agent. This was established by testing the (PSE) for any cytotoxic activity against the following human tumor cell lines: Liver Carcinoma Cell Line (HEPG2); Colon Carcinoma Cell Line (HCT_{116}); Cervical Carcinoma Cell Line (HELA); and Breast Carcinoma Cell Line (MCF7).

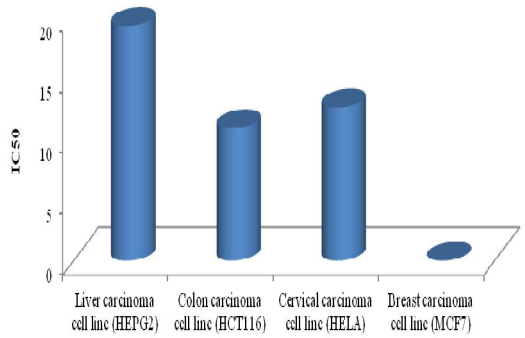

Figure 2: Anticarcinogenic activity of roasted peanut skin extract on some cell line carcinomas.

Figure 2 represent the effect of (PSE) on the human carcinoma cell lines tested and the results are indicated by the IC50, which is the dose of the compound (PSE) which kills surviving cells up to 50%. The smaller the concentration or dose the more effective is the compound. Looking at Figure 2, the following could be observed:

That (PSE) was most effective on Colon Carcinoma Cell Line (HEPG2) with an IC50 = 10.9 µg/ml. This means that at this dose of PSE, 50% of the surviving cells were killed.

PSE was next more effective on Cervical Carcinoma Cell Line (HELA)with a bit higher IC50 = 12.6µg /ml.

Liver Carcinoma Cell Line (HEPG2) needed a higher dose of (PSE) to reach IC50. IC50 = 19.3µg/ml.

PSE had no effect on Breast Carcinoma Cell Line (MCF7).

These results indicate that (PSE) possess potential anticarcinogenic properties, but as recommended by the Biology Department, National Cancer Institute, Cairo further pharmacological investigations in vitro and in vivo are required to confirm the activity of the tested extract.

Francisco and resurrection (2009b) reported the presence of five phenolic compounds, two stilbenes and eight flavonoids in peanut skin extract gallic, protocatechuic, epigallactocatechin, catechin, β-resorcyclic (internal standard), caffeic, procyanidin B_2, epicatechin, epigallocatechingallate, p-coumaric, ferulic, piceid, epicatechingallate, catechingallate, resveratrol and quercetin. While (Win *et al.,* 2011) found that p-hydroxybenzoic acid and resveratrol, chlorogenic acid, and p-coumaricacid, Ferulic acid and epicatechin were present in the skin of peanuts. Many of these compounds are reported to exhibit anticarcinogenic properties (Block *et al.,* 1992; Potter *et al.,* 2002; Soobrattee *et al.,* 2005; Srinivasan *et al.,* 2007; Actis-Goretta *et al.,* 2008; Taha *et al.,* 2012).

Conclusion

It can be thus concluded that peanut skin, although it constitutes the least part of the peanut yet it is the most valuable part due to its biological activity. It displays high antioxidant activity and is a potential anticarcinogenic agent. Roasting of the whole seed increases the antioxidant activity of the skin. Thus roasted peanut skin extracts can be used safely in the edible oil industry to delay its oxidation. It can be applied in other food industries as a natural antioxidant instead of synthetic antioxidants. Further biological studies are expected to show positive results.

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A Conceptual Framework of the Relationships between Family Functioning, Alexithymia and Emotional Intelligence among Early Adolescents in Tehran-Iran

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Abstract: Emotional intelligence structure is a concept with little empirical research, particularly in relation to the link between family circumstance and personality characters and with respect to gender, number of sibling, family income, and family educational status of early adolescents. In the present study, the researchers attempted to show that regarding to the definition and foundation of a conceptual framework how would design a conceptual framework according the relationships between family functioning, alexithymia and emotional intelligence variables, among Iranian early adolescents. Due to the lack of research on family functioning, alexithymia and their influences on early adolescents' emotional intelligence in Iran, there is much need for research that explores those factors of influence on early adolescent's emotional intelligence, specifically.
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Keywords: Family Functioning, Early Adolescent's Emotional Intelligence, , Alexithymia, Children's Alexithymia, Emotional Quotient, Conceptual Framework, Family Background

1. Introduction

Emotional intelligence is a set of abilities that include conception, emotion appraisal and expression, emotion management and regulation, as well as emotion utilization for affective involvement (Goleman 1995); these abilities had been the focus in this research. The family has the highest effect on the individuals and it can mould their behaviors at any moment. A behavior which is created in relation to other family members is not limited to a normal agreeable behavior. The family can form abnormal behaviors, too (Sanaei, 2000). Considering, alexithymia is not classified as a mental disorder in the DSM-IV. It refers to the difficultly to identify, describe feelings to other people and it is a dimensional personality characteristic that varies in severity from person to person thus, family as a first circumstance can create this kind of characters. In addition, the treatment by parents to their children and how they react to their interests and activities, as well as children treatment to one another, emotion and information exchange among them, emotional protection to one another, and the relationships of the family members' with outsiders may also influence the children's emotional intelligence (Naghavi, 2010). The family functioning construct is a relatively new concept with little empirical research, particularly related to the link between seven specific sub-components of the family function (dysfunction) and their emotional intelligence's early adolescent.

A conceptually similar emotional intelligence construct is alexithymia. A comparison of the definitions of emotional intelligence and alexithymia suggests that the two constructs are closely related (Parker *et al*., 2001). Meanwhile, the emotional intelligence construct emerged from an integration of an array of research findings on how people appraise, communicate and use emotion (Salovey & Mayer, 1989, 1990). Although psychological systems have negatively looked into emotions, the attention given to emotions and feelings can be regarded as the core and basis of psychology and one can therefore look for mental disturbances roots in emotional perturbations like fear, anxiety, depression and alexithymia (Naghavi et al., 2010). Moreover, there is empirical evidence indicating that alexithymia is associated with the difficulties in discriminating among different emotional states (Bagby *et al.,* 1993). In research studies by Salovey and Mayer (1989, 1990), the overlapping emotional intelligence and alexithymia constructs were acknowledges, and the researchers made attempts to empirically evaluate the relationships between the two constructs. One possible explanation for this is that these investigators have yet to introduce a standardized method for assessing emotional intelligence.

It is understood from the previous studies that emotional intelligence is associated with some factors, such as family function and some personality characters like alexithymia. This research studied the relation between family functioning and emotional intelligence so as to develop and expand the concept of emotional intelligence in the family. In other words, the importance of family functioning on

alexithymia and emotional intelligence has been found to be very significant. It is expected that this research would identify different family functioning dimensions have influences on early adolescents' emotional intelligence. Furthermore, with respect to the current context of Iran, the society is changing from a traditional to a modern society. In this situation, family are facing different and more challenging roles and responsibilities compared to those were in the past. With their increased level of knowledge and growing mobility, they have become more participative in parenting, which could lead them to have more sensitivity to developing their children's emotional, personality, social, and so on. However, they may lack knowledge of how to go about it. This has created a gap in the body of knowledge, for this and many other reasons stated earlier on, the current study investigated on the relationship between family functioning, alexithymia and emotional intelligence of early adolescents.

2. Relationship between Family Functioning and Emotional intelligence

There has been a growing interest in the issues of family functioning and emotional intelligence of early adolescents (Tamplin, Goodyer & Herbert, 1998; Gottman, 1997; Walsh, 1993, Patterson, 1995; Ozbaci, 2006; Manuel, 2002; Yamada, 2004) and the factors influencing them (Goleman, 1996; Mayer & Salovey, 1990; Carson & Parke, 1996; Palmer *et al*., 2007; Brudy & Hall, 2000; BarOn, 1997; Martinez-Pons, 1997; Schutte, 1998) to develop more integrated theories of development (McMaster, 1995; Epstein, Bishop, & Levin, 1960; Goleman, 1995; Tamplin *et al*., 2002). In fact, emotional intelligence (EI) has recently attracted a lot of interest in research on family functioning. Several new findings recently obtained have shown that parents with emotional intelligence are helpful. That is how family members deal with each other's feelings, and apart from possessing a basic role in their direct behaviours towards their children, they also model such interactions to them. As indicated earlier on, early adolescents pay attention to the emotional interactions of the family. Gottman (1997) pointed out that good parenting requires not only intellect but also involves emotion. In the last decade or so, science has discovered a tremendous amount of roles emotions play on our lives. Meanwhile, researchers have found that more than IQ, emotional awareness and the ability to handle feelings can determine success and happiness in all lifestyles, including family relationships. For parents, this quality of emotional intelligence (as many now call it) means being aware of early adolescence's feelings and also being able to empathize, soothe, and guide them. For early adolescents who learn most lessons about emotions from their family, this includes the ability to control impulses, delay gratification, motivate them, read other people's social cues, and cope with life's difficulties. In addition, early adolescents, whose parents consistently practice emotion coaching, have better physical health and score higher academically than those whose family do not offer such guidance.

According Yamada (2004), children of depressed family are at increased risk of emotional difficulties and behavioural problems. Nonetheless, little is known about the effects of maternal depression on early adolescence's emotional intelligence. The purpose of this research was to examine several broad dimensions of emotional intelligence in children aged 7 to 8 years old whose mothers were with or without any background of depression. The preliminary findings indicated that gender differences in children's emotional intelligence, with an advantage for girls and an association between infant temperament and children's abilities of emotional intelligence at 7 year old. According to Naghavi & Ma'rof (2012), today in the Iran, Iranian families have started to take on roles vastly different from family of previous generations. Moreover, family takes on ever more responsibility for raising their early adolescents than in the generations that preceded them. As a result, they discovered that introverted children with high level of emotional intelligence were influenced more by the family environment as compared to extroverted children with low emotional intelligence. Therefore, the purpose of the study was to determine and describe the correlations between family functioning and emotional intelligence in the context of Iran.

3. Relationship between Emotional Intelligence and Alexithymia

A conceptually similar emotional intelligence construct is alexithymia. A comparison of the definitions of emotional intelligence and alexithymia suggests that the two constructs are closely related (Parker *et al*., 2001). Meanwhile, the emotional intelligence construct emerged from an integration of an array of research findings on how people appraise, communicate and use emotion (Salovey & Mayer, 1989, 1990). The salient features of the alexithymia construct include the difficulties in identifying and describing subjective feelings, a limited imaginative capacity, and an externally oriented style of thinking (Taylor *et al*., 1991, 1997). Moreover, there is empirical evidence indicating that alexithymia is associated with the difficulties in discriminating among different emotional states (Bagby *et al.,* 1993), and with a limited ability to think about and use

emotions to cope with stressful situations (Scheaffer, Mendenhall & Ott, 1990; Parker *et al*., 1998). In research studies by Salovey and Mayer (1989, 1990), the overlapping emotional intelligence and alexithymia constructs were acknowledges, and the researchers made attempts to empirically evaluate the relationships between the two constructs. One possible explanation for this is that these investigators have yet to introduce a standardized method for assessing emotional intelligence. In addition, the definition of the constructs was recently operationalized by Schutte *et al*. (1998) who had developed and validated a 33-item self-report scale. In a mixed, but rather small, university student and community sample (n=25), this scale correlated strongly and negatively (r=-0.65) with the 26-item Toronto Alexithymia Scale.

4. Relationship between Family Functioning and Alexithymia

The record of studies on the relationship between family functioning and alexithymia was that there are both direct and indirect family influences on alexithymia. Lumley, Mader, Gramzow, and Pepineau (1996) showed the family and parental correlations of alexithymia in their research entitled, "Family factors related with alexithymia characteristics". This research composed of two parts; namely, the relation between the cognitive and emotional characteristics of alexithymia and family malfunctioning and mothers' alexithymia, and both were studied. In the first part, 127 young people were assessed using alexithymia scale (TAS-20). To assess impaired imagination, Cohen's Scored Archetypal Test, comprising of nine factors, was used and the FAD instrument was also applied to assess family malfunctioning. In the second part, 80 of their mothers filled in the TAS-20 about themselves. The correlation of mothers' alexithymia characteristics was studied using the same characteristics in their children. The results showed that the overall family pathology is associated with alexithymia. In particular, the difficulty in identifying feelings is associated with the affective involvement of a malfunctioning family, thinking with external orientation and partial control of family performance, and defective imagination with inefficiency in solving family problems. In the second part, the characteristics of mothers' alexithymia were shown to have a meaningful correlation with their children's alexithymia. This finding also showed confused family functioning and mothers' alexithymia are responsible in generating the characteristics of their children's alexithymia.

Tamplin *et al*. (1998) conducted a research with the title, "Family functioning and parents' general health in families having basically depressed adolescents". Using FAD measuring instrument and GHQ, they compared family functioning and parents' general health in families with depressed adolescents (n = 60) and with a control group (n = 34). The average FAD scores and general health for the clinical group were found to be meaningfully worse than that of the control group. In 56% of the clinical group's families and 29% of the control group's families, the practical criteria of malfunctioning were observed. In addition, Naghavi (2011), demonstrated that early adolescent develop specific alexithymia characteristics as a result of family dysfunction in emotional or cognitive domains in her study. Berenbaum and James (1994) demonstrated the relation between alexithymia and family ambience in their research entitled, "Associates and retrospective history of alexithymia." Their research was carried out among college students and the findings revealed that higher degrees of alexithymia had a meaningful correlation with decreased expression in the family and lower emotional security feeling in childhood. Therefore, this paper presents the conceptual framework of the relationship between family functioning, alexithymia, family background and emotional intelligence of early adolescents.

5. Conceptual Framework

Miles and Huberman (1994) defined a conceptual framework as a visual or written product, one that "explains, either graphically or in narrative form, the main things to be studied—the key factors, concepts, or variables—and the presumed relationships among them". The conceptual framework developed for this study integrates the family functioning model, alexithymia and emotional intelligence theory. Figure .depicts the conceptual framework developed for the present study. The theoretical perspectives and literature review guide the identification of variables and the development of the conceptual framework.

As illustrated in Figure 1 the conceptual framework of this study focused the factors affecting the skills of the early adolescents' emotional intelligence. This chart is divided in two parts; the first part is related to the assessment of the relationship between gender, family size, family income, family education status, and early adolescents' emotional intelligence, whereas the second part indicates the relationship between family functioning, alexithymia and early adolescents' emotional intelligence. Hence, the conceptual framework describes the relationships of family functioning, alexithymia and emotional intelligence. The framework, which is based on a theoretically derived conceptualization, incorporates family and

early adolescence. According to the relevant literature, early adolescents learn to express, understand, and regulate their emotions in interactions with their parents and siblings. Furthermore, it indicates that families are strong shapers of early adolescence's performance and their emotion is an important aspect of family functioning (Dunsmore & Halberstadt, 1997; Stover, 2003). Therefore, the second part of the conceptual framework illustrates the relationship between family as emotional coaches and early adolescents' emotional intelligence and their social behaviours. Hence, the focus of the conceptual framework is on the relationships between the independent variables of family functioning (namely, problem solving, communication, roles, affective responsiveness, affective involvement and behavioural control) and alexithymia (i.e. difficulty in identifying feelings, difficulty in describing feelings and thinking with external orientation) as well as dependent variables of emotional intelligence (which include emotional conception and appraisal, emotion regulation and emotion utilization).

In this research, the relationships between the demographic variables and the key variables were also examined. As for the association between gender and emotional intelligence, according to the conceptual framework shown in Figure 1, there are several notable differences between the male and female adolescents. However, statistically significant gender differences do exist for several factors natured by emotional intelligence and girls were found to have higher emotional intelligence than that of boys (Katyal & Awasthi, 2005).

As for family size, family income and family education status in relation to emotional intelligence, there are many assumptions about them. Some existing research indicated slight but significant relationships between emotional intelligence and family size, family income, and family education status. According Naghavi & Ma'rof (2012), parental emotion affect on early adolescent's emotion and social behaviors by its emotional regulation. Hughes and Carolyn (2002) pointed that our first parents during childhood are our siblings. Our relationships with them, even during childhood, include components which will later become significant in our relationships as adults. Some of these components include mutual dependence, role division, emotional communication and problem solving, agreement and conflict, as well as cooperation and mental health. In addition, studies around the world have consistently shown that family income is related to early adolescence's cognitive social development, emotional intelligence, personality characters and academic achievement. Similarly, Blau, (1999) believe that early adolescence of lower-class families are more likely to have low emotional intelligence and social performance and do poorly on standardized tests as compared to those early adolescents in the middle- and upper-class families. Furthermore, some researchers have found that higher educated family evinces higher emotional intelligence of early adolescents. Wiltfang & Scarbecz, (1990) have shown the importance of family environment, so the definition also covers the characteristics that determine the social status of the family, such as education level, occupational status and professions of the parents, as well as the quality of the residence, the working conditions of the parents and the relationships between siblings.

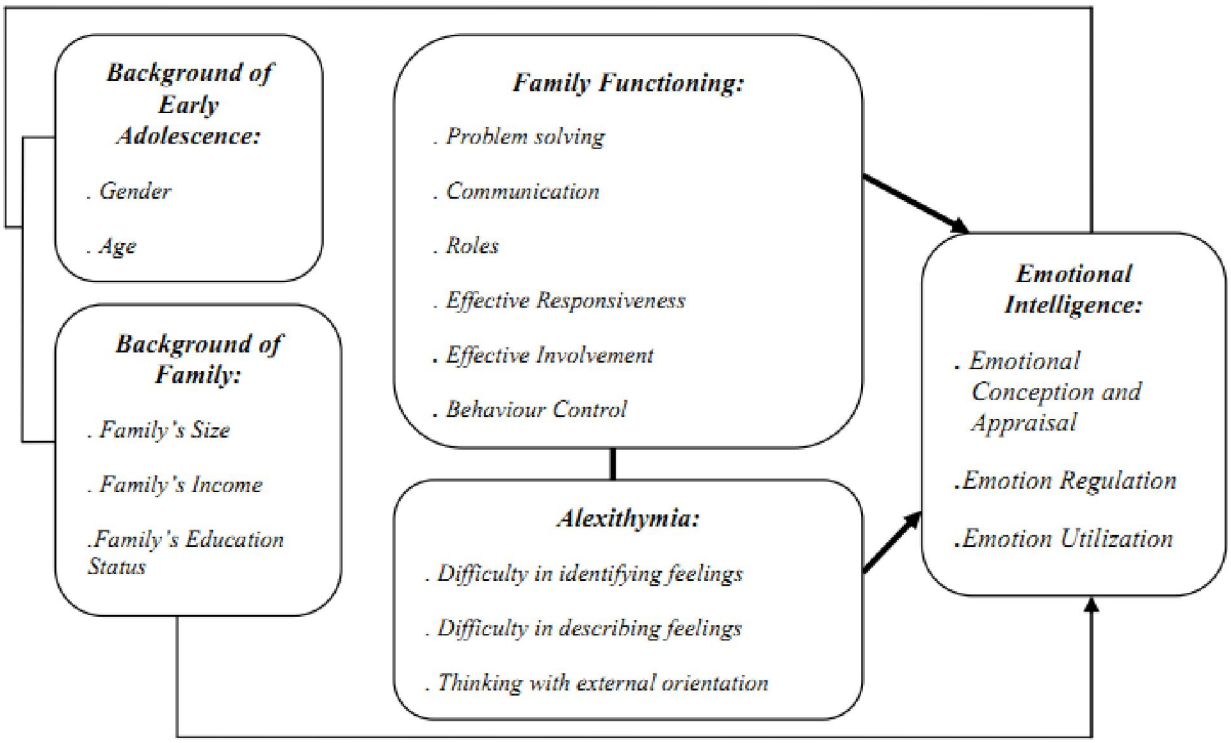

Figure 1: A Diagram of the Conceptual Framework of the Relationships between Family Functioning, Alexithymia and Emotional Intelligence among Early Adolescents

6. Conclusion

Regarding in relation to the above discussion, the related literature has indicated that early adolescents' emotional intelligence is influenced by some factors such as the background of earl adolescents and their families, family functioning and alexithymia. Hence, this paper attempted to, shows that how those factors would influence on early adolescents' emotional intelligence.

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Family Size and Construct of the Early Adolescent's Emotional Intelligence

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Abstract: Emotional intelligence is a set of abilities that include conception, emotion appraisal and expression, emotion management and regulation. The emotional intelligence structure is a concept with little empirical research, particularly in relation to the link between family environment and personality characters and with respect to family members of early adolescents. For this reason, the specific objective of this research is to determine the relationship between family size and early adolescent's emotional intelligence.The present research was carried out among 234 Iranian students in the second and grades of guidance schools (age 12-15) in Tehran, Iran. The students (girls and boys) were clustered through random and multistage sampling. Data were collected using the family background questionnaire and Schutte's (1998) Emotional Intelligence Scale. Results of multiple comparisons of LSD indicate that there is significant difference between groups of family size. Consequently, multi comparisons of LSD confirmed the results of the ANOVA. The findings indicate that the early adolescents, whose families have less members, have higher emotional intelligence.
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Keywords: Early Adolescent's Emotional Intelligence, Family Size, Family Members, Emotional Quotient (EQ), Family Size and Emotional Intelligence.

1. Introduction

Emotional intelligence refers to understanding the feelings of oneself and of others, is related to people, and one's ability to adapt to coping with the immediate surroundings to be more successful in dealing with environmental demands (Bar-On & Parker, 2006; Goleman, 1995). Among positive family factors for the adaptability of adolescents with life stresses are secured interest relations with caretakers during childhood, powerful family functioning and parents' effective attendance as a family subsystem. Thus, the family with multi-functional aspects serves as a fundamental dimension in the development of early adolescents' emotional intelligence. Hughes and Carolyn (2002) pointed that our first parents during childhood are our siblings. Our relationships with them, even during childhood, include components which will later become significant in our relationships as adults. Contrarily, Naghavi (2011) have shown that dysfunctional family affective responsiveness was related to difficulty in describing feelings.

Our first partners in our childhood are our siblings. Our relations with them, even during childhood, include certain components which will later become significant in our relationships with others as adults. These components include mutual dependence, role division, emotional communication, and problem solving, agreement and conflict, as well as cooperation and mental health (Hughes & Carolyn, 2002). There are many assumptions about emotional intelligence and family size. Some existing research indicates a slight relationship between emotional intelligence and family size.

Ozabaci (2006) carried out a study on the effects of family size and the number of siblings on emotional intelligence among 274 parents who are mothers and fathers of elementary school students. As a result, he found out that the family environment in which the foundations of emotional intelligence are first laid is a setting the child grows up in and acquires information relating to life. In more specific, family environment bearing healthy and high quality characteristics affects the development of the child in many ways like the ego concept of the child and his/her emotional and social development. Meanwhile, the social status of the family, the residence, relations within the family, the number of siblings and the relations among the siblings determine the characteristics of the family environment and emotional intelligence.

Important variations in emotional intelligence exist based on family structure. For example, children in different birth-order positions may have different opportunities, such as the difference in the availability of their family resources, the availability of parental time, energy, and attention, the quality of the relationship with parents, and other family members who have influence on younger siblings' emotional intelligence outcomes (Cicirelli, 1994; Lu, Donald & Treiman, 2008).

According to Naghavi, Ma'rof, Asgari & Mirza (2012), family education of mutual learning would be provided to help students learn how to appropriately deal with people.

Ritcher, Ritcher, Eisemann & Mau (1997) have suggested that large families pose a risk factor for children's mental health and behaviour, which may have negative effects later in life. Fisher (1984) believe that a large family size is often related to undesired family conditions such as overcrowding and low financial status, poor parental behaviour, parental crimnality and sibling delinquency, inadequate parental supervision and discipline, and lack of attention, affection and family interaction.

On the contrary, Zarinah, Rozumah, Krauss & Rumaya (2006) found that number of children was not significantly related to child's academic achievement. According to the study by Busard (1965), as the number of children increases in the family, parents' view toward raising children and circumstances in which their kids grow also changes. In crowded families, especially with over 6 children, family roles are specified more clearly, everybody's tasks are determined, and a more precise and authoritative discipline is exerted (Tahurian, 2005 cited in Khosravi, 2008).

Naghavi & Ma'rof (2012), demonstrate that For early adolescence, who learn most lessons about emotion from their family, it includes the ability to control impulses, delay gratification, motivate them, read other people's social cues, and cope with life's difficulties.

In fact, larger families having larger number of children and/or extended relatives living with them are thought to dilute family resources by spreading themselves among several children. These limit the quantity and quality of the interactions between the children and their parents, and they may affect some early adolescents' characters. In industrialized nations, having more siblings may reduce their opportunities of education (Lu, Donald & Treiman, 2008). In other words, children from large families benefit less than those from small families from parental resources even if the same resources are available for all of them (Powell & Steelman, 1999).

However, the key is, if negative resources like alcohol drinking and drug issues, or mental problems within the family are also diluted as a function of family size, it is plausible that under certain negative circumstances, having a larger number of siblings might be advantageous (Downey, 1995; Powell, Steelman, 1999). In this line; Golestan, Haslinda, Nobaya & Anjomshoa (2010), believe that Family interventions would be focused on family members; this is able to lead to less conflict and more positive family environment.

Consequently, some existing research has indicated a slight relationship between family size and their early adolescents' emotional intelligence. This indicates that family structure may influence positively and negatively on early adolescents' emotional intelligence. Meanwhile, the variables of the family background, such as the family size, are the important factors that affect emotional intelligence development of their children Thus, with respect family size, it is one way of those emotional skills that can be developed in teenaged children. The present study explored the interaction between family's size and early adolescents' emotional intelligence.

In addition, the treatment by parents to their children and how they react to their interests and activities, as well as children treatment to one another, emotion and information exchange among them, emotional protection to one another, and the relationships of the family members' with outsiders may also influence the children's emotional intelligence (Naghavi, 2010). Although a body of relevant research literature is available, the findings of such research studies which investigated the effects of family's size on early adolescents' emotional intelligence were derived mainly from western-based samples that are socially and culturally different from the Iranian sample.

2. Materials and Methods

The purpose of this study was to examine the relationship between family size and the early adolescent's emotional intelligence among Iranian guidance schools students in Tehran, Iran. The schools were chosen based upon their location and programs of study. The population of research involved in this study consisted of all the Iranian students who enrolled in guidance schools of Tehran (234 students, academic year 2010-2011).

The data were collected using (Schutte, 1998) Emotional Intelligence Scale for assessing early adolescence's emotional intelligence. To identify the difference between emotional intelligence and family size of early adolescents.

The emotional intelligence scales used to assess emotional intelligence, i.e. Schutte's Emotional Intelligence Self-measuring Scale (introduced by Schutte and her colleagues in 1998 and Mayer and Salovey's original emotional intelligence model, 1990), was used to measure emotional intelligence, which includes emotional conception and appraisal, emotion regulation and emotion utilization. This scale includes 33 self-report items. This scale includes 33 self-report items. Some examples of the items included in the scale are:

A. I can easily identify my emotions and feelings.

B. I can persuade myself by imagining success in work.
C. I admire others when they do something good.

The subject selected his/her degree of agreement or disagreement by any of these sentences in a five-point Likret scale, from strongly disagreed = 1 to strongly agreed = 5. In this study, the reliability for the emotional intelligence test was obtained by using Crombach's alpha, $\alpha = 0.84$.

In addition, the demographic questionnaire was also used to gather relevant background information of the subjects in this research.

Considering the question and research hypothese, the following statistical method is use to analyze data: Descriptive statistics was provided to show the variation in the estimated means and standard deviations for each of the dependent and independent variables across the sample. In this research for multi comparisons of variabels LSD "*Least Significant Difference*" test was used. The LSD test was used for determine the difference between the mean score of emotional intelligence of the groups of family size as the dependent variables.

3. Results and Discussion

Description of the participants

The study was among 7150 girls and boys Iranian students. After determining the sample gathering, 4 regions selected random among Tehran's 19 educational regions. Then, among the guidance schools of each region, 2 schools are selected by simple random method: one girls' school and one boys' school. In each school, pupils are selected from grade 3 and grade 2 by simple random method. The sample (234) consisted of the guidance schools pupils (12-15 years old). The respondents (234) for this study were the early adolescence with 116 boys and 118 girls.

This research studied the effects of family size on the early adolescents' emotional intelligence. Hence, a descriptive analysis of early adolescents' emotional intelligence with respect to family size was obtained. Table 1 presents the descriptive information of the early adolescent's emotional intelligence, according to their family size.

In this research, 3 groups of family size (being the only child, with one sibling in the family, and with two and more siblings in the family) were compared; however, the analysis for this particular hypothesis concerned with the question of the difference between early adolescents' emotional intelligence and the statistics dealing with the three samples mean by family sizes. The mean score for the emotional intelligence of early adolescents, with respect to their family size (being the only child, with one sibling in the family, and with two and more siblings in the family), as presented in Table 2 are M=127.87 (SD=5.94), M=116.78 (SD=1.73) and M=109.78 (SD=3.70), respectively. The appropriate statistical method, i.e. the analysis of variance (ANOVA), was used to test the difference between emotional intelligence of early adolescents in relation to their family size. The ANOVA statistical method for the equally mean value scores of early adolescent' emotional intelligence was also conducted by using SPSS software. The obtained results indicated that there is a statistical significant difference between early adolescents' emotional intelligence in term of their family size ($F=216.69$, $p<0.01$). Table 2 presents the results from the ANOVA which tested the difference between the mean of the early adolescents' emotional intelligence for the three groups of family sizes.

A significant difference only suggested that there is a significant difference between the group means. However, it does not identify the group means that are significantly different. Hence, to determine the groups that are significantly different, the LSD Test was used. Table 3 presents the LSD test for the analysis difference between early adolescents' emotional intelligence according to family sizes. The results of the multiple comparisons of the LSD indicated that there is a significant different between the group of family sizes (sig=0.000).

Consequently, the multi comparisons of the LSD confirmed the result of the ANOVA (ANOVA's comparisons between the groups of family sizes). In other words, early adolescents' emotional intelligence is significantly different according to their family size, and this supports the findings obtained in the study by Ozabaci (2006) which suggested that the social status of the family, the residence, relationships within the family, number of siblings and their relationships determine the characteristics of the family environment and emotional intelligence.

According to a study by Fazelinia (2001), one of the social harming factors to a family is children's multiplicity. Decreases of parent-child interactions, decreases of the chances of being together, as well as listening and positive attentions in crowded families are probably some reasons for the decrease in emotional intelligence in such families. The result indicated that there is a slight difference between the levels of emotional intelligence of early adolescents according to their family sizes. Moreover, early adolescents' emotional intelligence showed a different ranking for the family size groups with the only child (M=127.87, SD=5.94) than those with more than one sibling in the family (M=116.78, SD=1.73), and more than two and more siblings in

the family (M=109.78, SD=3.70), respectively. This means that the early adolescents, who live in the family with smaller size, have higher emotional intelligence.

Table 1: A summary of samples for early adolescents by demographic variables

Demographic Variables	*Frequency*	*Percentage (%)*
Sample	*234*	*100.00*
Early Adolescent's Gender		
Male (boy)	*116*	*49.6*
Female (girl)		
Total	*118*	*50.4*
Family Size	*234*	*100*
The only child	*148*	*64.1*
Has one sibling in the family	*52*	*22.5*
Has two and more siblings in the family	*31*	*13.4*
Total	*231*	*100*

Table 2: Descriptive information of early adolescents' emotional intelligence in terms of family size

Family size	*Emotional Intelligence*		
	Mean	***N***	***SD***
Being the only child	*127.87*	*148*	*5.94*
With one sibling in the family	*116.78*	*52*	*1.73*
With two and more siblings in the family	*109.78*	*31*	*3.70*

Table 3: Summary information of ANOVA for early adolescent's emotional intelligence by respect to family size

	SS	***Df***	***MS***	***F***	***Sig***
Between Groups	*10935.34*	*2.00*	*5467.67*	*216.69*	*0.00*
Within Groups	*5753.09*	*228.00*	*25.23*	*216.69*	*0.00*
Total	*16688.42*	*230.00*			

Note: *P<0.01*

Table 4: The multiple comparisons of the LSD test between the mean of early adolescent's emotional intelligence summary according to the different groups of family sizes

Multiple comparisons for the LSD test, Dependent Variable = EI of early adolescents						
Being the only child, with one sibling in the family, and with two and more siblings in the family		***MD***	***SE***	***Sig***	***95% co. Level***	
I=family size	***J=family size***				***Lower***	***Upper***
Being the only child	***One sibling in the family***	*11.084**	*.810*	*.000*	*9.49*	*12.68*
	Two and more siblings in the family	*18.090**	*.992*	*.000*	*16.14*	*20.05*
With one sibling in the family	***only child***	*-11.084**	*.810*	*.000*	*-12.68*	*-9.49*
	Two and more siblings in the family	*6.490*	*1.140*	*0.10*	*-4.76*	*9.25*
With two and more siblings in the family	***only child***	*-18.090**	*.992*	*.000*	*-20.05*	*-16.14*
	One sibling in the family	*6.490*	*1.140*	*.010*	*9.25*	*-4.76*

Note: *The mean difference is significant at 0.05 level*

4. Conclusion

Based on the findings of the current research, has shown that a greater percentage of smaller family size of early adolescents' scores appeared in higher categories of social skills and emotional intelligence than those from bigger families. This means that adolescents living in small family size have more emotional intelligence scores. According to Ozabaci (2006), the social status of the family, the residence, the relationships within the family, the number of siblings and the relationships among the siblings determine the characteristics of the family environment

and emotional intelligence. So, regarding to this finding, it is important to consider early adolescents' activities should be provided because providing a positive family environment is not only the responsibility of the parents. The interaction between parents and early adolescents would be increased to enhance life adjustment abilities; these include engaging them in the school's parenting activities to improve early adolescents' emotional intelligence and promote the ability to recognize and control personal emotions, understanding their early adolescents' personal characters and abilities, training their early adolescents to possess multiple capabilities and interests, development their early adolescents' learning activities, and understanding what they/he/she are/is learning by providing some help to them.

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Visfatin and Fetuin-A: Novel Markers for Endothelial Dysfunction in Chronic Kidney Disease

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Abstract: Background: Endothelial dysfunction (ED) has a major role in the cardiovascular outcome of patients with chronic kidney disease (CKD). The hallmark of endothelial dysfunction is impaired nitric oxide-mediated endothelial-dependent vasodilatation. Visfatin is an adipocytokine that has recently generated much interest and could contribute to endothelial dysfunction. Fetuin-A may be one of the contributing factors for the development of ED in CKD patients. In addition, fetuin-A 256Ser/ Ser (allele G) might affect serum fetuin- A levels. The aim of the present work is to study the role of visfatin and fetuin-A in patients with different stages of CKD in correlation with the level of NO (nitrite /nitrate) as a settled marker of endothelial dysfunction. Also, to study the relation between fetuin-A gene polymorphisms and the susceptibility to ED in patients with CKD in different stages; and identifying the effect of fetuin-A gene polymorphisms on the level of serum fetuin-A in CKD patients. **Methods**: the present study included sixty patients at different stages of CKD with age range from 18 to 60 years old. All patients were non-diabetic and arranged in five groups according to the stage of CKD assessed GFR from stage 1 to 5 representing the groups from I to V. Serum visfatin and serum fetuin-A were measured using ELISA technique and serum NO was measured as (total nitrate and nitrite) using the Griess reaction. Serum levels of glucose, triglycerides, total cholesterol and HDL-cholesterol were estimated by enzymatic colorimetric methods. LDL-cholesterol was then calculated using Friedewald's formula. Genotyping for the common functional polymorphisms on fetuin- A (Thr256Ser) using polymerase chain reaction (PCR) technique was performed. **Results**: A statistically significant elevation of serum total nitrate and nitrite and serum visfatin in CKD patients compared to controls respectively, while serum fetuin-A showed statistically significant decrease in CKD patients compared to the control group. Serum total nitrate and nitrite levels were significantly increased in all stages of CKD, while serum visfatin was significantly increased in stages 2 to 5 of CKD. Serum fetuin-A showed significant decrease in stages 2 to 5 of CKD. There was no statistically significant difference between the studied CKD cases and the control group as regards to the frequencies of the three genotypes of fetuin–A (C → G); Thr256Ser polymorphism. In both CKD patients and the control group, the distribution of the fetuin-A (C → G); Thr256Ser gene polymorphisms did not show significant correlation with low serum fetuin-A levels. A significant positive correlation was found between serum levels of total nitrate and nitrite and serum levels of (visfatin, triglycerides, total cholesterol, LDL-cholesterol), while A significant negative correlation was found between serum levels of total nitrate and nitrite and serum levels of (fetuin-A and HDL-cholesterol) in CKD patients. Stepwise regression analysis revealed that the strongest predictors of endothelial dysfunction were found to be serum visfatin and HDL-cholesterol as they could explain significantly 52% of the changes in total nitrate and nitrite. **Conclusion:** The results of the present study suggest that high serum levels of visfatin and total nitrate and nitrite in CKD patients may contribute to impaired endothelial functions in CKD. Visfatin and fetuin-A may be novel markers for endothelial dysfunction in CKD patients and in diagnosis of early stages of CKD and they may play a role in uremia-related atherosclerosis. The distribution of the fetuin-A (C → G); Thr256Ser gene polymorphisms may does not affect serum fetuin-A levels.

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Keywords: Endothelial dysfunction, visfatin, NO,CKD, fetuin-A , gene polymorphisms of fetuin-A

1. Introduction

In renal failure, endothelial dysfunction (ED) and atherosclerosis are almost universal, as well as cardiovascular complications. Cardiovascular diseases (CVD) remain the major cause of morbidity and mortality in chronic kidney disease (CKD) patients[(1)] . Traditional cardiovascular risk factors including dyslipidemia, hypertension, smoking and diabetes mellitus are highly prevalent in these patients [(2)]. Nontraditional biomarkers, for example, markers of inflammation, ED, myocardial necrosis and left ventricular remodeling , have been associated with increased cardiovascular event rates and mortality risk in population with and without CKD[(3)].

Endothelial dysfunction (ED) represents an obligatory, prodromal phase in the atherosclerotic process. Endothelial dysfunction may be also responsible for accelerated atherosclerosis in patients with CKD. Endothelial dysfunction is characterized by shift of the action of the endothelium towards reduced vasodilatation and a proinflammatory state denoting impairment of endothelium-dependent vasodilatation[4] . The hallmark of endothelial dysfunction is impaired nitric oxide-mediated endothelial-dependent vasodilatation[5] . The endothelium-derived nitric oxide (NO) is synthesized from the substrate L-arginine via endothelial NO synthase (eNOS) and plays a crucial role in regulating a wide spectrum of functions in the cardiovascular system, including vasorelaxation, inhibition of leukocyte-endothelial adhesion, vascular smooth muscle cell (SMC) migration and proliferation, as well as platelet aggregation. Physical or biochemical injury to the endothelium impairs production and/or function of endothelium-derived vasoprotective mediators of vascular health, such as NO, resulting in increased vascular contraction, enhanced thrombus formation and exacerbated SMC proliferation and migration. It is, therefore, not surprising that loss of endothelial NO function is associated with several cardiovascular disorders, including atherosclerosis [6,7] .

The adipose tissue is a complex organ with function far beyond the mere storage of energy. Indeed, it secretes a number of adipokines. Visfatin is one of the adipokines (also known as adipocytokines) which is a family of secreted proteins released by fat cells that regulate a variety of physiological and pathological processes[8,9] . Visfatin was found to be up-regulated in visceral fat in parallel with insulin resistance[10,11].Visfatin is also called nicotinamide phosphor-ribosyltransferase (Nampt) an enzyme that catalyzes the first step in the biosynthesis of nicotinamide adenine dinucleotide (NAD) from nicotinamide. Visfatin which is protein in nature has also been reported to be a pre-B cell colony-enhancing factor (PBEF) that promotes B cell maturation and inhibits neutrophil apoptosis [12,13] . Therefore, visfatin/PBEF/Nampt appears to be a multifunctional protein acting as a hormone, cytokine and/or enzyme [14, 15]. Nicotinamide phosphoribosyltransferase (Nampt) is the rate-limiting enzyme that catalyzes the first step in the biosynthesis of NAD from nicotinamide. This protein was originally cloned as a putative pre-B cell colony-enhancing factor (PBEF) and also found to be a **vis**ceral **fat**-derived adipok**in**e (**visfatin**). [16]. As a multifunctional protein, visfatin plays an important role in immunity, metabolism, aging, inflammation and responses to stress [17] . However, the pathophysiological role of visfatin is not completely understood .Visfatin is implicated in the transcriptional regulatory activity of a variety of cellular processes, including stress, cytokine responses, differentiation and metabolism [18,19] . One function of visfatin is the regulation of inflammatory and immunomodulating processes. Visfatin can induce the cellular expression of inflammatory cytokines, such as (interleukin-1β) IL-1β, Tumour necrosis factor-α (TNF-α) and (interleukin-6) IL-6 [20] . Deteriorating renal function may increase overall inflammatory responses because of the decreased renal clearance of factors that are directly or indirectly involved in inflammation. Inflammation has also been implicated in vascular diseases as acute myocardial infarction and thrombotic stroke. Chronic renal disease is a highly associated with atherogenic disease and subclinical systemic inflammation [21] . Visfatin may play an important role in atherogenesis and plaque destabilization and due to the fact that endothelial cell damage or injury is invariably associated with clinical conditions such as thrombosis, hypertension, renal failure and atherosclerosis, visfatin may be considered as a novel modulator for endothelial dysfunction [22] .

Fetuin-A is an acidic glycoprotein synthesized by the liver, it is expressed also in osteoblasts, the tongue and the placenta [23,24].Fetuin-A is part of the cystatin superfamily of cysteine protease inhibitors [25] The fetuin-A gene is located at chromosome 3q27 [26]

Fetuin-A, plays an important role in the development of specific organs (bone marrow, brain, gonads and liver).[27]Fetuin-A is a negative acute phase protein.[28] Fetuin-A has role as a protease inhibitor, and acts as an inhibitor of matrix metalloproteinases. [29] Fetuin-A down regulates the synthesis of proinflammatory cytokines and prevents excessive inflammation [30] . Fetuin-A acts as circulating inhibitor of calcification. [31]

In renal failure, hyperphosphataemia was recognized as an independent risk factor for cardiovascular disease[32] . It was believed that the mineral homoeostasis which is disturbed in CKD, results in an elevated serum calcium phosphate (Ca-Pi) product sustaining calcium phosphate precipitation [33] .

Fetuin-A accumulates in the skeleton due to a high affinity to hydroxyapatite. Fetuin-A binds to bone morphogenic protein-2 and transforming growth factor β inhibiting mineralization and suppressing the expression of bone matrix proteins. It inhibits the de novo formation and precipitation of the apatite precursor mineral, basic calcium phosphate (BCP) [34] Fetuin-A can inhibit undesirable calcification in circulation without inhibiting bone mineralization [35] It was hypothesized that fetuin-A on the surface of these mineral colloids has two functions (i) it reduces diffusion of ions to the mineral core and (ii) it prevents particle aggregation [36] . So that fetuin-A may act as a systemic inhibitor of ectopic calcification.

Defective endothelial function, an initial step in the development of atherosclerotic plaque, is prevalent in moderate to advanced CKD [37] . These data in CKD patients indicate that fetuin-A may be one of the contributing factors for the development of endothelial dysfunction in CKD patients[38] , specific polymorphisms of fetuin-A gene were demonstrated to influence circulating levels of fetuin-A [39] .

The aim of the study was to assess the role of serum visfatin and fetuin-A as novel markers of ED in patients with different stages of CKD, in correlation with the marker of endothelial damage NO (total nitrates and nitrites); as well as studying the relation between fetuin-A gene polymorphisms and the susceptibility to ED in patients with CKD in different stages and the effect of fetuin-A gene polymorphisms on the level of serum fetuin-A.

2. Materials and Methods

The present study included sixty patients at different stages of CKD defined according to the National Kidney Foundation (NKF) – Kidney Disease Outcomes Quality Initiative (KDOQI) classification[40] with age range from 18 to 60 years old. All patients were non-diabetic and arranged in five groups according to the stage of CKD assessed by glomerular filtration rate (GFR) as follows:

Group I: stage 1(GFR≥90 ml/min/1.73m^2).
Group II: stage 2 (GFR from 60 to 89 ml/min/1.73m^2).
Group III: stage 3 (GFR from 30 to 59 ml/min/1.73m^2)
Group IV: stage 4 (GFR from 15 to 29 ml/min/1.73m^2).
Group V: stage 5 (GFR <15 ml/min/1.73m^2).

Each group included twelve patients. Also, **Group VI** twelve matched healthy volunteers with matched age and sex were included to serve as healthy controls.

The patients were chosen from the main University Hospital of Alexandria. In order to minimize any confounding effects of conditions that may influence ED, patients with overt congestive heart failure, valvular heart disease, acute coronary syndrome, atrial fibrillation, smokers, nephrotic syndrome, patients taking angiotensin converting enzyme inhibitors, angiotensin receptor blockers, statins or supplemental vitamin pills were excluded. In addition, patients with a prior diagnosis of diabetes or with a fasting glucose level greater than 126 mg/dl were also excluded. The subjects were told to avoid foods high in nitrate (i.e. spinach, beets, cabbage, cauliflower and lettuce) for 3 days before taking blood samples and were asked to fast overnight before sampling blood [41] .

The study was conducted in accordance with the Local Ethics Committee of the Faculty of Medicine, University of Alexandria; an informed consent was obtained from all patients included in the study.

All patients included in this study were generally evaluated depending on the following:

I- Clinical Evaluation: Includes proper history taking and clinical examination particularly stressing on vital sign measurement (blood pressure, pulse and temperature).

II- Calculation of GFR from serum creatinine levels using Cockcraft and Gault (C&G) formula [42]

Glomerular filtration rate (GFR) was determined for proper diagnosis, selection and grouping of cases and controls.

Cockcroft-Gault GFR = (140-age) X (Wt in kg) X (0.85 if female) / (72 X Cr).

Wt=weight, Cr= serum creatinine in mg/dL.

III- Laboratory Investigations

A) Sample collection, storage and preparation:

Five milliliter venous blood were withdrawn from every patient and control in the morning after over night fasting. 500 µl whole blood was separated in an eppendorf containing ethylenediamine tetraacetic acid (EDTA) 5% and stored at –20°C for genotyping for the common functional polymorphisms on fetuin- A (Thr256Ser) using Polymerase chain reaction (PCR) technique.

The remaining sample was transferred into disposable plastic tube and was allowed to clot, then was centrifuged at 1200 rpm for 10-15 minutes to separate the serum. The serum was then divided into four aliquots which were kept frozen at -20°C until use. The following laboratory investigations were carried out:

B) Routine Laboratory Investigations:

They included estimation of serum glucose [43], creatinine [44] , triglycerides [45] , total cholesterol [45] and high-density lipoprotein (HDL) cholesterol[46] using enzymatic colorimetric methods. Low-density lipoprotein (LDL) cholesterol was calculated by Friedewald's formula. [45]

C) Specific laboratory tests:

1. Determination of serum visfatin [47] using enzyme linked immunosorbant assay (ELISA) (RayBio® Human Visfatin Enzyme Immunoassay, US) and serum fetuin-A[48] also using ELISA (BioVendor Human Fetuin-A ELISA,Germany).
2. DNA extraction and genotyping for the common functional polymorphisms on fetuin- A (Thr256Ser) using PCR technique [49] .
3. Estimation of NO (nitrate and nitrite) based on the Griess reaction [50,51] .

DNA extraction from blood, amplification of fetuin -A gene by PCR and (Thr256Ser) mutation analysis by restriction endonuclease enzyme sacI[49]

DNA extraction:

DNA was purified from whole blood using Axygen Prep Blood Genomic DNA Miniprep Kit for the purification of genomic DNA from whole blood. This method was based on the efficient release of genomic DNA from anti-coagulated whole blood by a special cell lysis and heme/protein precipitation buffer coupled with the selective adsorption of the genomic DNA to a special AxyPrep column. The purified genomic DNA was eluted in a low-salt Tris buffer containing 0.5 mM EDTA which enhanced DNA solubility and helped to protect the high molecular weight DNA against subsequent nuclease degradation. The eluted genomic DNA was subjected to PCR amplification of the fetuin-A gene.

PCR amplification

DreamTaq™ Green PCR Master Mix (fermentas life sciences)

DreamTaq™ Green PCR Master Mix (2X) was a ready to use solution containing DreamTaq™ DNA polymerase, optimized DreamTaq™ Green buffer, MgCl2 and dNTPs. The master mix was supplemented with two tracking dyes and a density reagent that allow for direct loading of the PCR product on a gel. The dyes in the master mix did not interfere with PCR performance and were compatible with downstream applications such as restriction digestion.

For a total 25µl reaction volume:

DreamTaq™ Green PCR Master Mix (2X) 12.5 µl.
Forward Primer 1µl.
Reverse Primer 1 µl.
Extracted DNA 5 µl .
Water nuclease-free 5.5 µl.

Primers:

A pair of primers was utilized to amplify the fetuin-A gene. The lyophilized primers were purchased from Fermentas Life Sciences. The lyophilized primers were reconstituted by addition of sterile water to a final concentration of 50 picomoles/µl and distributed in aliquots and stored at –20°C.

Primer sense sequence:

F5-GTCACCCCTCCTTGTAAC-3
[Tm (thermodynamic) = 45.6°C]

Reverse primer antisense:

R5-CCCCAATGAGACCACA-3.[Tm = 46°C]

Protocol of amplification:

Tubes were transferred to the thermal cycler (Biometra) where the PCR conditions were as follows:

- Initial denaturation at 95°C for 5 minutes.
- 35 cycles of denaturation at 94°C for 1minute, annealing at 59°C for 1 minute, extension at 72°C for 1 minute.
- Final extension at 72°C for 15 minutes.

Mutation analysis of fetuin-A Thr256Ser (c.766C >G) polymorphism [49]

For mutational analysis of fetuin-A Thr256Ser (c.766C > G); FastDigest® SacI restriction enzyme 100 µl (for 100 reactions) was supplied with 1 ml of 10X FastDigest® Buffer. They were supplied from (Fermentas Life Sciences) and stored at -20°**C.**

Protocol for Fast Digestion of DNA:

Total volume: 30 µl;

- Water nuclease-free 17 µl.
- 10X FastDigest® buffer 2 µl.
- PCR product 10 µl.
- FastDigest® enzyme 1 µl.

They were mixed gently and incubated at 37°C for 60 min.

The digested products were separated on 1.5% agarose gel. Allele C does not contain the *Sac*I site remain undigested as 405 bp fragments, whereas allele G yields 193- and 212-bp fragments.

For Detection of the digested products PCR marker (Fermentas Life Sciences), the DNA fragments range from 50bp to1000 base pairs was used and Gel electrophoresis was performed:

- 12µl of the digested product was slowly loaded into the slots of the submerged gel using a micropipette.
- 8 µl of the TE buffer +2 µl of the50bp ladder+2 µl of loading dye 6X (Amresco) was slowly loaded into one of the slots the submerged gel using a micropipette.
- A 302 nm ultraviolet transilluminator was used for visualization of the DNA bands. Allele C does not contain the *Sac*I site remain undigested as 405 bp fragments whereas allele G yields 193- and 212-bp fragments and they were photographed.

Determination of serum nitric oxide metabolites (nitrite and nitrate) concentrations

I- Nitrate assay [50]: Serum nitrate was quantitated colorimetrically through nitrate reduction by NADPH-dependent nitrate reductase. Serum samples were incubated with FAD (0.2 mmol/L), NADPH (12 mmol/L) and nitrate reductase from Aspergillus species (500 U/L). The reaction was allowed to develop in the dark because of photosensitivity of FAD. Then, the absorbance of samples was recorded at wave length 340 nm.

At 25° C, 100 µl of serum sample were mixed with 250 µl of 100 mmol/L potassium phosphate buffer (PH 7.5), 50 µl of distilled water, 50 µl of 0.2 mmol/L FAD and 10µl of 12 mmol/ L NADPH. 40 µl of 500 U/L nitrate reductase were added and immediately mixed. The reaction was allowed to develop in darkness. After 45 minutes incubation, the absorbance was recorded at 340 nm.

Calculation:

Nitrate concentration in the sample was calculated form the following equation:

Nitrate concentration = Δ Absorbance × factor

Where:

Δ Absorbance = ASB- AS- ARB

ASB =Absorbance of sample blank.

AS =Absorbance of sample

ARB =Absorbance of reagent blank

Factor = VT/VS × 1/L × 1/ε 340 nm × 10^3 = 0.833

VT = total volume of reaction mixture (500 µls)

VS = sample volume

L = light path [1cm, ε 340 nm = millimolar absorpitivity of NADPH at 340 nm (6.22 L. $mmol^{-1}$ cm^{-1})]

10^3 = conversion from mmol/L to µmol/L

II- Nitrite assay [51]: Serum nitrite was quantitated colorimetrically after reaction with Griess reagents. Griess assay reagents (1% sulfanilamide, 0.5% naphthylethylenediaminedichloride and 2.5% phosphoric acid) were first mixed and incubated with samples or standard to form a purple azo dye. Then the absorbance was measured at wave length 543 nm. Nitrite concentration was determined from a linear standard curve.

0.4 ml of each sample was mixed with 0.8 ml of freshly prepared 1% sulfanilamide in 2.5% phosphoric acid and 0.8 ml of 0.5% naphthylethylenediaminedichloride in 2.5% phosphoric acid. The absorbance was measured at 543 nm after incubation for 10 minutes at room temperature in dim light. Concentration was determined from a linear standard curve between 2.5 and 30µM sodium nitrite. NO was estimated as the sum of total nitrates and nitrites.

Statistical analysis

The study data were statistically analyzed using the Statistical Package for Social version program (SPSS program-version 10.0 – SPSS Inc., Chicago, IL. USA). The data were expressed as median, mean ± standard deviation (SD) or proportions (%). Between-groups comparisons were assessed for nominal variables with the χ2-square test while for quantitative variables differences among the groups were analyzed by Mann-Whitney test and Kruskal–Wallis test. Correlations between variables were analyzed by using Pearson's correlation coefficient. Statistical significance was assessed at $P < 0.05$. All calculated P values were two-tailed.

3. Results

The Distribution of patients according to the etiology of CKD in the present study is shown in Table I: 24 patients (40%) were due to hypertension,18 patients (30%) had glomerulonephritis, 10 patients (16.7%) had reflux nephropathy , 2 patients (3.3%) had autosomal dominant polycystic kidney and in 6 patients(10%) the etiology of CKD was unknown.

Table I. Distribution of patients according to the etiology of CKD

The etiology of CKD	Frequency	Percent (%)
Hypertension	24	40
Glomerulonephritis	18	30
Autosomal dominant polycystic kidney	2	3.33
Reflux nephropathy	10	16.67
Unknown	6	10
Total	60	100

In the present study, the age range in the patient group was from 20 to 59years with a mean value of 40.25 ± 10.8 years, while the range in the control group was from 27to 55 years with a mean value of 42.17± 9.321years. Statistical comparison of the subjects' age, showed no significant difference between the two studied groups (Z = 0.932, *p*= 0.338). CKD patients included 31 males (51.7%) and 29 females (48.3%) while the control group included 5 males (41.7%) and7 females (58.3%). Statistical comparison of the subjects' sex, showed no significant difference between the two studied groups (χ^2 =0.4, *p* =0.527). Serum levels of glucose, triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol are shown in Table II. Statistical comparisons between CKD patients and the control groups as regards serum levels of glucose, triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol were not significant at $p > 0.05$.

In CKD patients, serum total nitrate and nitrite concentration ranged between 32.4 -132.3 µmol/l with a mean value of 76.57±20.38µmol/l (median value of 71.35µmol/l). In the control group, serum total nitrate and nitrite concentration ranged between 31.2 - 53.7µmol/l with a mean value of 42.77±8µmol/l (median value of 41.75µmol/l). There was statistically significant elevation of serum total nitrate and nitrite concentration in CKD patients compared to the control group (Z=-5.101, $p < 0.001$). (Table II)

As regards serum visfatin concentration in CKD patients, it ranged between 21-80ng/ml with a mean value of 46.34±15.52 ng/ml (median value of 43

ng/ml). In the control group, serum visfatin concentration ranged between 21-42 ng/ml with a mean value of 31.62±7.35 ng/ml (median value of 31.7ng/ml). There was statistically significant elevation of serum visfatin concentration in CKD patients compared to the control group (Z=-3.224, *p* =0.001). (Table II).

Serum fetuin-A concentration ranged between 0.01-0.47 g/l with a mean value of 0.23±0.09 (median value of 0.21g/ml) in CKD patients. In the control group, serum fetuin-A concentration ranged between 0.24-0.45 g/l with a mean value of 0.33±0.05 (median value of 0.32g/l). There was statistically significant decrease of serum fetuin-A concentration in CKD patients compared to the control group (Z=-3.325, *p* =0.001). (Table II).

As regards statistical comparison in between CKD groups, in Group V there was statistically significant elevation of serum total nitrate and nitrite levels and serum visfatin levels in comparison with (Group I,II and III); $p < 0.05$. Also, in Group IV there was statistically significant elevation of serum total nitrate and nitrite levels in comparison with Group I and significant elevation of serum visfatin in comparison with Group I and Group II; $p \leq 0.05$(Table III.)

In Group III there was statistically significant elevation of serum visfatin levels in comparison with Group I ; $p \leq 0.05$,but no statistically significant difference was found between Group III or II; $p > 0.05$. (Table III.)

As regard serum fetuin-A levels there was statistically significant decrease in Groups III, IV and V in comparison with Group I; $p < 0.05$. (Table III.)

Table II: Statistical comparisons between CKD patients and the control group as regards routine laboratory data, serum levels of total nitrate and nitrite, visfatin and fetuin-A.

Parameters	CKD patients (n=60)	Control (n=12)	Statistical test	*P* value
Serum glucose(mg/dl) Mean ± SD	83.57±6.63	80.25±5.53	Z=-1.707	0.088 NS
Median	81	85		
Range	70- 99	70-88		
Serum triglycerides(mg/dl) Mean ± SD	161.43±66.96	130.85±59.56	Z=-1.463	0.143 NS
median	164.9	150		
range	68-276	71.3-250		
Serum total cholesterol(mg/dl) Mean ± SD	173.4±46.01	150.5±24.3	Z=-1.709	0.087 NS
Median	173.44	150		
Range	99-291	99.2-189.9		
Serum LDL-cholesterol (mg/dl) Mean ± SD	114.13±45.39	88.37±35.89	Z=-1.712	0.087 NS
Median	107.6	91.42		
Range	34.44-191.5	34.44-165		
Serum HDL-cholesterol(mg/dl) Mean ± SD	43.48±45.39	50.2±10.45	Z=-1.501	0.133 NS
Median	42.6	54.1		
Range	12-67	32.5-67		
Serum total nitrate and nitrite (µmol/l) Mean ± SD	76.57±20.38	42.77±8	Z=-5.101	<0.001**
Median	71.35	41.75		
Range	32.4 -132.3	31.2-53.7		
Serum visfatin (ng/ml) Mean ± SD	46.34±15.52	31.62±7.35	Z=-3.224	0.001**
Median	43	31.7		
Range	21-80	21-42		
Serum fetuin-A (g/l) Mean ± SD	0.23±0.09	0.33±0.05	Z=-3.325	0.001**
Median	0.21	0.32		
Range	0.01-0.47	0.24-0.45		

Z: for Mann-Whitney test. SD: Standard deviation. NS:Nonsignifiant. **: Highly significant ($p \leq 0.01$).

Table III: Statistical comparison between different groups of CKD as regards serum total nitrate and nitrite (µmol/l), serum visfatin(ng/ml) and serum fetuin-A(g/l)

Parameters	Groups						χ^2 *p* value
	Group I Stage1 (n=12)	**Group II Stage2 (n=12)**	**Group III Stage3 (n=12)**	**Group IV Stage4 (n=12)**	**Group V Stage5 (n=12)**	**Group VI Control (n=12)**	
Serum nitrate & nitrite(µmol/l) Range Mean± SD Median	32.4-122 66.08± 23.65 62.2	51 -96 71.5±15.30 65.75	50.3-96.1 73.56±15.24 69	58.40-100.3 80.9±15.75 82.45	64-132.3 92.89±21.54 90.54	31.20-53.7 42.77±8 41.75	χ^2=35.48 p<0.001**
Z_1 (p)		-0.925 (0.378) NS	-0.925 (0.378) NS	-1.993 (0.045) *	-2.714 (0.006) **	-3.205 (0.001) **	
Z_2 (p)			-0.290 (0.799) NS	-1.328 (0.198) NS	-2.483 (0.012) *	-3.984 (<0.001) **	
Z_3 (p)				-1.328 (0.198) NS	-2.483 (0.012) *	-3.984 (<0.001) **	
Z_4 p)					-1.213 (0.242) NS	-4.158 (<0.001) **	
Z_5 (p)						-4.157 (<0.001) **	
Serum Visfatin(ng/ml) Range Mean± SD Median	21-46 35.08±8.87 39	27-48 38.75±6.9 40	30-61 44.42±9.3 40	23-80 55.82±20.32 60.45	38-80 57.65±14.92 60.50	21-42 31.63±7.35 31.70	χ^2=25.52 p=0.001**
Z_1 (p)		-1.131 (0.266) NS	-2.009 (0.045) *	-2.545 (0.011) *	-3.124 (0.001) **	-1.069 (0.291) NS	
Z_2 (p)			-1.253 (0.219) NS	-2.197 (0.028) *	-2.862 (0.003) **	-2.196 (0.028) *	
Z_3 (p)				-1.740 (0.089) NS	-2.117 (0.033) *	-2.692 (0.007) **	
Z_4 (p)					-0.029 (0.977) NS	-2.714 (0.007) **	
Z_5 (p)						-3.637 (<0.001)**	
Fetuin-A (g/l) Range Mean± SD Median	0.13-0.44 0.30±0.08 0.31	0.17-0.47 0.26±0.10 0.21	0.14-0.29 0.22±0.07 0.23	0.15-0.30 0.20±0.05 0.20	0.01-0.35 0.19±0.11 0.20	0.24-0.45 0.33±0.05 0.33	χ^2=21.59 p=0.001**
Z_1 (p)		-0.752 (0.478) NS	-2.428 (0.014) *	-2.656 (0.007) **	-2.369 (0.017) *	-0.895 (0.378) NS	
Z_2 (p)			-1.158 (0.266) NS	-1.677 (0.094) NS	-1.273 (0.219) NS	-2.314 (0.021) *	
Z_3 (p)				-0.145 (0.887) NS	-0.347 (0.755) NS	-3.094 (0.001) **	
Z_4 (p)					-0.058 (0.977) NS	-3.868 (<0.001)**	
Z_5 (p)						-3.235 (0.001) **	

χ2: Chi square for Kruskal Wallis test.
Z_1 : Z for Mann Whitney test between group I and other groups.
Z_2: Z for Mann Whitney test between group II and other groups.
Z_3: Z for Mann Whitney test between group III and other groups.
Z_4: Z for Mann Whitney test between group IV and other groups.
Z_5: Z for Mann Whitney test between group V and other groups.

* : Statistically significant ($p \leq 0.05$).
**: Highly significant ($p \leq 0.01$).
SD: Standard deviation.

Matrix correlations between age, serum levels of total nitrate and nitrite, visfatin, fetuin-A, triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol in CKD patients are recorded in Table 4. A significant positive correlation was found between serum levels of total nitrate and nitrite and

serum levels of visfatin, triglycerides, total cholesterol and LDL-cholesterol; (r = 0.475; p <0.001, r = 0.320; p =0.006, r = 0.258; p = 0.029 and r=0.346; p =0.003; respectively), while a significant negative (inverse) correlation was found between serum levels of total nitrate and nitrite and serum levels of fetuin-A and HDL-cholesterol (r = -0.325; p =0.005 and r = -0.283; p =0.016; respectively). A significant positive correlation was found between serum levels of visfatin and serum levels of triglycerides, total cholesterol and LDL-cholesterol (r = 0.453; P<0.001, r = 0.391; p =0.001and r = 0.463; p = <0.001; respectively), while a significant negative (inverse) correlation was found between serum levels of visfatin and fetuin-A (r = -0.269, p =0.022). Also, A significant negative (inverse) correlation was found between serum levels of fetuin-A and serum levels of triglycerides, total cholesterol and LDL-cholesterol (r = -0.310; p =0.008,r = -0.256; p =0.03 and r = -0.420; p = <0.001; respectively), while a significant positive correlation was found between serum levels of fetuin-A and HDL-cholesterol (r = 0.416, p <0.001).(Table 4)

Univariate correlation between serum levels of total nitrate and nitrite and visfatin in CKD patients showed a significant positive correlation between the two parameters in CKD patients (r^2 = 0.225; p <0.001) (Figure 1). While, a univariate correlation between serum fetuin-A and both serum levels of total nitrate and nitrite and serum levels of visfatin with in CKD patients showed a significant negative correlation between the two parameters (r^2 = 0.106, p=0.005; r^2 = 0.091, p =0.01, respectively) (Figures 2,3).

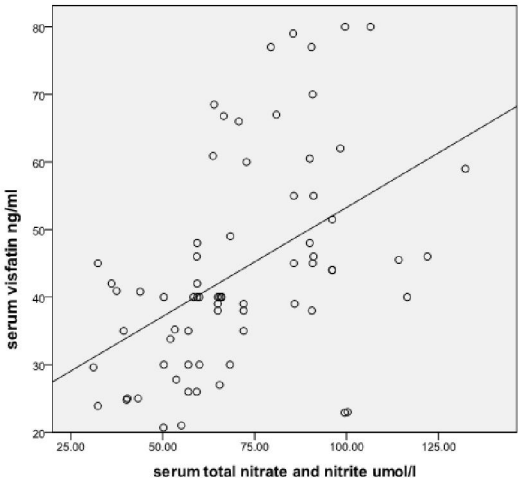

Figure (1). Univariate correlation between serum levels of total nitrate and nitrite and visfatin in CKD patients. r2 = 0.225, p<0.001

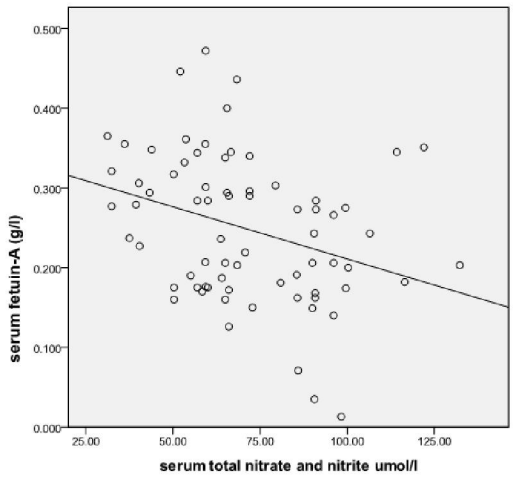

Figure (2). Univariate correlation between serum levels of total nitrate and nitrite and fetuin-A in CKD patients. r2 = 0.106, p=0.005

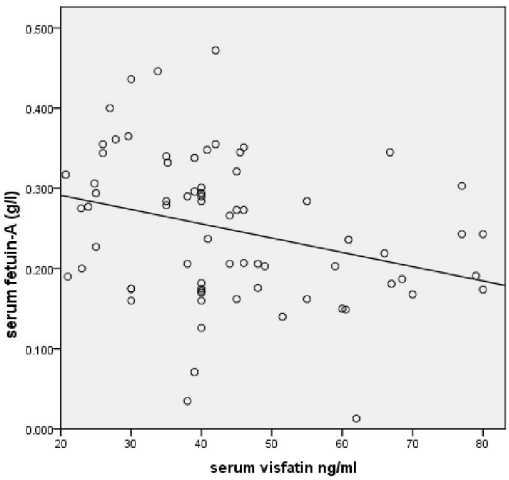

Figure (3). Univariate correlation between serum levels of visfatin and fetuin-A in CKD patients. r2 = 0.091, p=0.01

Table IV. Matrix correlation between age, serum levels of total nitrate and nitrite, visfatin, fetuin-A, triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol in CKD patients.

		Total nitrate and nitrite μmol/l	Visfatin ng/ml	Fetuin-A (g/l)	Triglycerids (mg/dl)	Total cholesterol (mg/dl)	LDL-cholesterol (mg/dl)	HDL-cholesterol (mg/dl)
Age	r	0.110	0.188	0.100	0.198	0.185	0.197	-0.009
	p	0.403 NS	0.151 NS	0.447 NS	0.129 NS	0.158 NS	0.132 NS	0.943 NS
Total nitrate and nitrite (umol/l)	r		0.475	-0.325	0.320	0.258	0.346	-0.283
	p		<0.001 **	0.005 **	0.006 **	0.029 *	0.003 **	0.016 *
Visfatin (ng/ml)	r			-0.269	0.453	0.391	0.463	-0.194
	p			0.022 *	<0.001 **	0.001 **	<0.001 **	0.102 NS
Fetuin-A (g/l)	r				-0.310	-0.256	-0.420	0.416
	p				0.008 **	0.03 *	<0.001 **	<0.001 **
Triglycerides (mg/dl)	r					0.626	0.926	-0.378
	p					<0.001 **	<0.001 **	0.001 **
Total cholesterol (mg/dl)	R						0.704	-0.125
	p						<0.001 **	0.294 NS
LDL-cholesterol (mg/dl)	r							-.555
	p							<0.001 **

r: Pearson correlation coefficient. NS: non significant *: Statistically significant ($p \leq 0.05$). **: Highly significant ($p \leq 0.01$).

In order to clarify predictors of endothelial dysfunction in the CKD patients, a stepwise regression model was done. The strongest predictors of endothelial dysfunction measured as serum total nitrate and nitrite were visfatin and HDL-cholesterol (beta= 0.635, p <0.001 and beta=-0.388, p =0.0039; respectively) ,while r^2 of the model was 0.52. This means that 52% of the changes in serum total nitrate and nitrite could be explained and accounted by serum visfatin plus serum HDL-cholesterol.

Serum total nitrate and nitrite can be calculated from the equation:

Y= a + b1x1+ b2x2

Y= serum total nitrate and nitrite umol/l

a=60.339, b1=0.635, x1= serum visfatin ng/ml, b2=-0.388, x2= serum HDL-cholesterol (mg/dl)

Results of gene polymorphism on fetuin –A (C⟶ G); Thr256Ser (Table V, Figure 4)

Restriction enzyme analysis was attempted for detection of fetuin-A gene restriction fragment length polymorphism(C ⟶G); Thr256Ser at *Sac*I recognition site. Allele C does not contain the *Sac*I site and remained undigested as 405 bp fragments, whereas allele G contained the *Sac*I recognition site and yielded 193- and 212-bp fragments. There are three possible genotypes for the fetuin-A: i.e. Thr/Thr**(**Allele C), Thr/Ser (heterozygote) and Ser/Ser (Allele G); (homozygote for absence of the sacI site, heterozygote and homozygote for the presence of the sacI site; respectively) (Figure 4).

The frequencies of the three genotypes in the CKD patients were: Thr/Thr **(**Allele C) 45 patients(75%), Thr/Ser (heterozygote) 12 patients (20%) and Ser/Ser (Allele G) 3 patients(5%), while in the control group, the frequencies were Thr/Thr**(**Allele C) 8 subjects(66.7%), Thr/Ser (heterozygote) 3subjects (25%) and Ser/Ser(Allele G) 1 subject (8.3%). There was no statistically significant difference between CKD patients and the control group according to the frequencies of the three fetuin–A genotype polymorphisms (C ⟶G); Thr256Ser ($\chi 2$=0.414, p =0.813) (Table V).

Table V: Statistical comparison between CKD patients and the control group according to the frequencies of fetuin –A genotype polymorphisms (C ⟶G); Thr256Ser .

	Thr/Thr (Allele C)	**Thr/Ser (Heterozygote)**	**Ser/Ser (Allele G)**	**total**	χ^2	**(*p*)**
cases	45(75.0%)	12(20.0%)	3(5.0%)	60(100%)	0.414	0.813 NS
control	8 (66.7%)	3(25.0%)	1(8.3%0)	12(100%)		
Total	53(73.6%)	15(20.8%)	4(5.6%)	72(100%)		

χ^2 =Pearson Chi-Square. NS: non significant

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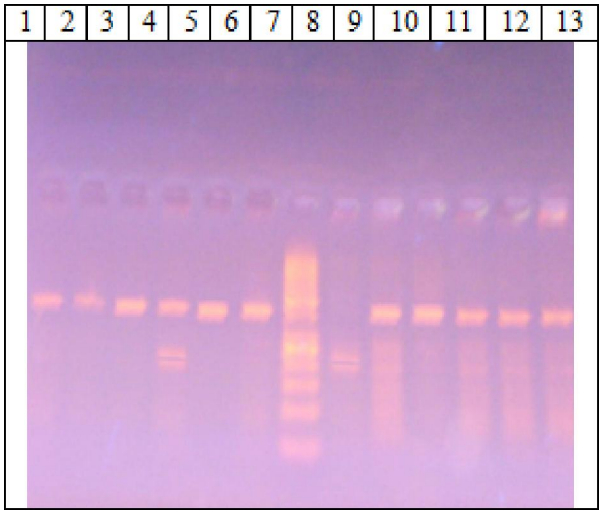

Figure 4: Ethidium bromide stained agarose gel electrophoresis of PCR amplified fetuin-A gene (C ⟶ G); Thr256Ser following sac I restriction digestion for some samples.

Lane 7: DNA size marker 50 – 1000 bp.
Lane 8: Allele G (Ser/Ser) homozygote for the presence of the sacI site which digests the 405 bp fragment into 193 and 212 bp fragments in a homozygote patient.
Lane 1,2,3,5,6,9,10,11,12,13: Allele C (Thr/Thr) undigested as 405 bp. fragments homozygote for absence of the sacI site.
Lane 4: heterozygote patient (Thr/Ser).

Results of the effect of fetuin-A (C ⟶ G); Thr256Ser gene polymorphisms on the level of serum fetuin-A (Table VI).

In both CKD patients and the control group, the distribution of the fetuin-A gene polymorphisms (C ⟶ G); Thr256Ser did not show statistically significant difference between fetuin-A gene polymorphisms and serum fetuin-A levels ($\chi 2$=4.305;P=0.116, $\chi 2$=1.756; p =0.416; respectively).

Showing:

Table (VI). Statistical comparison between the distribution of the fetuin-A (C ⟶ G);Thr256Ser gene polymorphisms and median serum fetuin-A levels in CKD patients and the control group.

Fetuin-A (g\l)	Thr/Thr (Allele C)	Thr/Ser (Heterozygote)	Ser/Ser (Allele G)	$\chi 2$	(p)
CKD cases(n)	45	12	3	4.305	0.116 NS
median(g\l)	0.243	0.179	0.19		
Control(n)	8	3	1	1.756	0.416 NS
median(g\l)	0.324	0.348	0.279		

χ^2: Chi square for Kruskal Wallis test. NS: non significant.

4. Discussion

Endothelial dysfunction (ED) represents an obligatory, prodromal phase in the atherosclerotic process. Endothelial dysfunction may be also responsible for accelerated atherosclerosis in patients with chronic renal failure. Indeed, chronic renal disease is a highly atherogenic disease and state. Thus, the presence of even minor kidney dysfunction has recently been recognized as a significant risk factor for subsequent CVD and death [52] . The etiology of ED is complex and involves dysregulation of multiple pathways. The hallmark of endothelial dysfunction is impaired nitric oxide-mediated endothelial-dependent vasodilatation, which may be attributed to decreased production or activity of nitric oxide[53] . To assess the generation of nitric oxide (NO); measurement of NO production in vivo is difficult because of its short half-life. Consequently, its metabolite total serum nitrate and nitrite has been used as a surrogate marker for estimating NO production [54] .

The present study showed significant elevation of total serum nitrate and nitrite in CKD patients compared to control group (Z=-5.101, $p < 0.001$). In all CKD groups serum total nitrate and nitrite levels was statistically significant than the control group ($\chi 2$=35.48, $p < 0.001$). This is in accordance with several studies [55-57] that reflected the elevation of NO in chronic renal failure and hemodialysis. To our knowledge this study is pioneer to demonstrate significant elevation of total serum nitrate and nitrite in early stages of CKD stage I and II (group I and II CKD patients). The underlying molecular mechanisms for the reduced action of NO has been investigated and attributed to a decrease in NO bioavailability which may be caused by decreased expression of eNOS, lack of substrate or cofactors for eNOS, alteration in the signaling pathways activating eNOS, and/or accelerated degradation of NO by reactive oxygen species [58], increased inflammation-induced inducible nitric oxide synthase (iNOS) expression and thus NO.

Recently **Ueda *et al.*** [59] has looked for CKD reported increased asymmetric dimethyl arginine (ADMA) levels in CKD (even in CKD stage I patients) who did not have classical CVD risk factors, suggesting that NO metabolism is impaired in CKD. ADMA, an endogenous inhibitor of NO, its increase is associated with reduced nitric oxide synthesis and vascular dysfunction [59] . In the present study a significant positive correlation was found between serum levels of total nitrate and nitrite and triglycerides, total cholesterol, LDL-cholesterol. (r = 0.320, p =0.006; r = 0.258, p = 0.029; r=0.346; 0.003, respectively), while a significant negative correlation was found between serum levels of total nitrate and nitrite and HDL-cholesterol (r = -0.283, p =0.016, respectively). In agreement with our result **Patil *et al.*** [60] found that altered nitric oxide level may be related to advanced endothelial dysfunction, increased nitric oxide end-products associated with low HDL levels and increased oxidative stress and suggested that elevated levels of NO promote the peroxidation of the lipid moiety and induce immune responses and inflammatory reactions that cause cell damage. Meanwhile HDL cholesterol is considered as an independent, strong inverse predictor of cardiovascular events [61] .

In the present study we measured circulating levels of the intracellular enzyme visfatin, hypothesized to be a marker of endothelial cell damage [62] .Compared to healthy controls, visfatin concentrations were found to be increased significantly in CKD patients compared to control group (Z=-3.224, p =0.001). When the different groups of patients were compared visfatin concentrations were found to be increased in all CKD stages except stage 1(group I) (χ2=21.59 , p =0.001). This result is in agreement with the result of previous recent studies.[63]In the present study a significant positive correlation was found between serum levels of visfatin and triglycerides, total cholesterol, LDL-cholesterol (r =0.453, p < 0.001;r=0.391, p =0.001;r = 0.463, p = < 0.001,respectively). This finding in accordance with **Mu J *et al.*** [64] who found similar results and gave the hypothesis that visfatin is associated with atherosclerosis in patients with CKD.

Visfatin also functions as a proinflammatory adipocytokine that is secreted by neutrophils in response to inflammatory stimuli and upregulates the production of cytokines in the monocytes. It was reported that visfatin promotes angiogenesis which can be associated with CKD-related vascular dysfunction [65] . Moreover visfatin was upregulated in the foam cell macrophages within human unstable carotid and coronary atherosclerotic lesions that play a role in plaque destabilization [66] . It is suggested that visfatin may play an important role in uremia-related atherosclerosis.

Kim *et al.* [67] investigated the effect of visfatin on vascular inflammation, a key step in a variety of vascular diseases. Visfatin induced leukocyte adhesion to endothelial cells and the aortic endothelium by induction of the cell adhesion molecules (CAMs); intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1). Promoter analysis revealed that visfatin-mediated induction of CAMs is mainly regulated by nuclear factor-κB (NF-κB). Furthermore visfatin was found to stimulate ROS generation in endothelial cells. Based on visfatin enhanced NAD(P)H dependent ROS production in endothelial cells these observations, visfatin may be able to accelerate vascular diseases through ROS overproduction by inducing vascular damage. In addition it was demonstrated that antioxidants blocked visfatin-induced NF-κB activation and CAM expression [67,68] .

We extend this finding by showing an independent positive association between serum levels of visfatin and total nitrite and nitrate (r=0.475, p < 0.001). This observation may be due to the fact that a dysfunctional adipose tissue signaling-reflected by elevated visfatin in CKD may directly affect the vascular endothelium, causing its dysfunction. It appears likely that reduced glomerular filtration rate can contribute to the accumulation of adipocytokines. This could explain the marked dysmetabolism in chronic kidney disease. The kidney plays an important role in the regulation of adipokines, and altered renal handling of these substances might contribute to an increase in the uraemia-associated increased risk of cardiovascular disease and mortality [69] .

Recently, visfatin has been reported to be inversely correlated with functional changes in the endothelium as assessed by flow-mediated vasodilatation of the brachial artery in CKD and early diabetic nephropathy. Our results are supported by **Yilmaz *et al.*** [70] who documented that endothelial function improved during the first month after renal transplantation, and the degree of improvement correlated to reductions in circulating visfatin. In view of these results, serum visfatin is recommended as one of the most promising markers of ED in CKD patients.

Fetuin-A is a circulating protein mostly synthesized in the liver and ubiquitously present in the extracellular space [71]. Fetuin-A is known to be an important inhibitor of vascular and soft tissue calcification. It has been proposed as a protective agent by binding hydroxyapatite structures and causes solubilization of calcium phosphate salt [72].

In the present study serum fetuin-A concentrations were found to be significantly decreased in CKD patients compared to the control group (Z=-3.325, p =0.001), when different stages of

CKD patients were compared fetuin-A concentrations were found to be decreased in all CKD stages except stage 1 (group I) in comparison with the control group ($\chi 2=21.59$, $p=0.001$). Low serum fetuin-A concentrations were observed relatively early in CKD, beginning at stage 2 (group II). This observation indicates that vascular calcification and CVD are likely to develop early during the progression of CKD which is further substantiated by parallel development of endothelial dysfunction in these patients.

Given the importance of vascular calcification in the development of high rate of CVD, several investigators studied the role of fetuin-A in CKD, mostly in patients with advanced disease.[73] In a cross-sectional study, **Ketteler *et al.,*** [74] have shown that chronic hemodialysis patients had lower levels of fetuin- A concentrations than healthy controls. They found fetuin-A deficiency was associated with inflammation and mortality in patients on chronic hemodialysis. Also an Egyptian study was conducted in Cairo University by **El-Shehaby *et al.,*** [75] and revealed that serum fetuin-A was significantly lower in hemodialysis patients than the control subjects and a significant association between low levels of fetuin-A and high calcium score and valvular calcification was found.

To investigate the relationship between fetuin-A and a few of the mechanisms involved in the development of atherosclerosis, a significant negative correlation was found between serum levels of fetuin-A and serum levels of triglycerides, total cholesterol and LDL-cholesterol ($r = -0.310$; $p =0.008$,$r = -0.256$; $p =0.03$, $r = -0.420$; $p <0.001$; respectively), while a significant positive correlation was found between serum levels of fetuin-A and HDL-cholesterol ($r= 0.416$, $p <0.001$) in CKD patients.

In parallel with the previous results, in patients with metabolic syndrome, increased levels of fetuin-A were strongly associated with the components of the metabolic syndrome and with an atherogenic lipid profile [71] . Furthermore, **Stenvinkel *et al*.**, [76] reported that low fetuin-A levels were associated with malnutrition, inflammation and cardiovascular mortality. It was found that fetuin-A serum levels were significantly lower in CKD patients with calciphylaxis compared with other CKD subjects; in addition the inability of human uremic plasma to inhibit the precipitation of calcium and phosphorus is corrected by the addition of fetuin-A which accounts for more than 50% of the precipitation inhibitory effect of serum [77,78]. In support of these observations, **Huang *et al.***[79] found an inverse relationship between the extent of coronary calcification and endothelium-dependent flow mediated dilatation (FMD) in 124 patients with suspected coronary artery disease. These studies have led to the conclusion that there might be a biological link between vascular calcification which is associated with low levels of calcification inhibitors and derangement in endothelial function in CKD patients [79] .

This study also demonstrated the relationship between fetuin-A levels and endothelial dysfunction as there was a significant negative correlation between serum levels of fetuin-A and serum levels of total nitrate and nitrite and visfatin ($R=-0.264$; $p =0.025$,$r=-0.269$; $p =0.022$; respectively). A possible explanation for this correlation is that fetuin-A administration significantly inhibits TNF-α in an experimental model [80] and TNF-α suppressed visfatin gene expression in an *in vitro* study [16], in addition visfatin [81] and NO may be up regulated while fetuin-A is down regulated during the process of ED. [80] In contrast to this study **Uz *et al.,*** [81] failed to find a correlation between serum visfatin and serum fetuin – A.

Lower fetuin-A levels could contribute to endothelial dysfunction in CKD patients by several potential mechanisms. First, fetuin-A is a negative acute-phase reactant, given the importance of inflammation in endothelial dysfunction, low levels of fetuin-A might simply reflect an inflammatory condition. [38] Second, Calcification has been shown to be initiated with the release of membrane-bound vesicles; which normally contain local calcification inhibitors, from vascular smooth muscle cell (VSMC). Reduced levels of calcification inhibitors, such as fetuin-A, would lead to both vesicle and VSMC calcification [82]. **Cola *et al.,*** [83] have suggested that prolonged exposure of stressful stimuli to endothelial cells may lead to expression of genes that promote vascular calcification. It has also been shown that VSMC have a potential to transform to osteoblast- like cells [84].

Studies have reported relationships between fetuin-A and coronary artery score as well as carotid plaque in CKD patients; carotid intima-media thickness (CIMT) has been shown as a powerful predictor of cardiovascular events in the general population [85]. **Zoccali *et al* .,** [86] have shown that CIMT is a predictor of adverse cardiovascular events in patients on hemodialysis. **Caglar *et al.,***[38] suggested that fetuin-A levels might be another contributor for the development of CIMT supporting the potential role of fetuin-A in the development of accelerated atherosclerosis. Recent studies have demonstrated a relationship between vascular calcification and endothelial dysfunction, indicating a mechanistic relationship between endothelial dysfunction and vascular calcification, at least in part mediated through calcification inhibitors.[87] Given the importance of the role of fetuin- A in the development of calcification, one may suggest that low fetuin-A levels might contribute to endothelial dysfunction by

inducing calcification. Assessment of serum levels of fetuin-A may be of value to identify those subjects at higher risk of development and progression of vascular lesion and may be a novel therapeutic approach.

In order to clarify predictors of endothelial dysfunction in the CKD patients a stepwise regression model was done. Variables expected to influence the endothelial dysfunction represented as serum total nitrate and nitrite and the predictors were (the age, serum levels of triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, fetuin–A and visfatin) were included. According to this model the strongest predictors of endothelial dysfunction, evaluated as serum total nitrate and nitrite, were visfatin and HDL-cholesterol (beta= 0.635, p <0.001 and beta=-0.388, p =0.0039; respectively), the model demonstrated that 52% of the changes in serum total nitrate and nitrite could be explained and accounted by serum visfatin plus serum HDL-cholesterol and an equation was developed to estimate serum total nitrate and nitrite from knowing the values of serum visfatin and HDL-cholesterol. According to the results of this study, serum levels of visfatin and HDL-cholesterol were found to be the most important predictors of endothelial dysfunction.

While several studies [74,75], including this study, reported low fetuin-A levels in CKD patients, the mechanisms that result in decreased fetuin-A concentrations are not clear. In an attempt to know more about the pathogenesis of increased risk of ectopic calcification and cardiovascular events in patients with renal failure, restriction enzyme analysis was done for detection of restriction fragment length polymorphism of fetuin-A gene; (C→G);Thr256Ser at *SacI* recognition site known to be associated with a higher cardiovascular mortality risk in the hemodialysis population.

There was no statistically significant difference between CKD patients and the control group according to the frequencies of the three fetuin–A genotype polymorphisms (C→G);Thr256Ser (χ2=0.414, p =0.813) In both CKD patients and the control group, the distribution of the fetuin-A (C→G);Thr256Ser gene polymorphisms did not show statistically significant relationship between serum fetuin-A levels and the three genotypes in CKD and the control group (χ2=4.305; p =0.116, χ2=1.756; p =0.416; respectively). Consequently, neither altered (C→G); Thr256Ser polymorphism of the fetuin-A gene appears neither to affect serum fetuin-A levels nor to have prognostic effect for the progression to cardiovascular disease in this population.

It should be noted that to our knowledge there are only two previous studies [76,88] on the association of fetuin-A (C→G); Thr256Ser gene polymorphisms with serum levels of fetuin-A in CKD. **Stenvinkel *et al.*,** [76] demonstrated that Swedish dialysis patients with fetuin-A 256Ser/ Ser (allele G) had lower serum fetuin- A levels and associated with higher cardiovascular mortality rates. However, **Cozzolino *et al.*,** [88] found that: In both Italian hemodialysis patients and the control group, the distribution of the fetuin-A gene did not show significant association between low serum fetuin-A levels and fetuin-A (C→G); Thr256Ser gene polymorphisms had no statistically significant difference between distribution of fetuin-A (C→G);Thr256Ser gene polymorphisms in hemodialysis patients and the healthy controls was found.

Probably, the reason that some recent clinical results are different from our findings may be explained by the differences in races. Another reason could be contributed towards numerous factors that lead to marked arterial calcification observed in CKD patients: all the 'classic' risk factors for atherosclerosis plus 'uraemia-associated' risk factors, such as duration of dialysis, uraemic toxins, inflammation and increased serum levels of phosphate, calcium-phosphate product and parathyroid hormone .[89]Finally, other fetuin-A single nucleotide polymorphisms not yet known may affect its serum level, however fetuin-A remains an important protective factor against arterial calcification, even if its definitive role remains to be elucidated [87] .

Conclusion

In conclusion, the present study showed that:

- Visfatin and fetuin-A may be novel markers for endothelial dysfunction in chronic kidney disease patients, and may be help in diagnosis of early stages of CKD. Visfatin and fetuin-A may play an important role in uremia-related atherosclerosis. However, serum total nitrate and nitrite is the best one of the studied markers in diagnosis of early stages of CKD.
- The distribution of the fetuin-A (C→G); Thr256Ser gene polymorphisms does not affect serum fetuin-A levels.

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Primary Assessment of the Biological Activity of Jojoba Hull Extracts

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Abstract: To our Knowledge no work up to the present moment has been reported in the literature on the phenolic extracts of jojoba hull. Thus the aim of the present work was to add value to this waste product by investigating the potentiality of different jojoba hull extracts as neutraceuticals. The efficiency of methanol, ethanol, acetone, isopropanol and ethyl acetate at concentrations of 100, 80, 70, 60, and 50% to extract phenolic compounds were investigated. Results revealed that 60% acetone extracted optimum phenolic compounds (13.9 mg/g hulls). Extraction at room temperature yielded more phenolic compounds than extraction at 45°C. On the other hand, 70% methanol extract of jojoba hulls exhibited the highest AOA (95.33%). The 70% methanol extract was added to a butter cake at 100 and 200 ppm as well as 200 ppm BHT. The cake was stored at room temperature and the butter analyzed every week for acid, iodine and peroxide values. Results proved that the addition of methanol extract delayed the oxidation of butter. The extracts of jojoba hulls exhibited different levels of antimicrobial activities on five food borne pathogenic bacteria. The 70% methanol extract of jojoba hulls showed potential as anti-carcinogenic agent on four different cell line carcinomas.
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Keywords: Jojoba hulls, Phenolic compounds, Antioxidant, Antimicrobial, Anti-carcinogenic.

1. Introduction

Due to the health awareness of the global population and the interest in a clean environment, the possibility of turning waste products into valuable nutraceuticals became desirable. Also substituting synthetic pharmaceuticals with neutraceuticals of plant origin became much advisable.

Seed hulls are among the food industry waste products that have proved to contain beneficial compounds such as phenolic compounds. Seed hulls reported to contain phenolic compounds include sunflower hulls (Mohamed and Taha, 2005), rice hulls (Asamarai *et al.,* 1996), buckwheat hulls (Watanabe, 1997), navy bean hulls (Onyenecho and Hettiarachchy, 1991), rapeseed hulls(Amarowicz *et al.,* 2000), peanut hulls (Duh and Yen, 1995), and sesame coat (Chang *et al.,* 2002).

Simmondsia chinensis (link) Schneider (syn. S. California nut, Buxus chinensis link) (Family simmondsiaceae) is a perennial dioeciously evergreen shrub, endemic to Sonoran desert of Arizona, California and New Mexico. It is now commercially cultivated in many countries all over the world. It is better known as jojoba (pronounced ho-ho-ba). Natives of California highly prized the fruit for food and the seed oil as medicine for cancer, kidney disorder, obesity, sore heart, warts and wounds **(**Leung and Foster, 1996**).**

Actually jojoba oil which is a wax ester and not oil is the most valued part of the seeds. Jojoba oil is used as a replacement for whale oil and its derivatives (Undersander *et al.,* 1990). Jojoba oil is used greatly in skin care (Gunstone, 1990). Jojoba oil is sought greatly by cosmetic manufacturers (Wisniak, 1994) Jojoba biodiesel has been explored as a cheap, sustainable fuel that can serve as a substitute for petroleum diesel (Bouaid *et al.,* 2007). The meal remaining after the removal of the oil from jojoba seeds represent a potential amendment for animals/ or humans. The jojoba meal contain toxic compounds simmondsin and its derivatives (kolodziejczyk *et al.,* 2000), which should be removed before being used as food or feed. No attention has been given to jojoba seed hull to our knowledge up to the present day.

Phenolic compounds exhibit a wide range of physiological properties, such as antioxidant, anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardioprotective and vasodilatory effects (Benavente-Garcia, 1997; Samman *et al.,* 1998; Puupponen-Pimiwaw *et al.,* 2001; Manach, *et al.,* 2005). There are Several types of phenolics including simple phenolic compounds, such as the cinnamic acids (I) or aldehydes (II) and polyphenolics, such as the 'condensed' (III) and 'hydrolysable' tannins (IV) (Haslam, 1981) The main phenolic subclasses in oilseed products are phenolic acids (hydroxylated derivatives of benzoic and cinnamic acids), coumarin, flavonoid, tannins and lignin group of compounds (Shahidi and Naczk, 2003).

Extraction of phenolic compounds from plant material is influenced by extraction conditions, i.e. solvent polarity, particle size, concentration, temperature and time. The impact of the extraction of

phenolic compounds on the analysis has often been overlooked as substantial variations in the extraction procedures; and solvents are documented in the recent literature (Antolovich *et al.,* 2000). Several solvents such as methanol, ethanol, acetone, water, ethyl acetate; and to a lesser extent, propanol, dimethyl formamide, dimethyl sulfoxide and their combinations have been used for the extraction of different classes of phenolic compounds (Antolovich *et al.,* 2000; Naczk and Shahidi , 2004; Parejo *et al.,* 2004; Vrhovsek *et al.,* 2004).

The aim of the present investigation is to achieve the effective utilization of a wasted material from an industrial oil crop (jojoba seed). Synthetic pharmaceuticals are being replaced nowadays with phytochemicals from natural sources. Therefore the biological activity of the jojoba seed hull extracts will be studied. Several solvents as well as several solvent concentrations, followed by several extraction times will be investigated to determine optimum conditions for the extraction of phenolic compounds from jojoba hulls. The antioxidant activity of the phenolic extracts will be determined. The effect of the methanol extract of jojoba hull will be evaluated as an antioxidant in butter cake during storage. Then the antimicrobial effect of the different phenolic extracts and anticarcinogenic activity of the methanol extract will be examined. If the phenolic extracts of the jojoba hulls prove to have the previous biological activity, it will certainly be a great profit to the human health and the pharmaceutical industry.

2. Materials and Methods

Jojoba hulls: were obtained from the Egyptian Natural Oil Co., (NATOTL), Ismailia Branch and Farm Factory 10th of Ramadan City Egypt. It belonged to the crop of 2010. The hulls were ground using a Wiley mill to pass through 60 mesh screen. The ground hulls were defatted with n- hexane in a Soxhlet apparatus and saved for further work.

Wheat flour 72% extractions: were obtained from South Cairo Mills Co., Egyptian Ministry of Supply and Trade. Moreover, Fresh butter, vanilla and baking powder were purchased from the local market.

Microorganisms: were obtained from the Microbiological Resources Center (Cairo MIRCEN) Faculty of Agriculture, Ain Shams University: *E.coli* 0157:H7 ATCC 51659, *Staphylococcus aureus* ATCC 13565, *Bacillus cereus* EMCC 1080, *Listeria monocytogenes* EMCC 1875 and *Salmonella typhimurium* ATCC25566.

Cell line Carcinoma: Different cell line carcinomas included: Liver Carcinoma Cell Line (HEPG2), Colon Carcinoma Cell Line (HCT), Cervical Carcinoma Cell Line (HELA), and Breast Carcinoma Cell Line (MCF7) were supplied and used in The National Cancer Institute, Biology Department, Cairo, Egypt.

Analytical methods:

Moisture, ash, protein, oil and crude fibre were determined in jojoba hulls according to (AOAC, 2005). Dietary fibre fractions were determined as recommended by Goering and Van Soest (1970).

Analytical methods were carried out on different crude phenolic extracts of jojoba seed hulls. Total phenolic compounds were determined by the Folin Ciocalteu method according to Hung *et al.,* (2002) and measured as gallic acid equivalent. Antioxidant activity was determined by two methods: Free radical scavenging activity according to Kuda *et al*. (2005) where crude phenolic extracts were dissolved in methanol to obtain a concentration of 500 ppm. 0.2 ml of this solution was completed to 4 ml by MeOH and 1 ml of DPPH (6.09×10^{-5} mol/L) was then added. The second method used is the coupled oxidation of β- carotene/ linoleic acid method described by Al-Shaikhan *et al.,* (1995). Determination was done at a concentration of 500ppm of each phenolic extract and 200 ppm BHT. Acid value, iodine value and peroxide value were determined in butter according to AOAC, (2005). Anticarcinogenic activity was determined in the National Cancer Institute (Biology Department) on several cell lines by the measurement of potential cytotoxicity of the phenolic extracts which was carried out by the Sulfo-Rhodamine-B stain (SRB) assay, according to the method of Skehan *et al.,* (1990). Antimicrobial activity for different extracts was tested against five pathogenic bacterial strains using the disc diffusion method as described by Kotzekidou *et al.*, (2008).

Effect of solvent type, solvent concentration, temperature and time on the extraction of phenolic compounds:

A detailed study using extracting solvents with different polarities was carried out. These solvents included ethanol, methanol, acetone, isopropanol, and ethyl acetate at concentrations of 100%, 80%, 70%, 60%, and 50%. These experiments were carried out at room temperature. Two grams jojoba hulls were extracted with 100 ml of each solvent in a shaking water bath for 24 hours. The solution was then filtered and filtrate saved. The residue was re-extracted with 50 ml of same solvent under same previous condition. The first and second filtrates were combined, evaporated under vacuum using a Buchi-rotary evaporator, to dryness. The dried extract was used to determine phenolic content and antioxidant activity.

Same experiment was carried out at 45°C for 1, 3, 6, 9, and 12 hrs, and at a meal: solvent ratio 1:75 w/v in just one extraction. 70% ethanol, 70% methanol, and 60% acetone were chosen to be examined in this experiment.

Preparation of fortified butter cake:

Butter cake was fortified with jojoba hull extract (70% methanol) with the aim of testing the power of the hull extract to inhibit oxidation of the butter in the cake. The ingredients of butter cake are given in Table 1 according to Mizukoshi *et al.* (1979) with little modification.

The natural antioxidant from jojoba hull extract was added to the butter cake mixture at 100 and 200 ppm levels and compared with synthetic antioxidant (BHT) at level 200 ppm. The product was baked at 191°C ± 5°C for 25 min. in an electric oven and the cake was stored at room temperature for three weeks. The control sample was made by the same method but without any added antioxidant.

Table 1: Ingredients of butter cake

Ingredients	Weight/g
Flour	200
Sugar	250
Whole egg	150
Vanilla	1
Baking Powder	13
Water	40
Fresh butter	100

The cake was extracted every week by soaking in n-hexane at room temperature for 48 hrs. The extract was filtered and evaporated to dryness. Extracted butter was analysed for its acid value, iodine value and peroxide value.

Statistical analysis:

The results are presented as average ± standard deviation (St. Dev.). All results were evaluated statistically using one way analysis of variance according to McClave & Benson, (1991).

3. Results and Discussion

Chemical composition of jojoba hulls is represented in Table 2. Results in Table are self-explanatory. Verbiscar and Banigan, (1978) are the only available authors to analyse jojoba hull and reported 10.7% moisture, 7.0% protein, 0.7% oil, 4.4% ash and 15.6% crude fibre. Our values are within the same range.

Table 2: Chemical Composition of jojoba seed hulls

Composition (%)	Jojoba hull ± = St. Dev.
Moisture	7.92 ± 0.324
Protein	3.34 ± 0.535
Oil	1.76 ± 0.461
Ash	2.15 ± 0.436
Crude fiber	17.13 ±0.333
Nitrogen free extract	67.70 ± 0.228
Simmondsin	0.19 ±0.662
Dietary Fiber Fractions	
Cellulose	17.29 ± 0.516
Hemicellulose	1.34 ± 0.852
Lignin	35.29 ± 0.458
Neutral detergent fiber (NDF)	54.17 ±0.277
Acid detergent fiber(ADF)	52.83 ± 0.739
Acid detergent lignin (ADL)	35.54 ± 0.981

Optimization of the extraction of phenolic compounds from jojoba hulls.

Since the solubility of phenolic compounds (PC) in general is governed by their chemical nature which may vary from simple to very highly polymerized substances, and also because the solubility of PC is affected by the polarity of solvent(s) used, it was advisable to first examine the suitability of the type of solvent for optimum extraction of phenolic compounds from jojoba hulls. Results of the solvent extraction of jojoba hulls with methanol, ethanol, acetone, isopropanol and ethyl acetate at concentrations 100%, 80%, 70%, 60%, and 50% are represented in Table 2. Results reveal a significant difference ($p<0.05$) between all extracting solvents at all concentrations except for 60% methanol and 60% isopropanol that showed no significant difference between them. Highest phenolic extraction was achieved with acetone at 80, 70, 60, and 50% reaching 9.93, 12.48, 13.90, and12.74 mg PC/g hulls, respectively. As for methanol and ethanol highest amount extracted 10.09 and 10.70 mg PC/g hulls, respectively, was accomplished with 70% solvent concentration.

Acetone probably extracted more PC because it is more polar than the other solvents. Kim *et al.* (2007) developed a method of designing solvents for the optimal extraction of bioactive ingredients from mulberry leaves using an alcohol-water binary solvent. From their study they reported that the extraction efficiency of the bioactive ingredients was correlated with the solvent polarity. This finding is in agreement with our results. Taha *et al.* (2011) studied the optimization of phenolic compounds and chlorogenic acid extraction from sunflower meal. They found that 80% acetone extracted maximum phenolics and chlorogenic acid when applying conventional, microwave-assisted, and ultrasound-

assisted extractions. Sun *et al.* (2006) prepared oat groat phenolic extracts using acetone, methanol and hexane. The acetone extracted highest amount of phenolic compounds. Acetone extract exhibited highest radical scavenging activity and highest Trolox value. Sun *et al*., (2006) and Taha *et al* (2011) findings are in agreement with our results.

Table 3: Effect of different solvents at different concentrations on the yield of phenolic compounds (mg/g) extracted from jojoba hulls at room temperature

Extracting Solvent	Solvent Concentration				
	100%	80%	70%	60%	50%
Methanol	6.05 ^{a} ± 0.04	7.79 ^{b} ± 0.04	10.09 ^{c} ± 0.03	9.70 b ± 0.03	9.30 d ± 0.03
Ethanol	3.32 ^{b} ± 0.04	7.27 ^{c} ± 0.03	10.70 ^{b} ± 0.03	9.50 c ± 0.05	9.61 c ± 0.04
Acetone	1.30 ^{c} ± 0.40	9.93 ^{a} ± 0.04	12.48 ^{a} ± 0.05	13.9 a ± 0.04	12.74 a ± 0.04
Isopropanol	1.11 ^{d} ± 0.03	5.46 ^{d} ± 0.07	7.80 ^{d} ± 0.03	9.7 b ± 0.04	4.20 e ± 0.03
Ethyl acetate	1.01 ^{e} ± 0.08	3.84 ^{e} ± 0.04	6.30 ^{e} ± 0.03	4.09 d ± 0.05	9.71 b ± 0.04
LSD at 5%	0.087	0.081	0.062	0.073	0.059

Different letters in each column indicates significant differences between solvents at (P<0.05) for each extraction concentration.

Time and temperature are other parameters that can affect extraction of the PC. The previous experiment was carried out at room temperature. Table 4 show the effect of temperature (45°C) and time of extraction (1, 3, 6, 9, and 12 hrs) on the amount of extracted PC from Jojoba hulls, using the more efficient three solvents according to results in Table 3. It is clear that there is no significant difference between the PC extracts at 6, 9, and 12 hrs, when using 60% acetone, 70% methanol or 70%ethanol. This could be explained by Fick's second law of diffusion revealing that final equilibrium will be attained between the solution concentrations in the solid matrix and solvent after a particular duration (Pinelo *et al.,* 2006). At 1 and 3hrs less PC were extracted by the three solvents. This result indicated that at 45°C, extraction for 6 hours is suitable for maximum extraction. Comparing extraction at room temperature for 2 nights while shaking, with extraction at 45°C for 1-12 hrs, it is clear that more PC were extracted at room temperature. 60% acetone extracted 10.47mg PC/g hulls at 45°C for 6 hours and 13.90 mg PC/g hulls at room temperature for 2 nights, 70% methanol and 70% ethanol at 45°C for 6 hrs extracted 8.53 and 8.20 mg PC /g hulls, respectively; and at room temperature for 2 nights extracted 10.09 and10.70 mg PC/ g hulls, respectively. It is expected that increase in extraction temperature is directly proportional to increase PC yield. Al-Farsi and Chang (2007) reported that increased temperature could promote the phenolic extraction by increasing both diffusion coefficient and solubility of phenolic compounds in extraction solvent. Besides that, intense heat from solvent was also able to release the cell wall phenolics and bounded phenolics by breaking down of cellular constituents (Wang *et al.,* 2008).The findings of the previous authors are contrary to our results. On the other hand, (Mueller-Harvey, 2001) reported that some phenolic compounds decomposed rapidly under high temperature and thus causes a reduction of the antioxidant capacity of a plant extract. This statement is in agreement with our results. We believe that perhaps some of the phenolic compounds were degraded by heating, or 6h extraction time might not be enough to solubilize the entire PC From jojoba hulls.

Table 4: Effect of time on the yield of phenolic compounds extracted from jojoba hulls at 45°C

Time(h)	60% Acetone	70% Ethanol	70% Methanol
1	8.77 ^{c} ± 0.15	5.90 ^{c} ± 0.09	5.40 ^{c} ± 0.20
3	9.73 ^{b} ± 0.15	7.50 ^{b} ± 0.20	6.53 ^{b} ± 0.35
6	10.47 ^{a} ± 0.25	8.53 ^{a} ± 0.15	8.20 ^{a} ± 0.20
9	10.63 ^{a} ± 0.15	8.63 ^{a} ± 0.15	8.27 ^{a} ± 0.21
12	10.63 ^{a} ± 0.15	8.63 ^{a} ± 0.15	8.27 ^{a} ± 0.21
LSD at 5%	0.321	0.282	0.438

Different letters in each column indicates significant differences between time of extraction at (p<0.05) for each investigated solvent

Antioxidant activity of Phenolic compounds extracted from jojoba hulls.

Phenolic substances possess many biological effects which are mainly attributed to their antioxidant activities in scavenging free radicals, inhibition of peroxidation and chelating transition metals (Bahman *et al.,* 2007). For examples, flavonols, cinnamic acids, coumarins and caffeic acids are well known polyphenolic compounds with strong antioxidant properties. Hence play an

important role in protecting food, cells and organs from oxidative damage (Osawa, 1999). These compounds (phenolic substances) all share the same chemical patterns, with one or more phenolic groups for hydrogen proton donors and neutralize free radicals (Pajero *et al.,* 2002; Milliauskas *et al.,* 2004; Atoui *et al.,* 2005; Galvez *et al.,* 2005).

Table 5 show the antioxidant activity of the different phenolic extracts resulting from extraction of jojoba hull with different solvents at room temperature. The antioxidant activity was determined by the DPPH scavenging method and the β- carotene/ linoleate method. In terms of antioxidant activity, the free radical scavenging activity (FRSA) is shown for three concentration levels of the phenolic extracts (25µl, 50µl, 100µl.). Statistical analysis of the FRSA made clear that there is no statistical difference (p<0.05) between the FRSA of the 70% methanol, 70% ethanol, and 60% isopropanol extracts of jojoba hulls and BHT at the three examined concentrations. A significant difference at (p<0.05) between 60% acetone jojoba hull extract and the other extracts and BHT at 25 and 50µl concentrations was detected, but no significant difference was found between all extracts and BHT at 100µl concentration, except 70% ethyl acetate which was significantly different at 100µl concentration. 60% acetone extract exhibited highest FRSA at 25 and 50µl concentration (59.93 and76.67 FRSA %, respectively), even higher than BHT (54.67 and73.35 FRSA %, respectively). At 100µl concentration the FRSA of the 70% methanol extract was the highest (81.19 FRSA %). In accordance the AOA as determined by the β-carotene/ linoleate method was also highest for 70% methanol extract of jojoba hulls (95.33%) compared to BHT (93.88%).These results led to the choice of the 70% methanol extract of jojoba hulls for further work. But in fact the methanol, ethanol and acetone extracts of the PC of jojoba hulls all possess very promising antioxidant properties. These results are in agreement with the results of other authors who reported on the efficiency of methanol extracts from seed hulls as antioxidants (Yen and Duh, 1995; Kyung *et al.,* 2006; Taha *et al.,* 2011). Seed coat plays an important role in protecting seeds from oxidative damage because the seed coat possesses large quantities of endogenous antioxidants such as phenolic compounds (Moure *et al.,* 2001).

Table 5: Antioxidant activity of jojoba hull phenolic extracts as determined by FRSA and β-carotene /linoleate methods

Phenolic extract	FRSA%		AOA%	
	25µl	50µl	100µl	
70% Methanol	52.66 ^{b} ± 2.83	66.44 ^{c} ± 1.90	81.19 ^{a} ± 4.06	95.33 ^{a} ± 1.53
70% Ethanol	54.23 ^{b} ± 2.95	67.03 ^{c} ± 3.25	80.74 ^{a} ± 4.11	89.66 ^{b} ± 1.53
60% Acetone	59.93 ^{a} ± 2.70	76.48 ^{a} ± 2.31	80.64 ^{a} ± 4.05	85.0 ^{c} ± 2.00
60% Isopropanol	53.28 ^{b} ± 3.00	69.67 ^{bc} ± 3.80	78.97 ^{a} ± 3.30	80.33 ^{d} ± 2.52
70% Ethyl acetate	45.38 ^{c} ± 2.36	48.84 ^{d} ± 3.61	63.97 ^{b} ±4.05	75.0 ^{e} ± 3.00
BHT	54.67 ^{b} ± 3.40	73.35 ^{ab} ± 3.71	76.87 ^{a} ± 2.77	93.88 ^{a} ± 3.01
LSD at 5%	5.148	5.663	6.69	3.8

Different letters in each column indicates significant differences between different solvents at (p<0.05) for FRSA at different concentrations, and AOA.

Effect of methanolic extract of jojoba hulls on the oxidation of butter-cake stored at room temperature for three weeks.

Seventy percent methanolic extract of jojoba hulls containing PC with AOA were added to a cake with butter as an essential ingredient in the cake. The methanolic extract was added at 100ppm and 200ppm to the cake to see its power of inhibition of butter oxidation during storage. BHT (200mg) was used for comparison. Acid value (AV), iodine value (IV) and peroxide value (PV) of the extracted butter were determined every week and taken as an indication for oxidation of butter. Results are represented in Table 6.

Statistical analysis revealed no significant difference (p<0.05) between AV, IV, and PV of the butter extracted from the cakes with 200ppm methanolic extract of jojoba hulls and 200ppm BHT, at zero time, 1, 2 and 3 weeks storage. Also no statistical difference between IV and PV of the butter with added 100and 200ppm methanolic extracts after one week storage period. All other treatments showed significant differences between all of them. AV, IV, and PV indicated that the 70% methanolic extract from jojoba hulls when added to the cake delayed oxidation of the butter, when compared to cake with no additions (control). 200 ppm methanolic extract showed more efficiency as antioxidant than 100ppm and was comparable to 200ppm BHT.

Table 6: Chemical characteristics of butter cake fortified with methanolic extract of jojoba hulls and BHT during three weeks of storage at room temperature.

Storage period (week)	Treatment	Acid Value (%)	Iodine Value (g/100g)	Peroxide Value (ml.equi.O_2/Kg)
Zero time	Control (no addition)	$0.71^{h} \pm 0.03$	$37.60^{bcd} \pm 1.39$	$2.7^{fg} \pm 0.20$
	Methanol 100ppm	$0.73^{h} \pm 0.04$	$39.83^{ab} \pm 1.40$	$2.36^{g} \pm 0.14$
	Methanol 200ppm	$0.75^{h} \pm 0.03$	$40.90^{a} \pm 1.75$	$2.27^{g} \pm 0.25$
	BHT 200ppm	$0.74^{h} \pm 0.03$	$41.20^{a} \pm 1.67$	$2.27^{g} \pm 0.25$
One week	Control (no addition)	$1.81^{c} \pm 0.04$	$30.37^{f} \pm 1.40$	$5.36^{dc} \pm 0.47$
	Methanol 100ppm	$1.16^{ef} \pm 0.04$	$35.33^{cd} \pm 1.81$	$4.07^{ef} \pm 0.45$
	Methanol 200ppm	$0.92^{gh} \pm 0.04$	$37.13^{bcd} \pm 1.75$	$2.7^{fg} \pm 0.04$
	BHT 200ppm	$0.85^{gh} \pm 0.04$	$38.47^{abc} \pm 1.75$	$2.53^{fg} \pm 0.40$
Two weeks	Control (no addition)	$2.51^{b} \pm 0.22$	$24.40^{g} \pm 1.18$	$10.73^{b} \pm 1.16$
	Methanol 100ppm	$1.53^{d} \pm 0.04$	$30.77^{f} \pm 2.55$	$7.53^{c} \pm 0.04$
	Methanol 200ppm	$1.19^{ef} \pm 0.04$	$34.57^{de} \pm 2.43$	$5.17^{de} \pm 1.16$
	BHT 200ppm	$0.99^{fg} \pm 0.10$	$35.90^{cd} \pm 2.33$	$4.93^{de} \pm 1.05$
Three weeks	Control (no addition)	$3.88^{a} \pm 0.35$	$19.63^{h} \pm 1.33$	$15.37^{a} \pm 1.33$
	Methanol 100ppm	$1.91^{c} \pm 0.25$	$26.53^{g} \pm 1.75$	$9.67^{b} \pm 1.84$
	Methanol 200ppm	$1.50^{d} \pm 0.13$	$30.33^{f} \pm 2.26$	$6.1^{cd} \pm 1.20$
	BHT 200ppm	$1.34^{de} \pm 0.07$	$31.36^{ef} \pm 2.66$	$5.83^{d} \pm 1.30$
LSD at 5%		0.22	3.158	1.614

Different letters in each column indicates significant differences between control butter and butter with additions and BHT at ($p<0.05$) for different storage times.

Acid value (AV), IV, and PV of the control butter at zero time were 0.71%, 37.60 g/100g , and 2.7 ml equiv O_2/ Kg., after three weeks of storage they changed to 3.88%, 19.63 g/100g, and 15.37ml. equiv. O_2/kg, respectively. Comparing AV of control (3.88%) after three weeks to 70% methanol extract 100 and 200ppm and BHT 200ppm it can be seen that the AV reached 1.19, 1.5, and 1.34%, respectively, IV reached 26.53, 30.33 and 31.36 g/100g, respectively compared to control 19.63 g/100g. While PV reached to 9.67, 6.1 and 5.83 ml equiv. O_2/Kg, respectively, in comparison to control (15.37ml equiv. O_2/kg). The increase in AV when compared to control at zero time is explained by the hydrolysis of the oil to free fatty acids which will lead to further formation of aldehydes and ketones (Kun, 1988). IV is the measure of unsaturation of any oil and the decline in iodine value is sometimes used to monitor the reduction of dienoic acids during the course of oxidation. Thus the decrease in IV during the whole storage period could be attributed to breaking of double bonds of unsaturated fatty acids of the lipid. Rehman (2006) added citrus peel extract to refined corn oil and stored at 25 and 45°C for 6 months to examine antioxidant capacity of the extract. He found that similar to our results a gradual increase in both the AV and PV, along with a decrease in IV. Last is the PV which is applicable for the early stages of lipid oxidation. It measures the peroxides which are the primary oxidation products. The PV of the butter increased during the three weeks but comparing PV of butter with methanolic extracts to control shows how the methanolic extract was a shelter that resulted in much less rancidity of the butter. On the other hand at end of the three weeks it became clear from the results that the addition of the 70% methanolic extract of jojoba hulls (100 and 200ppm) and 200ppm BHT delayed the rancidity of the butter.

Antimicrobial activity of phenolic compounds extracted from jojoba hulls.

There is considerable interest in the possible use of natural compounds as alternative food additives. They are used to prevent the growth of food borne pathogens or to delay the onset of food spoilage. Many naturally occurring compounds such as phenols (phenolic acids, polyphenols and tannins) have been considered in this context. Phenolics are being used in foods mainly for purposes such as antioxidants and other than antimicrobial agents (Nychas, 1995). Thus it seemed worthwhile to evaluate the phenolic extracts as antimicrobial agents.

The phenolic extracts of jojoba hulls using different extracting solvents were tested for their antimicrobial activity (AMA) against five bacterial strains using the disc diffusion method. The five bacteria were *Escherichia coli* 0157:H7 ATCC 51659, *Staphylococcus aureus* ATCC 13565, *Bacillus cereus* EMCC 1080, *Listeria monocytogenes* EMCC 1875 and *Salmonella typhimurium* ATCC25566. Comparing the effect of the different solvent hull extracts (80% methanol, ethanol, acetone, isopropanol and ethyl acetate) on the five bacteria strains, it is clear that the five extracts exhibited various degrees of inhibition against the 5 bacteria strains as presented in Table 7. Highest inhibition of *Bacillus cereus* was attained with ethyl acetate

jojoba hull extract, followed by isopropanol, methanol, acetone then ethanol with inhibition zone diameters 14.3, 10.8, 10.3, 8.3, and 8mm, respectively. *Staphylococcus aureus* show to be inhibited in decreasing order by methanol >isopropanol > acetone > ethanol > ethyl acetate extracts with inhibition zone diameter 15.5 >14 > 13.3 >13 >2.3mm, respectively. As with *Bacillus cereus* highest inhibition of Salmonella *typhimurium* was reached with ethyl acetate extract with 16.5mm inhibition zone diameter followed by ethanol extract with 11.6 mm inhibition zone diameter. Other extracts resulted in less zone inhibition between 9.7-5mm. For *Listeria monocytogenes highest* zone inhibition was with ethanol extract 16mm and lowest zone inhibition resulted from isopropanol extract10mm. *Escherichia coli* were inhibited by four extracts only and their inhibition zone diameters were in the following order 21.3 > 16.3 > 15.6 > 2.5mm for acetone, isopropanol, methanol and ethyl acetate, respectively. Ethanol extract exhibited no effects on *E. coli.*

The overall results indicated that different bacteria species exhibit different sensitivities towards phenolics. In the present work Gram-positive and Gram-negative microorganisms were affected by the phenolic extracts of jojoba hulls. Estevinho *et al.,* (2008) reported that the susceptibility of bacteria to phenolic compound and Gram reaction appears to have influence on growth inhibition. The inhibitory effect of phenols could be explained by interactions with the cell membrane of microrganisms and is often correlated with the hydrophobicity of the compounds (Sikkema et al., 1995; Weber and De Bont, 1996). Phenolic compounds could have an activating or inhibiting effect on microbial growth according to their constitution and concentration (Rauha *et al*., 2000; Reguant *et al*., 2000; Alberto *et al*., 2001; Estevinho *et al*., 2008; Rodríguez Vaquero *et al*., 2010).

Anticarcinogenic activity of a methanolic extract from jojoba hulls

This evaluation was carried out in the National Cancer Institute, Biology Department, Cairo. The experiment was done by the Sulfo-Rhodamine-B stain (SRB) assay. Jojoba hull extract (70% metabolic) has been evaluated as a chemopreventive agent. This was established by testing the jojoba hull methanolic extract for any cytotoxic activity against the following human tumour cell lines: Liver Carcinoma Cell Line (HEPG2); Colon Carcinoma Cell Line (HCT); Cervical Carcinoma Cell Line (HELA); Breast Carcinoma Cell Line (MCF7).

Figure 1 represents the effect of jojoba hull methanolic extract on the human carcinoma cell lines tested and the results are indicated by the IC50, which is the dose of the compound (jojoba hull phenolic extract) which kills surviving cells up to 50%. The smaller the concentration or dose the more effective is the compound. Looking at Figure 1, the following could be observed:

Methanolic extract of jojoba hulls was most effective on HELA cell line carcinoma with an IC 50 = 9.75µg / ml

IC 50 for HEPG was attained with 15.8 µg/ml of methanolic extract.

Following is IC 50 = 16µg/ml for MCF7 cell line.

Least affected was HCT cell line with IC 50 =19.8µg/ml.

These results indicate the potentiality of the 70% methanolic extract of jojoba hulls as an anticarcinogenic agent. This effect is probably due to the phenolic content of the jojoba hulls. The anticarcinogenic activity of phenolic compounds has been documented (Owen *et al.,* 2000; Cai *et al.,* 2004; Kerr and Sarangarajan, 2007). The Biology Department –National Cancer Institute-Cairo recommended that further pharmacological investigations *in vitro* and *in vivo* are required to confirm the activity of the tested 70% methanolic extract of jojoba hulls.

Conclusion

It can be concluded from this study that jojoba hull extracts are a very promising source of bioactive compounds with antioxidant, antimicrobial and anticancer properties. This neglected waste product from an industrial oilseed crop should be given more attention to get optimum benefits for the pharmaceutical (nutraceutical) industry.

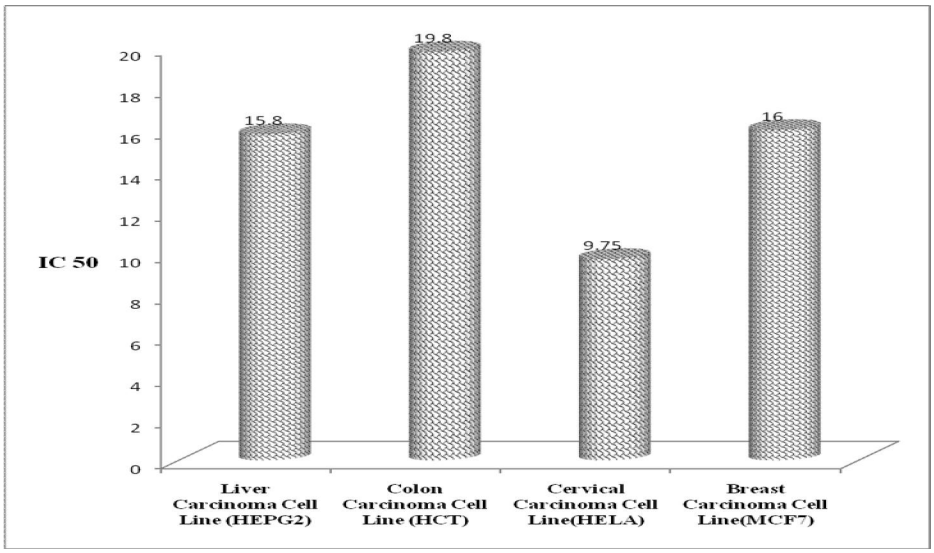

Figure 1: Anticarcinogenic effect of 70% methanolic extract of jojoba hulls.

Table 7: The effect of different jojoba hull extracts on the inhibition of some food borne pathogenic bacteria.

Jojoba hull extract	Strain/Inhibition zone diameter (mm)				
	B.c	St	Sa	Lis	E.coli
80% Methanol	10.3	15.5	9	11.6	15.6
80% Ethanol	8	13	11.8	16	-
80% Acetone	8.3	13.3	5	10.5	21.3
80% Isopropanol	10.8	14	9.7	10	16.3
80% Ethyl acetate	14.3	2.3	16.5	12.6	2.5

B.c= (*Bacillus cereus* EMCC1080), St.= *Staphylococcus aureus* ATCC 13565, Sa= (*Salmonella* typhymurium ATCC 25566), Lis = *Listeria monocytogenes* EMCC 1875, E. Coli = *Eschirechia.coli* 0157:H7 ATCC 51659

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Does JAK2 V617F Mutation in Egyptian Patients with First Episode Venous Thromboembolism Contribute to the Hypercoagulable State and Interact with other Thrombophilic Factors?

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Abstract: ***Background***: The term thrombophilia includes any inherited and acquired disorders associated with an increased tendency to venous thromboembolism (VTE). Inherited thrombophilia is one of the main determinants of VTE, and the presence of inherited thrombophilic defects exposed carriers to increased risks for VTE compared with non-carriers .The *JAK2*^{V617F}mutation is present in the majority of patients with polycythemia vera and essential thrombocythemia, which are myeloproliferative neoplasms frequently associated with arterial and venous thromboembolism. Whether JAK2V617F mutation is associated per se with hypercoagulability remains unclear. Our aim was to clarify the contribution of JAK2V617F to a Hypercoagulable state, as well as its interaction with other thrombophilic factors in patients with venous thrombosis (lower Limb deep venous thrombosis and pulmonary embolism). ***Material and Methods:*** The study subjects were 106 Egyptian patients diagnosed as having first episode venous thromboembolism based on Doppler ultrasound +/- computed tomography and pulmonary angiography from January 2010 to December 2011 (54 men and 52 women); median age 39.5; age range from (14–80 years). They were compared with sixty healthy controls sex and age matched. Full history was taken and the clinical and laboratory data were reviewed. All patients and control groups were subjected to assays for factor V Leiden mutation, prothrombin gene mutation G20210A, protein C activity, protein S activity, antithrombin III level, serum homocysteine, anticardiolipin IgG and Ig M antibodies and lupus anticoagulant to evaluate the Hypercoagulable state and the prevalence of JAK2 V617F mutation was detected by two round allele-specific polymerase chain reaction in both patients and control subjects. Samples positive for the mutation were subsequently analyzed via ARMS-PCR. ***Results*****:** Among the 106 patients, eighty-four had deep venous thrombosis, sixteen had pulmonary embolism and six with concomitant deep venous thrombosis and pulmonary embolism. Twenty five patients were positive for factor V Leiden , three were positive for prothrombin gene mutation ,four had protein S deficiency (three patients had only protein S deficiency and one with factor V mutation) ,six patients had protein C deficiency ,only one had antithrombin III deficiency. Fourteen patients were positive for anticardiolipins and seven for lupus anticoagulant (2 patients were positive for lupus anticoagulant alone and five with positive anticardiolipins) and seven had hyper-homocysteinemia. Jak2V617F mutation was detected in six patients (5.7%) and was positive in two subjects in the control group(3.3%). ***Conclusion*****:** Factor V Leiden is the most common inherited thrombophilic defect in Egyptians. The presence of inherited thrombophilic defects exposed carriers to increased risks for VTE compared to non carriers .The prevalence of jak2 V617F mutation in one hundred and six Egyptians presenting with first episode venous thromboembolism is low (5.7%) and is statistically insignificant in comparison to the controls and has no association with any of the thrombophilic risk factors.

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Key words: Thrombophilic factors, Jak2 V617F mutation, Venous thromboembolism.

1. Introduction

Venous thrombosis is now considered a multicausal disease. Gene-gene interactions and environmental risk factors increase the risk of venous thrombosis [1].As venous thrombosis is mostly caused by disturbances in the plasma coagulation system, abnormalities of coagulation factors are mostly risk factors for venous thromboembolism (VTE) .Inherited and acquired thrombophilia may per se account for a sizeable fraction of first episode venous thromboembolism in unselected series of patients [2].

JAK2 is a cytoplasmic tyrosine kinase that plays a central role in signal transduction from multiple hematopoietic growth factor receptors[3]. A gain-of-function mutation in the gene encoding the Janus kinase2 (JAK2) that results in a valine-to-phenylalanine substitution at position 617(V617F) has been described in patients with Philadelphia-negative MPN (more than 90% of patients with polycythemia vera and in about 50% of patients with essential thrombocytosis and primary myelofibrosis) [4-6] .

It has also been reported as a marker of occult myeloproliferative disorder (MPD) in patients with splanchnic venous thrombosis. The mutation causes constitutive activation of *JAK2*, which results in myeloproliferation independent of cytokines, mobilization of blood cell progenitors, and the spontaneous formation of endogenous erythroid colonies [7]. Limited data are available regarding the prevalence of the *JAK2V617F* mutation in patients with thrombosis outside the splanchnic region.

Whether JAK2 V617F mutation is associated per se with hypercoagulability remains unclear. The contribution of JAK2V617F to a hypercoagulable state, as well as its interaction with other thrombophilic factors has yet to be clarified

Our aim was to clarify the contribution of JAK2V617F to a Hypercoagulable state, as well as its interaction with other thrombophilic factors in patients with venous thrombosis (lower Limb deep venous thrombosis and pulmonary embolism).

2. Material and Methods

Patients and control subjects

Full history from all subjects, with an emphasis on personal history within 4 weeks before taking the blood sample, of circumstantial predisposing factors of venous thromboembolism (*ie*, surgery, immobilization, pregnancy, postpartum, trauma, oral contraception, varicose veins and any of arterial thrombosis) or malignancy within the last five years .

Patients

The study patients presented to the Medical Research Institute, Alexandria University, with first episode of venous thromboembolism for a thrombophilic workup from January 2010 to December 2011.

Our patients were 56 men (52.8%) and 50 women (47.2%) (median age, 39.5 years; age range, 14 to 80 years). Eighty-four patients had lower limb deep venous thrombosis (DVT) and 16 patients had pulmonary embolism (PE) and six with concomitant DVT and PE based on Doppler ultrasound and/or computed tomography and pulmonary angiography.

Patients with provoked venous thromboembolism include patients with a history of leg fracture or lower-extremity plaster cast, pregnancy and puerperium (up to 6 weeks from delivery), oral contraceptive intake, hormone replacement therapy, trauma, prolonged bed immobilization (>10 d), and long period of travel (>8 h) or surgery using a general anesthetic in the 3 months before the initial event.

Control subjects

Sixty subjects age and sex matched were randomly selected. None of these subjects had a history of venous thromboembolism, as determined by a structured questionnaire.

All patients and controls were subjected to complete blood count, thrombophilic workup included testing for deficiencies of antithrombin, protein C, and protein S and for lupus anticoagulant positivity before the start of anticoagulants for patients. In addition, testing for anticardiolipin antibodies (IgG and Ig M), serum homocysteine and for the presence of factor V Leiden and FII A^{20210} mutations and *JAK2* V617F mutation were done.

Blood Collection and Coagulation Tests

Blood samples were collected into vacuum tubes containing 0.129 mol/L trisodium citrate and were centrifuged at 2,000*g* for 15 min to obtain platelet-poor plasma after obtaining informed consent.

Deficiency of protein S is documented by decreased activity < 55% and was performed on a coagulometer (Acticlot ® protein s), protein C deficiency by decreased activity of protein C <60% via an amidolytic chromogenic assay (ACTICHROME® Protein C), antithrombin III deficiency by decreased activity < 75% by chromogenic assay (ACTICHROME® ATIII) and positive anticardiolipin Ig G(≥10 GPL U/ml) or Ig M (≥7 GPL U/ml) determined by enzyme-linked immunosorbent assay ((Orgentec Diagnostica GmbH), positive lupus anticoagulant (greater than 2 S.D.`s above the mean of the normal reference) (DVV test) or increased serum homocysteine level (> 15 µmol/L)(Axis® homocysteine EIA)

DNA Extraction and Analysis

DNA was extracted from EDTA anti-coagulated blood using genomic DNA purification kit (Fermentas) ·Factor V Leiden mutation and Factor 2 (prothrombin gene) mutation were done by allele specific polymerase chain reactions[8].

JAK2 V617F Mutation Screening

Analysis of the V617F (1848G>T) mutation was done on genomic DNA by using two –round allele-specific Polymerase chain reaction (AS-PCR) and (ARMS-PCR).

Two-round AS- PCR for the detection of the JAK2-V617F mutation

The primary AS-PCR was performed in a 25 µL reaction mixture using previously published primers which were a common reverse primer (R) (5'-CTGAATAGTCCTACAGT GTTTTCAGTTTCA-3'), a forward mutant specific

primer (FM) (5'-AGCATTTGGTTTTAAATTATG GAGTATATT-3'), and a forward internal control primer (FC) (5'-ATCTATAGTCATGCTGA AAGTAGGAGAAAG-3') . The primary AS-PCR master mix consist of 100 ng of genomic DNA, 2.5 mmol/l MgCl2, 0.2 mmol/l each of dNTP, and 0.625 U of Platinum Taq DNA Polymerase (Fermentas) together with 10 pmol /ul of R, 8 pmol /ul of FM and 2 mol/ul of FC. The PCR was carried out in a Primus 25 advanced thermal cycler (PeqLab , Biotechnologie GmbH). The PCR condition comprised an initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 2 min.

The secondary AS-PCR reactions using annealing temperatures between 63° and 65 ° to avoid non-specific binding of the primers were performed. The secondary AS-PCR was performed in a 25 µl reaction mixture consisting of ingredients as the primary AS-PCR except for the use of 2.0 mmol/l of MgCl2, 1 pmol/ul of R, 1.8 pmol/ul of FM, 0.2 pmol/ul of FC, 30 cycles of amplification step, and 1 µl of PCR products from the primary AS-PCR as a template.

PCR products from primary and secondary AS-PCR were analyzed on a 1.5% agarose gel in 0.5X TBE buffer and visualized after staining with ethidium bromide (EtBr). Mutant and internal control products were 203 bp and 364 bp in length, respectively. A 203-bp PCR product represented the presence of the mutant allele whereas a 364-bp PCR product was always seen even with or without mutant allele. Addition of the second round AS-PCR increase the detection sensitivity of JAK2 mutant allele[9].

ARMS PCR for the detection of JAK2-V617F mutations

Samples positive for the mutation were subsequently analyzed via ARMS-PCR, using the methodology by **Nadali *et al.*** [10] using 4 primers as follows; a forward outer primer, a reverse outer primer, a forward inner wild type specific primer and a reverse inner mutant specific primer. The forward primer from one set and the reverse from the other generate a control 463-bp band in all cases. The reverse inner mutant specific primer and the forward outer primer generate a 279-bp mutant fragment. In the presence of the wild-type JAK2, the reverse outer primer and the forward inner wild type specific primer produce a fragment of 229-bp

Statistical Analysis

Data were fed to the computer using the Predictive Analytics Software (PASW Statistics 18). Qualitative data were described using number and percent. Association between categorical variables was tested using Chi-square test**.** When more than 20% of the cells have expected count less than 5, correction for chi-square was conducted using Fisher's Exact test.

Quantitative data were described using median, minimum and maximum as well as mean and standard deviation. The distributions of quantitative variables were tested for normality using Kolmogorov-Smirnov test, Shapiro-Wilk test. D'Agstino test was used if there was a conflict between the two previous tests**.** If it reveals normal data distribution, parametric tests was applied. If the data were abnormally distributed, non-parametric tests were used.

For abnormally distributed data, Mann-Whitney Test (for data distribution that was significantly deviated from normal) were used to analyze two independent population.

Odd ratio (OR) and 95% Confidence Interval were used. Significance test results are quoted as two-tailed probabilities. Significance of the obtained results was judged at the 5% level.

3. Results

In this study one hundred and six patients were enrolled (median age 39.5years, age range (14–80 years). Fifty-four were males and fifty-two females; In most patients with inherited thrombophilia, the first thrombotic event occurred before the age of 45 years. Eighty-four patients had lower limb deep venous thrombosis and sixteen patients had pulmonary embolism and six with concomitant DVT and PE) (Table 1).

The presence of circumstantial risk factors for venous thromboembolism among patients is demonstrated in (Table 2) and the prevalence of inherited and acquired thrombophilic risk factors alone or in combination in the patient group is demonstrated in (Table 3).

Inherited Thrombophilic Risk Factors

Factor V Leiden was the most common inherited thrombophilic defect among patients (23.6%) while it was ten percent among controls. The prevalence of carriers for the FV Leiden mutation were significantly significant between the patient group and the control group (p = 0.031). There were 3 carriers of the FII A^{20210} mutation among the

patients group (2.8%) while no mutation was found in the control group.

Protein C deficiencies were observed in 6 patients (5.7%) and in none of the control group, while protein S deficiencies were observed in 4 patients (3.8%) and in one of the control group (1.7%) with p value 0.088 and 0.655, respectively. One patient had antithrombin III deficiency (0.9%) and no antithrombin III deficiency could be detected in the control group.

Acquired thrombophilic risk factors

Fourteen patients were positive for anticardiolipins either Ig G or M (13.2 %) and seven were positive for lupus anticoagulant (6.6%),none of these were found in the controls.

Eight had hyper- homocysteinemia (7.8%) in the patient group and four had hyper-homocysteinemia in the control group(6.7%) but the difference was statistically insignificant.

JAK2 V617F mutation was positive in 6 patients (5.7%) and in two of the control subjects (3.3%) and the difference is statistically insignificant (Table 4) .

Figure (1) shows Jak 2 V617F mutation by two-round (AS-PCR) (Figure 1) , While figure (2) shows Jak 2 V617F mutation by (ARMS-PCR).

The risk of VTE is 2 to 3-fold higher among patients with factor V Leiden mutation ,while 1 to 2 fold higher among patients with factor II mutation, protein C deficiency, antithrombin III deficiency , positive for lupus anticoagulant and anticardiolipins. (Table 5)

There was no association between Jak2 mutation and other thrombophilic factors (Table 6).

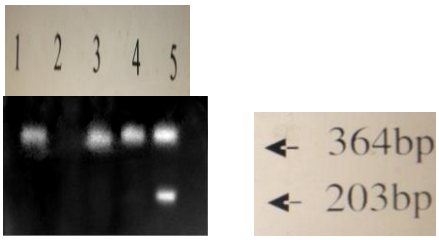

Figure (1) : Agarose gel analysis for the detection of JAK2V617F mutation in genomic DNA by Two – round Allele specific PCR. lanes 1,3,4,5, samples from patients .Lanes 1 ,3and 4 show a single band (364 bp) which is a wild type band and acts as an internal PCR control, lane 2 is the negative control. And lane 5 show mutant band (203 bp) carried by the patient. Arrows indicate the product of amplification.

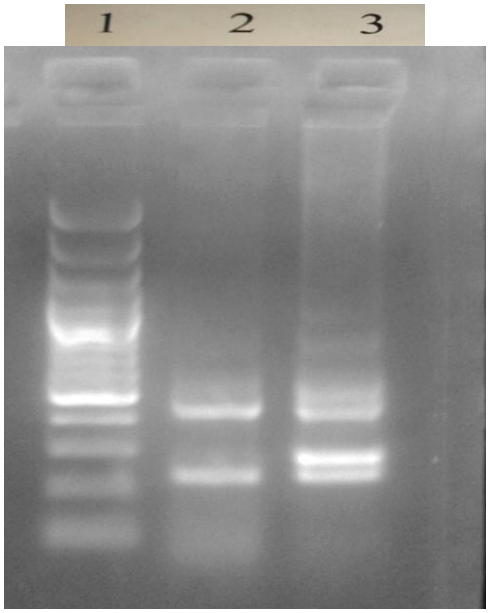

Figure (2): The JAK2V617F mutation in genomic DNA by ARMS-PCR assay.
Lanes 1 is 100–base pair (bp) markers; lane2 is negative control; lane 3 is patient positive for Jak2 mutation with 3 bands

Table (1): Characteristics of 106 patients with venous thromboembolism

Median age, years (range)	39.5 (14-80)
Sites of thrombosis -DVT -Pulmonary embolism -Concomitant DVT and PE	 84(79.2 %) 16 (15.1 %) 6 (5.7 %)
Complete blood counts Mean hemoglobin, g/l (range) Mean WBC, $x10^9$/l(range) Mean platelet, $x10^9$/l(range)	 12 (8-17.5) 6(3-17) 200 (70-480)

Table (2): Prevalence of Circumstantial Thrombophilic Risk Factors in 106 patients with venous thromboembolism

Risk Factors	Patients(n = 106)
Recent surgery	(7)6.6%
Puerperium	(1)0.9%
Fracture & immobilization	(6)5.7%
Cancer	(9)8.5%
Use of oral contraceptives	(2)1.9%
Obesity	(32)30.2%
Idiopathic	(43)40.6%

Table (3): Prevalence of Inherited and Acquired Thrombophilic Risk Factors Alone or in Combination in 106 patients with venous thromboembolism

Risk Factors	Patients(n = 106)%
FV Leiden mutation only	(24) 22.6%
FII A^{20210} mutation only	(3)2.8%
Antithrombin III deficiency	(1)0.9%
Protein C deficiency only	(6)5.7%
Protein S deficiency only	(3)2.8%
Anticardiolipins antibodies only	(9)8.4%
FV Leiden + protein S deficiency	(1)0.9%
Lupus anticoagulant alone	(2)1.9%
Lupus anticoagulant +anticardiolipin antibodies	(5)4.7%
Hyperhomocysteinemia	(8)7.5%

Table (4):Comparison between the two studied groups according to different Laboratory parameters

	Patients		Control		FEp
	No.	%	No.	%	
Factor V	25	23.6	6	10.0	p = 0.031*
Anticardiolipins IgM/IgG	14	13.2	0	0.0	0.002*
Lupus anti-coagulant	7	6.6	0	0.0	0.049*
Protein C deficiency	6	5.7	0	0.0	0.088
Protein S deficiency	4	3.8	1	1.7	0.655
Anti thrombin III deficiency	1	0.9	0	0.0	1.000
Factor II mutation	3	2.8	0	0.0	0.554
Hyper-homo-cysteinemia	8	7.5	4	6.7	1.000
Jak2 V617F mutation	6	5.7	2	3.3	0.712

p: *p* value for Chi-square test
FEp: *p* value for Fisher Exact test
*: Statistically significant at $p \leq 0.05$

Table (5): Relative risk for thrombosis associated with inherited and acquired risk factors among patients and control groups

	Patients (n = 106)		Control (n = 60)		OR	95%CI (lower- upper)
	No.	%	No.	%		
Factor V mutation						
-ve	81	76.4	54	90.0		
+ve	25	23.6	6	10.0	2.778*	(1.069-7.220)
Anticardiolipins IgM/IgG						
-ve	92	86.8	60	100.0		
+ve	14	13.2	0	0.0	1.652*	(1.453-1.879)
Lupus anti-coagulant						
-ve	99	93.4	60	100.0		
+ve	7	6.6	0	0.0	1.606*	(1.423-1.813)
Protein C deficiency						
-ve	100	94.3	60	100.0		
+ve	6	5.7	0	0.0	1.600*	(1.419-1.804)
Protein S deficiency						
-ve	102	96.2	59	98.3		
+ve	4	3.8	1	1.7	2.314	(0.253-21.189)
Anti thrombin III deficiency						
-ve	105	99.1	60	100.0		
+ve	1	0.9	0	0.0	1.571*	(1.400-1.764)
Factor II mutation						
-ve	103	97.2	60	100.0		
+ve	3	2.8	0	0.0	1.583*	(1.408-1.779)
Hyper-homo-cysteinemia						
-ve	98	92.5	56	93.3		
+ve	8	7.5	4	6.7	1.143	(0.329-3.966)
Jak2 V617F mutation						
-ve	100	94.3	58	96.7		
+ve	6	5.7	2	3.3	1.740	(0.340-8.905)

Table 6:The association between Jak2 V617F mutation and other thrombophilic factors

	Jak2				FEp
	-ve (n = 100)		+ve (n = 6)		
	No.	%	No.	%	
Obesity					0.665
-ve	69	69.0	5	83.3	
+ve	31	31.0	1	16.7	
Malignancy					0.421
-ve	92	92.0	5	83.3	
+ve	8	8.0	1	16.7	
Leg cast					1.000
-ve	99	99.0	6	100.0	
+ve	1	1.0	0	0.0	
Bed rest					1.000
-ve	95	95.0	6	100.0	
+ve	5	5.0	0	0.0	
Surgery					1.000
-ve	93	93.0	6	100.0	
+ve	7	7.0	0	0.0	
Puerperum					1.000
-ve	99	99.0	6	100.0	
+ve	1	1.0	0	0.0	
Factor V mutation					1.000
-ve	76	76	5	83.3	
+ve	24	24	1	16.7	
Anticardiolipins IgM/IgG	24				0.582
-ve	87	87.0	5	83.3	
+ve	13	13.0	1	16.7	
Lupus anti-coagulant					1.000
-ve	93	93.0	6	100.0	
+ve	7	7.0	0	0.0	
Protein C deficiency					1.000
-ve	94	94.0	6	100.0	
+ve	6	6.0	0	0.0	
Protein S deficiency					0.211
-ve	97	97.0	5	83.3	
+ve	3	3.0	1	16.7	
Anti thrombin III deficiency					1.000
-ve	99	99.0	6	100.0	
+ve	1	1.0	0	0.0	
Factor II mutation					1.000
-ve	97	97.0	6	100.0	
+ve	3	3.0	0	0.0	
Hyper-homo-cysteinemia					1.000
-ve	92	92.0	6	100.0	
+ve	8	8.0	0	0.0	

FEp: *p* value for Fisher Exact test

4. Discussion

Hypercoagulable conditions are a group of inherited and acquired disorders that predispose to venous thromboembolism. Laboratory-based testing has made it possible to identify a predisposing genetic cause in up to 50% of patients with venous thromboembolism. Factor V Leiden is the single most common inherited thrombophilic defect[11].

Forty-three patients in this study do not have obvious risk factor for thrombosis (40.6%), while more than half of the thrombotic events occur in association with circumstantial risk factors; Richard (2003), reported that twenty –three percent of his patients with first venous thromboembolism had undergone surgery within 2 months, 18% had malignancy, 15% developed VTE during a hospitalization for medical illness, 2% had major trauma, and a 41% were idiopathic [12] .

Factor V Leiden is present almost exclusively among Caucasians, with a prevalence of 5% in the general population with European ancestry and18% among patients with VTE; the risk of VTE is 2 to 7-fold higher among heterozygotes ,while the prothrombin 20210A allele is present in 2% of healthy individuals and in 7% of patients with VTE and the risk of VTE is 2 to 3-fold higher among heterozygotes [13] .

Our findings as regards to factor V Leiden mutation correspond with Margaglione *et al.* who found that patients with deep venous thrombosis (DVT) of the lower extremities (n = 346) or with additional PEs (n = 175) showed similar prevalence of FV Leiden mutation (24.3% and 16.6%, respectively) but FII A^{20210} mutation was(14.2% and 12.6%, respectively) in both groups which was higher than that reported by us [14].

The prevalence of protein C, S deficiencies, antithrombin III among our patients were 5.7%, 3.8% and 0.9% respectively, this goes in agreement with others [15] .

Screening for the *JAK2*V617F mutation has been carried out in several retrospective cohorts of patients with venous or arterial thrombosis in whom there was no apparent reason to postulate that myeloproliferation would be hidden as after splanchnic venous thromboses. Not surprisingly, these studies found that the mutation either was absent or was present at a very low prevalence (≤1%), not higher than that found in healthy people. For instance, Pardanani *et al.,* who screened in phase I 434 patients who had developed venous thromboembolism, stroke, or myocardial infarction, found the mutation in only 5 patients (1.1%) [16] .

Jak2 V617F mutation was detected in 3.3% healthy controls in Egyptian control subjects while in a Chinese study, the mutation was detected in blood samples of nearly 1% of 3935 study participants who were healthy or had miscellaneous diseases apparently unrelated to chronic myeloproliferative disorders [17] .

Remacha et al reported that of 295 patients with venous thrombosis, only 1 was found to be positive for the $JAK2^{V617F}$ mutation, although the study later revealed that the patient had occult MPD[18]. Ugo *et al.,* reported a low prevalence of JAK2 V617F mutation in 392 unselected unprovoked VTE patients without overt myeloproliferative disease 1%. [19].

Taken together, all of these studies further add to the dilemma of whether to include this screening test in routine thrombophilia screening. *JAK2* V617F mutation has been found in up to 40% of patients with splanchnic venous thrombosis. In contrast, the prevalence of the mutation has been reported to be low did not exceed 1·5% in patients with non-splanchnic venous thrombosis and without overt MPNs[20].

Our findings correspond with those previously reported (**Pardanani *et al.*, 2008**, **Remacha *et al.*, 2007**; **Ugo *et al.*, 2008**; **Regina *et al.*, 2007**;; **Rossi *et al.*, 2007**; Za *et al.* ,2009; Rodger *et al.*, 2011;) confirming a low frequency of the *JAK2* V617F mutation among patients with unprovoked venous thrombosis of the legs and without overt MPNs [16,18-23].

In the studies done by (Remacha *et al.*, 2007; Regina *et al.*, 2007; Za *et al.*, 2009) patients have been included irrespective of the presence of known conditions of inherited and acquired thrombophilia[18,20,22].

Conclusion

In recent years, the ability to diagnose inherited genetic defects and common acquired conditions predisposing to thrombosis has greatly increased. Venous thromboembolism is now understood to be a complex interaction of genetic and environmental factors leading to thrombosis. Carriership of FV Leiden mutation identifies an at-risk condition for venous thrombosis and is the most common inherited thrombophilic defect. Screening for the *JAK2*V617F mutation is not recommended as part of the battery of investigations that are performed to understand the mechanism of unexplained venous thrombosis. The yield in terms of positivity is likely to be very low, statistically insignificant when compared with the controls. It will definitely not be cost effective and it has no association with other thrombophilic factors.

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Investigation and intervention on the psychological status of families with Hepatolenticular Degeneration children

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Abstract: Objective To investigate the psychological status of family with Wilson's disease children, interventions to alleviate chronic sorrow of their families. **Methods** 25 children with Wilson's disease were randomly divided into 13 control group parents, and 12 observation group, was evaluated for both groups were applied ABQ parents of children with mental status; the control group received routine care in the observation group to the targeted group intervention and individual intervention, 3 months after evaluation by the scale effect. **Results** The parents of children with the observation group questionnaire sadness, anger, pain and the total degree of improvement was significantly better than the control group (all P <0.05). **Conclusion** Presence of chronic sorrow the family in children with Wilson's disease, early assessment and implementation of targeted intervention, will help ease psychological burden of parents of children with epilepsy to reduce their family of chronic sorrow.

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Key words: Wilson's disease; chronic sorrow; Families of children with Wilson's disease; Adapted Burke Questionnaire (ABQ); Intervention

1. Introduction

Hepatolenticular Degeneration (HLD), also known as Wilson disease (WD) is an autosomal recessive copper metabolism disorder, the worldwide incidence of it is 1/100 000 to 1/30 000 [1]. The pathogenic gene carrier of the disease is about 1/90. It has a higher prevalence in the Chinese population mainly occurs in adolescents. The main clinical manifestations are characterized by tremors, muscle rigidity, and unclear articulation, mental disorders and cirrhosis of the liver. HLD is one of the few treatable genetic diseases of the nervous system; the key is early diagnosis, early treatment, late diagnosis or inappropriate treatment lead to disability or even death.

However, in clinical care, we found that most parents can not correctly treat the pathological behavior of the children. The lack of disease-related knowledge leads to incorrect responses, and thus can not provide a good family support to the children, impairs of treatment outcome and quality of life of the children. Olshansky [2] proposed the concept of chronic sorrow, a cycle of recurrent pain or sorrow of parents or caregivers, which can occur in different periods with children with severe or chronic disease. If the sorrow last more than 2 to 3 months, it becomes chronic sorrow. In this study, we investigated and analyzed the psychological condition of the families of HLD children and take specific care measures to improve the psychological status of the parents and caregivers.

Object and Methods

1.1 Object 25 diagnosed HLD cases in the neurology clinic out-patient and ward of our hospital from July 2006 to July 2010, 19 males and 6 females, aged 2 to 19 years, the average age is 9.3 years. Three were brothers in the 25 cases. The first symptoms, in 13 cases (52.0%) were physical disabilities, in 6 cases (24.0%) were mental disorders, in 6 cases (24.0%) were liver damage. The age of onset was 17 years of age, duration ≥ 1 month. Among the 25 parents, 11 were mothers, 14 were fathers, the average age of them is 35.34 years, and they all were the primary caregivers of the children. Education: junior high school and below were six (24.0%), high school were 12 (36.0%), college and above were 7 (40%). 25 parents were randomly assigned into two groups, 12 were in the observation group, and 13 were in the control group. The difference was not significant (P> 0.05) compared the proportion, age, education level and the children disease level of the two groups of parents.

1.2 Methods

1.2.1 Psychological status assess of the parents Adapted Burke, Questionnaire (ABQ) questionnaire were used [4]. The author modified some individual projects based on the specific situation of China, including eight kinds of emotional state, i.e., sadness, shock, anger, denial, pain, despair, fear and guilt. Take 0 = strongly to 3 = very strong, four evaluation scores ranged from 0 to 24 points, the higher score means higher sad degree. Reliability

Cronbach's alapha value is 0.935 [3].

1.2.2 Interventions

The control group received conventional treatment and care, and regular follow-up. The observation group received three months targeted family intervention according to the results of the assessment as follows:

1.2.2.1 Collective intervention ① HLD knowledge seminars by professionals for parents: twice a month, each time 60 ~ 90min, for three consecutive months, including the knowledge of HLD disease, medication knowledge, the importance and methods of diet care, the activities of daily living, and the role of harmonious family environment to control symptoms. After each lecture, we organized the parents to discuss and exchange the opinions and answer their questions. ②Setting up HLD hotline in the outpatient (opening every Saturday 8:30 to 11:30), neurology physicians of our hospital is responsible for answering and explaining, the parents of HLD children can ask their questions by telephone

(1) **Psychological exchange** Family members with HLD children inevitably generate anxiety and fear. By the communication with them we found that the main reasons of anxiety and fear is the lack of understanding of the disease, the feeling of guilty for children and worried whether the brothers or sisters of HLD children is sick too when they know the HLD is a genetic disease. We explained the pathogenesis of the disease to family members of patients to inform them that the disease is curable genetic disease, and told them the treatment for the patient and the screening methods for other family members. This eased the anxiety of the families and gave them confidence to face the disease [4].

(2) **Treatment guiding** The families should develop a compliance behavior to the doctors, do not abuse their own drugs; do not change the dose and time. The drug should be stored in a cool, dry, dark, and fixed place. We explained the adverse reactions that may occur after taking the drug and told parents if the children feel sick they should come to hospital for medical treatment timely. The meals should for the HLD children should be light, easily digestible and rich in vitamins and fiber. The patients should maintain a low copper diet, do not drink the high-copper-containing water, do not eat high copper food such as seafood, nuts, mushrooms, beans and their products, do not use copper pots when cooking. Adding trace elements such as zinc, iron and calcium is recommended because they are antagonist of copper and can promote copper excretion.

(3) **Home care** Using a wet mopping for the floor, opening doors and windows regularly to ventilation. We suggested that the families purchase of air-cushion mattress, place intensity-regulate light fixture at bedside of the patients and the placed the patients in the low-rise building and low beds with shelf and air cushion mattress. We informed the potential security risks to the families to improve their security awareness, introduced the reasons early performance and dangers of pressure ulcers to the families. If the patient is bedridden, families should learn the expectoration method by chest percussion, which is an effective way to help the patients' sputum discharge.

1.2.2.2 Individual interventions Disease education and psychological counseling of the parents were specified to five specialist nurses for the specific circumstances of each family twice a month; each nurse was responsible for 3-4 families. Including: ① Adjustment of family communication, and learn to communicate with the children; to create a harmonious family environment, strengthen the exchange of feelings; to adjust expectations for children, such as the correct treatment of children with academic and daily living skills. ② The guidance for the parents of children to correctly identify adverse drug reactions, the recurrence symptoms and the corresponding approach, to improve the ability of parents to solve problems. ③ Answering the questions of parents of children patiently, to provide targeted guidance for children with behavioral skills and social function and rehabilitation. ④ Arrangement more than 2 times family gatherings during the intervention (including children), to encourage parents to discuss their care experience and the existing problems, to establish mutual support networks. Exchange contact means between nurses and patients to communicate at any time when problem happens.

1.2.3 Methods of evaluation After three months continuous intervention ABQ questionnaire is used to evaluate the intervention effects.

1.2.4 Statistical methods SPSS software was used for statistical analysis. Repeated measures analysis of variance was used.

2. Results

ABQ score before and after the intervention of the two groups of parents (Table 1)

Table 1. ABQ scores between two groups before and after intervention. $\bar{x} \pm s$

Group		Sadness	Shock	Anger	Denial	Pain	Despair	Fear	Guilt	Total
Control	Before	2.79±1.2	2.08±1.2	2.11±0.9	2.66±1.6	2.98±1.4	2.24±1.6	2.13±1.3	2.34±1.4	2.32±1.2
***n*=13**	After	2.44±1.2	1.67±0.9	2.38±1.2	2.45±1.3	2.92±1.3	2.65±1.3	2.19±1.2	2.84±0.5	2.15±1.5
Observation	Before	2.57±0.8	1.99±1.3	2.40±1.4	2.09±0.2	2.58±1.2	2.04±1.1	1.95±0.3	2.37±1.7	2.28±1.0
***n*=12**	After	1.64±1.1*	1.29±1.7	1.32±1.1*	1.15±1.4	1.77±0.9*	1.18±1.6	1.76±1.3	2.21±1.1	1.36±0.9*

Note: The two groups main effect, * P <0.05.

The results showed that: the improvement was significantly better than the control in the observation group in the scores on sadness, anger, pain, and the total (P <0.05).

3. Discussion

3.1 The reasons of family chronic sorrow

Chronic sorrow is a cycle, recurring pain or sorrow of parents or caregivers. It is also reported in the parents of children who suffered from mental retardation, developmental disabilities, early maturity, Down syndrome, neural tube defects, and chronic disease. Similarly, in the caregivers of adult patients with Parkinson's disease, multiple sclerosis, Alzheimer's disease and cancer it is also reported[5].In this study chronic sorrow may be related to the following factors: ① The HLD is a congenital genetic disease, parents tend to think that the pain of disease to the children is bought by themselves, so they feel pain, low self-esteem and guilt [2]. ②Knowing little about the disease: multi-system damage caused HLD, the adverse effects of the drug, and psychiatric symptoms coupled with the worry about the physical and mental development of the children, after their studies and to join the army, employment, daily living, emotional issues all these things increase the chronic sorrow in the parents of HLD children [6] ③ Economic issue is also a reason of chronic sorrow. Long-term drug application, as well as to deal with the attendant adverse effects, and periodic review of the inspection fees: all these things above give the family enormous psychological pressure and economic pressure. ④The children are very young, lack of self-expression and they can not take care of themselves, they need the care of family members for a long time. The parents have to spend much time to accompany their children so they do not have enough time with their r jobs and learning. If this last for a long time, chronic sorrow happens. All these above give the parents of HLD children many psychological problems.

3.2 Reasonable intervention to alleviate the psychological pressure of the family members of HLD patients

Medical staff should give the parents of HLD children system and specification family support and health education, to help to improve the level of awareness of the disease and care of the children; should inform the parents the good prognosis of adherence treatment in this disease, so that they can see the prospect of treatment, and establish the confidence to adhere treatment, and adhere to the standardized systematized treatment; should let children get a good therapeutic effect[7], thus may improve the family atmosphere, to the ultimate improve the negative psychology of parents of children. Table1 showed that in sadness, anger, pain, as well as total scores the observed group of parents improved significantly better than the control group (P <0.05), suggesting that a reasonable intervention can effectively alleviate the chronic sorrow in the family with HLD children.

The warmth and good care of the family has a large impact on the treatment and rehabilitation of the patient member. Family not only can protect and promote the function of members of the health, but also can provide all the necessary care and support to sick members. Therefore, being a nurse, we should give mental and psychological care and support to the family members of patients, and have conversation with them by charisma and good interpersonal skills , to establish a harmonious relationship of mutual trust, so that they are willing to accept the views of nurses, and consciously coordinate with the care and guidance.

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Changes in psychological states of caregivers of patients with moderate or severe Alzheimer's disease following Memantine therapy

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Absract: ***Objective*:** To assess the psychological states of caregivers of patients with moderate or severe Alzheimer's disease and to explore the effects of memantine therapy for patients. ***Methods*:** 40 patients with moderate or severe Alzheimer's disease and their caregivers were studied. Patients were treated for 6 months with open-label memantine. Caregivers were assessed at baseline and at the end of the sixth month (month-6). Their psychological states were assessed by: Symptom Checklist 90, Self-Rating Anxiety Scale (SAS), and Self-rating depression scale(SDS). The difference of their psychological states between different time points and the average normal psychological state of Chinese people (hereby referred to as "Chinese normal scale") were analyzed by T-test. ***Results:*** There were significant differences in depression, anxiety, hostility, paranoia, and total SCL-90 scale between baseline and month-6 (all $p<0.05$).When compared to normal SCL-90 scale, there were significant differences in all of emotional states except compulsion, phobia, and psychosis at baseline, where no significant differences in all of them between month-6 and Chinese normal scale. There are significant differences in SDS and SAS scale of caregivers between baseline, month-6, and Chinese normal scale .***Conclusions*:** Caregivers of patients with moderate to severe Alzheimer's disease may have worse psychological states than average normal population and memantine therapy for AD paitents maybe alleviate these problems.
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Key Words: Alzheimer's disease; caregiver; psychological states; memantine

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive and functional impairment, and behavioral and psychological symptoms of dementia (BPSD). While BPSD are highly prevalent at all stages of dementia, they are particularly common in severe dementia, with 90% of individuals with severe disease exhibiting one behavior and 50% having at least four[1]. AD caregivers are often subject to enormous stressors and are at high risk for depression with nearly half of caregivers in some studies meeting formal diagnostic criteria for depression[2]. Caregivers also show increased utilization of health services and psychotropic medications, and one study reported that caregivers who reported distress were 63% more likely than non-caregivers to die within 4 years. Thus, adverse effects of care giving seem to be especially pronounced among dementia caregivers, even after controlling for intensity of care giving involvement[3].

The current study examined the effectiveness of memantine on psychological problems of caregivers of patients with moderate or severe Alzheimer's disease. The objective is to confirm the effectiveness of memantine in the real world setting and to determine whether treatment with memantine would be associated with decreases in nursing burden, caregiver distress and use of as required medications.

2. Methods

2.1 Subjects

All AD patients come from consecutive AD patients of neurological outpatient clinics and ward from September 2010 to September 2o11. Each subject underwent a comprehensive diagnostic screening assessment including physical and psychiatric examinations, as well as a review of his/her medical history. All patients were residing in Zhengzhou, met NINCDS-ADRDA criteria[4] for probable Alzheimer's disease, had moderate to severe AD
as demonstrated by a score of 0–15 on the Mini-Mental State Examination (MMSE). Their caregivers who interested in and agreed to the study were recruited.

2.2 Study design

Patients meeting entrance criteria were treated for 6 months with open-label memantine. The memantine (Ebixa) used in this study was provided by Lundbeck Denmark, Inc.. The memantine dose was administered beginning at 5mg once daily for 1 week and increased to 10 mg twice daily for the remaining weeks in the increment of 5mg/week increase. Caregivers were assessed two times: at baseline and month-6. Psychological states were assessed used: ① Symptom

Checklist 90（SCL-90）[5], which assesses 10 behaviors occurring in caregivers: somatization, compulsion, interpersonal sensitivity, depression, anxiety, hostility, phobia, paranoia, and psychosis; ②Self-Rating Anxiety Scale (SAS) [5]; ③Self-rating depression scale(SDS) [5].

2.3 Data analysis

All data were shown as mean ± SD and difference between baseline, month6 and Chinese normal scale were analyzed by T-test with SPSS 14.0 software. The P value less than 0.05 was considered to be significantly different.

3. Results

Forty AD patients and their caregivers who met the including criteria were enrolled . table 1 summarized their socio-demographic characteristics. The mean age of patients is 73.30±7.54 and of caregivers is 61.55±5.77. Percentage of male in patients is 40.25% whereas in caregivers is 55.00%. For education level, most of patients are less-educated and most of caregivers are moderate-educated. With regard to relation with patients, 62.5% of caregivers is spouse of patients and 20% is children of patients.

Table 2 shows the difference in caregivers' SCL-90 scales between baseline and month 6, ,normal scale . There are significant difference in depression, anxiety, hostility, paranoia and total SCL-90 scale between baseline and month-6. When compared to Chinese normal scale, there are significant difference in all of items except compulsion, phobia and psychosis at baseline. There is no significant difference in all of items between month 6 and normal scale .

Table 3 shows the of SAS,SDS scale of caregivers between baseline, month 6 and Chinese normal scale . There are significant difference in depression, anxiety scale of caregivers between baseline, month 6 and Chinese normal scale.

Table 1. Socio-demographic characteristics of AD patients and caregivers

	AD patients (*n*=40)	AD caregivers(n=40)
Age (years)	73.30±7.54	61.55±5.77
Gender		
Male	17 (40.25%)	22 (55.00%)
Education (years)		
≤6	19 (47.50%)	13 (32.5%)
6~12	12 (30.00%)	21 (52.5%)
＞12	9 (22.50%)	6 (15.00%)
Relation to patient		
Spouse		25 (62.5%)
Sibling		3 (7.5%)
Children		8 (20.0%)
Others		4 (10%)

Table 2. Comparison of SCL-90 scale of caregivers between baseline, month-6, and Chinese normal scale

	AD caregivers (*n*=40)		Chinese normal (*n*=1388)	t1	t2
	baseline	month-6			
Somatization	1.38±0.58	1.33±0.25	1.37±0.48	-	2.01[b]
Compulsion	1.63±0.70	1.61±0.69	1.62±0.58	-	-
Interpersonal Relation	1.68±0.32	1.64±0.25	1.65±0.51	-	2.03[b]
Depression	1.79±0.56	1.53±0.61	1.50±0.59	2.68[a]	2.72[a]
Anxiety	1.56±0.24	1.38±0.28	1.39±0.43	2.67[a]	2.65[a]
Hostility	1.55±0.39	1.49±0.57	1.48±0.56	2.65[a]	2.05[b]
Phobia	1.26±0.48	1.24±0.42	1.23±0.41	-	-
Paranoia	1.49±0.45	1.42±0.49	1.43±0.57	2.66[a]	2.64[a]
Psychosis	1.30±0.48	1.28±0.42	1.29±0.42	-	-
Total	138.02±34.12	130.11±30.46	129.96±38.76	2.70[a]	2.69[a]

Note : t1—baseline vs. month 6; t2—baseline vs normal model. a—p＜0.05, b—p＜0.01

Table3. Comparison of SAS, SDS scale of caregivers between baseline., month-6, and Chinese normal scale

	AD caregivers (*n*=40)		Chinese normal (*n*=1388)	t1	t2
	baseline	month-6			
SAS	31.57±5.62	29.12±3.03	29.78±0.46	3.13[a]	5.30[a]
SDS	36.30±6.48	34.14±5.67	33.46±8.55	4.03[a]	4.36[a]

Note : t1—baseline vs. month 6; t2—baseline vs. normal model. a—p＜0.01

4. Discussion

The current study indicated that caregivers of patients with moderate or severe Alzheimer's disease had worse psychological states than normal population. These problems are: depression, anxiety, hostility, and paranoia. Psychiatric and behavioral symptoms occur in the majority of patients with AD over the course of the illness50–52 especially in moderate or severe states, with symptoms of depression among 20%– 50%; patients with AD; agitated or aggressive behaviors appearing in 70%; and delusions or hallucinations in as many as 30%–50% [6].

In fact, depression and psychosis are included as descriptors in Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, criteria for AD. One recent study have shown that the direct influence of patients' cognition on caregiver burden is limited and rather mediated by other disease indicators. Both ADL-abilities and behavioural disturbances are important predictors of perceived caregiver burden, where the latter has the strongest effect [7].Another study[8] indicated that more severe psychiatric and behavioral problems, along with decreased quality of life were all significantly associated with higher levels of burden, and depression among caregivers.

The most frequently used pharmacological treatment for Psychiatric and behavioral symptoms is antipsychotics, particularly atypical antipsychotics. Although their use has been supported by evidence from randomized controlled trial (RCT) data, there remain concerns about potential side effects, such as cerebrovascular adverse events, extra pyramidal side effects and metabolic effects[9].

The results of this study showed that memantine can alleviate the worse psychological states of caregivers of patients with moderate or severe AD . The best-studied treatment for moderate to severe AD is the non-competitive NMDA receptor antagonist memantine, which has been shown to be efficacious in RCTs. With regard to its effect on BPSD, a pooled analysis of the effect of memantine treatment in three large 6-month RCTs in moderate to severe AD patients with agitation and aggression or psychosis showed an advantage for memantine over placebo on the Neuropsychiatric Inventory (NPI) agitation/aggression subscale at week 12 and weeks 24/28[10]. The decreased agitated and aggressive behavior in institutionalized patients with moderate to severe AD following treatment with memantine was accompanied by improvements in nursing burden and decreased psychotropic use[11].

In conclusion, this study indicate that caregivers of patients with moderate to severe Alzheimer's disease may have worse psychological states than normal population and memantine therapy for AD patients maybe alleviate these problems.

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Improvement of soft soils using reinforced sand over stone columns

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Abstract: In the present study, a series of laboratory model tests have been developed to study the behavior of unreinforced and geogrid-reinforced sand bed resting on stone columns. It has been observed that the soft clay is improved with stone columns. The diameter of stone columns has been taken as 50 mm, three stone columns have been used in the study with spacing of 75mm, while the footing is represented by a plate of 350x250x10mm for all the model tests carried out. Load was applied to the soil bed through the footing until the total settlement reached at least 5% of footing length. The influence of the thickness of unreinforced as well as geogrid-reinforced sand bed and the number of geogrid reinforcement on the performance of stone columns have also been investigated. The inclusion of geogrid layer within sand bed also increases the load carrying capacity and decreases the settlement of the soil. However multilayer reinforcement system is effective to transfer the stress from soil to stone columns. Significant improvement in load-carrying capacity of soft soil is observed due to the placement of sand bed over stone columns. Single layer reinforcement with stone columns is very effective to reduce the total settlement as there is considerable reduction in the total settlement due to stone column itself . The inclusion of reinforcement in the sand bed decreases significantly the depth of sand layer.
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Key word: geogrid; geogrid reinforcement; sand bed; sand layer; soil

1. Introduction

One of the techniques extensively used in soft soils is the use of stone columns. The use of stone columns can accelerate consolidation of the soft ground and consequently accelerate the strength gain of the surrounding soft soil. It has been used to increase the bearing capacity of soft soils and reduce the settlement of superstructures constructed upon.

In recent years, many studies have been carried out to understand the behavior of foundation reinforced by stone columns without considering the inclusion of geosynthetic reinforcement[1-5].Many researchers have studied the load-settlement behavior of single or multilayer geosynthetic-reinforced granular beds resting on soft soil without stone columns inclusions [6-15] .Most of the works reported in the literature are developed for foundations either reinforced by stone columns or geosynthetic layers. Limited studies have been done on the combined use of geosynthetic reinforcement and stone columns [16]. Han and Gabr [17] presented a numerical analysis of single layer geosynthetic-reinforced pile-supported earth platform over soft soil. Deb et al. [18] developed a lumped parameter model for single layer geosynthetic-reinforced granular fill-soft soil with stone columns. However, in the field multilayer geosynthetic reinforcements can be used along with stone columns. Thus, it is necessary to study the multilayer geosynthetic-reinforced granular fill resting over soft soil improved with stone columns. One of the techniques extensively used in soft soils is vibroreplacement, which consists of replacing some of the soft soil with crushed rock or gravel to form an array of stone columns beneath the foundation. Although the use of conventional stone columns in soft soil deposits was found to benefit foundations in many respects, Madhav and Miura, (19).The degree of improvement of a soft soil by stone columns is due to two factors. The first one is inclusion of a stiffer column material (such as crushed stones, gravel, sand) in the soft soil. This is largely reported in the literature [20- 25]. The second factor is the densification of the surrounding soft soil during the installation of the vibrocompacted stone column itself and the subsequent consolidation process occurring in the soft soil before the final loading of improved soil. The experimental work performed by Wattes et al. [26], and Vautrain [27] verifies that the installation of vibrocompacted stone columns leads to an improvement of the in situ soft soil characteristics and consequently, enhances the load displacement response of reinforced soil, Guetif, et al [28]. However, Greenwood [29] proposed an empirical method for estimating the reduction of settlement of reinforced soil taking into account the installation process of stone columns. In the present study, laboratory model tests have been conducted on three stone columns to study the effect of reinforcement and number of reinforced layers as well as unreinforced sand bed on settlement response. The

maximum number of the reinforced layers and unreinforced sand bed has also been determined.

2 Materials

2.1Clayey soils

The properties of clay have been presented in table 1. Unconfined compressive strength (UCS) tests were carried out on clay samples. Water content of the clay was maintained at 25% throughout the series of tests. The bulk unit weight of the clay at 25% water content was determined to maintain identical unit weight in all the tests.

2.2 Sand

A commercially available graded sand were used to prepare the sand bed placed below the clay bed and over the stone column- improved soft clay. The average particle size of sand was ranging between 1-4mm . Crushed stone materials of size 2—8 mm were chosen to prepare the stone column, the particles were generally sub-angular. Sand &stone properties are represented in table2.

To maintain same unit weight of sand in each test, the required weight of sand in each layer was calculated based on bulk unit weight. The sand was poured in two layers. Each layer was compacted with steel hammer to achieve the required thickness .The same procedure was used for stone columns, but the stone was poured in five layers.

2.3. Geogrid

Biaxial geogrid was used as a reinforcement layer. The properties of geogrid reinforcement have been presented in Table 3.

Table 1. Engineering properties of clayey soil

Property	Soil
Classification	CL
Colour	Brown
Liquid limit%	45
Plastic limit%	20
Plasticity index%	25
Optimum moisture content%	18.0
Maximum dry unit weight	17 KN/m³
Specific gravity	2.63
Bulk unit weight at 25%water content	19.2KN/m³

3 Testing Program

3.1. Experimental Setup

To prepare the soil bed, a rectangular tank of 1000 mm x 250 mm size and 500 mm high was used in all the tests. , a thin-walled aluminum tube measuring 50 mm in outside diameter was pushed slowly through the clay sample to a depth of 35cm. Centrality was achieved by using a guide attached to the top of the cylinder. The sample within the tube was retrieved, creating a cylindrical cavity of 50 mm diameter at the centre of the clay. Three cylindrical cavities were achieved representing the stone columns with 50mm diameter &spacing 75mm. The stone column was installed up to 35cm depth in clay bed. Compaction were used to the clay, stones and sand to achieve the required density of the materials. Steel plate of 350x100 mm and thickness 10 mm was used as footing to apply the load. Dial gauges were used for measuring the settlement of footing during the application of load. The diameter of stone columns was chosen to be 50 mm each, in all the tests and the depth of clay bed was maintained at 350 mm ; below the clay bed, a 50mm sand bed was at the bottom of the container. The first test was carried out on clay bed without any improvement techniques and the load-settlement behavior was investigated. Other tests were carried out on soft soil improved by stone column alone and on soft soil improved by stone column along with unreinforced and geogrid-reinforced sand bed. Plate. 1. shows the schematic diagram of the test setup. Summary of the tests conducted has been presented in fig 2, 3, 4, 5, and 6.

Table 2. Properties of sand &stone

Parameters	Values	
	Sand	stone
Specific gravity	2,7	2.67
Maximum dry unit weight	19.2 KN/m³	17.5KN/m³
Bulk unit weight at 65% relative density	17.9 KN/m³	16.1KN/m³

Table 3. Properties of grogrid

Parameter	Value
Aperture size	135x135mm
Thickness	1.omm
Weight	285gm/m²
Strain at failure	3.5%
Elastic axial stiffness at 1% strain	300 KN/m
Maximum tensile strength	8.5 K N/m

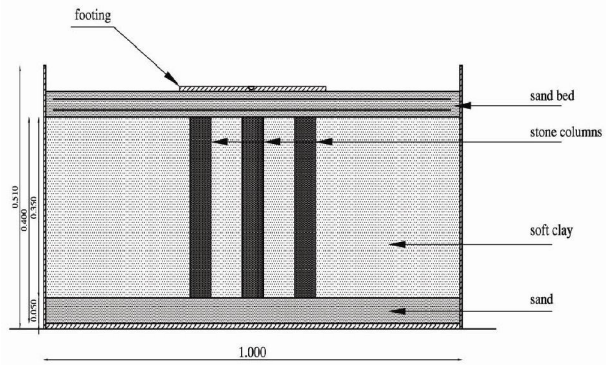

Plate. 1 schematic diagram of the apparatus

3.2. Preparation of Clay Bed

In all the tests, identical technique was to prepare the clay bed. To maintain similar properties throughout the tests, clay bed was prepared at 25% water content in all the cases. The bulk unit weight at 25% water content was found as 19.2 kN/m3. To maintain same unit weight of clay in each test, the container was filled in five equal layers of 70 mm thickness and the required weight of clay in each layer was calculated. Each layer was compacted with steel hammer to achieve the required thickness.

3.3. Column Construction

A replacement technique was considered the most easily repeated method for column installation in very soft soils. After preparing the clay bed of 350 mm over a sand bed 50mm thickness at the bottom of the container, three cylindrical holes of diameter 50 mm &spacing of 75mm were dug at the centre of the clay bed by steel pipe of 50 mm diameter and a depth of 350mm. The unit weight of stones was determined and using the known volume of the hole, the total weight of stone required to fill up the hole was determined. Total weight of stone material was divided into five equal layers to fill up the hole. Each layer of stone was poured and compacted with steel bar in such a manner that the finished height of each layer of stone column was 70 mm.

3.4. Preparation of Sand Bed

The weight of sand required to form a certain thickness of the bed for the lower and upper bed was determined by using the known unit weight of sand. For different thicknesses of sand, the required weight of sand was calculated and preparation of bed was carried out in layers. Each layer was compacted with a hammer with equal efforts of compaction to achieve the required depth of sand bed.

3.5. Testing Procedure

Loading was applied through a footing resting on the prepared soil bed and resistance offered by test bed with or without stone column was measured with the help of proving ring. Load was applied in equal increments and each increment of the load was maintained until negligible change in the settlement was observed. The settlement due to increment of each equal interval of loading step was observed through three mechanical dial gauges having least count of 0.02 mm fixed on the footing. Loading was applied until the total settlement of the footing attained was at least 5% of footing length.

4. Results and Discussions

4.1. Thickness of Sand Bed

To determine the optimum thickness of unreinforced sand bed, the thickness of sand bed was varied from 20mm to 80mm.The load carrying capacity at 175mm settlement has been calculated. From Fig. 1, it has been observed that the placement of sand bed over stone column-improved soft clay increases the load- carrying capacity of the improved soil . As compared to unimproved clay bed, an improvement of 75% in load- carrying capacity has been observed when the clay bed is improved with stone column only. As compared to unimproved clay bed, 100,126,140,167&180% improvement in load-carrying capacity has been observed when unreinforced sand bed of 20,40,50,60&80mm is placed over stone column-improved soft clay respectively. Fig. 1 shows the load settlement characteristics of the unreinforced sand bed of different thicknesses placed over stone column-improved clay.For 20,40,50,60&80 mm sand bed thickness, a loading intensity of 0.6 kN, as compared to unimproved soil, the settlement has been reduced by 63%, 69.2%,80%,87% and 90.7% respectively. For 1.0KN the settlement has been reduced by 60%, 74.8%,80%,85.6% and 88% respectively. The increase of sand bed thickness increases the load-carrying capacity also the settlement reduction increases up to a thickness of 60mm whereas beyond this value the reduction of settlement decreases and the increase of thickness is insignificant.

The thickness of sand bed was taken 60mm in the study .The reinforced geogrids was taken 1,2,3 &4 layers.

Fig. 2 shows the load-settlement characteristics of the geogrid reinforced sand bed of 1,2,3&4 reinforced layers placed over stone column-improved clay. The improvement in load-carrying capacity at 175 mm settlement is 180%&200% when unreinforced and geogrid reinforced sand bed with optimum number of layers has been placed over stone column improved soft clay respectively.

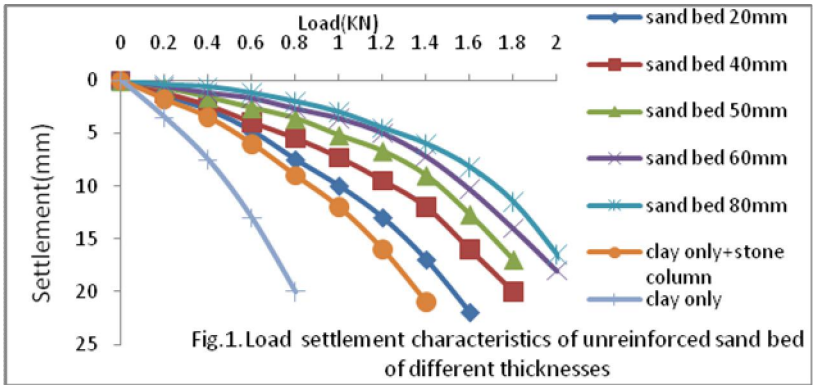

Fig.1.Load settlement characteristics of unreinforced sand bed of different thicknesses

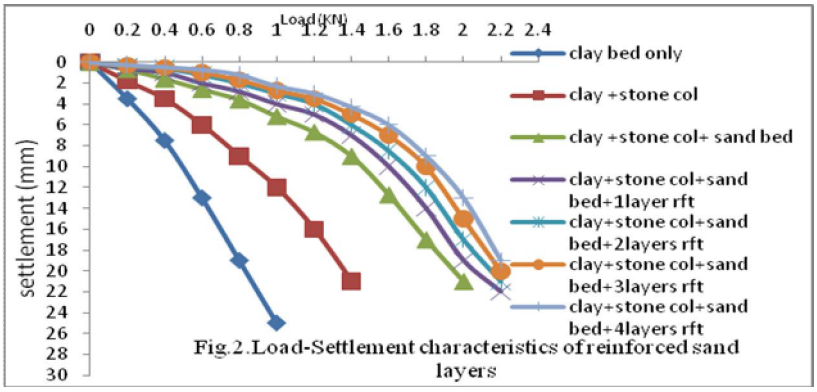

Fig.2.Load-Settlement characteristics of reinforced sand layers

4.2. Number of Geogrid-Reinforced Sand Bed

It has been observed that as the number reinforcement layer increase, the reduction in settlement increase. To determine the optimum number of the geogrid-reinforced sand bed, 1, 2, 3, and 4 layers of geogrids were used. Results obtained when using three layers of reinforcement are nearly close to those obtained with four layers.

A loading intensity of 0.2 kN, as compared to unimproved sand bed, the settlement has been reduced by 15.3%, 43.2%,57% respectively .For 0.6 kN, as compared to unimproved sand bed, the settlement has been reduced by 23%, 53.8%,61.5% respectively. For 1.0KN the settlement has been reduced by 31%, 43%,47% respectively.

The presence of reinforcement layers in sand bed increases the load-carrying capacity also the settlement reduction increases with the increase of number of geogrid layers up to a value of 3 layers, whereas beyond this value the reduction of settlement is insignificant. At low sand bed thickness, large deflection has occurred in the geogrid reinforcement directly underneath the footing. The large deflection of the geogrid reinforcement would mobilize the membrane action and induce more mobilized tension in the geogrid layer. The vertical component of the tensile force acting in the geogrid reinforcement partially counterbalances the superimposed load exerted by the overlying soil. As a result, the vertical stress is reduced in the zone below the reinforcement due to combined action of mobilized tension in the reinforcement and membrane action in its curvature [31- 34] (Burd, ; Lee et al.; Basudhar et al., Deb et al,) However, when the sand bed reinforcement layers increases, a major portion of the shear failure zone of the soil is developed above the reinforcement layer and the deflection of the reinforcement also decreases. This led to reduction in the utilization of membrane action and less mobilized tension in the geogrid has been induced [32](Lee et al.). This phenomenon reduces the effectiveness of the geogrid layer causing reduction in bearing capacity. Thus, the stone column under geogrid-reinforced sand bed produces less bearing capacity than that under geogrid-reinforced sand bed. Studies show that as the thickness of the reinforced sand bed is equal to or greater

than the optimum thickness of the unreinforced sand bed, the bearing capacity of unreinforced and reinforced sand bed is almost same [32](Lee et al). This is due to the fact that as the thickness of the reinforced sand bed increases, the deflection of the reinforcement decreases and the effectiveness of the reinforcement also decreases. When the thickness of the reinforced sand bed is equal to or greater than the optimum thickness of the unreinforced sand bed the effectiveness of the reinforcement is almost insignificant. Thus, the geogrid-reinforced sand bed with 60 mm thickness will almost same bearing capacity as compared to that under an

unreinforced sand bed with 80mm thickness. The improvement in load-carrying capacity, as compared to unimproved soft clay, at 175 mm settlement is 180% and 200% when unreinforced and geogrid-reinforced sand beds with optimum thickness have been placed over stone column-improved soft clay, respectively. [32] Lee et al. reported similar observation based on numerical and model studies of strip footing resting on reinforced- granular fill-soft soil system without stone column inclusions. Due to presence of stiffer stone column in the soft clay, lower optimum thickness of the sand bed has been required as compared to the optimum thickness under without stone column condition to get the maximum improvement in load-carrying capacity of improved ground. However, from the present study and the results reported by [32]Lee et al., it has been observed that the ratio of optimum thickness of the unreinforced to geogrid- reinforced sand bed is almost similar for both the cases under with and without stone columns [34](Deb et al).

4.3. Thickness of reinforced sand bed

Fig. 3. shows the load-settlement characteristics of the geogrid reinforced sand bed of different thicknesses placed over stone column-improved clay . To determine the optimum thickness of the geogrid-reinforced sand bed, 3 layers of geogrid reinforcement was chosen with 20,40,50,60,80mm of sand bed. It has been observed that as the thickness of reinforced sand bed increases the load-carrying capacity increases up to a depth of 60mm whereas beyond this value ,the increase of the thickness of the sand bed is insignificant. When the thickness of the reinforced sand bed is equal to or greater than the optimum thickness of the unreinforced sand bed the effectiveness of the reinforcement is almost insignificant (Lee et al.,).The improvement in load-carrying capacity, as compared to unimproved soft clay over stone column and geogrid-reinforced sand beds which have been placed over stone column-improved soft clay at 17.5 mm settlement with thickness of sand bed 20mm is 35.7% and 138% respectively, for 40mm thickness, 50% and 163% respectively, for 50mm thickness, 57.5% and 175% respectively, for 60mm thickness, 64.5% and 188% respectively, for 80mm thickness, 71.4% and 200% respectively.

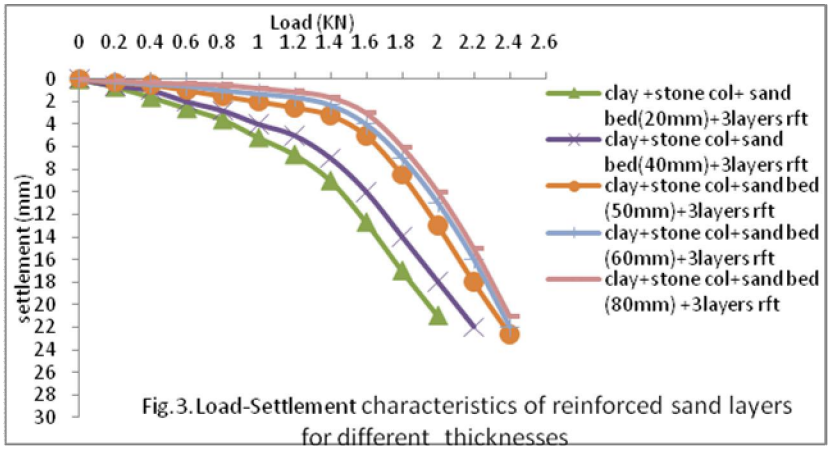

Fig.3.Load-Settlement characteristics of reinforced sand layers for different thicknesses

4.4. Length of geogrid

Fig. 4. shows the load-settlement characteristics of sand bed reinforced by geogrid reinforcement of various lengths. From the load- settlement characteristics, it has been observed that for a particular settlement, the load-carrying capacity increases as the length of the geogrid increases up to twice the length of the footing, whereas beyond this value the increase of length is insignificant. The length of geogrid used was 1L,1.5 L,2 L,2.5 L, while the sand bed was 60mm. Thus, the optimal extent of the reinforcement is twice the length of the footing; and, beyond this length any additional reinforcement is ineffective.

However, in the present study, the model container has been taken as sufficiently large to reduce the boundary effects. To reduce the scaling effects, the dimensions of the various components have been chosen proportionally with the prototype dimensions. In the present experimental study, small aperture size and thin model geogrid with relatively low stiffness has been used to avoid the size effect in the model experimental results. However, in case of field application comparatively large aperture size and thicker geogrids with higher stiffness are usually used. Thus, the chosen model geogrid properties used in the present experiments are suitable to achieve the same performance results as compared to full-scale geogrid. Thus, the results of the present laboratory model study are useful to investigate the behavior of the unreinforced and geogrid-reinforced sand bed resting over stone column-improved soft clay.

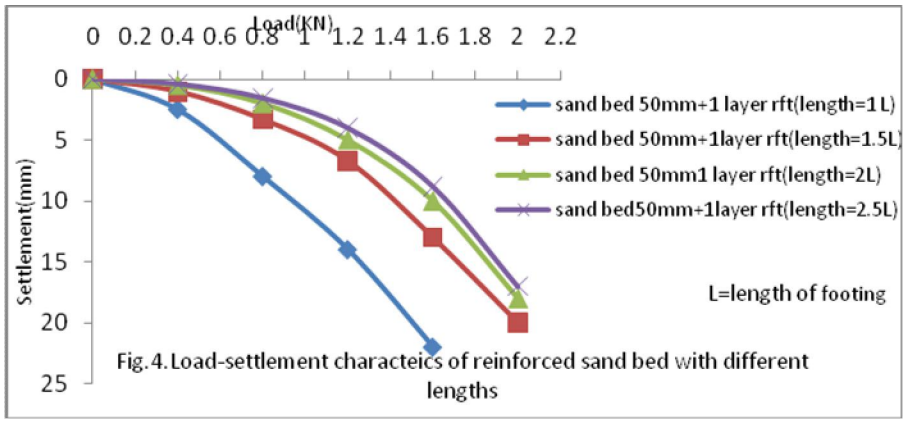

Fig.4.Load-settlement characteics of reinforced sand bed with different lengths

4.5. Load-settlement characteristics

Fig. 5. shows the load-settlement characteristics of the unimproved clay bed, clay bed improved by stone column alone and clay bed improved by stone column along with 60 mm thick unreinforced and geogrid-reinforced sand bed. The number of the geogrid layer has been taken as 1,2, & 3layers. The improvement in load-carrying capacities under different conditions has been computed at 175 mm settlement,5% of the footing length. From Fig. 5, it has been observed that the placement of sand bed over stone column-improved soft clay increases the load- carrying capacity of the improved soil and the use of geogrid layer within the sand bed is effective in further increment of the same. As compared to unimproved clay bed, an improvement of 75% in load- carrying capacity has been observed when the clay bed is improved with stone column only. As compared to unimproved clay bed, 140% improvement in load-carrying capacity has been observed when unreinforced sand bed is placed over stone column-improved soft clay and for reinforced sand bed the improvement is 150,175,200% for 1,2, & 3 reinforcement layers respectively. For a loading intensity of 0.5 kN, as compared to unimproved soil, the settlement has been reduced by 41.6%, 67%,83.3%,86.2% and 91.6% when the soil is improved by only stone column, by stone column along with unreinforced and geogrid-reinforced sand bed1,2,3 layers respectively. For a loading intensity of 1.0 kN, as compared to the presence of stone columns, unreinforced sand bed, reinforced sand bed with 1,2,3 layers, the reduction is 35.7%, 57.1% , 71.4%,78.5&85.7% respectively in settlement has been observed ; whereas for a loading intensity of 1.5 kN, the reduction in settlement is 29.1%,50%,62.5%,67% &79.2% respectively. Thus, it can be said that the geogrid reinforcement is more effective for higher loading intensity than for lower loading intensity. Similar behavior has been observed by Deb et al. [30] in the developed model for geosynthetic-reinforced granular fill-soft soil system with stone columns.

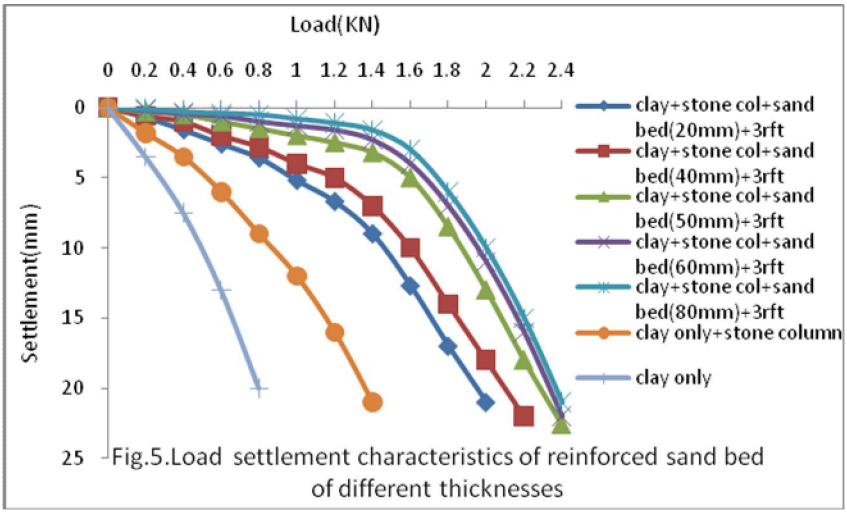

Fig.5.Load settlement characteristics of reinforced sand bed of different thicknesses

Conclusions

Based on the experimental results the following conclusions can be drawn:

I. The presence of stone columns in soft clay improves the load- carrying capacity and decreases the settlement of the soft soil. The placement of sand bed further increases the load-carrying capacity and decreases the settlement of the stone column-improved soil. The inclusion of geogrid as reinforcing element in the sand bed significantly improves the load-carrying capacity and reduces the settlement of the soil. As compared to unimproved soft clay, 75%, 140 % and 200% improvement in load- carrying capacity have been observed (at settlement equal to 5% of the footing length) when soft clay is improved by stone column alone, by placing of unreinforced and geogrid-reinforced sand bed of optimum thickness over stone column, respectively.

2. The optimum thickness of unreinforced sand bed placed over the stone column- improved soft clay is 1.6 times the optimum thickness of the geogrid-reinforced sand bed. The optimum thickness of unreinforced and geogrid-reinforced sand bed is 0.23 and 0.143 times the length of the footing, respectively.

3- It has been observed from the load- settlement characteristics that for a particular settlement, the load-carrying capacity increases as the length of the geogrid increases up to twice the length of the footing, whereas beyond this value the increase of length is insignificant.

4- The presence of reinforcement layers in sand bed increases the load-carrying capacity also the settlement reduction increases with the increase of number of geogrid layers up to a value of 3 layers, whereas beyond this value the reduction of settlement decreases.

5-, It has been observed that the placement of sand bed over stone columns-improved soft clay increases the load- carrying capacity of the improved soil and the use of geogrid layer within the sand bed is effective.

6-The sand bed layer below stone columns is effective to prevent any deformation to the stone columns due to loading of footing. The chosen model of the stone column properties used in the present experiments are suitable to achieve the same performance results as compared to full-scale stone columns.

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4/4/2012

A Proposed Computer-Based System Architecture for Crowd Management of Pilgrims using Thermography

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Abstract: Over the years, overcrowding and difficulties in crowd control have resulted in a number of fatal accidents during the Hajj. Despite many efforts and improvements for roads and footbridges, ensuring the safety of pilgrims continues to challenge especially with the annual increase of the number of pilgrims. The challenge has attracted many researchers who provided several methodologies for crowd monitoring and estimation of its density. This paper proposes to extend an earlier monitoring effort done by the same authors to develop a decision support system allows for close monitoring and control of crowd movements. It incorporates data acquisition and processing via several thermal cameras deployed as sensors at strategic points on Nafra (Arafat to Muzdalifah) access roads. The sensors are linked to an analysis module, which in turn measures crowd flow and density in real time. When crowds become too dense, an alarm is triggered according to different density levels. At this point, the integrated decision support system generates different alternatives to the controllers in order for them to take the appropriate actions. The paper illustrates the proposed system component. It also describes the architecture of each component as well as the architecture of the entire system. The system can contribute to provide complete safety for crowds during the Hajj event that attracts millions each year.
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Keywords: Hajj, Islamic informatics, crowd management, crowd density estimation, crowd monitoring, thermography.

1. Introduction

Once a year, around 3.0 million Muslims of every ethnic group, color, social level, and culture gather from all over the world in Makkah to perform the Fifth Pillar of Islam, Hajj. The pilgrimage involves a number of sacred rituals, and represents a profound personal and spiritual journey for Muslims. **Ben-Mahmoud *et al.* (2010)** have shown that the number of pilgrims will dramatically increase in the next few years to reach almost 3.75 million Muslims. Moving this giant number of people with uncontrolled manner resulted in many accidents in the past 20 years (**Still, 2011**). One area in particular has seen a number of fatalities: the city of Mina, located south-east of Mecca, during the ritual at Al-Jamarat. Despite continuous development of the pilgrim's additional access points, footbridges, and emergency exits, ensuring the safety of pilgrims continues to challenge the authorities.

Many researchers discussed the situation of Al-Jamarat rituals at Mina, and many efforts by the authorities have been made to provide safety to pilgrims during this ritual. However, after the sunset of the ninth day of Dhul-Hijjah pilgrims go to Muzdalifaduring "Nafra". The "Nafra" process includes the movement of 3.0 Million pilgrims from Arafat to Muzdalifa before sunrise using certain limited routes. Figure 1 shows the "Nafra" routes on a map and a view of pilgrims during the "Nafra". This is the focus of this paper.

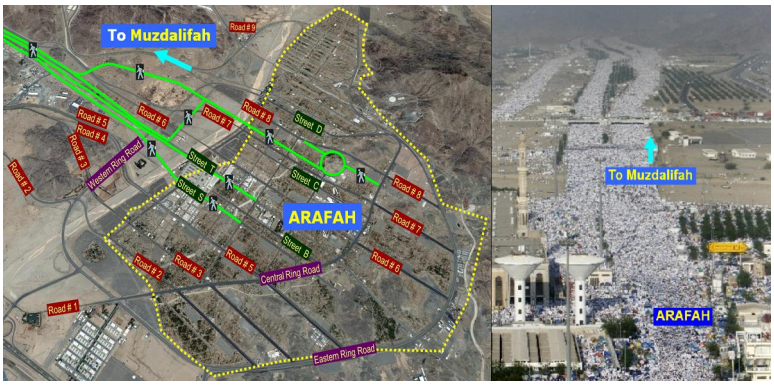

Figure 1: "Nafra" Routes

The main objective of this paper is to provide an integrated decision support system allows for close monitoring and control of crowd movements providing an early warning of any buildup for not only guidance of pilgrims and protection against accidents caused by overcrowding but also preserving a level of comfort during the movement to keep the sanctity of emotions at its best.

Moreover, managing millions of people gathered from diverse countries around the globe is not only a matter of placement them in the correct route. The gathered troops are different in nationalities and so in customs. Pilgrims coming from Gulf area prefer to move by cars or buses; pilgrims coming from India, Pakistan and Bangladesh prefer to move by walking together, and pilgrims that belong to Shi'a[1] also prefer to stay together. This makes the challenge more difficult in order to manage the moving of different troops together.

To control pilgrims, the Kingdom of Saudi Arabia, Ministry of Hajj has established six establishments to provide services for pilgrims plus GCC and interior establishment. Each establishment is responsible for managing pilgrims from different locations such as South Asian, Non-Arab Africans, South East Asians, Arabians, Iranians (Shi'a) and Turkish and Muslims of Europe, Americas and Australia. Each establishment has around 100 offices; each office is responsible for around 5000 pilgrims. These establishments will be the objective stack holders of the proposed crowd management system.

The proposed system incorporates data acquisition through real-time thermal video sequence analysis and processing, and management information distribution, via several thermal cameras deployed as sensors at strategic points on "Nafra" routes. The sensors are linked to an analysis module, which in turn measures crowd flow and density in real time. When crowds become too dense, an alarm is triggered according to different density levels. At this point the decision support system fires and provides operating authority by different alternate actions to regulate site access accordingly.

The proposed system architecture consists of two main components: the information management component, and the decision support system module. The information management module incorporates acquiring the required information about the crowd density and behavior, whether it's accelerating or decelerating in different roads on "Nafra" route. It uses infrared thermal video sequences (**Abuarafah *et al.*, 2012**) in monitoring process. Then it uses fuzzy logic to manipulate the outcome of the thermal video analyzer in integration with road parameters to deduce the status of each individual road in the route. The second component is the decision support module which integrates operations research with an expert system. The operations research works to determine pilgrims mass per minutes for each available road due to road parameters and possible time remains. The expert system divides hajj in homogeneous groups according to establishment and religious party. Then the decision support system works to generate alternate decisions based on closed roads, road priorities, and which group should move through which road. These alternatives will be available to the decision makers in each establishment to manage their crowds.

This paper is divided into four sections. The first section is this introduction. The second section discusses the crowd management issues and related work. The third section illustrates different component architecture. And the last section concludes the work and discusses the future of the research.

2. Related work

Generically, crowd can be defined as a large number of people gathered together with or without orderly arrangement. Crowd management is defined as the systematic planning for, and supervision of, the orderly movement and assembly of people. Crowd management involves the assessment of the people handling capabilities of a space prior to use (**Fruin, 1993**).

A moving crowd, even a large one, has the capacity to 'self organize' safely if the density is low enough. Under normal conditions, crowds have a spontaneous intelligence of their own, developing 'laminar flows', or streams, that keep everyone moving. As density increases, these smooth patterns start to disintegrate. **Helbing *et al.* (2007)** came up with some unexpected findings. As crowd density rose, they identified the onset of stop-and-go waves similar to those found in road traffic jams. This was followed by transition to a much more chaotic state, with outbreaks of panic as individuals lost control. This phenomenon – known as crowd turbulence – can trigger disasters.

Figure 2shows different level of crowds that can be noticed by human eye. In (a) the entire body is visible so there is no crowd; in (b), only body and head visible so overcrowding may occur. In (c) and (d) the crowd level need to be managed.

[1] Shia Islam (Arabic: شيعة, Shīʿah) is the second largest denomination of Islam. The followers of Shia Islam are called Shi'ites or Shias. "Shia" is the short form of the historic phrase Shīʿatuʿalī (شيعةعلي), meaning "followers of Ali", "faction of Ali", or "party of Ali".

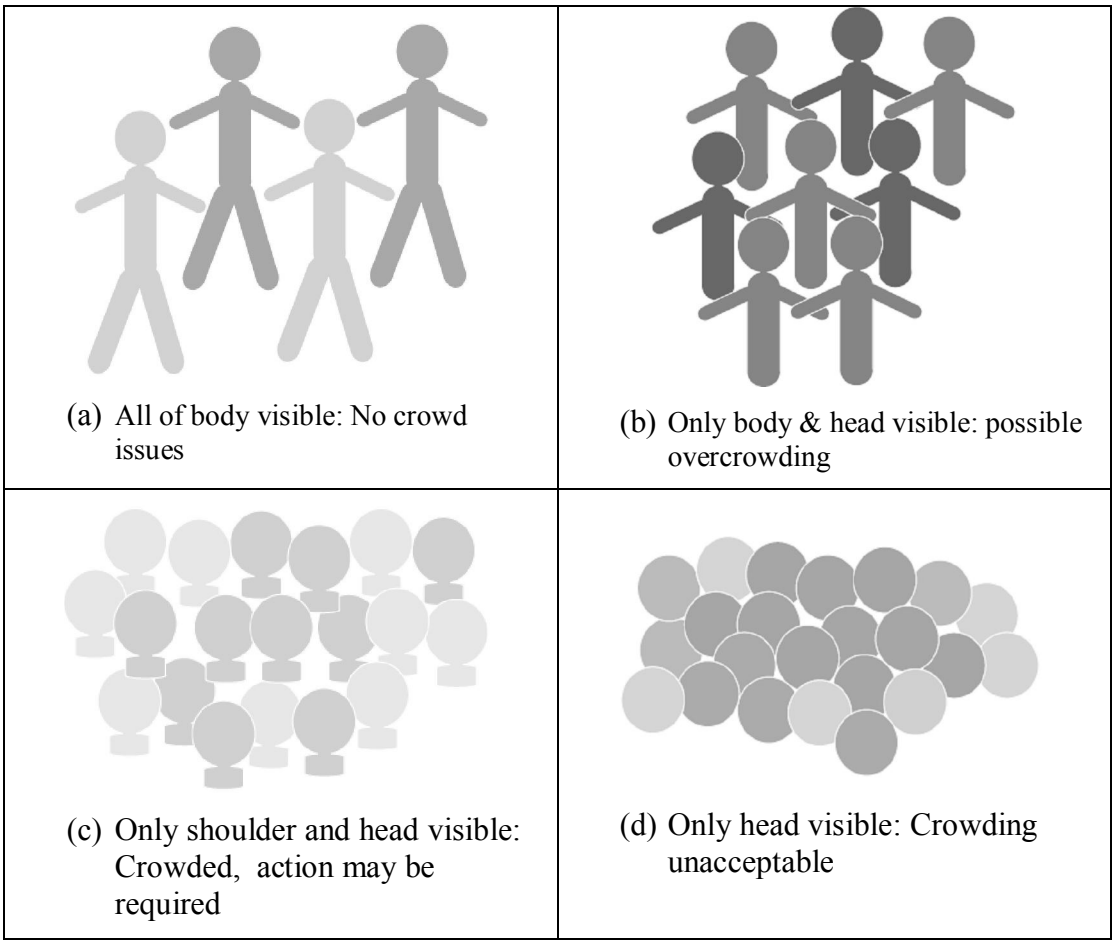

Figure 2: Different Levels of Crowd

Most major crowd disasters can be prevented by simple crowd management strategies. The primary crowd management objectives are the avoidance of critical crowd densities and the triggering of rapid group movement (**Fruin, 1993**). In the next two paragraphs, two crowd management systems have been studied.

Schubert *et al.* (2008) presented a decision support system for crowd control. Decision support is provided by suggesting a control strategy needed to control a specific disturbance situation. Control strategies consist of deployment of several police barriers with specific positions and strengths needed to control the disturbance. The control strategies are derived for a set of pre-stored example situations by using genetic algorithms where successive trial strategies are evaluated using stochastic agent-based simulation. The optimal control strategy for the current situation is constructed by the best linear combination of pre-stored example situations. The optimal strategy is given as the same linear combination of associated strategies. So, their system is using a decision making algorithm where a current situation is compared to all simulated situations.

A linear combination of control strategies, whose corresponding weighted superposition of simulated situations most closely resembles the current situation, is given as the required decision.

Deshpande and Gupta (2010) have proposed a computer based system combining fuzzy logic and Graphical Information System (G.I.S) to monitor and avoid the crowding disasters. They have proposed two-step mode. The first step is pre-disaster planning incorporating the determination of sensitive locations and space management, evacuation paths using (G.I.S) and management related arrangements. The second step is real time analysis of crowds to detect a possible emergency. Their system contains two modules. The first is a fuzzy inference system to determine crowding situations and plan of action. The fuzzy interface depends on the number of pixels and shape of objects to determine the crowd density. It also uses object characterization from the image to determine the speed of the crowd. The second is the determination of the shortest evacuation path for the

current area under surveillance. The shortest path is determined with the help of G.I.S. and the overall crowding situation. Their proposed system follows certain steps: acquiring basic information, formation of evacuation network, and calculation and decision.

3. Component Architecture

The proposed system consists of two main components. The first component is responsible for the information management. It incorporates data acquisition module through the use of several thermal cameras deployed at critical points on the target route. The thermal videos will be fed into an analyzer to calculate the density of the crowd in each road in the target route. The crowd analysis will be fed into a fuzzy logic module along with pre-stored information about roads geometry to devise the status of each road individually. The second component is the decision support system. It incorporates an operations research module that determines the pilgrims mass per minute for each available road due to road parameters and possible time remains. It also incorporates an expert system module that divides the pilgrims in homogeneous groups according to establishment, country, roads, and religious group. Then decision support component will generate different alternatives showing the closed roads, road priorities, and which group should move through which road. The alternatives will be used by the authorities to take the necessary actions. Figure 3shows a schematic diagram of the proposed system architecture. In the following sections, each module will be discussed.

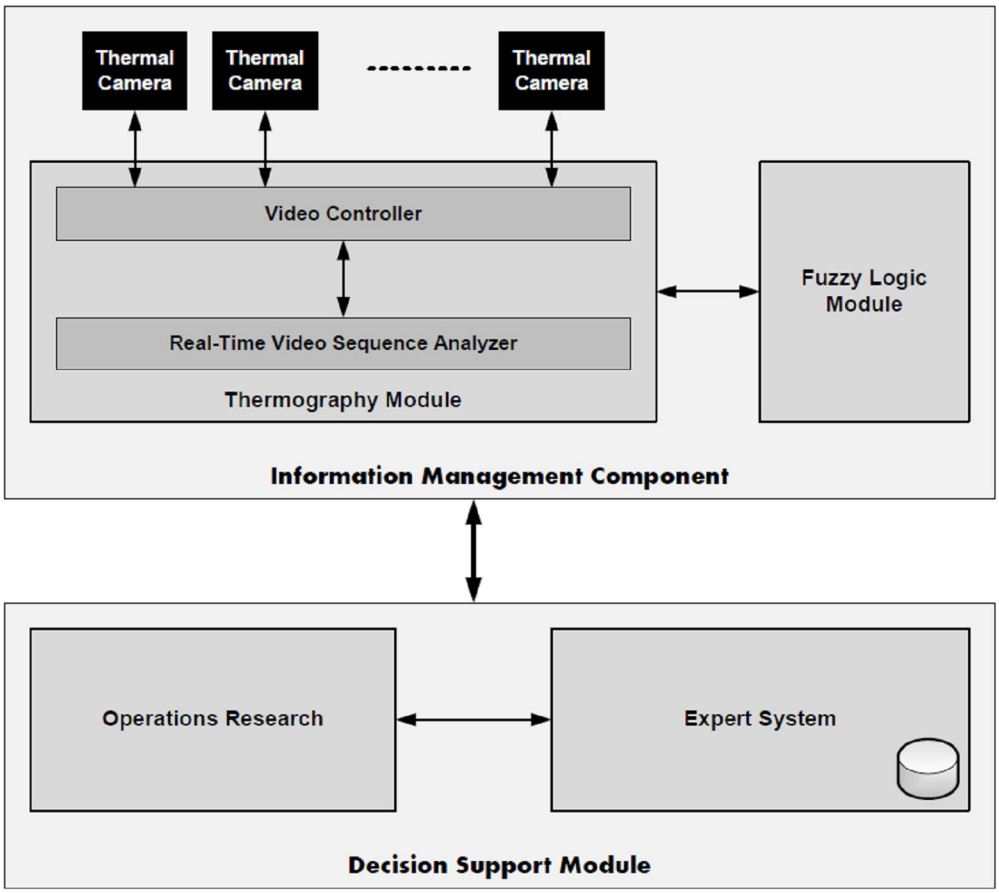

Figure 3: Proposed System Architecture

3.1. Information Management Component

The main objective of this component is to manage information about the crowd and the venue which is the "Nafra" route. The component is divided into two main modules: the thermography module and the fuzzy logic module.

The thermography module (**Abuarafah *et al.*, 2012**) uses a set of FLIR E60bx thermal camera deployed over an elevation about 10m above each pedestrian road in the route. The cameras will be connected to a controller. The controller collects different video sequences about each road in certain period of time and feeds the second module which is the video sequence analyzer with the collected video sequences and the necessary calibration information such as human temperature. The video sequence

analyzer module calculates the crowd density in real-time in pre-defined steps; every step includes a specific number of frames pre-defined in system configuration. The less number of frames in the step will result in increasing the accuracy of calculating the average crowd ratio. The module will indicate the crowd density percentage in different colors. In addition the module infers the movement behavior from whether it is accelerating or decelerating. Several reasons make thermography is the most suitable technique for the Hajj event. First thermal imaging is non-contact, i.e. uses remote sensing so it keeps the user out of danger. Meanwhile it does not intrude upon or affect the target at all so it keeps people privacy intact. Also the produced images allow for excellent overview of the target without the need of intelligent recognition of faces or body parts.

The second module in this component is the fuzzy logic component. This module manipulates the output of the analyzer in integration with road parameters such as road length and width to formulate the status of each road individually. The fuzzy logic module prioritizes the roads according to three parameters the crowd density on the road, the road length, and the road width. So, suppose that the crowd density is normalized among several roads; then the priority of them is arranged according to the shortest and widest one. The roads will be assigned a discrete number from 0 (lowest) to 10 (highest) to describe its priority

3.2. Decision Support Component

The decision support component consists of two modules: the operations research module and the expert system module.

The operations research module determines pilgrims mass per minute for each available road due to road parameters and possible time remains.

The expert system module plays a crucial rule in this problem to organize the pilgrims according to country, race, and religious party. Arrangement rules are stored in a knowledge base and the expert system inference engine integrates these rules with the operations research module output in order to do correct placement of different troops of pilgrims according to their preference.

The decision support component hence uses these rules to generate different alternatives to the authorities including the closed roads, other road priorities, and suggestions of which group should move through which road.

3.3.The Proposed End User Interface

Figure shows the proposed graphical user interface (GUI) of the described system. The screen is divided into three sections. The main section includes the map of the "Nafra" routes with different roads through the route. On the right hand side a real-time view of the current situation of each road appear at certain time interval. At the bottom, different alternatives are coming from the system including the closed roads, the road priorities and the suggestion of different actions.

Figure 4: The Proposed System GUI

Conclusion

In this paper, computer-based system architecture for crowd management of pilgrims during Hajj has been proposed. The system integrates real-time thermal video sequence analysis with decision support. The system provides real-time crowd density measurements and communications, making it possible for the security authorities to spot potential problems early and to interfere to reduce the risk to pilgrims.

For future research the implementation of the decision support module will be done and field experiment to the system will be evaluated.

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4/5/2012

Menopausal symptoms and the quality of life among pre/post menopausal women from rural area in Zagazig city

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Abstract: Introduction: "Menopause" denotes the final cessation of menstruation, either as a normal part of aging or as the result of surgical removal of both ovaries. **The aim**: this study aimed to investigate the impact of the menopausal symptoms on the quality of life of pre/post menopausal women from rural area, Zagazig city. **Research design:** a descriptive cross-sectional comparative study design was used in this study. **Settings**: This population based survey was conducted in one of rural district of Sheba, Zagazig city. **The sample**: consisted of 175; premenopausal (97) and postmenopausal (78) women whose ages ranged from 40-70 years old. **Tools**: data were collected by Menopause Rating Scale (MRS) and quality of life Brief (WHOQOL Brief). **Results**: the highest mean scores of menopausal symptoms were somatic symptoms and urogenital domains in postmenopausal women than in premenopausal women. There was a statistically significant difference between two studied groups in relation to their mean and standard deviation scores regarding their quality of life; physical, psychological and environmental domains. There was the significant negative correlation between MRS scores and WHOQOL- Brief scores in social, environmental domains, and overall mean score of quality of life for postmenopausal women. **Conclusion**: It can be concluded that post-menopausal women in the study subjects experience high prevalence of menopausal symptoms that adversely affected their quality of life. **Recommendation**: Further research addressing women's health needs is also essential for improving the quality of life of postmenopausal rural women.
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Key Words: Menopause, Severity of symptoms, Menopause Rating Scale (MRS), Quality of life, WHOQOL

1. Introduction

Menopause is defined as the permanent cessation of menses resulting from reduced ovarian hormone secretion that occurs naturally or is induced by surgery, chemotherapy, or radiation. Natural menopause is recognized after 12 months of amenorrhea that is not associated with a pathologic cause (Rahman *et al.,* 2010). Menopause is a physiological event in the women's life. It is caused by aging of ovaries which leads to decline in the production of ovarian Gonadotrophins, Estrogen and Progesterone. The deficiency of these hormones elicits various somatic, vasomotor, sexual and psychological symptoms that impair the overall quality of life of women (Dennerstein *et al.,* 2000 and Deeks & McCabe, 2004).

The mean age of the menopause in Egypt is 46.7 years, which is low compared to many countries, but this age has been rising in the past few years in the west, probably because of the different 'sociocultural attitudes' towards the menopause in different communities. The western women attitude towards the menopause is generally positive and about one-third of them considers the menopause as 'a normal physiological change'. Nevertheless, the Egyptian women need an awareness campaign about menopause in order to educate them about this important stage of their lives (Sallam *et al.,* 2006).

Menopause is a normal physiological change experienced by middle aged women. Some of the menopausal symptoms experienced by these women can be severe enough to affect their normal lifestyle. Unfortunately majority of those women are not aware of the changes brought about by menopause (Lu *et al.,* 2007 and Rahman *et al.,* 2010). It was also noted in some postmenopausal women with long term estrogen deficiency, changes to the cardiovascular or bone which leads to osteoporosis. It is well documented that menopausal symptoms experienced by women affect their quality of life (Dhillon *et al.,* 2006).

Symptoms experienced at menopause are quite variable, and the etiology of the symptoms is multi factorial. Also, menopausal symptoms can affect women's health and wellbeing ((Sievert, 2001 and Daley *et al.,* 2007). Some of the menopausal symptoms included: hot flushes, urinary incontinence and reduced sexual function (Greendale *et al.,* 1999). The nature, frequency and severity of symptoms vary not only among the individuals of the same population with different cultures, ethnicities and women from different countries, but also at different

stages of menopause (Randolph *et al.,* 2003). Several studies reported the experiences of menopausal symptoms of women from different parts of world and the significant impact of these symptoms on QoL of menopausal women at different status of menopause (Blumel *et al.,* 2000 and Fuh *et al.,* 2003).

The World Health Organization (1993) defines QoL as an individual's perception of their position in life in the context of the culture and the value system in which they live and in relation to their goals, expectations, standards and concerns can be applied to menopausal women. Also, the WHO identified four broad domains as being universally relevant for the quality of life, namely physical health, psychological well-being, social relationships, and environment (Hendry & McVittie, 2004 and Pensri *et al.,* 2007).

The menopause has been reported as one of the opportunities for women, to visit health-care services (Guthrie *et al.,* 2003). The health care of women during this stage requires special attention to the identification of their health needs in order to provide competent care (Gharaibeh *et al.,* 2010).

Aim of the study: To investigate the impact of menopausal symptoms on the quality of life of pre/post menopausal women from rural area in Zagazig city.

Research question:

Is there relationship between the menopausal symptoms on the quality of life of pre/post menopausal women?

2. Subjects & Method:

Research design:

A descriptive cross-sectional comparative study design was used in this study.

Study Settings:

This population based survey was conducted in one of the rural district of Sheba area, Zagazig city, Sharkia Governorate, Egypt.

Sample size:

The sample size was calculated through EPI info (Epidemiological information system) soft ware version 6. The estimated sample size was calculated to be 175 pre/post menopausal women, whose ages ranged between 40 and 70 years.

Study subjects:

The sample consisted of 175 women their ages ranged from 40-70 years old. The data was collected through 6 months started at October 2011 and finished at March 2012. These women from Sheba village. They satisfied the following exclusion criteria, they are free from ovarectomy hysterectomy, or other chronic diseases. The number of the study population (175) was determined according to the following procedure:- A multistage stratified random sampling technique was used for the identification of eligible women. At first stage, Sheba was selected randomly using lottery method. During the second stage of sampling, a name and address list of all the women aged 40 – 70 years was drawn from the maternal center health. In the third stage, out of established list, every fourth women was selected randomly. Initially for the selection of first number the lottery method was used for the first four numbers followed by every fourth number onward included into sample.

Ethical Consideration:

Both written and oral information about the reasons of the study were given in local language to women invited to participate in the present study. The participants were informed that their inclusion in the study will be voluntary and were given a guarantee of anonymity. They were informed that they were be free to withdraw from study and if any question they do not want to answer they can withdraw it.

Tools of Data Collection:

A structured questionnaire sheet was prepared by the researchers including 3 parts:

1) First part was used to collect the socio demographic data, including: age and level of education, occupation, marital status and family size.

2) The second part is a modified vision of menopausal rating Scale (MRS) (Heinemenn *et al.,* 2003), and menopausal symptoms list done by the researchers to assess the menopausal symptoms and severity (Schneider and Behre, 2002 and Germain *et al.,* 2001).

For purpose analysis (MRS) were further categorized according to 0 no, mild to moderate and sever to very sever. *Menopause Rating Scale:* consisted of 11 items assessing menopausal symptoms, divided into three subscales. A) Somatic: Hot flushes, heart discomfort, sleep problem and muscles and joint problems. B) Psychological: depression, irritability, anxiety and physical and mental exhaustion. C) Urogenital: Sexual problems, bladder problems and dryness of vagina. Each item can be graded from 0-4, (0= not present), (1=mild), (2=moderate), (3=severe), (4=very severe) (Heinemenn *et al.,* 2003). For the present study the MRS English version was translated into local language. In order to facilitate analysis and interpretation of the result, total scores in each area were 56, those who obtained scores less than 11 were considered to have no symptoms, 12 to 35 were mild and moderate symptoms and more than 36 were

considered to have severe and very sever symptoms.

Menopause status definition:

The menopause status was defined based on the reported length of time since last menstrual period. Women who reported the normal menstrual cycle for last three months were classified as Premenopause. Women who reported change in the length of menstrual cycle for at least seven days from baseline or change in the menstrual flow like lighter or heavier from baseline for last three months were classified perimenopause, those last menstrual periods occurred 12 months or more months ago were categorized as post menopause. Surgical menopause was defined as cessation of menstruation following either removal of ovaries (with or without hysterectomy) (Soules *et al.,* 2001).

3) Third part:
   WHOQOL questionnaire was modified by the researchers for the purpose of assessing the quality of life (QOL) for menopausal women.

WHOQOL Brief:

WHOQOL questionnaire has been developed in order to make a reliable, valid and responsive assessment of generic QOL that is applicable to the people living in different conditions and cultures. Two versions are available the WHOQOL with 100 items and 26 items short form version of WhOQOL 100 (Skevington *et al.,* 2004). We have used WHOQOL Brief (Urdu Version) for its brevity. The Urdu version is has been available with excellent reliability and validity (Khan *et al.,* 2003).

The WHOQOL Brief consists of four domains physical, psychological, social and environmental. The scores were calculated according to the standard methods that the raw scores were converted to transformation scores. The first transformation converts scores to range of 4-20 and the second transformation converts domain scores to 0 to 100 scale. Higher scores reflect better quality of life.

The WHOQOL Brief contained 26 items, categorized under 4 main domains Physical, Psychological, Social and Environmental. A separate 5 point scale ranging from never (4) to always (o point) was used for the measurement of each items. total score of each domain were 108 ; the higher score indicating a good QOL, a lower score indicating a poor QOL and high effect of menopausal symptoms on quality of life. Those who obtained scores from 0 to 33.3 % were considered (poor QOL), from 33.3 % to 66.7% were considered (average QOL) and more than 66.7% were considered to have (good QOL).

Validity and reliability

The questionnaire was translated into Arabic, and then reviewed by 5 experts (2 experts from community health nursing and 3 experts from obstetrics and Gynecology nursing) who conducted face and content validity of all item. All recommended modifications were performed. Degree of reliability alpha precision 88% of the study sample.

Pilot study

It was carried on 10% of the subject to test applicability and clarity of the tools, all recommended modification was performed through an extensive review of literature regarding menopausal symptoms and quality of life. After the development of the tool, the menopausal women who were taken from the previously mentioned setting.

Field work

The study was conducted during the period October 2011 to March 2012. Informed consent to participate in the study was obtained from the subjects. Modifications of the tools were done accordingly. Each subject was individually interviewed using the previously mentioned tool. Time consumed for each interview ranges from 30 to 45 minutes. The collected data were categorized, tabulated and made ready for use. The tools of data collection were translated into Arabic by the researchers, tested and verified by bilingual persons.

Statistical analysis

Statistical package for social sciences (SPSS) version 15.0 was used for data analysis. Results are presented as numbers (percentages) for qualitative variables and mean ± standard deviation for normally distributed quantitative variables are reported. Differences in proportion for menopausal status, demographic and health characteristics were assessed by Pearson Chi-square test and difference in mean score for quality of life were compared. Pearson coefficient of correlation (r) was determined among WHOQOL and MRS score. *P*-value less than 0.05 was considered as statistically significant.

3. Results

Table (1): demonstrates the soci-demography of the study subjects. The table showed that less than half of the studied subjects (44.0%) belonged to the age group ranged from 51-60 years old. The mean age of them was 54.0± 7.9 years, in addition to 32.0% of them have primary or preparatory school while 25.1% among the studied sample were illiterate, and less than two thirds (58.3%) of them were house wife and the rest of them were worker, Low proportion of women have high income . On the other hand, (70.8%) of them were currently married. Concerning

socioeconomic status, less than two thirds of the studied subjects (58.3%) belonged to poor socioeconomic status and more than one quarter of them (39.4%) belonged to middle socioeconomic class.

Table (1): Distribution of study sample according to their socio-demographic characteristics.

	Socio-demography	No (175)	%
Age	40-50	63	36.0
	51-60	77	44.0
	> 60	35	20.0
	X ± SD	54.02± 7.97	
Education	Illiterate	44	25.1
	Primary/preparatory	56	32.0
	Secondary	35	20.0
	University	40	22.9
Occupation	House wife	102	58.3
	Worker	73	41.7
Marital status	Single	4	2.3
	Currently Married	124	70.8
	Divorced	11	6.3
	Widow	36	20.6
Family size	2 to 4	15	8.6
	5 to 6	98	56
	6 and more	62	35.4
Crowded index	2-3	16	9.1
	4-5	19	10.9
	5or more	140	80.0
Income	Not enough	84	48.0
	Enough	57	32.6
	More enough	34	19.4
Socioeconomic status	Poor	102	58.3
	Middle class	69	39.4
	Upper class	4	2.3

Table (2): illustrated the severity of the menopausal symptoms among the studied groups. It can be observed that, the highest mean scores of menopausal symptoms were somatic symptoms and urogenital domains in postmenopausal women than in premenopausal women (10.46±6.28, 9.96±5.26 and 3.31±2.46, 2.69±1.96, respectively). While the mean scores of Psychological symptoms is the lower in postmenopausal women than premenopausal women (3.38±4.22, 4.22±3.66, respectively). The mean of the total MRS score was higher in postmenopausal women than premenopausal women (16.86±9.11, 17.15±11.21, respectively), and there is no statistically significant difference between two study groups regarding total MRS score.

Table (3): showed the quality of life among study subjects. according to the findings, there was a statistically significant differences between two studied groups in relation to their mean and standard deviation scores regarding their quality of life domains regarding physical, psychological and environmental domains (44.18±12.31 , 49.30±12.59, 43.13±11.01 & 40.17±13.15, 44.09±14.66, 38.58±12.82) respectively to premenopausal and postmenopausal women ($P = 0.3, 0.1$ & 0.1), as there was increase of mean scores of social domain in premenopausal women more than postmenopausal women (53.35 ± 19.38, 48.18 ± 18.34, respectively). However, there was no statistically significant difference according to social domain betweens two studied groups. The mean of the total quality of life scores was higher in premenopausal women than postmenopausal women (50.69±12.08, 45.15±13.75, respectively). However, there was a statistically significant difference between two studied groups in relation to their total mean score of quality of life.

The correlation between menopausal rating scale scores and WHOQOL- Brief scores is shown in **(Table 4)**. There was the significant negative correlation between MRS scores and WHOQOL-Brief scores in social, environmental domains, and over all mean score of quality of life for postmenopausal women. However, there wasn't significant negative correlation between MRS scores and WHOQOL- Brief scores in Physical and psychological domains of quality of life for postmenopausal women.

Table (5) displays the relationship between socio-demographic characteristics and quality of life. It was found that more than one third (27.3%) non- educated menopausal women had poor quality of life compared to educated women that had good quality of life, there was no statistical significance differences between quality of life and education. As regards occupation, more than one tenth (11.1%) of the worker also, had poor quality of life, while (5.6%)

were employed and had good quality of life, there was no statistical significance difference between quality of life and occupation. It was observed that, the poor quality of life for the subjects who family size less than four and poor socioeconomic, (26.7%, 18.6% respectively), there was no statistical significance differences between quality of life and family size and socioeconomic class. There was a statistical significance difference between quality of life and income.

Table (2): Distribution of the menopausal symptoms among two study groups.

Subscale and symptoms	Pre-menopause (97)		Post-menopause (78)		Test	P-Value
	No	%	No	%		
Somatic	**9.96±5.26**		**10.46±6.28**		**T= 0.57**	**0.56**
1. Hot flushes						
None	36	37.1	16	20.5		
Mild - Moderate	51	52.6	51	65.4	X2 = 5.94	0.05
Sever - Very sever	10	10.3	11	14.1		
1. Sweating						
None	27	27.8	12	15.4		
Mild- Moderate	61	62.9	52	66.7	X2 = 5.68	0.06
Sever- Very sever	9	9.3	14	17.9		
2. Heart discomfort						
None	42	43.3	50	64.1		
Mild - Moderate	42	43.3	19	24.4	X2 = 10.18	0.017*
Sever - Very sever	13	13.4	9	11.5		
3. Sleeping problem						
None	15	15.5	18	23.1		
Mild- Moderate	50	51.5	31	39.7	X2 = 3.31	0.24
Sever - Very sever	32	33.0	29	37.2		
11. Muscle and joint problem						
None	5	5.2	5	6.4		
Mild - Moderate	54	55.7	40	51.3	X2 = 0.38	0.82
Sever - Very sever	38	39.2	33	42.3		
Psychological	**4.22±3.66**		**3.38±2.5**		**T= 1.72**	**0.08**
4. Depressive mood						
None	37	38.1	45	57.7		
Mild- Moderate	44	45.4	22	28.2	X2 = 7.36	0.02*
Sever- Very sever	16	16.5	11	14.1		
5. Irritability						
None	28	28.9	34	43.6		
Mild- Moderate	49	50.5	33	42.3	X2 = 4.95	0.11
Sever - Very sever	20	20.6	11	14.1		
6. Anxiety						
None	48	49.5	41	52.6		
Mild- Moderate	38	39.2	28	35.9	X2 = 0.55	0.90
Sever- Very sever	11	11.3	9	11.5		
7.Physical and mental exhaustion	48	49.5	47	60.3		
None	38	39.2	23	29.5	X2 = 4.28	0.34
Mild- Moderate	11	11.3	8	10.3		
Sever- Very sever						
Urogenital	**2.69±1.96**		**3.31±2.46**		**T= 1.85**	**0.06**
8. Sexual problem	30	31.9	23	29.5		
None	61	62.9	50	64.1	X2 = 2.32	0.97
Mild- Moderate	6	6.2	5	6.4		
Sever- Very sever						
9. Bladder problem	42	43.3	32	41.0		
None	50	51.5	30	38.5	X2 = 11.01	0.006
Mild- Moderate	5	5.2	16	20.5		
Sever- Very sever						
10. Dryness of the vagina	33	34.0	21	26.9	X2 = 3.99	0.32
None	59	60.8	49	62.8		
Mild- Moderate	5	5.2	8	10.3	**T= 0.2**	
Sever- Very sever	**16.86±9.11**		**17.15±11.21**			**0.85**
Over all score						

Table (3): relation between quality of life and menopausal symptoms among two study groups.

domains	Pre menopause(97)	Post menopause (78)	T test	*P*-Value
Physical	44.18 ± 12.31	40.17 ± 13.15	2.07	.039*
Psychological	49.30 ± 12.59	44.09 ± 14.66	2.52	.012*
Social	53.35 ± 19.38	48.18 ± 18.34	1.79	.074
Environmental	43.13 ± 11.01	38.58 ± 12.82	2.79	.012*
Overall mean score	50.69±12.08	45.15±13.75	0.005*	2.83

Table (4): Pearson's correlations of quality of life and menopausal rating scale among two study groups.

Subscale and symptoms	Pre menopause(97)		Post menopause (78)	
	r	p-value	r	p-value
Physical	.04	0.67	-.01	0.89
Psychological	-0.17	.07	-0.14	0.23
Social	-0.36	0.000*	-0.36	0.000*
Environmental	-.078	0.44	-0.27	0.01*
Overall Mean Score	-0.14	0.18	-0.25	0.01*

Table (5): Relation between socio-demographic characteristics and quality of life of the study sample.

socio-demographic characteristics	quality of life			Total (175)	X2	p-value
	poor	moderate	good			
Age						
40-50	8(12.7%)	51(81.0%)	4(6.3%)	63(100.0%)		
51-60	9(11.7%)	64(83.1%)	4(5.2%)	77(100.0%)	10.19	.037*
>60	12(34.3%)	22(62.9%)	1(2.9%)	35(100.0%)		
Education						
Illiterate	12(27.3%)	29(65.9%)	3(6.8%)	44(100.0%)		
Primary\ preparatory	14(25.0%)	42(75.0%)	0	56(100.0%)	19.53	0.003
Secondary	2(5.7%)	31(88.6%)	2(5.7%)	35(100.0%)		
University	1(2.5%)	35(87.5%)	4(10.0%)	40(100.0%)		
Occupation						
House wife	21(20.6%)	76(74.5%)	5(4.9%)	102(100.0%)	3.02	0.55
Worker	8(11.1%)	61(83.3%)	4(5.6%)	73(100.0%)		
Marital status						
Single	2(50.0%)	2(50.0%)	0	4(100.0%)		
Currently married	14(11.3%)	103(83.1%)	7(5.6%)	124(100.0%)	0.07	11.63
Divorced	2(18.2%)	9(81.8%)	0	11(100.0%)		
Widow	11(30.6%)	23(63.9%)	2(5.6%)	36(100.0%)		
Family size						
2 to 4	4(26.7%)	11(73.3%)	0	15(100.0%)		
5 to 6	15(15.3%)	80(81.6%)	3(3.1%)	98(100.0%)	5.42	0.24
6 and more	10(16.1%)	46(74.2%)	6(9.7%)	62(100.0%)		
Crowded index						
2-3	0	14(87.5%)	2(12.5%)	16(100.0%)		
4-5	2(10.5%)	15(78.9%)	2(10.5%)	19(100.0%)	7.31	0.12
5or more	27(19.3%)	108(77.1%)	5(3.6%)	140(100.0%)		
income						
Not enough	24(28.6%)	60(71.4%)	0	84(100.0%)		
Enough	1(1.8%)	52(91.2%)	4(7.0%)	57(100.0%)	27.90	0.000*
More enough	4(11.8%)	25(73.5%)	5(14.7%)	34(100.0%)		
Socioeconomic status						

4. Discussion

Menopause is a transitional phase that is immediately prior to and after menopause, when clinical, biological, and endocrinological symptoms of menstrual cession commence, it occurring universally in all women who reach midlife. The timing of menopause as well as women's experience of menopausal symptoms varies between populations and within populations (Gharaibeh *et al*., 2010). The incidence of menopausal symptoms is influenced by socio-demographic/ sociocultural factors, economical stresses, general health status, and individual perception of menopause, genetic and racial differences and reproductive parameters like parity (Nisar & Sohoo, 2010).

The current study revealed that the mean age of study subjects was 54.0± 7.9 years, similar study conducted by Dhillon *et al.* (2006) and Palacios *et al.*

(2010) reported that the mean age at menopause was 51.14 ± 2.11years. This is slightly higher than studies done in Singapore (49.1 years), and Thailand (48.7 years). However, comparing our findings with previous researcher, ours still fall between the normal range of menopausal age. Another study conducted by Delavar & Hajiahmadi, 2011 who stated that the mean age in menopause was 47.7 years. This was similar to that reported by women from Shiraz (47.8 years), and Pakistan, however, it was lower than that of Iran (49.6 years), and the USA (51.4 years).The possible explanations for the relatively lower mean age in menopause were the differences in the definition of menopause, population sample and the survey method. Hormone replacement therapy in the perimenopausal period could be an important factor of delayed menopause in developed countries.

The number of highly educated women incorporated in the present study is less than the primary or preparatory and illiterate women, and less than two thirds of them were house wives and the rest of women were worker. Low proportion of women have high income this is agree with Rahman *et al.* (2011) who emphasized that the lowest proportion of women were highly educated (5.5%). However, 85.06% of the women were housewives and 14.93% of women were involved with paid work. The highest percentage of women was from families with average income, whereas the lowest were from families with high income and figures were 66.29% and 14.14% respectively.

The findings of the present study showed that the women in postmenopausal period suffered from severe different menopausal symptoms such as: musculoskeletal, hot flushes and sweating symptoms as well as sexual, bladder problem, dryness of vagina compared to premenopausal period. This may correlates with fluctuating levels of estrogen in the blood from premenopausal to postmenopausal period. While the Psychological symptoms either decline or remain stable in the postmenopausal women. These results are congruent with Ayatollahi *et al.* (2004) who found that the most common symptoms associated with menopause in Iranian women were reported as muscle pain (75.1%), night sweats (69%) and hot flushes (67.9%). Also In Malaysia, Jahanfar *et al.* (2006) who reported that the most common symptoms were found to be joint and muscle discomfort (84.3%), followed by anxiety (71.4%), physical and mental discomfort (67.2%), hot flushes and sweating (67.1%). These differences in frequencies of symptoms may be associated to differences of race, life style, culture, genetics and diet. For example musculoskeletal symptoms in women of menopausal age may be related to hormonal changes or, they may be due to women's roles within particular culture. In the study conducted by Waidyasekera *et al.* (2009) among Sri Lankan women the joint and muscle discomfort, physical and mental exhaustion and hot flashes were the most prevalent menopausal symptoms. This similar with Gharaibeh *et al.* (2010) who found that vasomotor signs were reported to have the highest scores for severity as manifested by hot flushes and night sweating. Also Ashrafi *et al.* (2010) showed that night sweats, joint and muscle pain and hot flashes are the most common symptoms associated with menopause in Iranian women. These findings were also noted by Rahman *et al.* (2010) emphasized that the frequency of sexual problems, bladder problems and vaginal dryness were experienced mainly by perimenopausal and postmenopausal group of women and it was also significant statistically in comparison to other menopausal status. This is disagreement with Dhillon *et al.* (2006) who reported that The classical presentation of menopausal symptoms; hot flushes, sweating and night sweats were noted to be lower (35.8%) in comparison to findings from studies done on western women which were reported to be from 45% to 75%. However, our findings of low menopausal classical symptoms were shared by studies done in other Asian countries

The present study found significant difference in the mean scores of the domain Physical, Psychological as well as environmental domain and the mean of the total scores of WHOQOL- Brief at different menopausal status, in postmenopausal compared to premenopausal women. This may be due to the high scores of MRS for different menopausal symptoms. We did not found significant difference in scores for social domain of WHOQOL- Brief which due to some factors like financial resources, access to health and social care. These results were supported by Nisar & Sohoo, 2010 who found significant difference in the mean scores of the domain (Physical, Psychological, Social) and the total scores of WHOQOL- Brief at different menopausal status, these findings were inconsistent with Ozkan *et al.* (2005) and Satoh & Ohashi (2005) who reported that there was no significant difference in the mean scores in the all domains and the total score of the quality of life. Also, in the study conducted by Yakout *et al.*(2011) who stated Poor scores for different items of quality of life were observed among the study subjects including physical, role, social and psychological limitation as well as sleep and energy.

Regarding the correlation between menopausal rating scale scores and WHOQOL- Brief scores, a negative significant relation was demonstrated between quality of life in social, environmental domains, and over all means score of quality of life and postmenopausal symptoms, where quality of life

adversely affected by postmenopausal symptoms among the postmenopausal women in the study subjects. This is in agreement with the results of Karaçam and Seker (2007) who observed a significant and moderately negative relation between total menopausal symptom scores and quality of life scores. On the same line, Nisar & Sohoo (2010) highlighted that there was a negative correlation between MRS scores and WHOQOL- Brief scores in all domains for postmenopausal women. Moreover, Yakout *et al.* (2011) emphasized that the negative significant relation was demonstrated between quality of life and postmenopausal symptoms, where quality of life adversely affected by postmenopausal symptoms among the postmenopausal Saudi women in the study subjects.

According to the relationship between socio-demographic characteristics and quality of life among the post-menopausal women in the study subjects, there were no statistical significance differences between quality of life and education, occupation, and family size. While there were a statistical significance differences between quality of life and age and family income. This contrast with Sahar *et al.* (2011) who found a significant correlation was observed between quality of life and their general characteristics including: education, occupation, cohabitation, family size as well as their gravidity. This also was asserted by Gharaibeh *et al.*(2010)) who stressed that a significant relationship between the severity and occurrence of menopausal symptoms and age, family income, number of children, perceived health status and menopausal status. in another study conducted by Yakout *et al.*(2011) who mentioned that a significant relation was observed between quality of life and their general characteristics including: education, occupation, cohabitation, family size as well as their gravidity.

5. Conclusion

Menopause is an important time in a woman's life. Her body is going through many changes that can affect her quality of life, her social life, her feelings about herself, and her functioning at work. It can be concluded that post-menopausal women in the study subjects experience high prevalence of menopausal symptoms that adversely affected their quality of life. The QOL of postmenopausal rural women were decreased in its scores due to the menopausal symptoms.

Recommendations

This study demonstrated the needs of the less developed community for more effort from health care providers to start further researches about the quality of life of menopausal women. Moreover, the health care provider should implement an educational program for women about the menopausal period and how to pass it safely.

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Differentially Expressed Homeobox Genes in Salivary Adenoid Cystic Carcinoma versus Normal Salivary Gland Tissue

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Abstract: The purpose of this study is to identify differently expressed homeobox genes in salivary adenoid cystic carcinoma (SACC) versus normal salivary gland tissue, and determine the effects of homeobox genes on oncogenesis and differentiation of SACC. Six paired tissue specimens of SACC and surrounding normal salivary gland tissue were obtained. Customized Oligo microarray was used to analyze differential homeobox gene expression. Data were scanned by Agilent Scanner. Real-time PCR was used for quantitative analysis of gene expression. The results showed that Homeobox genes TGIF and EVX1 were differentially expressed in SACC versus normal tissues. As regulators of cellular proliferation and differentiation, homeobox genes may be involved in SACC oncogenesis.

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Keywords: adenoid cystic carcinoma; homeobox gene; gene microarray

1. Introduction

Adenoid cystic carcinoma of the salivary gland is one of the relatively common malignant salivary gland tumors. It has a strong infiltration capability and a high metastatic rate. The occurrence and development of adenoid cystic carcinoma is a complex process involving a variety of factors and steps, changes in the expression of various genes, and adjustments between the structure and function of relevant rerecording determinants.

The homeobox gene family is a large gene family which encodes transcription factors.[1,2] It has been confirmed that homeobox genes exist in all eukaryotic cells. Homeobox genes are expressed during specific growing stages of an organism and in specific tissues. Homeobox genes control cell mitosis and control the synthesis of various molecules and hormones involved in conveying information at a local and remote level to adjust the growth and differentiation of the embryo. Mutation in the sequence of certain homeobox genes can result in phenotype change in normal cells. Abnormal expression of homeobox genes may trigger uncontrolled cell proliferation and may prevent cells from differentiating and maturing, resulting in tumor. Recent studies have shown that disregulation of homeobox genes expression is not only involved in developmental deformity but also in the occurrence and development of tumors, including mammary cancer, malignant melanoma, cervical carcinoma, leukemia, and prostate cancer.[3-11]

Microarray technology[12,13] has been developing rapidly since the inception of the concept of DNA microarray by Fodor in 1991.[14] The purpose of this research is to compare the difference in homeobox gene expression between adenoid cystic carcinoma of the salivary gland and normal gland specimens by using microarray, and determine the relationship between homeobox gene expression and the illness itself.

2. Material and Methods

2.1 Patients and specimens

2.1.1 Salivary adenoid cystic carcinoma (SACC) and normal salivary gland tissues

Six patients with adenoid cystic carcinoma of the salivary gland who attended the Department of Oral and Maxillofacial Surgery, West China College of Stomatology, Sichuan University in June 2006 were enrolled in this study. Two patients were men and four were women. The average age was 50.7 years (range: 31 to 68 years). The degrees of the cases were as the follows: two cases were T_2; three cases were T_3; and one case was T_4 (UICC [15] TNM by stages). The patient's illness was diagnosed as salivary adenoid cystic carcinoma (SACC). All patients received extensive resection on the primary tumor focus with no other treatment such as chemotherapy or local radiotherapy.

Specimen processing: a 5×5×5-mm^3 piece of tissue from the primary focus of the SACC was cut with an aseptic scalpel and the unnecessary portion was discarded; a piece of normal gland tissue (of the same size) was cut 2-cm away from the edge of the primary tumor focus. The specimen was washed with DEPC solution and cleaned gently with aseptic

etamine. The specimens were divided into two parts. One part was fixed in formalin solution for further evaluation by a pathologist; the other part was treated with DEPC solution and immediately frozen in liquid nitrogen for RNA extraction. The process was carried out in aseptic conditions and within 10 minutes of specimen collection.

2.1.2 SACC cells

Two cell strain of SACC were used in this study: ACC-2 cells (low metastatic cell strain) and ACC-M cells (high metastatic cell strain). The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

2.2 RNA extraction, purification, and testing

Tissues and cell specimens were put it in ammonia, and fully grinded and mixed in the machine. The mixture was moved into a 15-mL RNase free centrifugal tube and centrifuged. The supernatant was removed and put into precooled Trizol buffer for total RNA extraction. The mixture was then transferred into a new tube and RNA was extracted with 1.5 mL, chloroform, precipitated with avantin, and then dissolved in RNAse-free purified water. To prevent DNA contamination, the RNA solution was treated with RNase-free DNAse. The quality and consistency of the total RNA was assessed by spectrophotometry; RNA was then purified on oligo-dT affinity column and the RNA quality was tested by electrophoresis on 1% agarose gel (normal electrophotogram clearly showed 28S, 18S, and 5S ribosomal RNAs).

2.3 Preparing custom homeobox gene microarray

The network of American National Biological Information Center was used to retrieve human gene data, and all the members of the homeobox family were found by using 'homeobox' as keyword in the database search. According to the relevant biological software design 232 oligonucleotide microarray probes were needed. Eight custom microarrays were made by using the Oligo microarray and the omni Grid 100 sample-application device (Gene Machine Company). There were 14 positive spots and 1 negative spot. The first 7 spots in the first and second ranks of each matrix within the microarray were quality control spots, which were identical in every matrix: every gene was tested 4 times repeatedly in the same microarray.

2.4 Microarray hybridization

A direct cDNA labeling method was used. cy3 fluorescent label was used for the cDNA from the experimental group and cy5 was used for the cDNA from the comparator group. After labeling they were hybridized by using equivalent probes. The hybridized microarray was scanned, the data recorded, and the Cy3/Cy5 ratio was calculated. Genes with a ratio value of 0.5 to 2.0 were not considered to have a significant difference in expression; genes with a ratio ≥2 were considered upregulated, and those with a ratio ≤0.5 were considered downregulated.

2.5 Quantitative real-time polymerase chain reaction (PCR)

Cells (ACC-2 and ACC-M) and normal tissue specimens of salivary glands were used in the quantitative real-time PCR. Primers were designed based on mRNA sequences found in the Genebank. The sequences of the primers for each gene studied are listed in Table 1.

Table1.Primersequencesusedinreal-timePCR

Gene	Primer sequences (5'-3')	PCR product (bp)
EVX1	F: 5'-GCGGGTTTCCTTTCATCTTC-3' R: 5'-GCTGTCATCCTCCTGCTG-3'	112
TGIF	F: 5'-GGAGAGTCGGCTGTGAAG-3' R: 5'-AAGGATAGGCATTGTAACGG-3'	291

The real-time PCR amplification reaction was carried out by using 10.0 μL SYBR Premix Ex Taq, 0.8 μL of each specific primer, 1 μL cDNA template in a total volume of 20 μL. PCR was performed in a GeneAmp PCR SYSTEM 9600 (Perkin Elmer, Waltham, Mass). For signal detection, the GeneAmp PCR SYSTEM 9600 was programmed to perform an initial step of 2 minutes at 95 C, followed by 45 thermal cycles of 15 seconds at 95 C, 20 seconds at 62 C, and 20 seconds at 72 C. For quantification, a cycle threshold (Ct) value was used against a standard curve constructed after amplification of 10-fold serial dilutions of template copies. All reactions were performed in triplicate. Target gene mRNA levels were normalized to the mRNA level of a control/reference gene: 18s (endogenous house-keeping gene).

2.6 Statistical analysis

One-way ANOVA was used to analyze gene expression in normal tissue, SACC tissue, and cells by using the SPSS software version 12.0 (SPSS, Chicago, Ill). The alpha level was set at 0.05.

3. Results

3.1 Pathological analysis of clinical specimens

Specimen tissue sections were stained with HE. The cancer was proven to be SACC by clinical diagnosis. The tissues surrounding the cancer were normal salivary gland tissues.

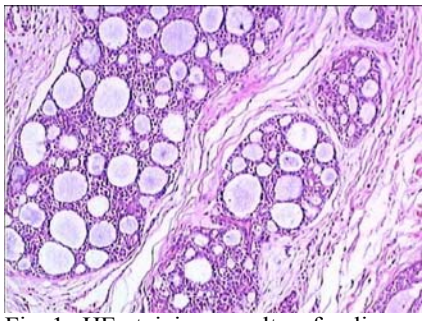

Fig 1. HE-staining results of salivary adenoid cystic carcinoma (300×)

3.2 RNA quality

Six groups of tissue specimens and two groups of cell specimens were evaluated. The total RNA were extracted and subjected to electrophoresis in 1% agarose gel. Clear zones of 28S, 18S, and 5S ribosomal RNA could be seen on the electrophoregram.

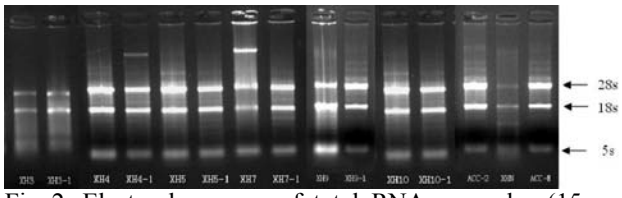

Fig 2. Electrophoregram of total RNA examples (15 specimens) : three zones as 28S, 18S and 5S can clearly be seen

3.3 Microarray results

The scanned image before microarray hybridization and the scanned image after hybridization showed that, in every group, the signal of the microarray was strong and the signals within each microarray was even. The correlation coefficients of two repeated points in two microarrays for the same sample were all above 80%.

3.4 Genes with differential expression

Eight paired specimens were studied. Data from the 8 hybridized microarrays were recorded and ranked by increasing ratio value. The ratio values were flagged as P.A or A, S/N≤2 or P≥0.05. Upregulated and downregulated homeobox genes on the microarray were selected, their sequence was retrieved, and the frequency of their appearance in the samples was counted (F, frequency) (Table 2).

Statistical analysis showed that, in tissue specimens, there were 9 upregulated homeobox genes whose frequencies were over 2. However, only one gene, TGIF, was upregulated both in cells and tissue specimens. Ten downregulated homeobox genes whose frequencies were over 2 were found in tissue specimens. Among them, EVX1 and IRX2 were downregulated both in cells and tissue specimens.

Table 2. Homeobox genes with differential expression between SACC and normal surrounding tissues

Differentially expressed genes	Frequency	Ratio (mean)	ACC-2	ACC-M
Upregulated		cy3/cy5 2.0		
DLX5 [a]	5	2.091	-	-
DBX1	4	2.119	-	-
EN1	4	5.193	-	-
IRX4	4	3.786	-	-
NKX6-1	4	4.119	-	-
DUXAP8	3	2.527	-	-
IRX3	3	7.617	-	-
ISL2	3	4.828	-	-
NKX6-2	3	4.914	-	-
TGIF	2	2.555	+	+
Downregulated		cy3/cy5 0.5		
EVX1 [a]	5	0.414	+	+
PITX1 [a]	5	0.166	-	-
DUXAP2	4	0.350	-	-
HOXB13	3	0.418	-	-
IRX2	3	0.227	+	+
ISL1	3	0.169	-	-
PITX2	3	0.305	-	-
PRRX1	3	0.315	+	-
SIX1	3	0.290	-	+
TLX2	3	0.325	-	-

[a]homeobox genes up-regulated or down-regulated in five microarray.

3.5 Quantitative real-time PCR

Quantitative real-time PCR results of the relative expression of EVX1 and TGIF in normal tissue and cells are shown in Table 3. There was no statistical difference in EVX1 expression between normal tissue and ACC-2. EVX1 expression was 200 times lower in ACC-M than in normal tissue (P<0.05) There was statistically significant difference in TGIF expression between normal tissue and ACC-M. TGIF expression was 8 times higher in ACC-M than in normal tissue (P<0.05)

4. Discussions

Genes which are differently expressed between malignant tissues and normal tissues may be involved in oncogenesis, cell multiplication and mitosis, and cell apoptosis. It may be closely related to malignant change and malignant progressing of adenoid cystic carcinoma of salivary gland tissue.

(1) DLX5 (distal-less homeobox 5) is a key differentiation factor which encodes a homeobox gene. This factor maintains the pluripotent differentiation potential and the self-turnover capability of embryonic stem cells. Reports have shown that this gene may be relevant to the pathogenesis of lymphatic leukemia.[16,17]

(2) DBX1 (distal-less homeobox) is the cognate frame of brain growth and a critical polyenergetic split determinant. It plays a critical regulatory role in the growing cycle of mammals.[18] Few reports have shown the relationship between DBX1 and oncogenesis of tumor.

(3) EN1 (engrailed homolog 1) plays an important role in the growing process of the rear somite in mammals. A study shows that transcription of the gene MENIN may be involved in the formation of multiglandular carcinoma, which is generally expressed in the form of medullary paraganglioma.

(4) EVX1 (eve, even-skipped homeobox, homolog 1) is located within chromosome No.7, and codes for a member of the even-skipped homeobox gene family. EVX1 protein may be an important transcription restraining determinant during embryonic development. So far, there is no relevant report on the involvement of EVX1 in the oncogenesis of human cancer.

(5) PITX1 (paired-like homeodomain transcription factor 1) encodes a member of the RIEG/PITX homeobox gene family. Members of this family are involved in organ growth and bilateral symmetry. Lord et al.[19] reported that the average level of PITX1 mRNA found in esophagus cancers associated with Barrett's esophagitis syndrome is much lower than that in Barrett's esophagitis syndrome (8.02) and normal esophagus (47, 46, $P<0.001$), which shows that PITX1 may play an important part in the process of occurrence and oncogenesis of esophagus Barrett's metaplasia-dysplasis – gland cancer.

In the present study, several homeobox genes, including DLX5, DBX1, PITX1 were differently expressed in SACC tissues vs normal salivary gland tissues, suggestion that these genes may be involved in oncogenesis in SACC. However, differential expression of these genes was not confirmed in cells. Of note, cells represent a 'single homogeneous component', while tissue specimens contain multiple cell types, including vessels, nerves, and extracellular matrix which may also be relevant to the tumor oncogenesis. These differentially expressed genes from tissue specimen may come from these different cell types within the tissue. The gene SIX1, which was differently expressed in ACC-M vs normal tissue but normal expressed in ACC-2 may be involved in the metastatic ability of cancer.

Two differentially expressed genes, TGIF and EVX1, were selected and their expression was evaluated using quantitative real-time PCR. TGIF was the only gene that was overexpressed in both SACC tissues and cells, although its frequency was relatively low. Several genes were downregulated in both SACC tissues and cells. Among them, EVX1 had the highest frequency. Cells (ACC-2 and ACC-M) were used to confirm EVX1 expression by real-time PCR because cells are more homogeneous; normal tissue was used as control. Real-time PCR confirmed that TGIF and EVX1 are differently expressed in SACC cells vs normal tissue.

In this study, we used microarray techniques and real-time PCR to identify the most relevant genes involved in SACC oncogenesis. TGIF and EVX1 were found to be differentially expressed in SACC vs normal salivary gland tissue. Further studies will be required to determine the mechanism and effects of these two genes on the oncogenesis of SACC.

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Linguistic Democracy in English Language Teaching

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Abstract: The major purpose of the present study was to examine English Language Teaching (ELT) in the light of democracy in Iran. To this end, first we have presented the major tenets of democracy, such as Equality and Freedom, then we have discussed these major principles in ELT in general, and finally linguistic democracy has been debated in the context of ELT in Iran. Our analyses show that linguistic democracy is not totally upheld in the field of English language education, requiring more attention and transformation. In the end, the results were discussed in the context of English language education in Iran and some suggestions were made.
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Keywords: Linguistic democracy; English language teaching; Iran

1. Introduction

The 20th century has probably been one of the most eventful eras in the world history witnessing a series of political upheavals including revolutions (Russian and Chinese), decolonization as a result of the end of the European empires (especially those of Britain and France), the two world wars, and the rise and fall of such metanarratives as Nazism, Fascism, and Communism. Nevertheless, when asked to pick out the one most significant of this century's events, a striking answer would be that of Kumar (1999), namely, the rise of democracy.

It sounds like a sensible answer though, when considering the ever-increasing impact democracy has ever since had on different aspects of today's life. Throughout its widespread presence, democracy was no more restricted to the political context of governing some people. As a matter of fact, far from having a solely political application, democracy has had the potential to be applied in a variety of other contexts such as cultural, social, and religious ones, to name a few. That is to say, it has practically turned into a popular method of reaching decisions in areas as diverse as entertainment, education, art, science, and theology.

In this study, we intend to examine English Language Teaching (ELT) in Iran to see whether the democratic ideas have permeated in ELT. To this end, we first present the core notions of democracy, then the manifestations of democratic notions are discussed in ELT, and finally ELT context is examined in Iran.

2. Democracy

The first democracy dates back to more than 2400 years ago when it is believed to have been used as the governing system in the Greek city state of Athens. Correspondingly, the term "democracy" originates from the Greek words "demos" meaning *people* and "kratos" meaning *power*, representing people's power, popular sovereignty, or rule by people (Hunt, Martin, Rosenwein, Hsia, & Smith, 2007).

As clear and familiar as it seems to be, this concept does not yield itself to one universally accepted definition (Fields, 1996, Prothro & Grigg, 1960). There are albeit a number of typical definitions proposed by different scholars all sharing some features which are regarded as the cornerstones of democracy. They include equality, freedom, majority rule/minority rights.

Equality: based on this principle, in a democratic society all citizens are equal before the law and they all enjoy equal rights, opportunities, treatment, and access to power and justice (Post, 2011, Diamond & Morlino, 2005). In addition, such society offers its people equal participation in the process of self-government in the forms of voting and communication within public discourse.

Freedom: this principle mainly entails three types of freedom: political, civil, and social; and includes such fundamental human rights as freedom of individual and political expression, freedom of thought and information, freedom of religion, freedom of speech, freedom of press, and freedom of association in public and private (Post, 2011, Diamond & Morlino, 2005).

Majority rule/ minority rights: as noted above, the term democracy means rule by people and, in practice, people are generally expressed through its majority; hence, majority rule forms the essence of the concept of democracy (Plattner, 2010). Yet, this principle goes on to include minority rights as well. The reason is that granting supreme power solely to majority rule would simply lead to oppressing and

tyrannizing the minority. Therefore, in this principle, majority rule is paired with minority rights in order to guarantee these rights.

In addition to these major principles, there are some core values which are considered to be essential particularly in the more modern approaches to democracy (Kupchan, 2012). **Pluralism** as the most important of these values denotes the fact that since democracy grants supreme authority to people and since people is represented by a totality of groups (social, ethnic, territorial, religious, etc.), diversity becomes an inevitable component of any democracy (Berndtson, 1999). **Tolerance** is another core value which is closely interwoven with the notion of pluralism in that in the existence of diversity of public interests and forms of their expression a system would not be able to work without the essential presence of tolerance and compromise.

As mentioned earlier, the political, religious, and economic circumstances in the 20th century ignited the spread of waves of democracy in diverse contexts with diverse applications. Nonetheless, its deep penetration in such a wide range of aspects of today's life has earned it a barrage of criticism calling it the "reigning dogma of our time" (Farrelly, 2011). Most of the critical views of democracy center around the belief that, to use Plato's words, "it is full of variety and disorder" and that it eventually leads to chaos (Hanford, 1916, p. 106).

3. Democracy in ELT

3.1. Equality

This primary democratic feature can be investigated in the context of English use and education in two main trajectories. In the first one, English is compared to indigenous languages in countries where English is a second or foreign language. In a democratic situation, while each of the co-existing languages is supposed to fulfill its own unique function in the given multilingual context, each one is expected to have a status equal to the others' and to be equally at disposal of the people. Nevertheless, a scrutiny of this issue reveals that English has seemingly taken on a hegemonizing role in multilingual contexts and created a linguistic hierarchy. This point is best elaborated on by Phillipson's *Linguistic Imperialism* published in 1992 (Bolton, 2004). In his book, Phillipson first discussed the systematic inequality between the English-speaking countries in the center and those on the periphery, which is evident in the political and economic hegemony of the west. He then went on to give his definition of *English Linguistic Imperialism:* "A working definition of English linguistic imperialism is that the dominance of English is asserted and maintained by the establishment and continuous reconstitution of structural and cultural inequalities between English and other languages" (p. 47, cited in Bolton, 2004, p. 348).

Based on linguistic imperialism, English has a hegemonizing role in the world today and that the powerful west imposes Standard English as the norm through which it exerts its domination (Davis, 2004). They also claim that the expansion of English, by decreasing local languages' central roles and functions, marginalizes them and will ultimately even lead to the demise of some of such indigenous languages. Supporting this notion, Rahman (1999) argued that English necessarily decultures people by replacing their most local cultural norms with Anglo-cultural ones (cited in Kirkpatrick, 2007).

In the same vein, Prodromou (1997) implicitly asserted the lack of democracy in English education context by stating that teaching and learning of English today brings about American hegemony and domination, explaining that, "English is both an instrument for furthering American interests and in turn is furthered by the successful promotion of those interests" (cited in Timmis, 2007, p. 129).

In the second trajectory, the so-called Standard English is compared to other varieties of English. Here again, in an ideal democratic situation, different varieties of English, including Standard English, are expected to enjoy equal power and status whereas the status quo seems to be otherwise.

A quick overview of English studies worldwide sheds lights on the persistent dominance of the traditional view, i.e. Standard English ideology, in the context of English use and education which has awarded the American and British English the authority to provide and prescribe the norms of usage in all international English using contexts (Bolton, 2004). The so-called Standard English is considered the only appropriate model used in teaching and the idea of using a localized variety of English as the model for teaching in the countries where they have one seems to be so abnormal that some linguistics including Prator (1968) even called it a *heresy,* arguing that such breaking away with the conformity to the native model would necessarily lead to a state of mutual unintelligibility (cited in Berns, 2006). Others like Quirk (2001) stated that it was the duty of linguists like him to make sure that a homogenous standard English is the only variety of English taught in international context (cited in B. B. Kachru, 2006).

The main functions of this ideology, which are virtually taken for granted nowadays, include aiming second language acquisition at the goal of *ambilingualism*, regarding *fossilization* as the final

fate of second language learners, and recognizing the varieties spoken by non-natives as *interlanguage* (Bhatt, 2001). Clearly enough, such prescription of a standard variety is in fact bestowing prestige to just one variety at the expense of suppressing all the others (Milroy & Milroy, 1999 cited in Davis, 2006). Nonetheless, Standard English keeps on acting as a benchmark against which all other varieties should be measured and a norm to which they all should conform. This explains the present circumstances in testing. The proficiency of English learners throughout the world has for long been assessed through centripetal-valued tests such as TOFEL, IELTS, etc. which imply an irrelevant *native* standard reference point against which the users of all other varieties of English should be tested (Jenkins, 2003).

This conformity is of particular importance to the supporters of Standard English ideology since, as we shall see later, they believe it would limit the offshoots and deviations from the norm and would consequently prevent fossilization of incorrect and inappropriate forms (Prator, 1968 cited in Berns, 2006). They even took a step further in defining Standard English as the usage of the educated –excluding regional dialects which he considered as uneducated speech –which has as its basis a common core of English (Quirk, Greenbaum, Leech, & Svartvik, 1972, cited in Davis, 2006). This common core contains the linguistic features that are present in and shared by all the varieties of English. Not surprisingly, British and American English are identified as the two manifestations of this Standard English. Davis (2006), very cleverly, referred to the discrepancy between this idea of a common core and the prescription of British and American English as the replacement of "One for all and all for one", by "All animals are equal, but some animals are more equal than others" (p. 514).

In the last four decades, however, along with the ignition of waves of democracy in the context of language education, there have been attempts to challenge the standard language ideology and replace it with the liberation linguistics ideology with the aim of rejecting Standard English as the norm and empowering the new varieties of English as well as their speakers (Bhatt, 2001; Bolton, 2004).

To this end, Kachru and Nelson (1996) suggested that a descriptive approach be applied to the world Englishes. It follows that, rather than dealing with the prescriptive rules of language usage and the way language should and should not be used, and considering diversities as incorrect ways, our approach to the present situation of English language should involve descriptive characterizations of language use and "the way language actually works" (p. 77). In short, it is time to replace the constant prescription of Standard English by its custodians with the descriptions of different varieties of English used around the world.

Furthermore, Halliday (2006) laid stress on the fact that the standard variety has "no intrinsic value" and that it is "just another dialect, but one that happened to be wearing a fancy uniform" (p. 350). Berns (2006) strongly questioned the validity of centripetal-valued tests which use Standard English as their yardstick. He argued that each setting has its own cultural and social values and since local norms are shaped in accordance with these values, each setting calls for its own nativized variety of English, the one that corresponds to its set of values and norms. As a result, it seems quite absurd to think that Standard English –which culturally represents the Judeo-Christian tradition –can be used cross-culturally and in different international settings without impeding successful communication and intelligibility.

Widdowson (2003) argued that the main importance of Standard English lies in a belief in its guaranteeing effective communication and standards of intelligibility. In his view, Standard English, which is usually defined in reference to its grammar and lexis, is primarily a written variety sanctioned for institutional use. He went on to explain that while being spoken with different accents, Standard English has a distinctive graphology and it is precisely because, as mentioned before, it is a written variety which has been designed for institutional purposes. Put simply, "good spelling represents conformity to convention and so serves to maintain institutional stability" (p. 38). Furthermore, he believed that Standard English is a *shibboleth*, marking the right sort of person. He elaborated on this issue arguing that while grammatical conformity, due to the in-built redundancy of language, is not crucial for effective communication, Standard English places much importance on it (rather than on lexis). The reason, according to Widdowson, is that grammar "is so often redundant in communicative transactions that it takes on another significance, namely that of expressing social identity" and so adopts the role of a distinguisher between members of the community and the outsiders (p. 39). The startling fact here is the existence of an implicit obligation of the membership of this community. In other words, you have just two choices: either you become a member of this community and enjoy its privileges including access to the institutions under its control, or, by persisting in your non-standard ways, you are marginalized and your ungrammatical speech and bad-spelt writing are assigned less importance and are not taken seriously.

Widdowson (2003), finally, striped the attitudinal goodness totally away from Standard English by noting the double standards concerning the issue. He elaborated on it explaining that the stability implied by Standard English is in contrast with the dynamic nature of language and that while Standard English calls for conformity, "proficiency only comes with nonconformity" (p. 42). So you are proficient in English to the extent that you do not conform to Standard English and do not submit to what it dictates to you. In other words, mastery means taking the possession of the language, bending it to your advantage, developing innovations in it, and being able to speak your mind rather than speaking the language.

3.2. Freedom

It should be noted that in the investigation of the second feature of democracy, i.e. freedom, in the context of English use and education, our concern is freedom of expression by which we mean the extent to which one is free to express themselves through a variety of English other than the so-called standard one. So, we had better set out with the question, how much have the non-standard or nativised Englishes actually been used in the body of English writings?

An examination of the growing bodies of literature in English reveals that most of the writers of such literary works are bi- or multilingual and "do not belong culturally to ... the Judeo-Christian tradition" (Kachru & Nelson, 1996, p. 84). Not surprisingly, far from representing traditional canons, the English such writers use is in fact a medium for indigenous expression and is, thus, "de-Anglicised", to use the term of Mesthrie and Bhatt (2008, p. 149). This is, of course, achieved through a range of different techniques and strategies including the use of local similes and metaphors, the translation of idioms and proverbs, the transfer of rhetorical devices, and the use of culture-specific speech styles (Kachru, 1986, cited in Mesthrie & Bhatt, 2008).

The reason, according to Thumboo (2006), could be that the multilingual and multicultural contexts inspire bi- or multilingual creative writers to reflect the same hybridity in their creative language through making use of some of the strategies and other resources present in their native language and literature. What is more, such non-English creative writers are even believed to be privileged over the English monolingual ones due to their "access to unique and specific linguistic configurations that are different from those of monolinguals in either language in their repertoires" (Yamuna Kachru, 2006, p. 375). Kachru and Nelson (1996), also, argued that the bilingual creativity of such writers reflect special linguistic, social and cultural features including mixing of codes, and nativization and acculturation of English in various other cultural settings. The interesting point about these features is that, while marking the text as something other than British or American, they "do not interfere substantially with transmission of message" (p. 76). The reason, of course, is the writers' making the context and action comprehensible to readers through using different strategies. Furthermore, old canons as reference points in the interpretation of such creative writings have been recently replaced by new ones since they were no more capable of accounting for the great cultural and social variations of these literatures.

Referring to the same point, Bolton (2006) asserted that today English has turned into *multi-canonical English* due to its nativization in un-English settings and, consequently, its presenting canons quite different from those of the Judeo-Christian tradition and the European cultural heritage. Moreover, Llamzon (1983) made use of a celebrated metaphor in which a new variety of English is likened to a transplant tree, and extended this metaphor by considering the creative writing and literary masterpieces in that variety as its fruits (cited in Bolton, 2004). This way, he most interestingly demonstrated that as fruits are a sign of maturity and vigor of a tree, creative writing and a local literature in English signal the achieved legitimacy and power of that variety of English.

All in all, considering the greatness of body of literature in nativized varieties, the context of English education seems to be more democratic regarding freedom of expression; however, a tricky question remains to be answered: “How much are these *non-standard* English writings taken seriously and given credit in more official contexts?” The context of publication can best shed light on this issue. Academic journals, particularly ISI ones, as an illuminating example, expect perfect conformity to Standard English and the traditional canons of Judo-Christian tradition. In fact, the one thing not cared for in such journals is multi-canonical English. Accordingly, not only are the articles written with slightest traces of local color and nativised dynamicity not appreciated by these journals, but they are not even taken seriously and are simply dismissed as “poorly written”. Still the striking fact is that such perfect conformity is sometimes demanded in non-native English journals in ESL and EFL countries, too.

3.3. Majority rule

To reiterate, majority rule, which forms the essence of democracy, denotes giving the authority

and ruling power to the majority while guaranteeing the rights of minorities at the same time. In order to investigate this democratic feature in the present context of English use and education, first we need to determine who constitute the majority and minority and what the ruling power refers to.

Since the time English took on the role of the language for international communication, new varieties of English started to spring up in different parts of the world as a natural result of its global spread. The remarkable point is that the speakers of these new Englishes who use English to communicate with fellow non-native speakers far outnumber its native speakers (Widdowson, 2003). Accordingly, the situations in which English is used as a lingua franca among its L2 speakers are much more common than the ones in which English is used between its L1 and L2 speakers (Jenkins, 2003). Therefore, no one can deny the fact that the majority of English speakers today are those with an L1 other than English.

As evident as this fact is, still the supreme power which is supposed to be the majority's is unquestionably given to the native speakers in the form of the authority to provide and prescribe the norms of usage in all international English using contexts. So, native speakers, who in fact constitute the minority, are believed to be the repository and guardian of the true language, as well as the standard setter (Davis, 2004). The manifestation of this fact is evident in virtually all English course books. According to Cook (2008), course books foster unfavorable images of second language users. That is to say, rather than representing positive images of successful L2 users that students could use as models, almost all these books show of L2 users is either ignorant tourists and foreigners, or students struggling to learn the language. And, it is not hard to guess the fascinating photos of which famous people they proliferate; monolingual ones, of course. Accordingly, "students never see successful L2 users in action and so have no role model to emulate other than the native speaker, which they will very rarely match" (p. 143).

This undemocratic situation has been, in the recent decades, frequently criticized by some scholars including Widdowson (2003) who strongly denied the native speakers' claim of the ownership of English language and their right to determine how it should be spoken around the world. In his book *defining issues in English language teaching*, he first referred to the common assumption that the native speakers of English are those living in England, where the language originated and that the very fact that they are native speakers, naturally gives them the authority to promote Standard English. Furthermore, Standard English is in fact the real and proper English whose privilege over other varieties lies in the fact that it guarantees clear communication and standards of intelligibility. While, based on this assumption, all those who are born to the language are considered to be native speakers and thus should have the authority to maintain Standard English, it is not actually the case; since the majority of English people, who speak some non-standard variety, are themselves instructed in Standard English at school. Based on this argument, he then concluded that the custodians of Standard English are not even natural native speakers but they are a minority of people, a particular self-elected subset of educated native speakers who have the power to impose this standard variety.

And as for the ownership of English, Widdowson (2003) did not deny the dual character of languages of every variety, i.e. performing communicative as well as communal functions, but asserted that no single community and culture has a right to claim the ownership of English due to the simple fact that it is an international language and thus, it transcends the traditional communal and cultural boundaries. He went on to explain that "the very fact that English is an international language means that no nation can have custody over it" (p. 43).

In the same vein, Jenkins (2003) argued that since English is used for international communication and is, thus, used among speakers from different nationalities, it simply makes no sense to talk of its non-native speakers. Representing this view, she listed some arguments against the use of the term *native* and *non-native* speaker of English, including: its assuming monolingualism to be the world's norm while the majority of people are bi- or multilingual, its disregarding the lingua franca function of English, its being offensive for the proficient users of English to be labeled as *non-native*, and more importantly, by proposing a simplistic view of what constitutes error in English language use, its bringing about problems with the international English testing since it implies an irrelevant *native* standard reference point against which the users of all other varieties of English should be tested.

Cook (1995), as well, made attempts to empower non-native speakers by proposing his *multi-competence* model (cited in Brown, 2007). According to the main tenet of this model, L2 users are quite different from monolingual native speakers and, thus, should not be compared to them; but should be considered in their own right. The main differences, as Cook put it, are as follows:

1. L2 users' knowledge of second language differs from that of native speakers. So, L2 users should not aim at the goal of passing for natives and should not, in turn, be demotivated on their failure in it.
2. L2 users' knowledge of their first language also differs from that of monolingual native speakers. It corresponds to the same familiar fact that L2 has always some effect on L1.
3. "L2 users think in different ways to the monolinguals" (p.196). Put simply, L2 user's mind is much more flexible than that of native speaker since they have access simultaneously to two competences rather than one; so, they have higher language and culture awareness.

Thus, "learning another language changes people in many ways… affecting not only the two languages but also the person as a whole" (p. 196). In short, this model regards L2 users superior to monolingual native speakers due to the merits mentioned above, and challenges the common assumption that the monolingual native speaker is the norm and a reference point against which L2 users should be measured.

3.4. Pluralism and tolerance

Clearly enough, a necessary condition for a democratic pluralism is the existence of a variety of discourses rather than just one, which inevitably would lead to dictatorship. This is not a sufficient condition, though. What makes it sufficient, as well, is a situation in which all the voices can be heard and that is why such pluralism calls for tolerance and compromise.

English as the language of international communication has for long been, and still is, spreading all over the world, and since any transmission of language brings about transformation (Widdowson, 2003), this spread has resulted in the existence of different varieties of English, each as a consequence of English contact with a certain language, culture and people. This undeniable hybridity fulfills the necessary condition of a democratic context of English use and education. As for the sufficient condition, these new Englishes need to be legitimate. However, the prevalent traditional view, that is, Standard English ideology, strongly denies the legitimacy of other varieties of English and even calls them the offshoots and deviations from the norm, to use Prator's words (1968, cited in Berns, 2006). The dominance of such traditional view can be witnessed in most publication including English textbooks and journals in which different varieties of English do not still seem to have gained legitimatcy. In some textbooks, for instance, there are some random exposures to new Englishes, but such exposures are so infrequent and limited that by no means represent the actual hybridity in the present context of English use.

Again, such undemocratic situation could not escape criticisms. It was most severely criticized by Kachru's theory of World Englishes (1982). *World Englishes* is defined as a theory used to "legitimate the Englishes spoken in the British non-white colonies" and the ideology behind it denies a special status for the native speakers of metropolitan English varieties and complains about these native speakers' discriminations against users of world Englishes (Davis, 2004, p. 442).The underlying philosophy of Kachruvian approach argues for the "importance of inclusivity and pluricentricity in approaches to linguistics of new varieties of English" and attempt to de-marginalize and legitimize the new Englishes (Bolton, 2004, p. 367). Also, according to Bhatt (2001), World Englishes paradigm discusses the global spread of English and the large number of functions it has taken on with increasing range and depth in diverse sociolinguistic settings around the world. This paradigm particularly emphasizes multilingualism, multicultural identities, multiple norms of use, and bilinguals' creativity. Moreover, having its theoretical and philosophical foundations in liberation linguistics, it severely problematizes the sacred cows of the traditional theoretical and applied linguistics including interference, interlanguage, native speaker, speech community, ideal speaker-hearer, Standard English, and traditional English canon.

This tension between the prescription of a world standard English and the legitimacy and autonomy of world Englishes calls to mind the double-voicedness of Bakhtin's (1994) centripetal and centrifugal forces. Centripetal forces, as a modernist feature, call for centralizing, homogenizing and convergence, which in the present context, contribute to the conformity to an authoritative and prescriptive standard variety which is believed to be the best. On the other hand, centrifugal forces, as a postmodernist feature, involve decentralizing and divergence and thus appreciate the diverse features and functions of English worldwide.

3.5. Criticism

As stated earlier, the main criticism leveled at democracy was a belief in its involving too much disorder and variety and its inevitably leading to chaos. Just the same concern has been expressed about a democratic context of English use and education.

It follows that, since different varieties of English have developed in different parts of the

world, naturally, these new Englishes are, with varying degrees, different from the ancestral one. Some people, especially the promoters of Standard English, have recently started to express their fear about this increasing diversity and movement toward divergence since they believe that if the center, i.e. Standard English, doesn't hold, "things fall apart, mere anarchy is loosed upon the world and we are back to Babel" (Widdowson, 2003, p. 36). In other words, English in that case divides up into mutually unintelligible varieties and therefore, loses its value as the international language. Albeit, regarding such fear quite unfounded, the adherents of World Englishes and liberation linguistics have not left it unanswered.

Language, according to Kirkpatrick (2007), has two important functions: communication and identity. He explained the link between function and variety through an "identity-communication" continuum. This continuum suggests that the identity function is highlighted when fewer people are involved in the act of communication and with closer social distance between them. Broad and informal varieties as well as job- and class-specific registers best express this function. In contrast, the more people who are involved in the act of communication, and the greater the social distance between them, the more the function of the language they use turns toward communication end. In other words, communication function assigns much importance to intelligibility and is usually associated with standard and educated varieties. Based on this continuum, Kirkpatrick also presented his view regarding the future of New Englishes. He argued that the mutual intelligibility of these varieties depends on the motivations of the speakers, i.e., their deliberate emphasis on, and the need they feel for, either communication or identity function, as well as on the listener's familiarity with the variety. Put simply, "all speakers of English are capable of being intelligible (or unintelligible) to speakers of other varieties if they are so motivated" (p. 35). He, further, argued that people highlight the identity function in communication within their speech community and communication function in communication between speech communities. He concluded that mutual intelligibility is guaranteed by the need for people to be able to communicate beyond their own speech community.

Referring to the same conflict between mutual intelligibility and group identity, Jenkins (2003) argued that in order for English to be able to function as the world's lingua franca, its different varieties need to be intelligible to each other and that "the main obstacle to such mutual intelligibility is identity" (p. 36).

Furthermore, Crystal (2003) argued that, as the case of mutual intelligibility does not happen for different dialects of the same language, it is very unlikely to happen for different varieties of English. Considering new Englishes as the international accents and dialects of English, he admitted that at times the speaker of a certain dialect might be unintelligible to the speakers of other dialects, i.e., usually when a need for identity is highlighted at the expense of a need for intelligibility. But this problem can resolve simply by the speaker's slowing down or reducing on difficulties over isolated lexical items. This way he illustrated that although "the need for intelligibility and the need for identity often pull people –and countries –in opposing directions", it is still possible for the two to co-exist happily (p. 127). He, also, took the bold step of assuring that even if the current spread of English and development of new Englishes resulted in their becoming mutually unintelligible, it would be nothing fatal since, in that case, a new World Standard Spoken English (WSSE) would arise and replace the myriad of Englishes.

Widdowson (2003) adopted a slightly different view by considering new varieties of English as *autonomous* languages which will ultimately reach the point of mutual unintelligibility. Distinguishing between language distribution, which involves conformity and adoption, and language spread, which involves adaptation and non-conformity, he explained that English is not so much distributed, as it is spread. He went on to argue that the varieties of English used for specific purposes which are considered as registers have already become mutually unintelligible, at least as far as lexis is concerned, with arising no complain or fear. He, then, called for the same tolerance to be extended to the same situation with local varieties, considered as dialects.

Similarly, Smith and Nelson (2006) regarded mutual intelligibility as a quite natural consequence of the global spread of any language and stated that it is not something about to happen in the future, but it has already happened and is clearly evidenced in the existence of English speakers in some parts of the world who have been unintelligible to other English speakers in other parts since about two centuries ago. They also asserted that there is no need for every English user to be intelligible to all the other English users and that it suffices for them to be intelligible only to those they wish to communicate with.

Finally, Smith's (1992) study deserves great attention here since it shed important light on the issues concerning intelligibility and native speakers (cited in Smith & Nelson, 2006). In this study, he first distinguished between three levels of

understanding, or intelligibility in a broad sense:
1. Intelligibility: recognition of the word/utterance.
2. Comprehensibility: assigning referential meaning to the word/utterance; locutionary force.
3. Interpretability: apprehension of the meaning behind the word/utterance; illocutionary force.

A quite noteworthy facet of this distinction is that, while in ESL and EFL teaching and learning the greatest stress is placed on the first two levels, the most important requirement of a successful communication is interpretability which is, in turn, achieved through gaining acceptable amount of situational, social and cultural awareness. Moreover, based on the startling result of this investigation, "native speakers (from Britain and the United States) were not found to be the most easily understood, nor were they, as subjects, the best able to understand the different varieties of English" (cited in Smith & Nelson, 2006, p. 441). This study had a promising result as well, namely, the one claiming that developing some familiarity with different varieties of English can easily solve the problem of their mutual unintelligibility.

4. Democracy in Iran's ELT

So far, it was tried to provide an overview of the overall context of English use and education with regard to democratic principles and values, as illustrated in Table 1. In this section, a more specific approach is adopted in the examination of the presence of democracy in Iran's formal and informal ELT.

Table 1. Democratic features and their manifestations in ELT.

Democratic feature	Manifestation in ELT	Aim
Equality	Liberation linguistics	Rejection of Standard English as the norm
Freedom of expression	Creative/bilingual's writing	Turning English into multi-canonical English
Majority rule	Death of native speaker	Rejection of native speaker's superiority and ownership of language
Pluralism & tolerance	World Englishes	Legitimizing new varieties of English

The formal educational system in Iran is somehow traditional and centralized: the government's central policies determine the "whats" and "hows" of teaching in the whole country and dictate them to all schools and teachers demanding complete conformity (Pishghadam & Mirzaee, 2008). Pragmatism, as a democratic notion, which empowers teachers by giving them the freedom to make local decisions and be more plausible and autonomous has no place in such a system. Also, students have no active participation and interaction in the process of making meaning; instead, during their non-constructivist education, they are just passive recipients of knowledge whose individual differences are ignored.

As for the informal context of ELT, far from liberation linguistics' view point, there is strong dominant belief in Standard English as the best variety of English, the norm, and the most appropriate model in teaching and yardstick in testing (Pishghadam & sabouri, 2011a, Pishghadam & saboori, 2011b). In fact, the Standard English ideology has so much dominance and penetration in this context that even if they were given the chance to use another model which might be more practical and useful in their local context, neither teachers nor learners would ever take it. In the same vein, New Englishes are generally regarded as incorrect, inferior, unimportant varieties; that is why the actual pluralism in the international context of English use is not indicated in the classrooms and, not surprisingly, most teachers and learners have negative attitudes towards new Englishes (lack of tolerance). In addition, native speakers are considered to be of higher status and superior to nonnative, bi- or multilinguals. Also, passing for native speakers is the ultimate goal and indicator of highest level of proficiency for the learners (Pishghadam & sabouri, 2011a, Pishghadam & saboori, 2011b).

Finally, for most local journals and publishers, perfect conformity to Standard English is a pre-requisite for publishing English articles and books. English textbooks are representative of the Standard English ideology and superiority of native speaker and do not include adequate exposure of different varieties illustrative of the hybridity in international English use.

5. Concluding Remarks

In a macro scope, it seems that democratic movements in ELT, which started to spread throughout the world about three decades ago, have ever since severely problematized the sacred cows, as Bhatt (2001) put it, of the traditional linguistics including native speaker, Standard English, and traditional English canon. Unfortunately, however, such movements have not developed in Iran's ELT strongly enough to challenge and set it free from the

prevalent traditional views yet.

So, in an attempt to democratize the context of Iran's ELT, there should be a paradigm shift in the educational system: it needs to be decentralized. It also needs to apply more constructivist collaborative methods of teaching to empower teachers and move toward meaningful learning to actively involve learners in the process of learning. Furthermore, rather than teaching and expecting complete conformity to American and British varieties, teachers are required to teach a negotiable variety of English and some strategies for negotiating understanding with those who cannot speak English well and those who speak a different variety. Such strategies are what Graddol (1998) and Willis (1999) called for in ELT (cited in Timmis, 2007). Also, considering the importance of "developing multilingual competence for transnational relationships", English teachers should adopt a multilingual and polyliterate orientation to writing in their classes (Canagarajah, 2008, p. 586). To achieve this goal, teachers should encourage their bilingual students' creative writing by teaching them the proper strategies such as code mixing and code meshing and that it would lead to the gradual pluralization of academic writing.

Moreover, test developers are required to change their benchmark, i.e. native speaker against which all the English speaking people are measured. They are, instead, expected to take the lingua franca status of English into account and develop comprehensive tests in accordance with its pluricentricity.

Finally, material developers are required to adopt a realistic view in taking proper account of pluricentral status of English in the textbooks through aiming for an intercultural communicative competence rather than a monolithic representation of native speaker culture. To this end, teamwork needs to be conducted in the preparation of the textbooks (or maybe localized ones) whereby the speakers of different varieties can contribute by representing their accents, creative writings, and pragmatic norms.

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