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Iraqi Journal of Medical Sciences

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An Overview on the Current and Futuristic Rapid Diagnostic Assays

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Abstract

There is now a huge need to innovate, design and use novel and practicable rapid diagnostic assays (RDA) to combat the grave challenge of emerging life-threatening and multiple drug resistant bacteria (MDR) bacterial pathogens. The conventional methods of diagnosing bacterial pathogens are well enough to give good specificity. However, the sensitivity of assays in terms of short time is still not well settled. The increased population of the world along with availability of global transport means resulted in the emergence of critical health situations that the current time-consuming means of diagnosis cannot cope with the timely management of diseases caused by serious human pathogens.

Introduction

The limitations of the conventional procedures for diagnosing bacteria are various depending on the type of the assay. For cultures, they are labor intensive, need special media, prolonged period of time to culture, some organisms are uncultivable on artificial media, and have potential health hazards. Other assays are considered semi-slow such as antigen detection and serology assays. For antigen detection assays, negative tests require confirmation and usually effected by poor specimen collection. Serological assays are unhelpful during early stage of infection and are not quite useful in immunocompromised patients [(1)].

Therefore, rapid diagnostic assays (RDA) have become a genuine need. The typical qualities of RDA are high sensitivity and specificity, high accuracy compared to gold standard, simple to perform, results in minutes to 1-2 hours, and cost effective. RDA without cultures are especially needed for microorganisms that grow poorly, cannot be cultured e.g. T. B., need for speed, there is critical case management (life threatening), and there is therapy failure (e.g Antibiotics resistance) [(2)].

Most pathogenic microorganisms need RDA for proper and rapid diagnosis. However, there are some pathogens that need RDA more than others. Those pathogens are usually fastidious bacteria e.g. C. trachomatis, Gonococci, pertussis, TB, M. genitalium, T. whipplei, C. burnetti, and B. henselae. Moreover, rapid Bacterial diagnosis is essential in life-threatening systemic infections e.g. N. meningitidis, S. pneumoniae, and H. influenza. Also, MDR bacteria are in urgent need for rapid diagnosis as most their infections need prompt intervention such as MRSA, multi-drug resistant TB [(3)].

Methods of RDA

The main currently used or developed methods of RDA are antigen detection assays, antibody detection (serology) assays, molecular detection

assays, and bacteriophage-based RDA. For antigen detection assays, they detect bacterial, viral or parasite antigen (surface antigen, soluble antigen) or toxin in biological fluids (CSF, blood, urine). The primary techniques are direct agglutination: slides, cards, latex agglutination: slides, cards, Immunochromatography: dipsticks and latex agglutination test. For serological assays, the main techniques are direct agglutination (antigen, latex + antigen), agglutination inhibition, immunodot, and immunochromatography. For molecular detection assays, the conventional and real-time PCR, hybridization probes, and DNA chip microarrays. PCR assays are most widely used because of their high sensitivity and specificity [(2)].

The advantages of molecular RDA are numerous including rapid, accurate, specific, sensitive, have powerful amplification potential useful for detecting traces of target pathogens nucleic acids. Moreover, molecular RDA does not require viable microorganisms and quantitative assessments are highly accurate [(4)]. One of the currently focused techniques using the molecular RDA is using microarrays in blood pathogens or those pathogens detected by cultures. Such microarrays are all-in-one kits able to detect specifically certain pathogens along with identifying their genotypes, antibiotic-resistant genes, virulent genetic makeup, mutated pathogens, and also able to quantitate the microbial load specifically. These all-in-one kits are capable to perform all these tasks in several hours rather than days or weeks as used to be before by using the conventional methods. However, there are some disadvantages for using molecular RDA including cost, false positives caused by amplification of contaminants, only sample from normally sterile sites should be considered for broad-range PCR, specimen is required to be refrigerated or stored in alcohol before processing, need training and highly qualified personnel, no antimicrobial sensitivity is available, and cannot differentiate viable versus dead microbes [(5)].

Phage-based RDT for Bacteria

Phages or bacteriophages = bacterial viruses. Phages are initially discovered in 1915 by Twort and independetly in 1917 by d'Herelle. It is estimated that every 2 days 50% of the world's population is destroyed by bacteriophages. Unfortunately, during Antibiotic era, phages were considered "non-conventional" medicine in spite of the continued use of phages in rapid diagnosis and in therapy in the former USSR: Eliave Institute in Tbilisi, Georgia (http://www.evergreen.edu/phage/home.htm).

There are numerous prospects for the Phage-based RDA (PBRDA) including phage-based rapid (1-60 minutes) detection methods were developed recently as both qualitative and quantitative assessments for several human pathogens such as *E. coli*, *E. coli O157:H7*, Methicillin-resistant staphylococcus aureus (MRSA), and other MDR bacteria. PBRDA are rapid, highly specific (up to species and strain) and unlike PCR, PBRDA can differentiate between viable and dead microbes. PBRDA are extremely sensitive, amplification effect is capable by using phage-based amplification means by which can detect up to 1-10 bacterial cells per sample. Also PBRA are good quantitative assays and there is possibility to develop phage lysins from used phages; these phage lysins are able to perform extra rapid assays, within seconds to 1 minute [(6-8)].

Phage lysins are conceived to be the ultimate goal for human hopes for a completely successful and resistance hassle-free bacterial therapy and extra-rapid diagnostic assays for human and animal pathogens. Phage lysins are cationic enzymes that have been designated using various names including phage-lysozyme, endolysin, lysozyme, lysin, phage lysine which are able to hydrolyze specific bonds in the murrain or peptidoglycan layer of the cell wall. Phage lysins are known to kill the target bacteria in few seconds. The interesting thing that their host range is wider than the corresponding phage particles which sometimes host range of phage lysins reach to the species level [(7,8)].

One of the PBRDA invented by us is the Lumulus amoebocyte lysate (LAL) phage-based rapid detection assay for pathogenic *E. coli*. LAL assay is able to detect traces of endotoxin by certain enzymatic reaction resulting in visible coloration measured by any spectrophotometer. It is traditionally used to measure any endotoxin contamination in food and pharmaceutical preparations. Specific designed phages lyse target Gram negative bacteria can lead to specific lysis which in turn leads to liberation of endotoxin. Hence, combing the use of specific phages with LAL resulted in inventing novel highly specific phage-based LAL rapid diagnostic assay for *E. coli*. Actually this novel approach can be simulated for any bacterial pathogen. The total assay time is 40-50 minutes only. It is specific assay at strain/ species level and can use a portable handy spectrophotometer. This assay proved to be successful for Gram negative bacteria (E. coli, other enterobacteriacae) and is a revolutionary step for rapid diagnosis at strain level [6-8].

References

1. Teles FS. Biosensors and rapid diagnostic tests on the frontier between analytical and clinical chemistry for biomolecular diagnosis of dengue disease: a review. Anal Chim Acta. 2010; 687: 28-42.
2. Tenover FC. Potential impact of rapid diagnostic tests on improving antimicrobial use. Ann N Y Acad Sci. 2010; 1213: 70-80.
3. Kim J, Yoon MY. Recent advances in rapid and ultrasensitive biosensors for infectious agents: lesson from Bacillus anthracis diagnostic sensors. Analyst. 2011; 135: 1182-1190.
4. Petrozzino JJ, Smith C, Atkinson MJ. Rapid diagnostic testing for seasonal influenza: an evidence-based review and comparison with unaided clinical diagnosis. J Emerg Med. 2009; 39: 476-490 e471.
5. Boonham N, Tomlinson J, Mumford R. Microarrays for rapid identification of plant viruses. Annu Rev Phytopathol 2007; 45: 307-328.
6. Jassim SA, Abdulamir AS, Abu Bakar F. Novel phage-based bio-processing of pathogenic Escherichia coli and its biofilms. World J Microbiol Biotechnol. 2012; 28: 47-60.
7. Methods for bacteriophage design: Patent no. WO2010064044, UK Application No. 0822068.3.International Application No. PCT/GB2009/051641. Filing date: 3rd December 2009. http://www.wipo.int/patentscope/search/en/WO2010064044
8. RAPID DETECTION OF BACTERIA. Patent no. WO2011098820, UK Application No. 1002295.2. Filing date: 11th February 2010. International Application No. PCT/GB2011/050249. Filing date: 11th February 2011. http://www.wipo.int/patentscope/search/en/detail.jsf?docId=WO2011098820&recNum=203&maxRec=7348&office=&prevFilter=&sortOption=&queryString=%28FP%2Fassay%29+&tab=PCT+Biblio

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Lectin Histochemistry of Tracheo-Esophageal Region in Chick Embryos

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Abstract

Background Glycosylation is an important modification involved during embryonic development. Lectins are specific carbohydrate-binding proteins; they can be employed as specific probes to localize defined monosaccharide and oligosaccharides on cell surface and on cytoplasmic structures, and in extracellular matrix.

Objectives The lectins (SBA, PNA, WGA, SWGA, UEA-I) binding were used as a sensitive, stable, and easy tool that can provide an extraordinarily sensitive detection for changes glycosylation and carbohydrate expression that may occur during embryogenesis and development of trachea-esophageal region.

Methods Fertilized chick eggs were incubated at 38 °C, embryos were fixed with Bouin's solution. Sections were treated with fluoresce ineisothiocyanate (FITC) labeledlectins.

Results The histochemical study during the 2nd and 3rd days of development revealed variable tempo-spatial variability of lectin bindings to the mesenchymal tissues and other embryonic structures at the trachea-esophageal region.

Conclusions The lectin bindings could be an indicator for the glycoconjucate changes that play an essential role in developmental phenomenon of trachea-esophageal morphogenesis by marking cellular differentiation, cellular migration, and cellular interactions.

Key words Trachea, esophagus, chick, embryo, lectin, histochemistry.

Introduction

The digestive and respiratory systems have different physiological functions and are generally considered to be and studied as two independent systems. Although at birth they are separated, they were derived from a common and transiently developed structure, the foregut, which is the anterior part of the gastrointestinal (GI) tract. The development of the foregut is not well documented in comparison to that of other parts of the digestive system [(1)].

Glycosylation is an important post-translational modification of proteins involved in cell-cell interaction during embryonic development. Specific carbohydrates moieties of the oligosaccharide side chains of the glycoconjugates are among the factors involved in these interactions and the developmental morphogenetic processes are correlated with changes in the sugar content of glycoconjugates located on cell surfaces or in extracellular matrix [(2)]. Moreover glycocojugates that are present in the extracellular matrix are involved in

regulatory cellular migration and play critical roles during early embryonic development [3].

Lectins are specific carbohydrate-binding proteins of non-immune origin, which agglutinates cells and/or precipitates polysaccharides or glycoconjugates", and does not have an enzymatic function. Lectins selectively and specifically bind non-covalently to carbohydrate residues without modifying them. An important property of a lectin is the ability to bind to carbohydrates [4,5]. It is for this reason that they can be employed as specific probes to localize defined monosaccharides and oligosaccharides on cell surface, on cytoplasmic and nuclear structures, and in extracellular matrix in cells and tissue from throughout the animal and plant kingdom, down to bacteria and viruses. Lectins have more than one binding site and therefore they are able to cross-link cells through interactions with carbohydrates in the cell membrane. They are sensitive, stable, and easy-to-use tools. Lectin histochemistry and cytochemistry can provide an extraordinarily sensitive detection system for changes in glycosylation and carbohydrate expression that may occur during embryogenesis, growth, and disease [6,7]. In the present study we used 5 lectins (Glycinmaximus (soya bean) SBA, Arachis hypogeal (peanut agglutinin) PNA, Triticum vulgaris (wheat germ agglutinin) WGA, Triticum vulgaris (succinylated WGA SWGA, Ulexeuropaeus UEA-I) in the study of trachea-esophageal region development.

Methods

Chick embryos: Fertilized chick eggs were obtained from a local hatchery and incubated at 38°C. Fifty chick embryos were removed out and were staged according to the criteria of Hamburger and Hamilton [8]. About 2-3 embryos for each of the different stages (stage 14 to 19) were used.

The tissues were fixed in Bouins solution for 8 hours at room temperature and were processed for paraffin sectioning [9]. All lectins used were obtained from Sigma. They were fluoresceine isothiocyanate (FITC) labeled. The procedure for Fluorescein Isothiocyanate Labelled Lectins was done according to Allison [10].

Results

Lectin Histochemical Bindings:

The binding pattern of the different lectins used in this study showed variable tempro-spacial criteria. These binding patterns were summarized in tables 1 and 2.

Table 1. The lectins binding pattern with epithelial and mesenchymal tissue of 2 day embryo

Lectin regions in 2 days	head mesenchyme	ateral to cardinal ventral vein	Perinotochord mesenchyme	Pharyngeal wall	Pharyngeal arch	Dorsomedial to cardinal vein	Traingular pharynx
SBA	O	*O	O	O	Oo	O	Oo
UEA I	*O	*O	O	*O	*O	*O	*O
PNA	O	*O	O	O	*	*	*
WGA	O	O	O	O	*	*O	*
SWGA	*O	*O	O	*O	*	O	*

O = Cell surface binding, *= Intracellular binding, Oo = extracellular, *O = Mixed intracellular and cell surface binding.

Table 2. The lectins binding pattern with epithelial and mesenchymal tissue of 3 day embryo

Lectin Regions in 3 days	Ventrolateral to cardinal vein	Head mesenchyme	Perinotochord mesenchyme	Slit-like pharynx	Pharyngeal Arch	Dorsomedial to cardinal vein	Tracheal bud	Mesenchyme around esophagus & bronchial buds
SBA	O	O	O	*O	*	Oo	*O	*O
UEA-I	*	*	O	*	*	*	O	O
PNA	*O	*O	O	*	*	*	*O	*O
WGA	*	*O	O	*	*	*O	*O	*O
SWGA	*O	*O	O	*	*	*O	*O	*O

O = Cell surface binding,* = Intracellular binding, Oo = extracellular, *O = Mixed intracellular and cell surface binding

The Lectin Binding During the 2nd Day of Incubation:

The binding f SBA during the 2nd day of development is shown in (Figures 1A, 1B, and 1C). The binding of UEA-I during the 2nd day was shown in (Figures 1D, 1E, and 1F). The PNA binding pattern in the same developmental period was shown in (Figures 1G, 1H, and 1I). That of WGA shown in (Figures 1J, 1K, and 1L). The binding of SWGA was shown in (Figures 1M, 1N, and O).

The Lectin Binding During The3rd Day of Incubation:

The lectin binding during the 3rd day of incubation was shown in (Figures 2A, 2B, 2C, and 2D) for SBA. The binding of UEA-I is shown in (Figures 2E, 2F, 2G, and 2H). The binding of PNA is shown in (Figures 2I, 2J, 2K, and 2L). WGA binding was shown in (Figures 2M, 2N, 2O and 2P). The SWGA binding pattern was shown in (Figures 2Q, 2R, 2S and 2T).

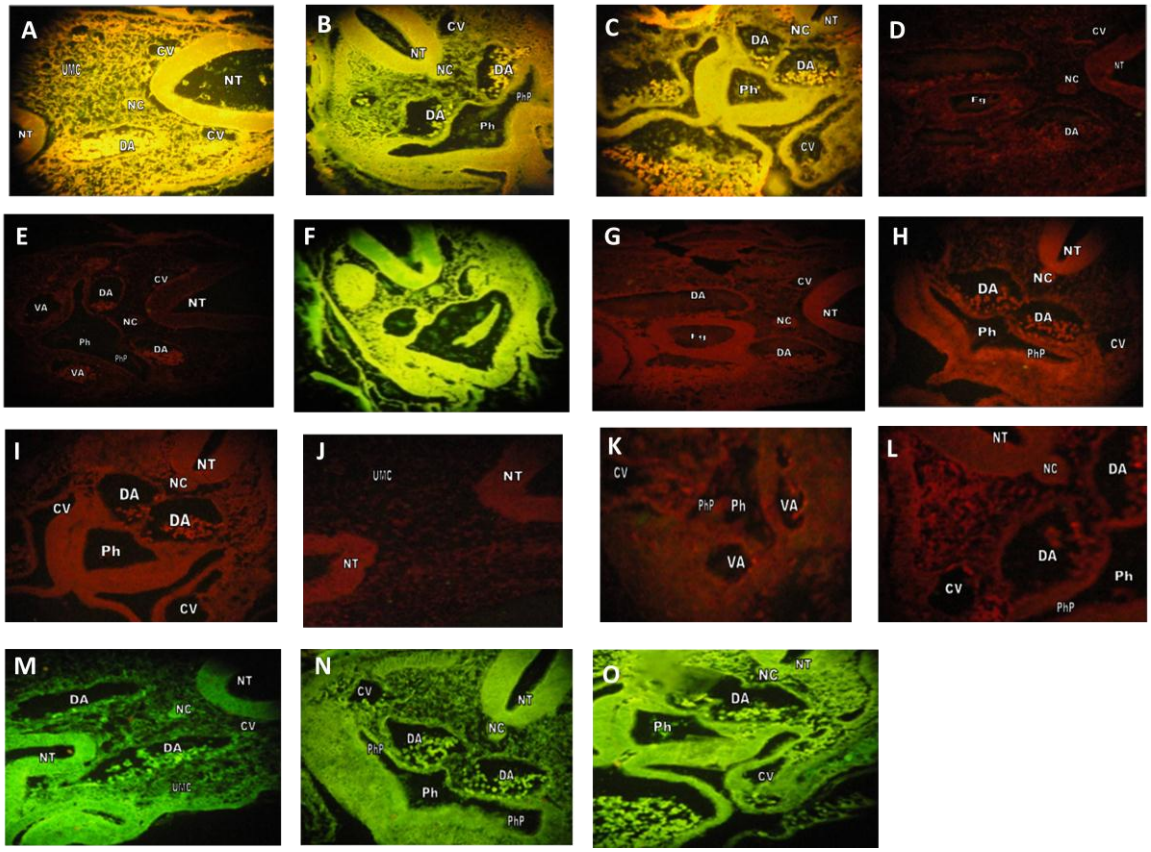

Figure 1. Transverse section of 2 day embryo, A-C incubated with SBA. 4X, D-F incubated

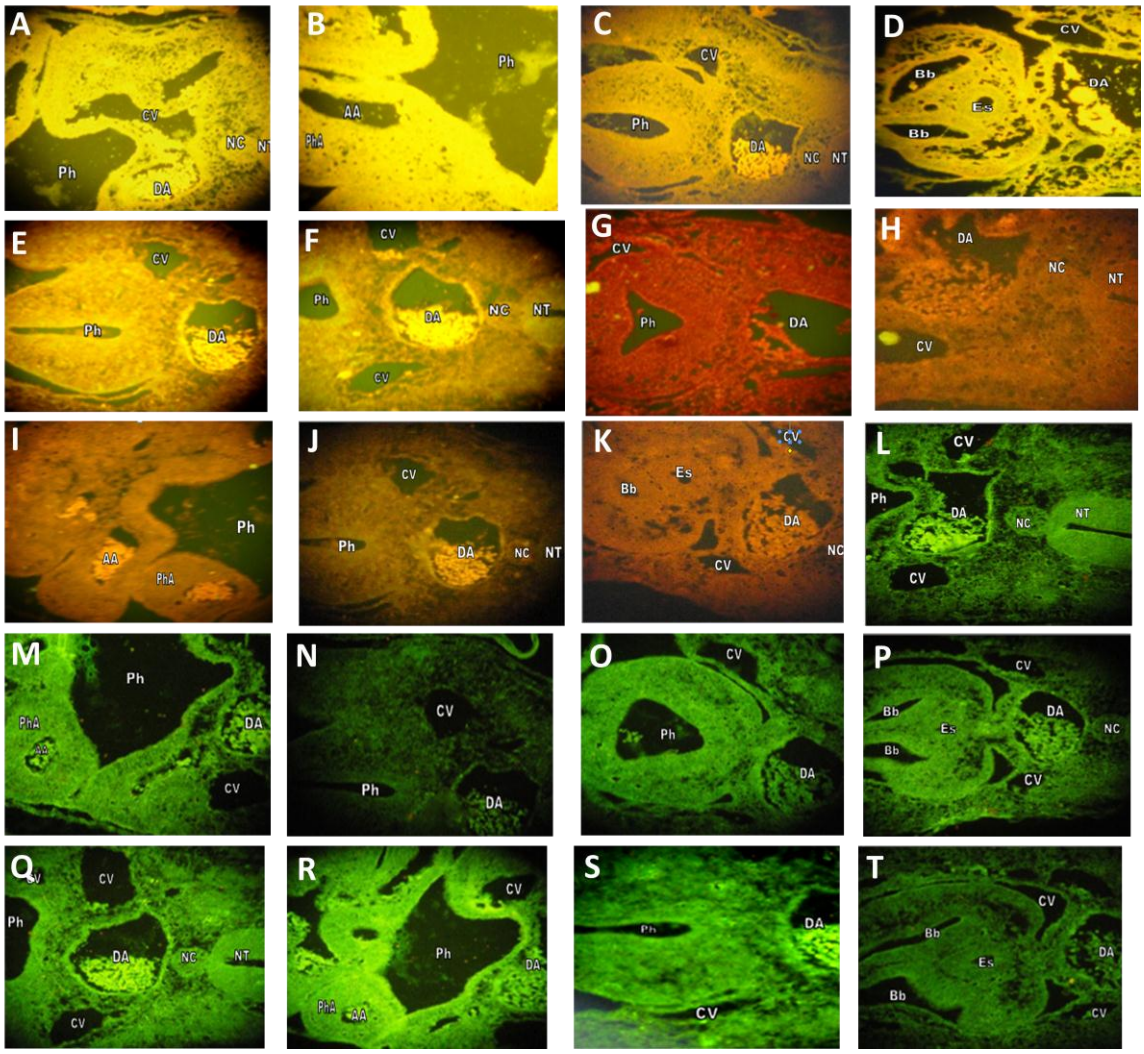

Figure 2. Transverse section of 3 day embryo, A-D incubated with SBA. 10X, E-H incubated with UEA-I. 10X, I-L incubated with PNA. 10X, M-P incubated with WGA. 10X, Q-T incubated with SWGA. 10X

Discussion

The Lectin Histochemical Reactions:

Thorpe *et al*. reported striking temporal and spatial patterning of specific carbohydrate sequences in the developing chick embryo [11]. The variable lectin bindings found in this study supported this concept and also supported the conclusion of Adiet *et al*. that Ulexeuropeus I (UEA-I), Glycine maximus (SBA), Arachishypogaea (PNA) and Triticumvulgare (WGA) binding showed a time-related variability of staining intensity and binding sites that depends upon the stage of differentiation and maturation [12].

The lectin binding to the mesenchymal tissue seen in this study supported the mesenchymal lectin binding reported by Gheri *et al*. [13]. The lectin binding pattern found in this study supported the view of Mohammad *et al*. reported that cell surface and extracellular glycoconjugate played an essential role in many developmental phenomenon as cell differentiation, cell migration, and cellular interactions [14].

The color of the fluorescence was reported to be variable, the fluorescence reactivity had been shown to be in different colors, green/ yellow and TRITIC or Texas Red (which fluoresces red) [7,15]. This conclusion was also found in the

fluorescence color of the lectin binding examined in this study, specially the fluorescence of WGA binding.

The lectin bindings should be logically considered at the mesenchymal tissues enclosing the embryonic structures around the developing pharyngeal and tracheo-esophageal regions.

Interpretation of the MesenchymalLectin Binding:

Around the Developing Cranial Part of the Foregut and Ventro-Lateral to the Cardinal Veins:

The Binding of PNA, SWGA, and UEA-I:

It seems that the mixed cell surface and intracellular binding of PNA and SWGA is a criterion of mesencymal tissues propagation ventrolateral to the deviated cardinal veins during the second and third days, the same criterion is seen at the mesenchyme propagation around the cranial part of the foregut at the third day.

The UEA-I binding varies at these mesenchymal regions showing temprospatial changes from mixed cell surface and intracellular binding at the second day to intracellular binding at the third day.

This conclusion goes with the concept of Zalik *et al*. reported that galactose specific lectin is expressed in spreading cells [(16)]. The PNA pattern binding (which is a galactose specific lectin could be related to spread of the mesenchymal tissues lateral to the deviated cardinal veins and around the developing cranial part of the foregut [(17)].

Also, according to this, conclusion the SWGA binding specific for N-acetylgalactosamine may be considered for the same interpretation as the binding pattern of SWGA simulate that of PNA [(18)]. The UEA-I binding specific for fucose may be related to cellular differentiation, this interpretation is based on the role of lectin binding in determination of the cellular differentiation [(19,20)].

Catt *et al*. stated that it is likely that in most tissues the high concentrations of lectin are particularly active in connective tissue during the extensive tissue reorganization [(21)]. The results of this study agree with that hypothesis.

The Binding of SBA and WGA:

SBA and WGA bindings showed variable tempro-spatial pattern that did not follow a conclusive chronological pattern in the mesenchyme around the cranial part of the pharynx and ventrolateral to the cardinal veins.

The variable SBA and WGA bindings at this mesenchyme are probably related to the masking effect described by Takahashi in the chick embryo [(22)].

Sinning *et al*. shown that SBA binding could be related to active proteins associated with mesenchyme formation that are localized to a particulate form of extracellular matrix, specific for matrix particulates in areas of the embryo that undergo an epithelial- mesenchymal interaction [(23)].

The results of this study reached a conclusion similar to that reported by Zschabitz *et al*. stated that WGA (Triticumvulgare) displayed a universal distribution of binding sites with differences in binding between focal areas of developing mesenchyme. These authors concluded that terminal sialic acid molecules, glucose, galactose-(β 1,4)-N-acetylglucosamine as well as galactose-(β 1,3)-N-acetylgalactosamine are diffusely distributed in mesenchymal tissue [(24)].

Dorsomedial to the Deviated Cardinal Veins:

The mesenchyme dorsomedial to the deviated cardinal veins lies on the sides of the neural tube represented the sclerotomal mesenchyme. It was described that the cells of the ventral and medial walls of the somites lose their compact organization, become polymorphous, and shift their position. These cells collectively known as the sclerotome that form a loosely woven embryonic connective tissue. This tissue will surround the spinal cord and the notochord to form the vertebral column [(25-29)].

In summary, during the 2nd day of development, the mesenchyme dorsomedial to the deviating cardinal veins showed cell surface reactivity with SWGA, and mixed reaction with UEA-I, and only intracellular reactivity with PNA. During the 3rd day of development, this mesenchyme showed

mixed reaction with SWGA, while PNA and UEA-I showed intracellular reaction. The binding at this mesenchyme showed cell surface SBA binding pattern during the second and third day of development. The WGA binding was of mixed cell surface and intracelluar pattern at the same period.

The PNA Binding:

This sclerotomal mesenchyme was shown in this study to be marked by intracellular PNA during the second and third day of development. This finding is in agreement with the suggestions of Gotz *et al*. whom reported conclusions supporting the results of this study; these authors reported that PNA and WGA were found in the developing sclerotomes [30].

Aulthouse and Solursh stated that PNA appears to be a marker for early precartilage cellular aggregates in chick embryo [31], also Zschabitz *et al*. suggested that in early stages of development, PNA selectively labeled the prevertebralblastema (the sclerotomes) [24].

Stringa *et al*. found specific binding to the lectin (PNA) in embryonic precartilage tissues and the developing somite. The hypothesis was that PNA-binding may be a characteristic of chondro progenitor cells in chick embryos [32]. Also, Mohammad *et al*. stated that PNA binding was found in the vertebral primordial at early stages [14].

The SWGA Binding:

The results of this study could support the hypothesis made by Griffith et al. that the undifferentiated mesenchymal transformation is accompanied by changes in the cell surface oligosaccharide complement of the differentiating cells. These authors stated that the pluripotential nature of the mesenchyme represents a developing system which is readily amenable to experimentation and should provide insights into the general mechanisms of cell differentiation and transformation [33].

The UEA-I Binding:

The binding pattern is probably a criterion of the mesenchymal tissue of the chick species during the third day of incubation, and thus this pattern could not be considered as a marker specific for the sclerotomal mesenchyme. This conclusion supported the finding of Zschabitz *et al*. that UEA-I failed to bind the vertebral primordial in rat embryo [24].

The SBA and WGA Binding:

The sclerotomal mesenchyme was shown in this study to be marked by cell surface and mixed cell surface and intracellular reactivity respectively during both the second and third days of development.

This pattern may be considered as a criterion of the sclerotomal cells of the chick embryo at this developmental period. This conclusion supported the finding of Gotz *et al*. that SBA did not react at all in the paraxial mesenchyme of the human embryos in comparison to chick embryos [30].

The Pharyngeal Arches:

The WGA binding maintained intracellular localization during the second and third days of development. The SBA pattern has been changed from extracellular binding at the second day to intracellular binding at the third day.

The PNA, SWGA and WGA Binding:

These lectins showed intracellular reaction with the mesenchyme of the pharyngeal arches during the second and third day of development, this pattern could be considered as a marker of this mesenchyme. This finding is in agreement with the report of Rojo *et al*., these authors stated that WGA reacted at every site of the bronchial region thus showing the presence of N-acetyl-D-glucosamine. These authors reported that other lectins, such as PNA and UEA-I, reacted also for a short time at some sites [34].

The same marker pattern of these lectins was found in the mesenchyme around the triangular pharynx at the second day and around the slit-like pharynx during the third day. This may indicate that this caudal pharyngeal lumen is surrounded by the mesenchyme of the pharyngeal arches. This interpretation depends on the consideration of Brooks and Leathem that the pattern of lectin binding could indicate the specific tissue type [6].

The UEA-I Binding:

The binding pattern of this lectin changes from a mixed cell surface and intracellular pattern at the second day to intracellular binding at the third day. Also this pattern was seen in the mesenchyme around the triangular and slit-like pharynx, and that could support the interpretation described with PNA, SWGA, and WGA binding.

The SBA Binding:

The pharyngeal arches mesenchyme changes the pattern of SBA from extracellular pattern at the second day to intracellular binding at the third day. Also the extracellular to intracellular changing pattern was seen in the mesenchyme around the triangular shaped pharynx at the 2nd day of developmentanl around the slit-like pharynx during the 3rd day, and that could supported the interpretation described with PNA, SWGA, and WGA binding.

Around The Respiratory Diverticulum, the Lung Buds, and The Esophagus:

The lectin binding pattern at the third day in these mesenchymal regions almost showed cell surface binding of the lectins with or without intracellular localization of pattern of the lectins used in this study. Such pattern could probably being considered to be specific to the type of the mesenchymal tissues at these regions.

These finding goes with the descriptions of Gheri *et al*. that lectin UEA-I was concomitant with the beginning of respiratory development [35]. The patterns of lectin binding appreciated in this study are the criteria that were applied as an indication of the topographic criteria or the type of the mesenchymal tissue separating the respiratory primordial from the esophagus.

References

1. Barbara P, Van den Brink GR, Roberts DJ. Molecular etiology of gut malformations and diseases. Am J Med Genet. 2002; 115: 221-30.
2. Miosge N, Gotz W, Quondamatteo F, et al. Comparison of lectin binding patterns in malformation and normal human embryos and fetus. Teratology. 1998; 57: 85-92.
3. Prattima NM, Avarham S. Carbohydrate-recognition and angiogenesis. Cancer Metastasis Rev. 2000; 19: 51-7.
4. Hernandez P, Martin O, Rodrigues Y, et al. Aplicaciones de laslectinas Rev Cubana Hematol Immun Hemoter. 1999; 15: 91-5. [Abstract]
5. Jörns J, Mangold U, Neumann U, et al. Lectin histochemistry of the lymphoid organs of the chicken. Anat Embryol. 2003; 207: 85-94.
6. Brook SA, Leathem AJC. Prediction of lymph node involvement in breast cancer by detection of altered glycosylation in the primary tumor. Lancet. 1991; 338: 71-4.
7. Brooks S, Leathem AU, Schumathem U. Lectin Histochemistry: A Concise Practical Handbook. Oxon UK: BIOS Scientific Publishers Ltd.; 1997.
8. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. J Morphol. 1951; 88: 49-92.
9. Robert SR. Experimental embryology techniques and procedures. 3rd ed. Minneapolis, Minnesota: Burgess Publishing Comp; 1965. p. 405-43.
10. Allison RT. The effect of various fixatives on subsequent lectin binding to tissue section. Histochemistry. 1987; 19: 65-74.
11. Thorpe SJ, Bellairs R, Feizi T. Developmental patterning of carbohydrate antigens during early embryogenesis of the chick: expression of antigens of the poly-N-acetyllactosamine series. Development. 1988; 102(1): 193-210.
12. Adi MM, Chisholm DM, Waterhouse JP. Histochemical study of lectin binding in the human fetal minor salivary glands. J Oral Pathol Med 1995; 24(3):130-135.
13. Gheri BS, Gheri G, Sgambati E, et al. Changes in expression of the oligosaccharides in the human fetal skin. Anat Anz 1997; 179(1):49-56.
14. Mohammed MHT, Mohammad RN, Mehdi D, et al. Distribution of specific glycoconjugates in early mouse embryonic notochord and paraxial mesenchyme. Iran Biomedical J. 2004; 9(1): 21-6.
15. Barahona JVR. Lectin and immune histochemical investigation on cellular alterations in chicken embryos following inoculation with Newcastle Disease Viruse (NDV) of different virulence. PhD Thesis, Univ. Veter. Med. Hannover; 2008. p. 172.
16. Zalik SE, Thomson LW, Ledsham IM. Expression of an endogenous galactose-binding lectin in the early chick embryo. J Cell Sci. 1987; 88: 483-93.
17. Nilsson CL. Lectin: Analytical technologies. USA: Elsevier. Florida State University. 2007.
18. Dudek ARW, Fix JD. Embryology, Board Review Series. 2nd ed. Wolters Klumer Company. Lippincott Williams & Wilkins, 1998.
19. Holthofer H. Vascularization of the embryonic kidney, detection of endothelial cells with Ulexeurepaeus-I. Cell Differ. 1986; 20: 27-31.
20. Schwechheimer K, Weiss G, Schnabel P, et al. Lectin target cell in human central nervous system and pituitary gland. Histochem. 1984; 80: 165-9.

21. Catt JW, Harrison FL, Carleton JS. Distribution of an endogenous beta-galactoside-specific lectin during fetal and neonatal rabbit development. J Cell Sci. 1987; 87: 623-33.

22. Takahashi H. The masking effect of sialic acid on Con A, PNA and SBA ectoderm binding sites during neurulation in the bantam chick embryo. Anat Embryol Berl. 1998; 185(4): 389-400.

23. Sinning AR, Hewitt CC, Markwald RR. A subset of SBA lectin-binding proteins isolated from myocardial-conditioned media transforms cardiac endothelium into mesenchyme. Acta Anat Basel. 1995; 154(2): 111-9.

24. Zschabitz A, Krahn V, Gabius HJ, et al. Glycoconjugate expression of chondrocytes and perichondrium during hyaline cartilage development in the rat. J Anat. 1995; 187: 67-83.

25. Lehmann FE. Further studies on the morphogenetic role of the somites in the development of the nervous system of amphibians. J Exp Zool. 1927; 49: 93-131.

26. Bagnall KM, Higgins SJ, Sanders EJ. The contribution made by a single somite to the vertebral column: experimental evidence in support of resegmentation using the chick-quail chimera model. Development. 1988; 103: 69-85.

27. Tam PPL, Trainor PA. Specification and segmentation of the paraxial mesoderm. Anat Embryol. 1994; 189: 275-305.

28. Elizabeth M, Kensicki M, Eisen JS. Sclerotome development and peripheral nervous system segmentation in embryonic zebrafish. Development. 1997; 124: 159-67.

29. Sadlar TW. Langman′ s medical embryology.10th ed. Montana: Twin Bridges; 2010.

30. Gotz W, Frisch D, Osmers R, et al. Lectin-binding patterns in the embryonic human paraxial mesenchyme. Anat Embryol Berl. 1993; 188(6): 579-85.

31. Aulthouse AL, Solursh M. The detection of a precartilage, blastema-specific marker. Dev Biol. 1987; 120(2): 377-84.

32. Stringa E, Love JM, McBride SC, et al. In vitro characterization of chondrogenic cells isolated from chick embryonic muscle using peanut agglutinin affinity chromatography. Exp Cell Res. 1997; 232(2): 287-94.

33. Griffith CM, Wiley MJ, Sanders EJ. The vertebrate tail bud: three germ layers from one tissue. Anat Embryol Berl. 1992; 185(2): 101-13.

34. Rojo MC, Bla´nquez MJ, Gonza´lez ME. A histochemical study of the distribution of lectin binding sites in the developing branchial area of the trout Salmotrutta. J Anat. 1996; 189: 609-21.

35. Gheri G, Sgambati E, Gheri S. Glycoconjugate sugar residue in the chick embryo developing lung: A lectin histochemical study. J Morphol. 2000; 243: 257-64.

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Influence of Cigarette Smoking on Seminal Plasma Soluble Fas as a Marker of Germ Cell Apoptosis

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Abstract

Background Male infertility constitutes about 50% of overall causes of infertility and apoptosis is known to have an essential role in the control of germ cell number in testis. Cigarette smoking is common in males at reproductive age. Studying the influence of smoking on apoptosis in male genital tract and its influence on fertility helps in the management of infertility in smokers.

Objectives To assess the influence of cigarette smoking on seminal plasma (SP) soluble Fas (sFas) and the correlation between SP sFas and serum sex steroidal hormones and conventional semen parameters in males of infertile couples.

Methods Seventy male partners of infertile couples (30 smokers and 40 non-smokers) were enrolled in this study. A subject was considered smoker if he had history of smoking of more 10 cigarettes per day for at least one year. Semen analysis was done according to World Health Organization (WHO) 2010. Specific kits were used for the measurement of SP sFas and serum testosterone and estradiol (E2).

Results In smokers' group, SP sFas was significantly ($p < 0.05$) negatively correlated with age and sperm motility and positively correlated with immotile sperm and round cell number.

Conclusion Germ cell apoptosis in smoker males of infertile couples is interrelated with sperm motility.

Keywords Male infertility, Smoking, Apoptosis, Seminal plasma sFas.

Introduction

Male infertility factor is identified in almost 50% of infertile couples while it is the sole cause in 20-30% of infertile couples; and a comprehensive male infertility evaluation is the goal to optimize a man's reproductive potential while maximizing his overall health [(1)].

Cigarette smoking is a widely recognized health problem and the highest prevalence of smoking observed in young adult males during their reproductive period [(2)]. Many literatures support the hypothesis that a significant correlation exists between tobacco smoking and altered reproductive physiology [(3)].

Apoptosis is an active, gene-directed cellular self-destruction which may occur in both physiologic and pathologic conditions [(4)]. It is thought to be one of the important factors in regulating the production of spermatozoa [(5)]. One factor implicated in sperm apoptosis is the cell surface protein, fibroblast associated (Fas). The interaction between Fas (CD95/Apo-1; a type I transmembrane glycoprotein receptor) and a cellular death inducing ligand (a type II transmemberaneglycoprotein; FasL) plays an important role in triggering the apoptotic pathway. Both Fas and FasL exist as membrane bound and soluble forms [(6)].

Human sFas is a 26-35 kD glycoprotein formed from cleavage of the specific extracellular region of FasL by the Matrilysin (protease enzyme) [7]. Soluble FasL is not as efficient as membrane-bound FasL in executing apoptosis [8,9] and can be antiapoptotic in some circumstances [10,11]. Previous reports have suggested that the Fas mediated system is implicated in the elimination of defective spermatozoa from the ejaculate and shows possible irregularities that could account for certain forms of male infertility [12].

Testosterone hormone is obligatory for spermatogenesis and the proper functioning of Sertoli cells [13]. It increases germ cell attachment to Sertoli cells and permit the release of mature sperms. In the absence of testosterone, spermatogenesis does not proceed beyond the meiosis stage. After withdrawal of testosterone, germ cells that have progressed beyond meiosis detach from supporting Sertoli cells and die, whereas mature sperm cannot be released from Sertoli cells resulting in infertility [14].

About 80% of the 17β-estradiol (E2) in the plasma of adult men is produced by extragonadal and extraadrenal aromatization of circulating testosterone and androstenedione by the enzyme aromatase particularly in the adipose tissue. The remainder (20%) comes from Leydig cells [15]. Some E2 is also produced by aromatization of androgens in testicular germ cells, sperms and Sertoli cells [16].

Estrogen is considered as an indispensable "male hormone" in the early spermatogenetic cycle as spermatogonial stem cell renewal is promoted by estradiol implantation and many clinical trials with aromatase inhibitors have resulted in a tendency to improve seminal parameters through suppression of estrogen-testosterone ratio, with an associated increase of follicular stimulating hormone (FSH) [17]. Additionally, estradiol and testosterone also inhibit luteinizing hormone (LH) in a negative feedback loop [18].

The aim of the present study was to evaluate the influence of cigarette smoking on the SP sFas levels in infertile males, and the correlation between SP sFas with serum testosterone and E2, and conventional seminal parameters in those subjects.

Methods

This study was done on seventy male partners of infertile couples attending to the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University. The couples had no history of pregnancy or abortion for more than one year from marriage with regular unprotected sexual intercourse and had no recent history or previous medical history of chronic disease. Their age range was (18-58 years with a mean of 32.5±7.81 years). The smokers involved in this study were having a history of smoking of at least 10 cigarettes per day for at least one year.

Semen sample was taken from each subject by masturbation after 2-7 days of abstinence. Conventional semen analysis was done for each sample according to the protocol of World Health Organization (WHO, 2010) [19], after incubation and liquefaction period (30-60 min). From 106 subjects enrolled primarily in the study, only 70 subjects included and divided into two groups (30 smokers) and (40 non-smokers); while those who were azoospermic or with severe oligozoospermia (sperm concentration less than 5 million sperm/ml) were excluded. Seminal plasma was collected after centrifugation of semen for 15 min. at 3000 rpm; and then frozen at -20^{o}C till analysis of sFas was done.

Seminal plasma sFas was measured using the kit of IBL International GMBH sAPO-1/FAS ELISA Enzyme immunoassay for the quantitative determination of human sAPO-1/Fas in human cell culture supernatants, serum, plasma or other body fluids ref. BE51901 (Germany).

Two ml of venous blood were drawn from anticubital fossa for the assay of serum testosterone and estradiol using the following kits: Serum total testosterone and E2 were assayed using {VIDAS® testosterone kit (Ref. 30 418, BioMérieux® SA, France), and VIDAS® estradiol II kit (Ref. 30 431, BioMérieux® SA, France)}, which are automated quantitative tests

for use on the VIDAS instruments for the enzyme immunoassay measure of total testosterone and E2 in human serum or plasma (lithium heparinate), using the ELFA (Enzyme Linked Fluorescent Assay)technique.

Statistical analysis

Data were analyzed using Microsoft Excel 2010 and SPSS Version 18. The results were presented as mean±standard deviation (SD). Normally distributed data were analyzed using unpaired student t-test while abnormally distributed data (skewed) were statistically analyzed using Mann-Whitney U test. Pearson correlation coefficient was used for correlations. A value of $P < 0.05$ was considered to be significant.

Results

Table 1 shows that there was no significant difference of age, body mass index (BMI) and serum sex steroidal hormones between smokers and non-smokers.

Table 1. Comparison between smokers and non-smokers infertile men parameters by unpaired t-test

Parameter	Infertile men		*P* value
	Smokers N = 30	Non-Smokers N = 40	
Age (yr)	33.27±8.22	31.93±7.55	0.4869
BMI (kg/m^2)	27.24±4.12	28.78±7.17	0.2624
Testosterone (T) (ng/ml)	5.57±2.88	5.67±3.22	0.8906
Estradiol (E2) (pg/ml)	37.82±22.57	39.65±21.4	0.7327
Testosterone/E2 ratio	168.46±90.14	151.51±67.44	0.3915

BMI: Body mass index, E2: Estradiol II

This study revealed that there was no significant difference in median of SP sFas between smokers and non-smokers infertile men ($P > 0.05$), table (2).

Table 2. Comparison of seminal plasma sFas between smokers and non-smokers groups by Mann-Whitney U test

Parameter	Smokers N = 21		Non-Smokers N = 36		*P* value
	Range	median	Range	median	
sFas (pg/ml)	0.0-298.0	32.0	0.0-712.0	4.0	0.653

It was also demonstrated that there was no significant difference between semen analysis parameters (volume, concentration, motility, morphology, agglutination and round cells) between smokers and non-smokers groups, table (3).

The age was significantly negatively correlated with SP sFas in smokers group (r = -0.474, $P = 0.03$) as shown in figure 1.

Figures 2 and 3 shows significant negative correlation between seminal sFas and progressive and non-progressive sperm motility percentage (r = -0.473, -0.448, P = 0.03, 0.042 respectively) and significant positive correlation with immotile sperm percentage and round cell no. in semen (r = 0.537, 0.712, P = 0.012, <0.001 respectively) in smokers group (figures 4 and 5), while these correlations were insignificant in non-smokers group.

Table 3. Comparison of semen parameters between smokers and non-smokers groups by unpaired t-test

Parameters	Infertile men		*P* value
	Smokers N = 30	Non-Smokers N = 40	
Semen Volume (ml)	2.25±0.82	2.09±0.78	0.4108
Semen pH	8.01±0.13	8.0±0.24	0.7222
Sperms Concentration (ml)	39.07±26.29	48.8±30.83	0.1595
Sperms Progressive motility (%)	33.53±17.83	31.28±16.58	0.593
Sperms Non-progressive motility (%)	21.0±12.63	20.88±9.38	0.9638
Immotile Sperms (%)	45.47±23.65	47.88±20.97	0.6599
Total sperm count/ejaculate	83.94±65.34	104.24±83.66	0.2586
Normal Sperms Morphology (%)	24.23±14.29	22.73±14.4	0.6647
Sperms Agglutination (%)	1.27±4.38	1.2±3.02	0.9432
Round cell count/HPF	9.13±7.77	9.35±7.26	0.9059

HPF= high power field

Discussion

In this study, there was no significant difference in median of SP sFas between smokers and non-smokers males of infertile couples ($P > 0.05$); however, the SP sFas was negatively correlated with age of smokers; this may be explained as cigarette smoking may enhance apoptosis process in the testis and genital tract with age [7] as sFas is considered antiapototic factor and the decrease in sFas level indicates increased apoptosis as mentioned above [10,11].

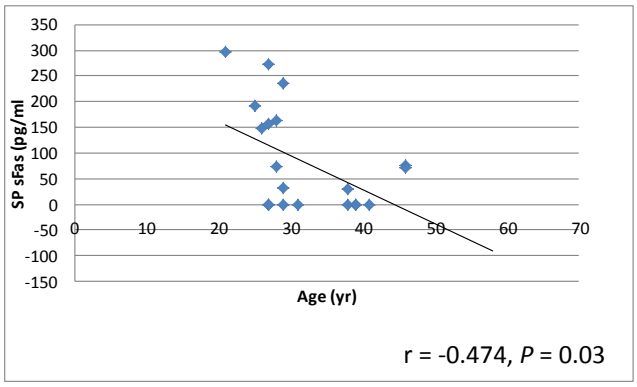

Figure 1. Correlation between age and seminal plasma sFas in smokers group

This study also showed that SP sFas was significantly negatively correlated with sperm motility and positively correlated with immotile sperms. This result disagree with Zedan et al [20], but it is go with Chen et al [21] who found inverse associations between percent apoptosis in ejaculated human semen and sperm motility, progressive motility, and morphology. It could be explained that increased antiapoptotic factor (sFas) and decrease apoptosis would lead to overcrowding of growing sperms and decrease space within seminiferous tubules and this causes increase percentage of defected sperms in the semen and increase immotile sperms and round cells (which are mainly immature germ cells) [22].

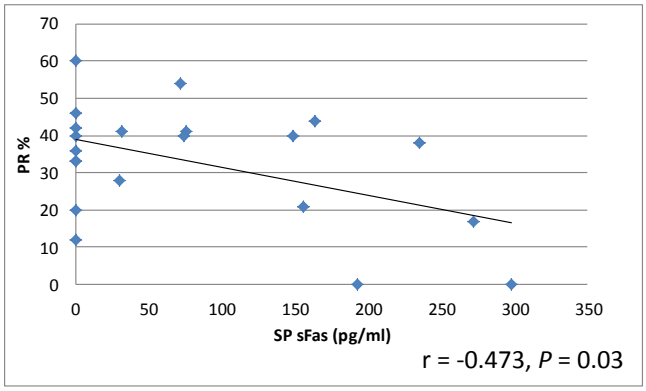

Figure 2. Correlation between seminal plasma sFas and sperm progressive motility percentage in smokers group

The hormones per se in this study had no significant correlation with semen parameters or with sFas. However, the total testosterone level and E2 in serum were higher in non-smokers than in smokers although the difference was non-significant, while the T/E2 ratio was higher

(but non-significant) in smokers than in non-smokers. These results go with Yardimci et al [23] who found significant decrease in total serum testosterone in smokers.

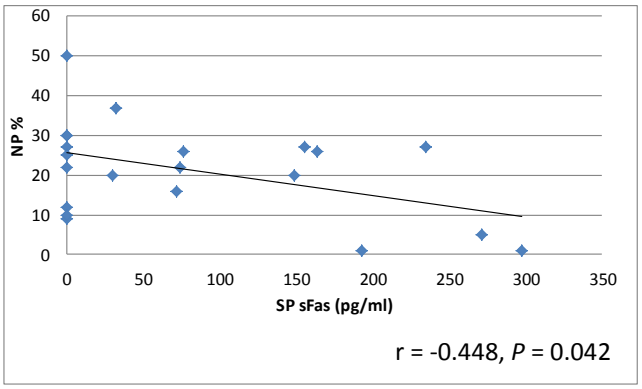

Figure 3. Correlation between seminal plasma sFas and sperms non-progressive motility percentage in smokers group

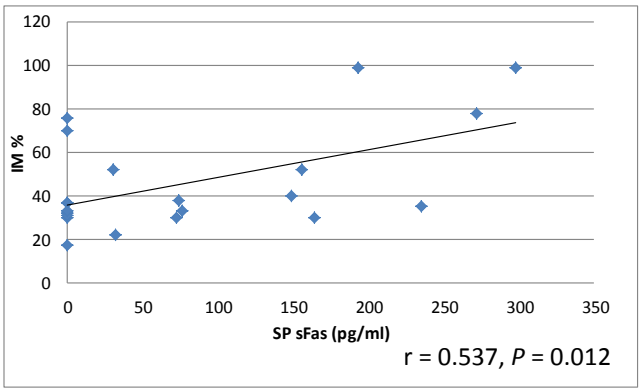

Figure 4. Correlation between seminal plasma sFas and immotile sperms percentage in smokers group

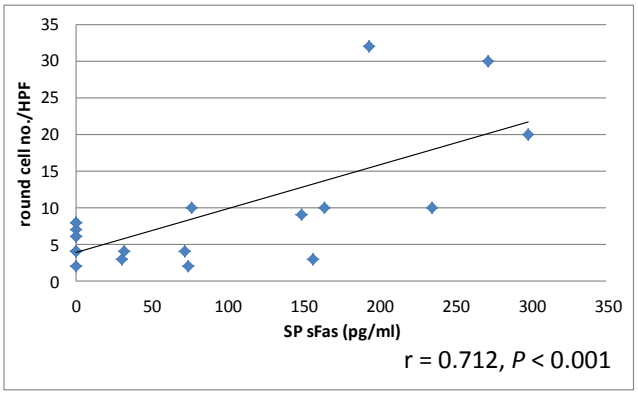

Figure 5. Correlation between seminal plasma sFas and round cell number in smokers group

The possible mechanism for these results, the smoking, over time, may cause degeneration of Leydig cells, which in turn reduce testosterone production. In contrast, English *et al* study showed a significantly higher total testosterone in smokers than non-smokers [24]. This maybe explained as the study was done on healthy subjects while in our study was on males of infertile couples. Another assumption is that man with high testosterone level may become addict to cigarette smoking. Regarding E2, Trummer et al (25) also had found no significant different of E2 level between smokers and non-smokers.

For semen parameters, the study showed no significant difference between smokers and non-smokers, these results were similar to the results of several other authors [26-29], but disagree with Künzle et al [30] who found that cigarette smoking was associated with a significant decrease in total sperm count (−17.5%), total number of motile sperm (−16.6%), significant reduction in the percentage of normal forms and sperm vitality, but ejaculate volume were slightly but non-significantly affected. This could be due to high number of subjects involved in their study compared with the low number in this study. On the other hand, Collodel et al [31] found that semen quality in infertile males seems not to be dramatically affected by cigarette smoking, only heavy smokers show significantly lower sperm concentration. The exact pathophysiology underlying cigarette smoking and sperm deterioration is unclear. Possible mechanisms include the influence of cigarette smoke on the function of Sertoli and Leydig cells and testicular microcirculation [30].

In conclusion, Germ cell apoptosis in smoker males of infertile couples is interrelated with sperm motility.

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References

1. Kuang W. The Initial Consultation for Male Infertility. In:Sabanegh ES (editor). Male infertility problems and solutions1st ed. USA: Humana Press (Springer Science+Business Media); 2011. p. 1-13.
2. Colgar AH, Jorsaraee GA. Marzony ET. Cigarette smoking and risk of male infertility. Pakistan J Biol Sci. 2007; 10(21): 3870-4.
3. Soares S. Cigarette Smoking and Fertility. Reprod Biol Insights. 2009; 2: 39-46.
4. Papathanassoglou ED, Moynihan JA, Ackerman MH. Does programmed cell death (apoptosis) play a role in the development of multiple organ dysfunction in critically ill patients? a review and a theoretical framework. Crit Care Med. 2000; 28: 537-49.
5. O'Neill DA, McVicar CM, McClure N, et al. Reduced sperm yield from testicular biopsies of vasectomized men is due to increased apoptosis. Fertil Steril. 2007; 87: 834-41.
6. El-Melegy NT, Ali M-E M. Apoptotic markers in semen of infertile men: Association with cigarette smoking. Intl Braz J Urol. 2011; 37(4): 495-506.
7. Powell WC, Fingleton B, Wilson CL, et al. The metalloproteinase matrilysinproteolytically generates active soluble Fas ligand and potentiates epithelial cell apoptosis. Curr Biol. 1999; 9: 1441-7.
8. Aoki K, Kurooka M, Chen JJ, et al. Extracellular matrix interacts with soluble CD95L: retention and enhancement of cytotoxicity. Nat Immunol. 2001; 2: 333-7.
9. O' Reilly LA, Tai L, Lee L, et al. Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. Nature. 2009; 461: 659-63.
10. Schneider P, Holler N, Bodmer JL, et al. Conversion of membrane-bound Fas (CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. J Exp Med. 1998; 187: 1205-13.
11. Knox PG, Milner AE, Green NK, et al. Inhibition of metalloproteinase cleavage enhances the cytotoxicity of Fas ligand. J Immunol 2003; 170: 677-685.
12. Kim SK, Yoon YD, Park YS, et al. Involvement of the Fas–Fas ligand system and active caspase-3 in abnormal apoptosis in human testes with maturation arrest and Sertoli cell-only syndrome. Fertil Steril. 2007; 87: 547-53.
13. Roades RA. Bell DR. Medical physiology - principles for clinical medicine. 4th ed. China: Wolters Kluwer business- Lippincott Williams and Wilkins; 2012. p. 682.
14. Walker WH. Non-classical actions of testosterone and spermatogenesis. Phil Trans R Soc B. 2010; 365: 1557-69.
15. Akingbemi BT. Estrogen regulation of testicular function. Reprod Biol Endocrinol. 2005; 3(51): 1-13.
16. Janulis L, Bahr JM, Hess RA, et al. Rat testicular germ cells and epididymal sperm contain active P450 aromatase. J Androl. 1998; 19: 65-71.
17. Aydos K, Yaman Ö. Aromatase inhibitors in male infertility. In: Colpi GM. Male infertility today. Milano: Fotolito E Stampa Grafiche Gelmini. 2004(4). p. 41-62.
18. Hsiao W, Schlegel PN. Assessment of the male partner. In: Kovacs J. The subfertility handbook – A clinician guide. 2nd ed. UK: Cambridge University Press; 2011. p. 43-59.
19. World Health Organization. WHO Laboratory Manual for the Examination and Processing of Human Semen. Geneva: WHO Press; 2010.
20. Zedan H, El-Mekhlafi A-W MM, El-Noweihi AM, et al. Soluble Fas and gonadal hormones in infertile men with varicocele. Fertil Steril 2009; 91(2): 420-424.
21. Chen Z, Hauser R, Trbovich AM, et al. The relationship between human semen characteristics and sperm apoptosis: A pilot study. J Androl. 2006; 27: 112-20.
22. Rodriguez I, Ody C, Araki K, et al. An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. EMBO J. 1997; 16: 2262-70.
23. Yardimci S, Atan A, Delibasi T, et al. Long-term effects of cigarette-smoke exposure on plasma testosterone, luteinizing hormone and follicle-stimulating hormone levels in male rats. Br J Urol. 1997; 79(1): 66-9.
24. English KM, Pugh PJ, Parry H, et al. Effect of cigarette smoking on levels of bioavailable testosterone in healthy men. Clin Sci. 2001; 100: 661-5.
25. Trummer H, Habermann H, Haas J, et al. The impact of cigarette smoking on human semen parameters and hormones. Hum Reprod. 2002; 17: 1554-9.
26. Kumosani TA, Elshal MF, Al-Jonaid AA, et al. The influence of smoking on semen quality, seminal microelementsand Ca^{2+}-ATPase activity among infertile and fertile men. Clin Biochem. 2008; 41: 1199-1203.
27. Chohan KR, Badawy SZA. Cigarette smoking impairs sperm bioenergetics. Int Braz J Urol. 2010; 36(1): 60-5.
28. Aghamohammadi A, Zafari M. The impact of cigarette smoking on sperm parameters: A cross sectional study. International Conference on Environmental, Biomedical and Biotechnology IPCBEE. 2011; 16: 51-84.
29. Aryanpur M, Tarahomi M, Sharifi H, et al. Comparison of spermatozoa quality in male smokers and nonsmokers of iranian infertile couples. Int J Fertil Steril. 2011; 5(3): 152-7.
30. Künzle R, Mueller MD, Hänggi W, et al. Semen quality of male smokers and nonsmokers in infertile couples. Fertil Steril. 2003; 79(2): 287-91.
31. Collodel G, Capitani S, Pammolli A, et al. Semen quality of male idiopathic infertile smokers and nonsmokers: An ultrastructural study. J Androl 2010; 31(2): 108-13.

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Histopathological Changes Induced by Single Dose of LD_{50} *Naja naja* Snake Venom on the Liver of Male Albino Rats

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Abstract

Background The common sign of snake envenomation is hepatotoxicity or liver injury that is dependent on quality and quantity of venom.

Objective To clarify the effect of intraperitoneal (i.p.) injection of LD50 dose of *Naja naja* snake venom on the hepatic tissues of albino rats after 3 and 24 hr from envenoming respectively.

Methods The rats were divided into 3 groups, the first group served as a control group, while the other groups 2 and 3 were treated with the snake venom (0.05 µg/g body weight i.p) and sacrificed by decapitation after 3 and 24 hours of the snake venom injection respectively. The livers were isolated and histological sections were prepared.

Results Intraperitoneal LD_{50} for Naja *naja* snake cobra was determined in rats to be equal to 0.05 µg/g body weight. Histopathological changes in liver tissues after 3 hr from injection were congestion of the central veins, congested liver sinusoids, leucocytes infiltration, cytoplasmic vacuolization and nuclear pyknosis, cellular swelling and necrosis of some cells. While histopathological changes in liver tissues after 24h from injection were the same signs in addition to cellular swelling, necrosis and damage of the injured hepatocytes with acute inflammation cells infiltration.

Conclusion The injection of $LD50_{dose}$ of *Naja naja* snake venomin rats can induce hepatic damage and hepatotixicity in albino rats.

Keywords *Naja naja,* snake venom, Rats, Liver, Histopathological changes.

Introduction

The venom of most snakes is highly phlogistic in humans [1]. Tissue changes following snake envenomation depend on the species of snake responsible for the bite, the composition of its venom and also the susceptibility of the tissue for a particular component of the venom [2]. *Naja naja* (cobra) is one of the most dangerous snake species in the world, where it provokes a high number of human deaths due to envenomations [3]. The main compounds of *Naja naja* venom are complex mixture of biologically active components comprising hydrolytic enzymes that cause several disorders such as hemorrhage, coagulation disturbances, edema and myotoxicity [4]. These enzymes are peptidases [5], phospholipases A_2 [6], metallopeptidases and non-enzymatic proteins/peptides like cardiotoxins, that caused hemolysis, local inflammation, depolarization, and contracture of smooth, skeletal and cardiac muscles [7], and small amounts of organic and inorganic molecules [8]. There are reports showing the effects of various snake venoms on liver tissues in rat that the venom causes damage of the hepatocyes [9-11]. The objective of this study is to

determine the histological alterations in the liver of rats following Naja *naja* envenomation in an attempt to improve our understanding of snake envenomation in rats.

Methods

Venom

Lyophilized crude venom of snake *Naja naja* venom was obtained from India (Sigma loeate Ltd). The crude venom was dissolved in phosphate buffered saline (PBS), pH 7.2. The determination of the median lethal dose LD_{50} of the snake Naja *naja* venom by intraperitoneal (i.p.) injection was carried on 40 adult healthy albino rats. The injected dose was 0.05 µg/g body weight of snake venom calculated according to the method of Meier and Theakston [12]. Results are shown in table (1)

Animals and Experimental design

Healthy adult albino rats of same age group (80±5 days) and weight (190±10 g) were taken from the High Institute for Infertility Diagnosis and Assisted Reproduction Technologies, AL-Nahrain University and the animals were housed in standard condition and fed with normal diet and water *ad libitum*. Animals were divided into three groups of 8 animals each. The first group, control, animals were injected i.p. with 0.1 ml in phosphate buffered saline and sacrificed 24 hr after injection. Groups two and three were injected i.p. with LD50 (0.05 µg/g body weight) of cobra venom and sacrificed at 3 and 24 hr after envenomation respectively. All animals were sacrificed, then liver was isolated and cut to small pieces from each experimental rats then transferred immediately to 10% formalin for 24 hr and dehydrated in ascending grades of ethanol (50-100%). Clearing was done in xylene and embedded in paraffin wax. Sections (4-5 µm thick) were prepared and then stained with hematoxylin and eosin (H & E) and methylene blue stain to be examined with light microscope.

Results

Venom Lethality:

The approximate i.p. LD_{50} for Naja *naja* snake cobra was determined in rats to be equal to 0.05 µg/g body weight, as shown in table 1.

Table 1. Determination of LD_{50} of *Naja naja* snake Cobra venom

Dose µg/g body weight	No. of animals	Survival (S)	Death (D)	% Mortality
0.02	8	8	0	0
0.04	8	5	3	37.5
0.06	8	3	5	62.5
0.08	8	1	7	87.5
0.1	8	0	8	100

LD_{50} = 0.05 µg/g body weight rats

Histological studies:

Light microscopic observation revealed that the control hepatic tissue (group 1) showed normal cells with prominent round nuclei and eosinophilic cytoplasm. The hepatocytes radiated towards a central vein and separated by blood sinusoids (Figures 1 & 2).

In the second group showed some histopathological changes were recorded after 3 hr from envenoming including some scattered of the hepatocytes suffering from cytoplasmic vacuolization, nuclear pyknosis, few cellular rarified of cytoplasmic components and central vein congestion. Congested blood sinusoids and lymphocytic infiltration were also recorded (Figures 3 through 6).

The liver tissues of the third group showed more severe histopathological changes were recorded after 24 hr from envenoming. These changes include extreme extend of the cellular swelling, necrosis and damage. Numerous inflammatory cells and Kupffer cells hyperplasia were noticed in between the necrotic hepatocytes (Figures 7 through 9). The injured hepatocytes were mostly infiltrated with numerous inflammatory cells (Figures 10 through 12).

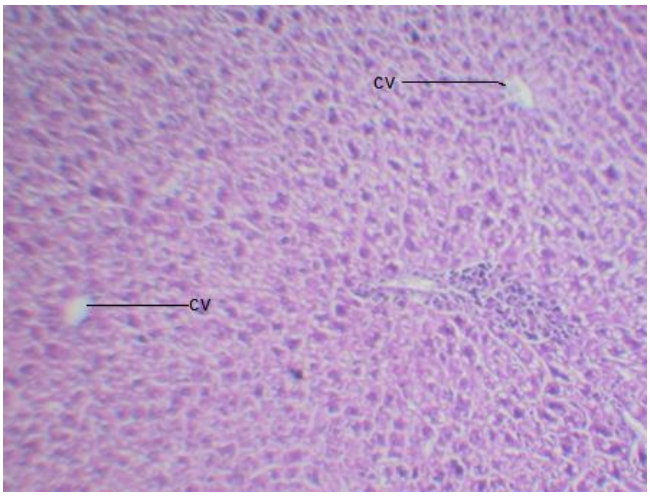

Figure 1. Liver section (from control) showing hepatocytes radiating from the central vein (CV) (H & E, 40X)

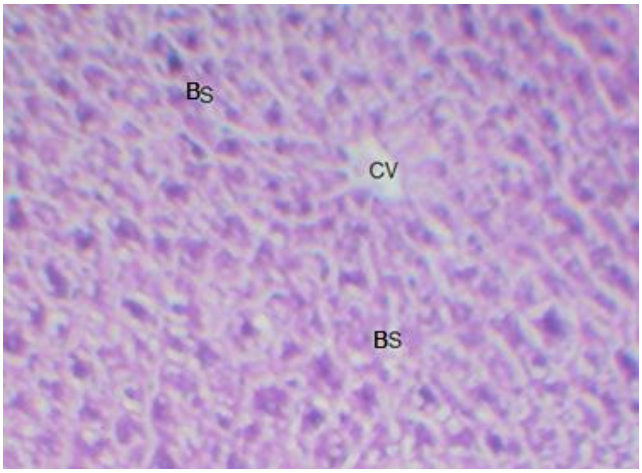

Figure 2. Liver section (from control) showing homogenously stained cytoplasm and normal nuclei, blood sinusoids (BS) and a central vein (CV) (H & E stain, X100)

Discussion

Several researches dealing with the effects of snake venoms in cells or tissues from the organs of rodents, like liver, kidney and muscle showed varying results, depending on the experimental concentrations, exposure time, site of injection, the species of the snake and the composition of the venom [13,14]. Snake venoms comprise complex mixtures of enzymatic and non-enzymatic proteins and small organic compounds.

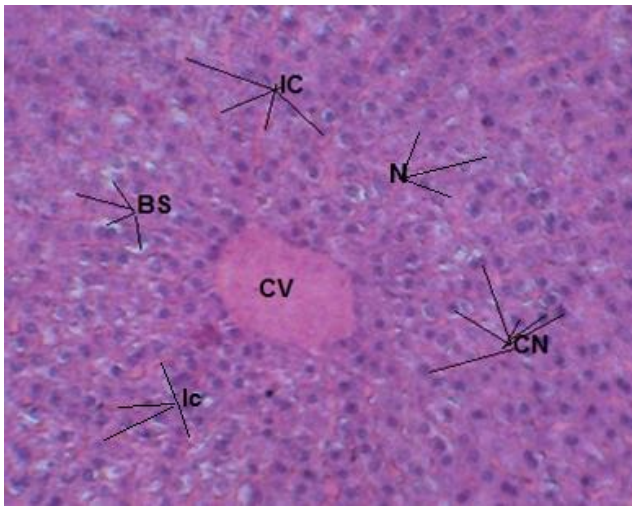

Figure 3. Section of liver tissues of rat after 3h from envenoming with LD50 snake venom showing condensed nuclei (CN) cellular necrosis (N) and number of inflammatory cells (IC), blood sinusoids (BS) and a central vein (CV) congested, (H & E, X100)

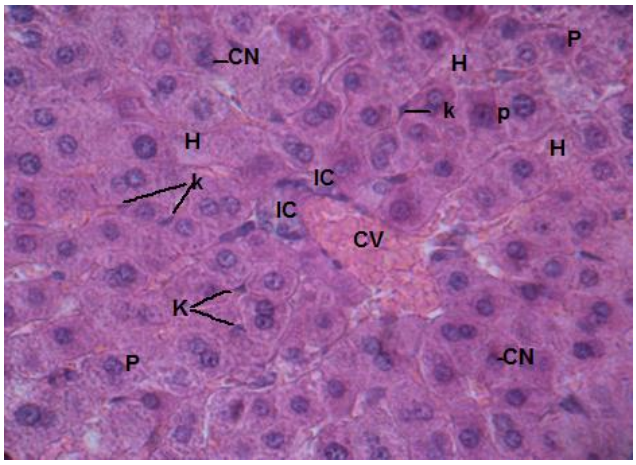

Figure 4. Liver section rat after 3h from envenoming with LD_{50} snake venom showing hydropic degeneration (H), increase number of kupffer cells (K), nuclear condensation (CN) and pyknosis (P), inflammatory cells (IC) and CV: central vein congested, (H & E, X200)

The pathology of envenomation includes both local and systemic effects such as neurotoxicity, myotoxicity, cardiotoxicity, coagulant disorders, hemorrhagic, hemolytic and edema forming activities [15]. Liver is considered as a target organ for envenoming by different types of snake venoms. Liver injury is among the common and most serious symptoms of cobra snake envenoming [16].

In the present work, the livers of rats after 3 and 24h envenomed with the LD_{50} of *Naja naja* snake

venom showed marked histopathological changes. The LD_{50} was selected for studying the histopathological changes associated with snake envenoming. These changes included congestion of intrahepatic blood vessels, increase in number of Kupffer cells, inflammatory cell, hydropic degeneration, variable degrees of cellular swelling, cytoplasmic changes, cellular necrosis and cellular damage.

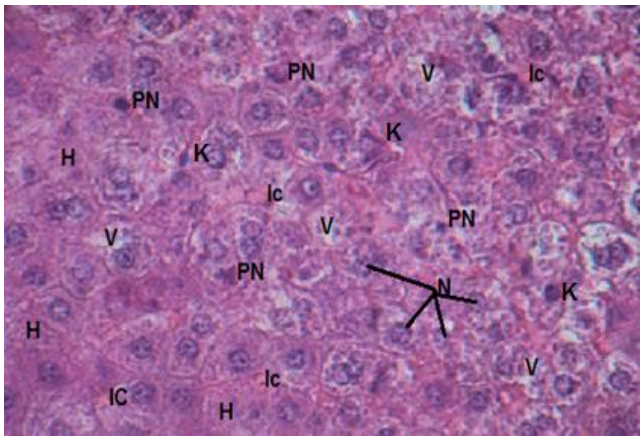

Figure 5. Liver section of rat after 3 hr from injection of snake venom showing hydropic degeneration (H), increase number of kupffer cells (K), variable sized nuclei (N), cytoplasmic vacuolization (v), pyknotic nuclei (PN) and inflammatory cell infilterations (Ic) (H & E stain, X400).

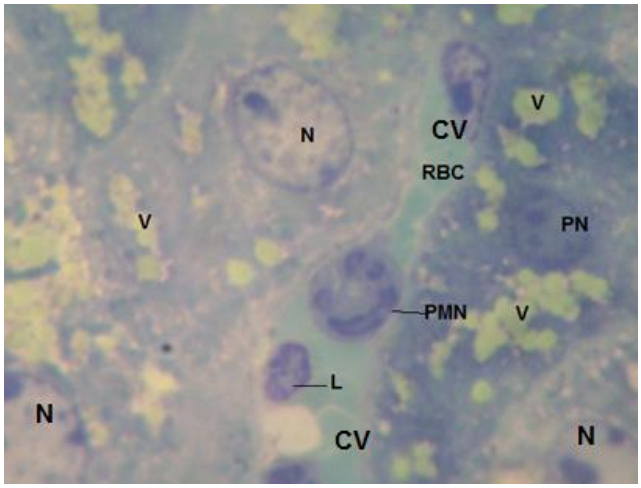

Figure 6. Section of liver tissues of rat after 3 hr from injection of snake venom showing hydropic degeneration (H), variable sized nuclei (N), cytoplasmic vacuolization (v), pyknotic nuclei (PN), lymphocyte (L), polymorph-onuclear cell (PMN) and central vein filled with erythrocytes (RBC) (Mathylene Blue stain, X1000).

The results of the present study were in agreement with those reported by Chethankumar [8] who observed that cellular swelling might be due to the action of *Naja naja* venom phospholipase, which causes disturbance of the cell membrane permeability with, which the Na^+/K^+ ATPase activities and consequent influx of Na^+ and water, induces changes to cellular membranes, especially those related to fatty acid changes in the major membrane phospholipids and eventually lead to cell death.

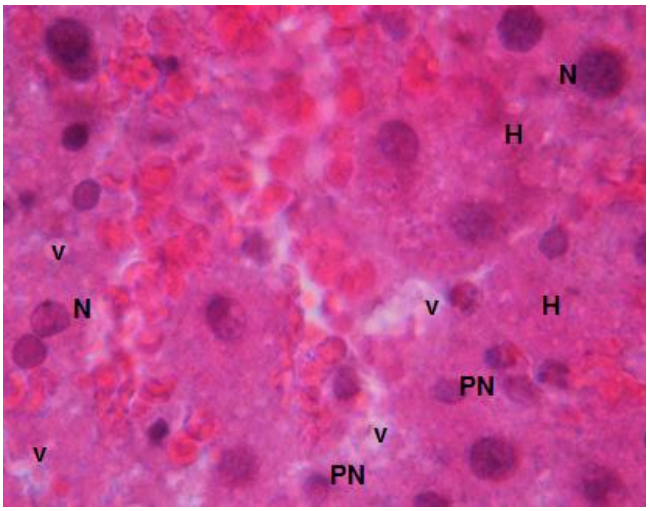

Figure 7. Section of liver tissues of rat after 24 hr from injection of snake venom showing hydropic degeneration (H), variable sized nuclei (N), cytoplasmic vacuolization (v), pyknotic nuclei (PN), central vein filled with erythrocytes and the sinusoids are filled with erythrocytes (H & E stain, X200).

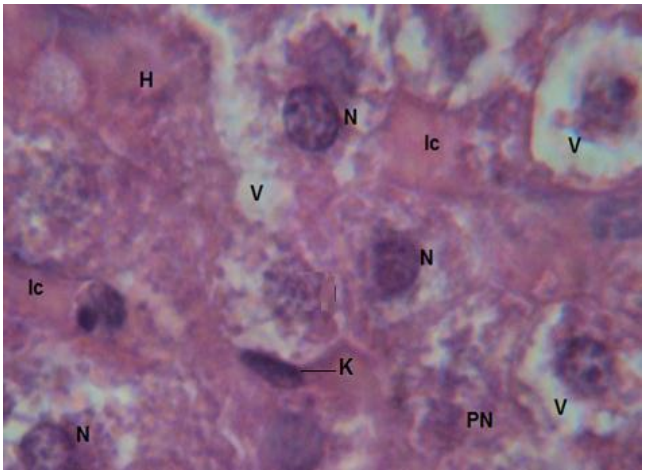

Figure 8. Section of liver tissues of rat after24 hr from injection of snake venom showing hydropic degeneration (H), kupffer cell (K), variable sized nuclei (N), cytoplasmic vacuolization (v), pyknotic nuclei (PN) and inflammatory cell infilterations (Ic) (H & E stain, X600).

In addition, Rahmy and Hemmaid [17] reported that snake (*Naja haje*) envenoming causes cellular swelling, cytoplasmic granulation and vacuolization in addition to intrahepatic hemorrhage, liver necrosis and activation and hyperplasia of the Kupffer cells. This activation of these cells might represent a defense mechanism of detoxification induced by the venom correlated with the degree of injury to the hepatic tissue which increases autophagy throughout the hepatic tissue.

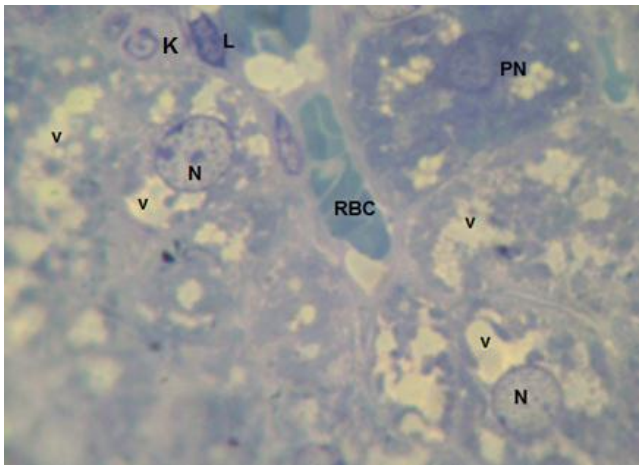

Figure 9. Section of liver tissues of rat after 24 hr from injection of snake venom showing hydropic degeneration (H), hypertrophy of kupffer cell (K), variable sized nuclei (N), cytoplasmic vacuolization (v), pyknotic nuclei (PN), leucocyte (L) and central vein with erythrocytes (RBC) (Mathylene Blue stain, X1000).

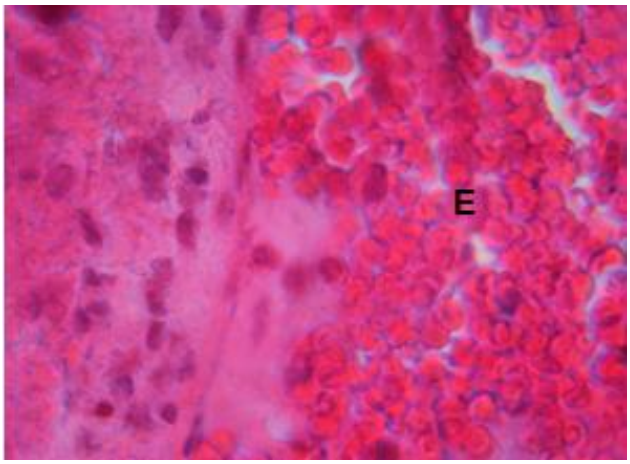

Figure 10. Section of liver tissues of rat after 24 hr from injection of snake venom showing erythrocytes (E) (H & E stain, X200).

Hanafy et al in 1999 [18] explained that *Cerastes cerastes* envenoming causes cellular swelling, cellular necrosis, nuclear pyknosis and presence of foci of damaged hepatic cells invaded with inflammatory cells. The appearance of vacuoles within the hepatocytes of the envenomed rats might indicate venom interference with mitochondrial and microsomal function that leads to disruption of lipoprotein and lipids accumulation. Similar findings were obtained by Abdel Ghani et al in 2009 [19] who attributed these changes to a hepatotoxic effect of the Naja Nigricollis venom and it is more likely to be described as cytoplasmic changes of some snake toxins.

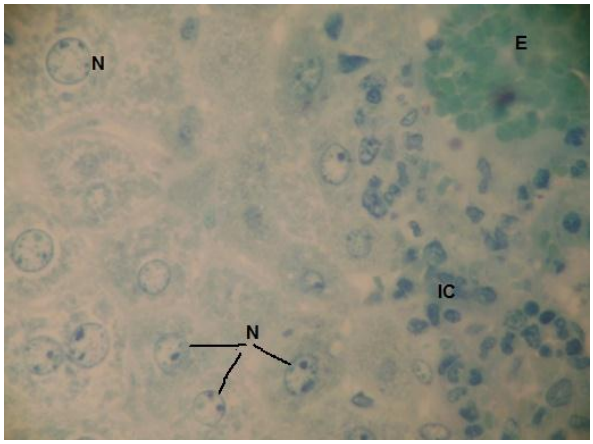

Figure 11. Section of liver tissues of rat after 24 hr from injection of snake venom showing erythrocytes (E), variable sized nuclei (N) and inflammatory cell infilterations (Ic) (Mathylene Blue stain, X100).

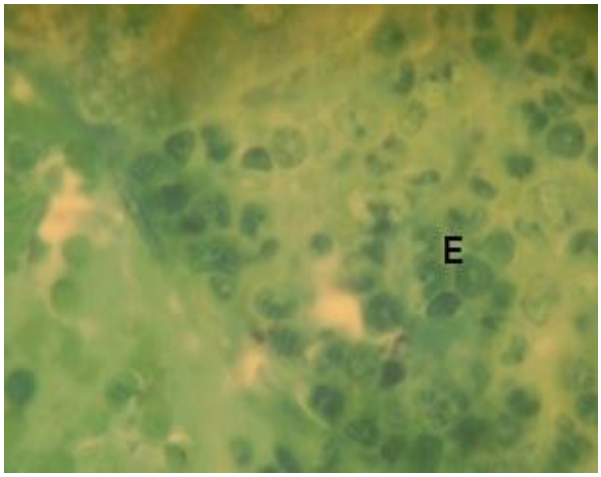

Figure 12. Section of liver tissues of rat after 24 hr from injection of snake venom showing erythrocytes (E) (Mathylene Blue stain, X400).

The present study has indicated that *Naja naja* envenomation causes acute toxic insult to the envenomated rats as a result of metabolic disturbance. More work is needed to illustrate the histochemical and ultrastructural alterations induced by *Naja naja* venom in the liver and other vital organs.

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References

1. Rosenfeld G. Symptomatology, pathology and treatment of snake bites in South America. In: Bëcherl W, Buckley E (eds). Venomous animals and their venoms. 2nd ed. New York: WB Saunders company; 1971. p.273.
2. Kamiguti AS, Theakston RD, Sherman N, et al. Mass spectrophotometric evidence for P-III/P-IV metalloproteinases in the venom of the Boomslang (*Dispholidus typus).* Toxicon. 2000; 38: 1613-20.
3. Li S, Wang J, Zhang X, et al. Proteomic characterization of two snake venoms: Naja naja atra and Agkistrodon halys . Biochem J. 2004; 384(1): 119-27.
4. Shashidharamuthy R, Mahadeswarasamy YH, Raqupathi L, et al. Systemic pathological effects induced by cobra (Naja naja) venom from geographically distinct origins of Indian peninsula. Exp Toxicol Pathol. 2010; 62(6): 587-92.
5. Dineshkumar P, Muthuvelan B. Isolation and Purification of L-amino Acid Oxidase from Indian Cobra Naja naja. Curr Res J Biol Sci. 2011; 3(1): 6-11.
6. Rudrammaji LM, Machiah K, Kantha T, et al. Role of catalytic function in the antiplatelet activity of phospholipase A2 cobra (Naja naja) venom. Mol Cell Biochem. 2001; 219(1-2): 39-44.
7. Binh DV, Thanh T, Chi P. Proteomic characterization of the thermostable toxins from Naja naja venom. J Venom Anim Toxins Incl Trop Dis. 2010; 16(4): 631-8.
8. Chethankumar M, Srinivas L. Gangliosides as potential inhibitors of Naja naja venom PLA2 (NV-PLA2) induced human erythrocyte membrane damage. Afr J Biochem Res. 2008; 2(1): 8-14.
9. Franca R, Veira R, Ferrari E, et al. Acute hepatotoxjcty of Crotalus durissus terrificus (South American rattl snake) venom in rats. J Venom Anim Toxins Incl Trop Dis. 2009; 15(1): 61-78.
10. Kiran K, More S, Gadag JR. Biochemical and clinicopathological changes induced by Bungarus caeruleus venom in a rat model. J Basic Clin Physiol Pharmocol. 2004; 15: 277-87.
11. Awadalla R, Rahmy TR, El-Shamy I. Intraperitoneal envenomation of rats with the LD50 of Echis carinatus snake venom: A Histological and histochemical Study. J Union Arab Biol. 1994; 1: 121-14.
12. Meier J, Theakston RD. Approximate LD50 determinations of snake venoms using eight to ten experimental animals. Toxicon. 1986; 24(4): 395-401.
13. Maria DA, Vassao RC, Ruiz IRG. Haematopoietic effects induced in mice by the snake venom toxin jararhagin. Toxicon. 2003; 42: 579-585.
14. Fox JW, Serrano SM. Exploring snake venom proteomes: multifaceted analyses for complex toxin mixtures. Proteomics. 2008; 8(4): 909-20.
15. Girish KS, Shashidharamurthy RS, Nagaraju TV, Kemparaju K. Isolation and characterization of hyaluronidase a spreading factor from Indian cobra (Naja naja) venom. Biochim. 2004; 86: 193-202.
16. Adzu B, Abubakar MS, Izebe KS, et al. Effect of Annona senegalensis rootbark extracts on Naja nigricollis nigricollis venom in rats. J Ethnopharmacol. 2005; 96(3): 507-13.
17. Rahmy TR, Hemmaid KZ. Histological and histochemical alterations in the liver following intramuscular injection with a sublethal dose of the Egyptian cobra venom. J Nat Toxins. 2000; 29: 21-32.
18. Hanafy MS, Rahmy NA, Abd El-Khalek MM. The dielectric properties of neutron irradiated snake venom and its pathological impact. Med Biol 1999; 44: 2343-64.
19. Abdel Ghani LM, El-Asmer MF, Abbas OA, et al. Histological and Immunohistochemical Studies on the Hepatotoxic Effects of the Venom of Naja Nigricollis Snake on albino mice. Egyp J Natural Toxins. 2009; 6(2): 100-19.

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Adsorption of Glimepiride on Activated Charcoal and Iraqi Kaolin from Aqueous Solution

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Abstract

Background Treatment of acute poisoning due to drug overdose in general depends on the prevention of further absorption of the drug and acceleration of elimination, using specific antidotes. Drugs adsorption is of significant importance in physical pharmacy for the preparation of physical antidotes.

Objective To investigate the adsorption of glimepiride from aqueous solution on two adsorbents (activated charcoal and Iraqi kaolin).

Method UV-Spectrophotometric technique was used to obtain the quantitive adsorption data at different conditions of pH and temperature.

Results The quantities of glimepiride adsorbed on activated charcoal and kaolin were increased with decreasing temperature. Adsorption isotherms of glimepiride on both surfaces were consistent with Freundlich adsorption isotherm. Thermodynamic functions (ΔG, ΔH and ΔS) were useful in describing the spontaneity of the adsorption process.

Conclusion The quantity of the drug that is adsorbed on activated charcoal was higher than that adsorbed on kaolin surface therefore; the activated charcoal is a better antidote.

Keywords Glimepiride, Adsorption, Activated charcoal, Iraqi kaolin

Introduction

Adsorption from solution is rather complicated in its theory due to the influence of solvent molecules and their competition with the solute species to be adsorbed. The porous and finely divided materials enhance adsorption due to their high surface area. Adsorption is usually accompanied by a decrease in free energy change and entropy of the system. This process is influenced by a number of factors such as concentration of adsorbate, surface area of adsorbent, temperature, ionic strength, pH solubility of adsorbate and adsorbent molecules [(1)].Through the citation of literature, it has been noticed that there are so many adsorption studies, some of these studies concentrated on the medical applications of the adsorption particularly in the treatment of poisoning and drug overdose [(1-13)]. These adsorption studies were carried out on different types of adsorbents such as kaolin, activated charcoal, attapulgite, talc, magnesium trisilicate and so many others. However the results of these studies indicated that the activated charcoal was the most active surface employed as adsorbent.

A number of studies were concentrated on the adsorption of heavy metals on clay minerals and oxides [(14)]. Recently an investigation was carried out on the adsorption of trimethoprim on cellulose acetate and attapulgite [(15)].

Kaolin was also used to adsorb toxic substances from the alimentary canal and is used in the treatment of diarrhea associated with food

poisoning [16]. Through the citation of literature it has been noticed also that no work has been done on the adsorption of the drug glimepiride, therefore it has been decided to investigate its adsorption on Iraqi kaolin and activated charcoal at different conditions.

Glimepiride is one of the third generation sulphonylurea, antidiabetic drug which stimulates insulin release. It is used for the treatment of non-insulin-dependent diabetes mellitus. Glimepiride is a constituent of an oral anti-diabetic medicine commercially available as Amaryl tablets. Over dosage of this drug may lead to sever and sometimes life-threatening hypoglycemia and require hospitalization even as a precautionary measure [17].

The aim of this investigation is to assess the abilities of both the Iraqi kaolin and the activated charcoal in adsorbing the over dosage of Glimepiride and to be used as antidote for such diabetic patients that may mistakenly ingest overdose of Amaryl or any other drug that contains Glimepiride.

If (x) mg of a drug is adsorbed from solution on (m) grams of an adsorbent, the extent of adsorption is given by (x/m) or (Qe). Two main theories have been adopted to describe the mechanism of adsorbate- adsorbent interaction.

1. The theory of Langmuir: Adsorption is limited to a monolayer of adsorbate molecules on a homogeneous adsorbent surface [18], (Equation 1):

Ce/Qe = 1/ab + Ce/a…… (1), where (a) and (b) are constants.

(Ce) is the equilibrium concentration of the drug in solution.

Plotting Ce/Qe versus Ce produce a linear relationship if Langmuir's isotherm is valid for the system under investigation.

2. Freundlich theory: It is an empirical equation (2) based on the assumption that the surface possessing heterogeneous adsorption sites of different potential energies and different geometrical shapes. These sites exhibit different affinities towards the same adsorbate molecules [18]

log Qe =log K+1/n log Ce…..(2), where (n) and (k) represent empirical constants.

The study of the temperature effects on adsorption will help also in evaluating the basic thermo-dynamical functions (ΔH, ΔS, ΔG) of the adsorption process.

The importance of these functions is to see the feasibility and spontaneity of the adsorption process through the sign and value of the free energy change (ΔG). The motive behind the spontaneous reaction is the tendency to decrease its free energy to the minimum value.

The enthalpy function (ΔH) is directly related to the electrostatic interaction during the adsorption process, whereas the entropy (ΔS) is a function which can be linked to the mobility of the adsorbate in the adsorption media and can be determined from Gibbs equation [19].

Methods

The instruments used were UV - VIS Spectrophotometer (UV-1800) Shimadzu, thermostated Shaker bath/GFL (D-3006), Germany, pH Meter/HM -73, TDA Electronics Ltd., Centrifuge /eppendorf 5804 R, electronic Balance/Sartorius Lab. BP 3015. The materials used are HCl (GCC) and NaOH (Emscope laboratories Ltd). The drug used was Glimepiride that is obtained from (Zhejiang jiangbei pharmaceutical co., ltd.) Germany.

Glimepiride is a white to yellowish–white powder, crystalline, odorless. Molecular Formula: C24H34N4O5S, Molecular Weight: 490.62.

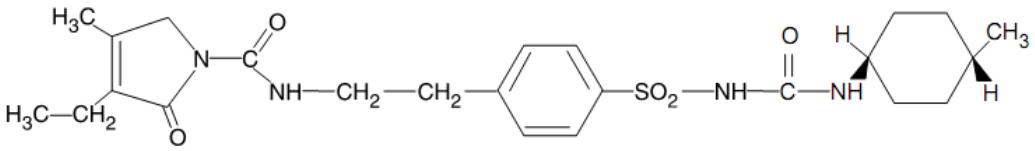

Figure 1. The structural formula of Glimepiride [20]

Adsorbents

Activated charcoal (fluka)

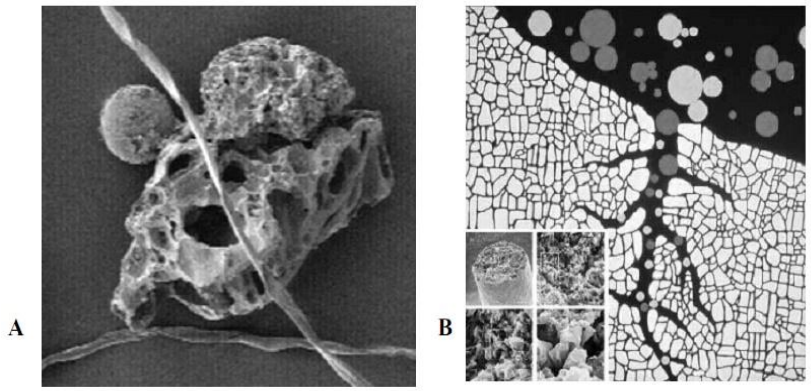

Figure 2. (A): Scanning electron micrograph of a particle of activated charcoal, (B): The pores that tunnel into the actual activated charcoal particles [21]

Iraqi kaolin: was obtained from (Dwaikhla) opened mine (north of Rutba) in the Iraqi Western desert supplied by the ''General Company for Geological Survey and Mining'', Baghdad, Iraq. The weight percentages of the Iraqi kaolin clay were:

SiO_2 (54.68%), Al_2O_3 (30.19%), Fe_2O_3 (1.02%), TiO_2 (1.00%) and loss on ignition (10.94%).

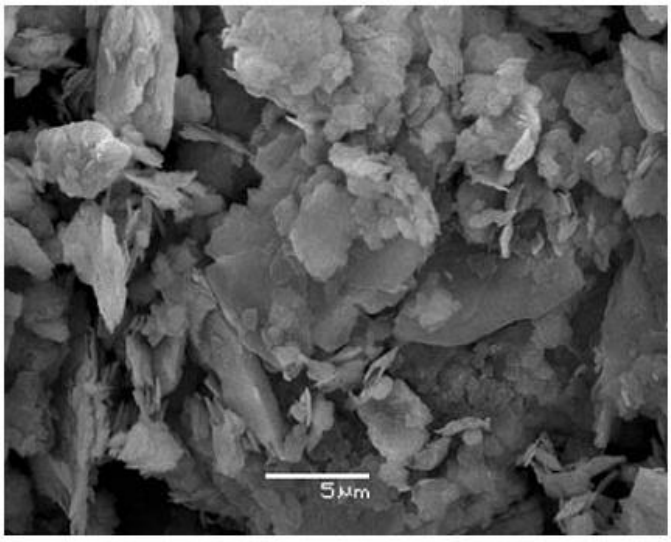

Figure 3. The shape characterization of kaolin [22]

Method

Kaolin and activated charcoal adsorbents were in powder form. Each of them was washed several times with excessive amounts of distilled water then dried at (170 ^{o}C) in the oven for three hours and kept in airtight containers. Each adsorbent was ground and sieved using Retsch test sieve 63µm.

A stock of (50 ml) aqueous solution of glimepiride drug (54 mg L^{-1}) was prepared and its (λmax) was determined. The maximum absorbance (λmax) was (200) nm. Various drug solutions with different concentrations were prepared by diluting the stock solution with distilled water (5.4, 16.2, 27, 37.8 and 48.6 mgL^{-1}).

In order to obtain the calibration curve for aqueous solutions of Glimepiride at pH =1 the absorbance values of these drug solutions were measured at the specific (λmax) using UV-Vis double beam Spectrophotometer and plotted versus the concentrations of these drug solutions (Figure 4).

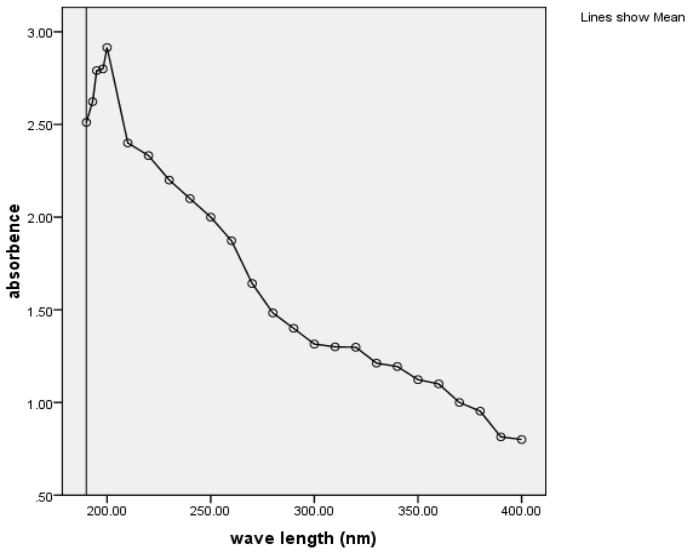

Figure 4. UV Spectra of aqueous solution of Glimepiride at pH=1

The time to reach equilibrium state, that is required for full saturation of adsorbent surface at 37 ^{o}C by the adsorbate has been determined by the following procedure: 50 ml initial concentration (54 mg/L) of adsorbate solution was shaken with (0.1 g) of each adsorbent. The absorbances of adsorbate solutions were measured by UV/Visible spectrophotometer at different intervals 10, 20, 30, 60, 90, 120……minutes until reaching equilibrium (no further uptake of adsorbate by adsorbent as the time proceeds).

A systematic procedure was followed to determine the adsorption isotherms for each pair of adsorbent -adsorbate systems. A volume of (50ml) of five different concentrations of drug solution (5.4, 16.2, 27, 37.8 and 48.6 mg/L) was shaken with (0.1 g) of adsorbent at a certain temperature in a thermostatic shaker. The speed of shaking was 60 cycles per minute. After the equilibrium time (30 min) elapsed, the mixtures were allowed to settle and the clear liquids were either centrifuged at 4000 round per minute (rpm) for 20 minutes or filtered using double filter papers T (whatman No. 42 Germany). The two methods of separation gave identical results. The absorbencies of the filtrate solutions were measured at (λmax). The equilibrium concentrations of the prepared solutions can be determined from the calibration curve using their absorbencies.

The adsorbed amount of the drug was calculated at certain conditions from the concentration of solution before and after adsorption according to equation (3):

$X_m = (C_o - C_e)\ V / m$……..(3), where C_o and C_e are the initial and equilibrium concentrations of drug solution (mg/L) respectively, V is the volume of solution in liter, X_m= the maximum quantity of adsorbate (in mg) that is adsorbed on the adsorbent at certain value of C_e that was fixed for all temperatures used in the study, (m) is the weight of adsorbent in grams.

X_m can be determined from equation (4):

$Q_e = X_m / m$……(4), where $Q_{e=}$ is the quantity of adsorbate (in mg) held by (0.1 g) of adsorbent.

The equilibrium constant (k) for the adsorption process at each temperature is calculated from equation (5):

K = (Qe) (0.1 g)/(Ce) (0.05 L)……(5), where (0.1 g) represents the weight of the clay that has

been used, (0.05 liter) represents the volume of the drug solution used in the adsorption process. The change in free energy (ΔG) could be determined from equation (6):

ΔG = - R T ln k…….(6), where R is the gas constant (8.314 J/mol. deg) and T is the absolute temperature.

The heat of adsorption (ΔH) may be obtained from equation (7):

In X_m = -ΔH/RT + constant …….(7)

The change in entropy (ΔS) can be determined from equation (8):

ΔG = ΔH – TΔS……(8)

Results

Adsorption isotherms

The adsorption isotherms of glimepiride on activated charcoal and kaolin at pH=1 and different temperatures are shown in figures 5 and 7. Figures 6 and 8 show a linear relationship between (log Qe) and (log Ce).

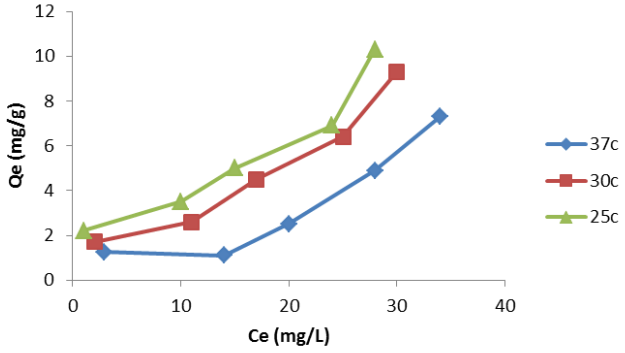

Figure 5. Adsorption isotherms of Glimepiride on activated charcoal at pH=1 and different temperatures (25, 30, 37 °C).

Effect of pH

The adsorption extent of glimepiride on activated charcoal and kaolin investigated at different pH values (1, 4 and 7). It was found that the drug quantity adsorbed increased with increasing pH of the solution. It is clear from figures 9 and 10 that pH does play an important role in the adsorption of glimepiride on activated charcoal and kaolin.

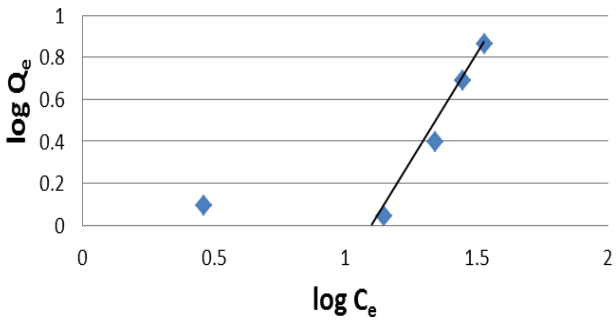

Figure 6. Linear form of freundlich isotherm of glimepiride adsorbed on activated charcoal at 37 °C and pH=1

Temperature effects and thermodynamic parameters

The general shapes of the adsorption of glimepiride on kaolin and activated charcoal at three different temperatures 37, 30 and 25ºc are given in figures 5 and 7. Figures 5 and 7 show also that the adsorption of Glimepiride decreases with increasing temperature.

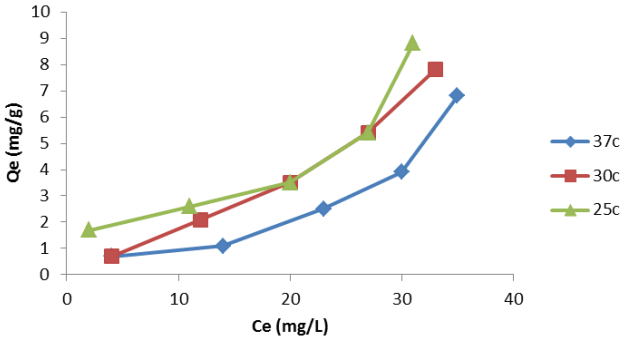

Figure 7. Adsorption isotherms of Glimepiride on kaolin at pH=1 and different temperatures (25, 30, 37 °C)

Table 1 gives X_m values at different temperatures at pH=1. Plotting (In X_m) versus 1000/T produced a straight line with a slope = - ΔH/R as shown in figures 11 and 12). Table 2 shows the basic thermodynamical values of adsorption of Glimepiride on kaolin and activated charcoal.

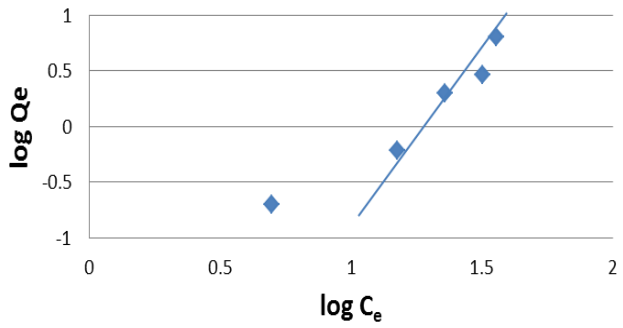

Figure 8. Linear form of freundlich isotherm of glimepiride adsorbed on kaolin at 37 °C and pH=1

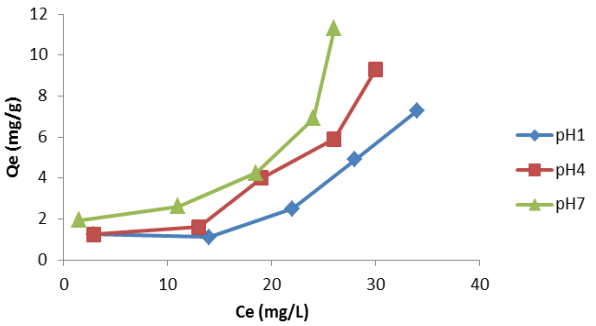

Figure 9. Adsorption isotherms of Glimepiride on activated charcoal at 37 °C and different pH (1,4,7)

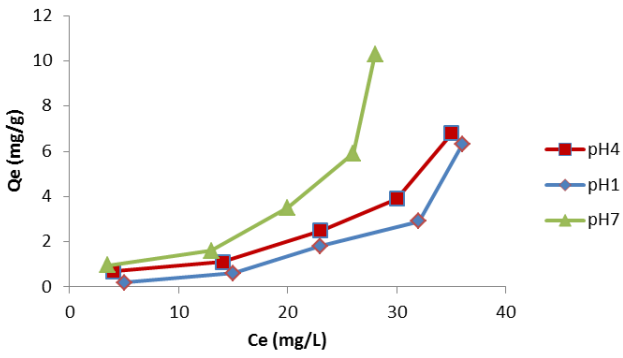

Figure 10. Adsorption isotherms of Glimepiride on kaolin at 37 °C and different pH (1,4,7)

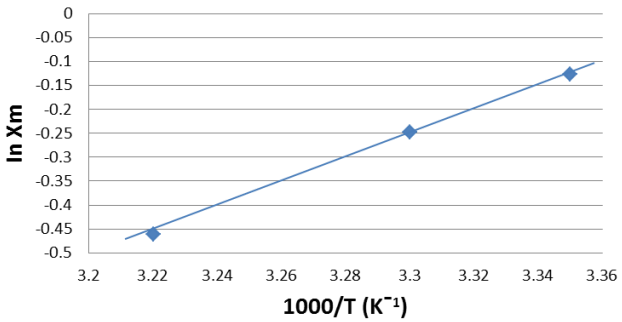

Figure 11. In Xm plotted against reciprocal absolute temperature for the adsorption of glimepiride on kaolin at pH=1

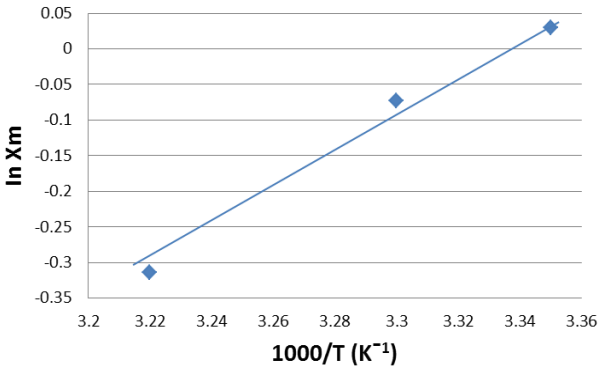

Figure 12. In Xm plotted against reciprocal absolute temperature for the adsorption of glimepiride on activated charcoal at pH=1

Discussion

Adsorption isotherm

The adsorption isotherms describe how adsorbates interact with adsorbents [23]. The relationship between the amount of substance adsorbed at constant temperature and its equilibrium concentration is called the adsorption isotherm [24]. The shape of the adsorption isotherms of Glimepiride on kaolin and activated charcoal was S type of Giles classification as shown in figures 5 and 7. This type of adsorption isotherm indicates a good applicability of freundlich assumptions. This indication can be seen clearly from the linear relationships between (log Qe) and (log Ce), Figures 6 and 8.

Surface imperfection and the presence of impurities can play an important role in the heterogeneity of the surface.

Table 1. Effect of temperature on the maximum adsorbed quantity for adsorption of glimepiride on activated charcoal and kaolin in pH=1

Adsorbent	T. ^{o}C	T. ^{o}K	1000/T $^{o}K^{-1}$	Xm (mg)	In Xm
Kaolin	25	298	3.35	0. 88	-0.1278
	30	303	3.30	0. 78	-0.2484
	37	310	3.22	0.63	-0.4620
Activated charcoal	25	298	3.35	1.03	0.0295
	30	303	3.30	0. 93	-0.0725
	37	310	3.22	0.73	-0.3147

Effect of pH

According to the concept that is known as "diffuse double-layer" the clay particles in aqueous solution are charged and can attract molecules either by electrostatic forces or by inducing dipole formation in the neutral molecule.

In general the pH of the solution affects the degree of ionization of the adsorbate molecules and the extent of dissociation of functional groups on the active sites of the adsorbent [(25)].

The maximum quantities of the drug adsorbed on both adsorbents followed the order: pH 7 > pH 4 > pH 1 (figures 9 and 10). This behavior can be attributed to the changes that may take place in the nature of adsorbent and the changes in the interactions between solvent-surface, solvent-solute and solute-surface species. The changes in these interactions are due to the changes in the degree of dissociation of acidic or basic functional groups of the solute, the solvent and the surface. This in turn affects the polarization of these species and subsequently affects the adsorption extent [(1)]. In addition, the solubility of Glimepiride may increase with decreasing pH because of the increase in the positive charges on the nitrogen atoms of the drug molecules which makes them more soluble and hence decrease the adsorption affinity toward kaolin or activated charcoal surface.

Effect of temperature & pH on thermodynamic parameters

The quantities of Glimepiride adsorbed on activated charcoal and kaolin were decreased with increasing temperature (figures 5 and 7). The increase in temperature may increase the solubility of the solute, hence decreasing its adsorption affinity towards the surface, in addition, to the increase in the kinetic energy of the species. Consequently, there is an increase in the entropy of the system, which results in a decrease of aggregate organization on the surface of the adsorbent.

The negative values of the ΔG for the adsorption of Glimepiride on activated charcoal and kaolin at pH=4 and pH=7 indicated that the adsorption process of Glimepiride is spontaneous, but the positive values of the ΔG at pH=1 in 37, 30 and 25 ^{o}C indicates that the adsorption process is nonspontaneous due to the small values (< 1) of the equilibrium constant. However, even with positive (ΔG) values there were some interactions.

The negative values of the ΔH for the adsorption of Glimepiride on activated charcoal and kaolin at pH=1, pH=4 and pH=7 at 25 ^{o}C indicated that the adsorption was an exothermic process. The positive values of ΔH at pH=1, pH=4 and at 37, 30^{o}C indicated an endothermic reaction, which could be attributed to the enlargement of pore size and/or activation of the adsorbent surface, creating some sort of interactions [(23).]

Table 2. Values of thermodynamic functions for the adsorption of glimepiride on activated charcoal and kaolin at different pH and temperatures

Adsorbent	ΔH(J/ mol)	ΔG(J/ mol)	ΔS(J/ mol k)	pH	Temperature
Activated charcoal	+811.50	+2178.72	-4.41	1	37 °C
Kaolin	+1191.08	+2705.74	-4.88	1	37 °C
Activated charcoal	+183.25	+1204.24	-3.36	1	30 °C
Kaolin	+626.22	+1887.43	-4.16	1	30 °C
Activated charcoal	-72.35	+760.40	-2.79	1	25 °C
Kaolin	+317.27	+1402.52	-3.64	1	25 °C
Activated charcoal	+187.47	+1232.06	-3.36	4	37 °C
Kaolin	+994.21	+2436.30	-4.65	4	37 °C
Activated charcoal	-809.85	-688.46	0.40-	4	30 °C
Kaolin	-985.95	-1116.82	+0.43	4	30 °C
Activated charcoal	-1355.47	-2241.69	+2.97	4	25 °C
Kaolin	-1051.76	-1314.66	+0.88	4	25 °C
Activated charcoal	-314.08	+361.20	-2.17	7	37 °C
Kaolin	-75.42	+791.02	-2.79	7	37 °C
Activated charcoal	-306.97	+353.04	-2.17	7	30 °C
Kaolin	-73.70	+773.15	-2.79	7	30 °C
Activated charcoal	-491.52	-51.08	-1.47	7	25 °C

The negative values of ΔS for the adsorption of Glimepiride on activated charcoal and kaolin at pH=1, pH=4 and pH=7 at 37 °C indicated a decrease in the degree of freedom of the adsorbed species [26]. The positive values of ΔS at pH=4 and at the temperatures 30 and 25 °C might be attributed to the more freedom of motion of the ions in the solution than that attached to the surface [27].

We conclude that Iraqi kaolin showed a moderate ability to adsorb the Glimepiride drug from its aqueous media, however, the activated charcoal showed a better performance, therefore, both adsorbents can be used as antidotes for dealing with a case of drug overdose. Moreover, adsorption isotherms of the drug Glimepiride on Iraqi kaolin and activated charcoal obeyed Freundlich isotherm model. These results indicated the surface heterogeneity of the adsorbents leading to different adsorption strengths from site to site and different affinities towards drug molecules. Furthermore, the drug quantities adsorbed on Iraqi kaolin and activated charcoal at 37 °C showed an increase in adsorption of the drug uptake with increasing pH of the solution. Finally, adsorption of Glimepiride on Iraqi kaolin and activated charcoal decreases with increasing temperature.

References

1. Abd Al-Hussien HK, Jasim SM, Issa SA. Adsorption of some drugs from aqueous solutions on kaolin clay. Iraqi J Med Sci. 2003; 2: 16-26.
2. Tsuchiya T, Levy G. Drug adsorption efficacy of commercial activated charcoal tablets *In vitro* and in man. J Pharmaceut Sci. 1972; 61(4): 624-5.
3. Cordonnier JA, Van den Heede MA, Heyndrickx AM. *In vitro* adsorption of tilidine HCl by activated charcoal. Clin Toxicol. 1986; 24(6): 503-17.
4. Dillon EC, Wilton JH, Barlow JC, et al. Large surface area activated charcoal and the inhibition of aspirin absorption. Ann Emerg Med. 1989; 18(5): 547-52.
5. Adediran GO, Tellaa C, Nwosu FO, et al. *In vitro* Adsorption of Chloroquine Phosphate on Pharmaceutical adsorbents. Centrepoint (Science Edition). 2007; 14(1&2): 31-8.
6. Tella AC, Owalude SO. Some Langmuir and Freundlich Parameters of Adsorption Studies of Chlorpheniramine Maleate. Res J Appl Sci. 2007; 2(8): 875-8.
7. Ganjian F, Cutie AJ, Jochsberger T. Adsorption of Cimetidine onto various pharmaceutical adsorbents. J Pharmaceut Sci. 1980; 69(3): 352-3.
8. Stankovicova M, Bezakova Z. Adsorption of basic esters of phenylcarbamic acid on activated charcoal. Acta Facultatis Pharmaceuticae Universitatis Comenianae. 2005; 52: 194-203.

9. Eboka CJ, Afolabi AB. *In vitro* adsorption of fluoroquinolones on some pharmaceutical adsorbents. Trop J Pharmaceut Res. 2006; 5(1): 533-8.

10. Khah AM, Ansari R. Activated charcoal: preparation, characterization and applications: A review article. Int J Chem Tech Res. 2009; 1(4): 859-64.

11. Decker WJ, Combs HF, Corby DG. Adsorption of drugs and poisons by activated charcoal. Toxicol Appl Pharmacol. 1968; 13(3): 454-60.

12. Roivas L, Neuvonen PJ. Drug adsorption onto activated charcoal as a means of formulation. Methods find Exp Clin Pharmacol. 1994; 16(5): 367-72.

13. Eddy NO, Ebenso EE, Ibok UJ. Adsorption and quantum chemical studies of the inhibitive properties of tetracycline for the corrosion of mild steel in 0.1 H_2SO_4. J Argent Chem Soc. 2009; 97(2): 178-94.

14. Sen TK, Mahajan SP, Khilar KC. Adsorption of Cu^{+2} and Ni^{+2} on iron oxide and kaolin and its importance on Ni^{+2} transport in porous media. Colloids and Surfaces A: Physicochem Eng Aspects. 2002; 211(1): 91-102.

15. Al-Bayati RA, Ahmed AS. Adsorption – Desorption of Trimethoprim Antibiotic Drug from Aqueous Solution by Two Different Natural Occurring Adsorbents. Int J Chem. 2011; 3(3): 21.

16. Onyekweli AO, Usifoh CO, Okunrobo LO, et al. Adsorptive property of kaolin in some drug Formulations. Trop J Pharmaceut Res. 2003; 2(1): 155-9.

17. Kiran T, Shastri N, Ramakrishna S, et al. Surface solid dispersion of Glimepiride for enhancement of dissolution rate. Int J Pharm Tech Res. 2009; 1(3): 822-31.

18. Glasstone S. Textbook of physical chemistry. 2nd ed. London: Macmilan and Co. Limited; 1962.

19. Baba AA, Adekola FA, Ogedengbe AS, et al. Thermodynamic studies of Lead and Cadmium by sphalerite ore in hydrochloric acid. Int J Chem. 2011; 21(3): 173-177.

20. Aventis Pharmaceuticals NJ, a member of the sanofi-aventis Group Bridgewater, NJ 08807 USA www.aventis-us.com., Rev. October 2005. " glimepiride Description".

21. Hattab AMGA. Adsorption of Some Fluoroquinolones on Selected Adsorbents, MSc Thesis, Faculty of Graduate Studies, AL-Najah National University, Nablus, Palestine, 2010.

22. Dohnalova Z, Svoboda L, Sulcova P. Characterization of kaolin dispersion using acoustic and electroacoustic spectroscopy. J Mining Metal. 2008; 44B: 63-72.

23. Sampranpiboon P, Charnkeitkong P. Equilibrium isotherm, thermodynamic and kinetic studies of Lead adsorption onto pineapple and paper waste sludges. Int J Energy Environ. 2010; 4(3): 91.

24. Karthikeyan G, Hang SS. Flouride sorption using morringa indicate-based activated charcoal. Iran J Environ Health Sci Eng. 2007; 4(1): 21-8.

25. Nandi BK, Goswami A, Das AK, et al. Kinetic and Equilibrium Studies on the Adsorption of Crystal Violet Dye using Kaolin as an Adsorbent. Sep Sci Technol. 2008; 43: 1382-403.

26. Maji SK, Pal A, Pal T, et al Adsorption thermodynamics of Arsenic on Laterite Soil. J Surface Sci Technol. 2007; 22(2-4): 161-76.

27. He J, Hong S, Zhang L, et al. Equilibrium and thermodynamic parameters of adsorption of Methylene Blue onto Rectorite. Fresenius Environ Bull. 2010; 19(11a): 2655.

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Pyocin-Based Molecular Typing of Local Isolates of *Pseudomonas Aeruginosa* Isolated from Blood Samples

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Abstract

Background *Pseudomonas aeruginosa* has assumed an increasingly prominent role as the etiological agent in a variety of serious infections in hospitalized patients. *Pseudomonas aeruginosa* strains produce three distinct types of bacteriocins (bactericidal substances). Bacteriocins of *P. aerugonos*a strains can be classified according to their morpology, or according to functions into pore forming pyocin and DNase activity–pyocins.

Objectives To type by a molecular method local isolates of *Pseudomonas aeruginosa* utilizing the genes encoding for the potent bacteriocin (pyocin).

Methods Fifty bacterial isolates of *P. aeruginosa* were re-identified by standard bacteriological methods and were subjected to PCR-amplification for the genes responsible for the production of three bacteriocins (pyocin S1, S2, and S3).

Results Out of the fifty local isolates of *P. aeruginosa* enrolled in this study there were forty five (45) isolates which showed the presence of the genes encoding for the two mentioned bacteriocins (S1 and S2) corresponding to 95% of the isolates and there were forty five (41) isolates showed the presence of the gene encoding for the pyocin S3 corresponding to 82% of the isolates.

Conclusions The present work showed a high genotypic relatedness of the studied clinical isolates of *P. aeruginosa* and it also emphasized the ability of the use of molecular typing of pyocins as more advanced and accurate methods for typing purposes and epidemiological studies.

Key words Pyocins, Bacteremia, bacteriocins, polymerase chain reaction (PCR), electrophoresis

Introduction

Pseudomonas aeruginosa has assumed an increasingly prominent role as the etiological agent in a variety of serious infections in hospitalized patients [(1)]. At particular risk are patients who have suffered major trauma or burns and are exposed to intensive care units [(2)]. Also at risk are normal individuals exposed to a compromising occupational or recreational environment.

Pseudomonas aeruginosa strains produce three distinct types of bacteriocins (bactericidal substances); proteins that have killing activity against strains of the same species. Bacteriocins of *P. aerugonos*a strains can be classified according to their morphology into three distinct types of bacteriocins designated R, F, and S pyocins, or according to functions into pore forming pyocin and DNase activity-pyocins [(3)].

Pyocins are composed of three functional domains, the receptor-binding domain, the translocation domain, and the DNase domain, as reported previously, pyocins S1 and S2 inhibit the lipid synthesis of sensitive cells [(4)].

S-type pyocins (S1, S2, AP41, and S3) cause cell death by DNA breakdown due to an endonuclease activity located at the C-terminal end of the larger component. However these compounds do not express any DNase when it is engaged in the complex [5].

Researchers stated that neither a revised pyocin typing technique nor O-serotyping provides all the requirements of the ideal typing system for P. aeruginosa. O-serotyping provides a rapid indication of antigenic differences when these occur. In an epidemic situation, however, the value of serotyping is limited unless the strains isolated belong to unusual serotypes. Pyocin typing requires a period of 24 h to achieve a result but provides adequate discrimination on which to base more confident epidemiological judgment [6].

Molecular techniques offer a considerable improvement, and can complement phenotypic data to obtain a better understanding of bacterial diversity [8]. PFGE is commonly employed, and has achieved widespread recognition as the 'gold standard' for *P. aeruginosa* DNA typing [9]. However, this method is limited by technical complexity, expense and prolonged turnaround times for results [8]. As an alternative, repetitive-element based PCR (rep-PCR) has shown considerable potential as a DNA typing tool in the laboratory (Lau et al., 1995; Rep-PCR assays utilize primers targeting highly conserved repetitive sequence elements in the bacterial genome. Two such groups of repetitive elements are the enterobacterial repetitive intergenic consensus (ERIC) sequences common to Gram-negative enteric bacteria, and the BOX elements, originally detected in *Streptococcus pneumoniae* [10].

This study was designed to find the frequency of a particular bacterial isolate associated with bacteremia as indicated by new PCR-based typing method for the genes encoding for pyocin S1, S2, and S3 located in the genome of local isolates of *P. aeruginosa*.

Methods

Sampling

This study recruited fifty septicaemic *Pseudomonas aeruginosa* isolates obtained from four sources, eighteen isolates were taken from the department of microbiology, College of Medicine, Al-Nahrain University, thirteen isolates from College of Science, Al-Mustansyria University, nineteen from department of pharmacology, Al-Kindey College of Medicine, Baghdad University, and three isolates from Al-Kadhiymia teaching hospital, Baghdad. These isolates were taken from patients of leukemia, lymphoma, urogenital, gastrointestinal, breast cancers, or from those suffering from burn infections and also from neonates. All of these isolates were collected in a period from February to December 2010. The isolates were subjected to various biochemical tests and standard bacteriological procedure to re-confirm the preliminary identification according to previous work [11].

1. DNA Extraction

The genomic DNA was extracted from bacterial cells using Wizard genomic DNA purification kits (Promega®, USA) and according to the manufacturer's instructions. Agrose gel (1.5%) electrophoresis was adopted to confirm the presence and integrity of the extracted DNA [12].

2. Primer

Primers were purchased from Bioneer® (South Korea) with melting temperatures and PCR product size of 58 ^{o}C; 278 bp and 53 ^{o}C; 541 bp for pyocin S1,S2 and pyocin S3, respectively.

To determine the type of S pyocin produced by different *P. aeruginosa* isolates, certain primers were used to detect the presence of the genes encoding for pyocin S1 and S2 which cannot be detected separately as they share the same antigenic properties, the3 used primers were as follows:

Because pyocins S1 and S2 share the same immunity protein, primers corresponding to the region encoding this protein were chosen [4].

For pyocin S1 and S2 immunity proteins, the following primers were selected:

a. S1S2imm-Fw (5-CACAAGGGAGGGAAGTGA-3).

b. S1S2imm-Rv (5-CGGCCTTAAAGCCAGGAA-3).

1. For pyocin S3 the following primers were selected:

a. RB-fw (5'-CGTATCACGAGACAGGCA-3').

b. RB-rv (5'-TGCCGCTTCTTCCGCTTT-3').

3. PCR Program

The preparatory step for PCR included the addition of 5 µl of the template bacterial DNA onto preloaded master mix eppendorff tubes followed by the addition of 1.5 µl (10 picomol\µl) of the specific primers, the final volume was completed to 20 µl by the addition of distilled water; finally the PCR program for pyocin S1 and S2 was run using the conditions mentioned in table 1.

Table1. Cycling protocol for the PCR amplification of pyocin S1 and S2 gene

Step		Temperature (°C)	Time	No. of cycles
Initial denaturation		94	30 seconds	1
First loop	Denaturation	94	30 seconds	30
	Annealing	528	30 seconds	
	Extension	72	30 seconds	
	Final extension	72	5 minutes	

For amplifying pyocin S3 gene, the primer concentration was 10 picomol\µl and the PCR mixture was prepared as for pyocin S1 and S2 while the cycling conditions run as mentioned in table 2.

Note: Cycling conditions were adopted as trial and error approach relying upon previous study [(4)].

Table2. Cycling protocol for the PCR amplification of pyocin S3 gene

Step		Temperature (°C)	Time	No. of cycles
Initial denaturation		94	30 seconds	1
First loop	Denaturation	94	30 seconds	30
	Annealing	56	30 seconds	
	Extension	72	30 seconds	
	Final extension	72	5 minutes	

4. Agarose Gel Electrophoresis

The preparation of reagents, buffers, stains, and the method of electrophoresing PCR products were prepared and done according to previous work. Agarose at 1.5, 1 grams was dissolve in 100 ml of 1X tris-borate EDTA buffer (or TAE) for PCR product and genomic DNA, respectively. The mixtures were then solubilized by heating with stirring, then they were left to cool at 60°C; ethidium bromide was then added to the mixtures and finally they were poured into the taped plate [(12)].

Results

The results of the current study revealed that all the fifty bacterial isolates (pre-identified as local isolates of *P. aeuginosa*) were re-identified as *P. aeruginosa* because they appeared as gram negative rods, capable of growth at 42°C, oxidase positive, sweet musty odor was produced, and were confirmed to be oxidative but not fermentative when applying oxidation/fermentation test.

The results of this study confirmed that all the 50 isolates enrolled in the study were viable and produced visible growth when activated and

sub-cultured; meanwhile the presence and integrity of their chromosomal DNA were also confirmed using agarose gel electrophoresis (Figure 1).

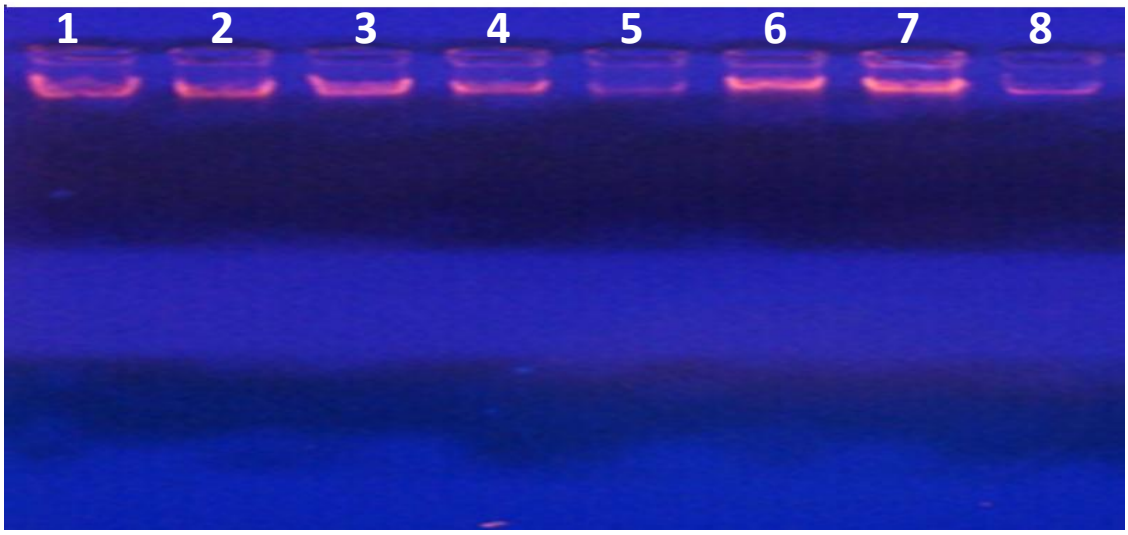

Figure 1. Chromosomal DNA bands on 1% agarose gel at 4V/cm for one hour

The results of PCR amplification for the genes encoding pyocin S1&S2 indicated that out of the fifty local isolates of *P. aeruginosa* enrolled in this study, there were forty five (45) isolates which showed the presence of the genes encoding for the two mentioned bacteriocins (S1 and S2) corresponding to 90% of the isolates (287 bp PCR products) while only five (5) isolates corresponding to 10% of the total fifty (50) isolates were recorded to be negative. The presence of the bands reflecting successful PCR amplifications and the absence of these bands indicated positive and negative results, respectively (figure 2).

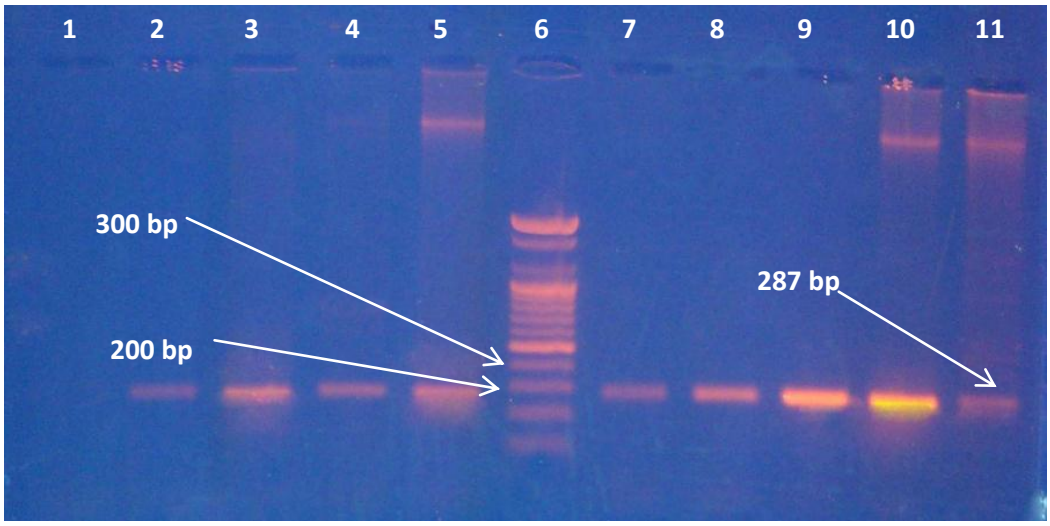

Figure2. Electrophoresis profile of PCR products of pyocin types 1 and 2 found in *P. aeruginosa*. From left to right: lane 2-5 represent the bands of PCR products belonged to four bacterial isolates, lane 6 represents the molecular size marker (100 bp) and lane 7-11 represent the bands of PCR products belonged to five bacterial isolates. Bands run on 1.5% agarose gel.

The results of PCR amplification for the gene encoding pyocin S3 revealed that out of the fifty local isolates of *P. aeruginosa* enrolled in the current study, there were forty one (41) isolates showed the presence of the gene encoding for the pyocin S3 corresponding to 82% of the isolates (415bp PCR products) while only nine (9) isolates corresponding to 18 of the total fifty (50) isolates were recorded to be negative; the positivity and negativity of PCR amplification was evidenced by the presence of the bands and the absence of these bands when amplification failed, respectively (Figure 3).

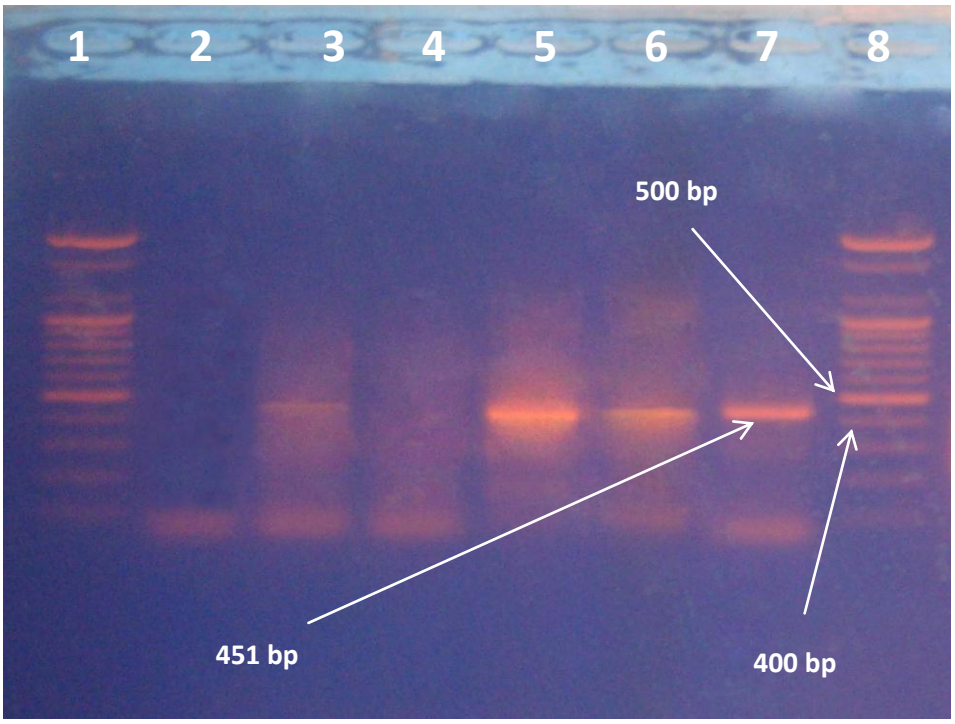

Figure3. Electrophoresis profile of PCR products of pyocin types 3 found in *P. aeruginosa.* From left to right: lane 1 and 8 represent the molecular size marker (100 bp), lanes2-7 represent the bands of PCR products belonged to six bacterial isolates. Bands run on 1.5% agarose gel.

Discussion

P. aeruginosa is an opportunistic pathogen that is able to cause severe invasive diseases in critically ill and immunocompromised patients [13]. It is a common pathogen in hospitals and particularly in intensive care units (ICU) that shows a high phenotypic diversity [14]. Moreover, high rates of resistance to antibiotics and frequent multi-drug resistance (MDR) are associated with nosocomial *P. aeruginosa* strains [15].

Molecular typing demonstrated that most *P. aeruginosa* isolates belonged to distinct genotype, demonstrating again that these methods had a higher discriminatory power than the phenotypic methods (antibiotyping and biotyping) [16].

Apart from discriminatory power, a suitable DNA typing assay must also have a high level of reproducibility, typeability and stability, low complexity and cost, as well as fast result turnaround times [17,18].

In a comparison among different molecular methods for typing bacterial strains, it was agreed that pulse field gel electrophoresis is the golden standard for typing purposes but the cost of PCR- based techniques is less and the shorter hands-on time for the PCR assays means that labour costs were significantly less, and training of personnel in this technology was simpler and more generic, compared to PFGE. The result

turnaround time for the rep-PCR assays was less than 10 h, which was considerably faster than PFGE (4-5 days). Also, as with all PCR-based techniques, the chance of generating artifact rather than detecting true genetic variation is greater if low-stringency PCR conditions are used such as those used in arbitrarily primed-PCR [19]. Rep-PCR assays use highly stringent conditions and therefore are more easily standardized [20]. However, optimization of all parameters of any DNA typing assay is essential to ensure optimal inter- and intra-laboratory standardization [18]. Overall, therefore, rep-PCR assays combine maximum discriminatory power, reproducibility, typeability and stability with cost-effective use of reagents and operator time.

In conclusion, the present work showed a high genotypic relatedness of the studied clinical isolates of *P. aeruginosa*. This finding might lead the way toward better approach for understanding the pathogenicity of the bacterial spp. Isolated from single particular clinical specimens and it may also provide a tool for more accurate typing method for this important nosocomial and opportunistic pathogen as compared to the conventional typing method; the presence of higher percentages of isolates producing pyocin S1,S2, and S3 may explain some the competing mechanisms exhibited by *P. aeruginosa* and this in turn can explain the dominance of a particular isolate in a particular clinical specimens.

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References

1. Neu HC. The role of *Pseudomonas aeruginosa* in infections. J Antimicrob Chemother ll. 1983; (Suppl. B): 1-13.
2. Sherertz RJ, Sarubbi FA. A three-year study of nosocomial infections associated with *Pseudomonas aeruginosa*. J Clin Microbiol. 1983; 18: 160-4.
3. Jachna-Sawicka KG, Bugalski EG. Bakteriocyny Paleczek Rodzaju *Pseudomonas.* Post Mikrobiol. 2005; 44(1): 17-27.
4. Denayer S, Matthijs S, Cornelis P. Pyocin S2 (Sa) kills *Pseudomonas aeruginosa* strains via the FpvA type I ferripyoverdine receptor. J Bacteriol. 2007; 189(21): 7663-8.
5. Michel-Briand Y, Baysse C. The pyocins of *Pseudomonas aeruginosa*. Biochimie. 2002; 84: 499-510.
6. Waite RD, Curtis MA. Pseudomonas aeruginosa PAO1 pyocin production affects population dynamics within mixed-culture biofilms. J Bacteriol. 2009; 191: 1349-54.
7. Ohkawa I, Maruo B, Kageyama M. Preferential inhibitionof lipid synthesis by the bacteriocin pyocin S2. J Biochem (Tokyo). 1975; 78: 213-23.
8. Kageyama M, Kobayashi M, Sano Y, et al. Construction and characterization of pyocin-colicin chimeric proteins. J Bacteriol. 1996; 178: 103-10.
9. Olive PBD. Principles and applications of methods for DNA-based typing of microbial organisms. J Clin Microbiol. 1999; 37: 1661-9.
10. Bertrand X, Thouverez M, Talon D, et al. Endemicity, molecular diversity and colonization routes of *Pseudomonas aeruginosa* in intensive care units. Intensive Care Med. 2001; 27: 1263-1268.
11. Holt JG, Krieg NR, Sneath PHA, et al. Bergey's Manual of determinative bacteriology, 9th (ed.), Awaverly Company: update. J Ant Microbiol Chemother 1994; 46(suppl. S1):1-7.
12. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual. 2nd edition, Cold spring harbor laboratory press, cold spring Harbor, New York, 1989.
13. Fonseca AP, Correia P, Sousa JC, et al. Association patterns of *P. aeruginosa* clinical isolates as revealed by virulence traits, antibiotic resistance, serotype and genotype. FEMS Immunol Med Microbiol. 2007; 51(3): 505-16.
14. Di Martino P, Gagniere H, Berry H, et al. Antibiotic resistance and virulence properties of *P. aeruginosa* strains from mechanically ventilated patients with pneumonia in intensive care units: comparison with imipenem-resistant extra-respiratory tract isolates from uninfected patients. Microb Infect. 2002; 4(6): 613-20.
15. Galvez A, Lopez RL, Abriouel H, et al. Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. Crit Rev Biotechnol. 2008; 28: 125-52.
16. Freitas AL, Barth AL. Typing of P. aeruginosa from hospitalized patients: a comparison of susceptibility and biochemical profile with genotype. Brazilian J Med Biol Res. 2004; 37: 77-82.
17. Tenover FC, Arbeit RD, Goering RV. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. Molecular Typing

Working Group of the Society for Healthcare Epidemiology of America. Infect Control Hosp Epidemiol. 1997; 18: 426-39.

18. Pfaller MA. Molecular epidemiology in the care of patients. Arch Pathol Lab Med. 1999; 123: 1007-10.

19. Tyler KD, Wang G. Tyler SD, et al. Factors affecting reliability and reproducibility of amplification-based DNA fingerprinting of representative bacterial pathogens. J Clin Microbiol. 1997; 35: 339-46.

20. Olive PBD. Principles and applications of methods for DNA-based typing of microbial organisms. J Clin Microbiol. 1999; 37: 1661-9.

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Detection of Nucleophosmin (NPM-1) and FLT3-ITD mutations in 30 Iraqi pediatric acute myeloid leukemia patients

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Abstract

Background Mutation within the FLT 3 and NPM 1 genes ranked within the most frequent recurrent known genetic markers in acute myeloid leukemia (AML) and show apparently opposite prognostic significance.

Objective To detect the frequency of FLT3-ITD and NPM-1 mutations in Iraqi pediatric AML patients using conventional polymerized chain reaction (PCR), and to relate their prevalence with the clinical presentation and the response to induction therapy.

Methods A prospective study of 30 children presented with AML and 16 children who were age and gender matched served as negative control for the mutation. AML cases were classified according to FAB classification. WBC count, platelet count and hematocrit were measured at diagnosis and after 30 days. Molecular analysis was done on peripheral blood or bone marrow aspirate samples by conventional PCR technology.

Results FLT 3-ITD mutation was detected in 3/30 (10%) patients, whereas NPM1 mutation was detected in 4/30 (13.33%) patients. Both mutations were detected in older age patients and predominantly in male. No significant correlation between each mutation and various hematological parameters, however WBC count was significantly higher in FLT-ITD unmutated patients. FLT3-ITD mutation was detected in M3 and M3 variant whereas NPM-1 mutation was detected in M2 and M3v. The three patients having FLT-3-ITD mutation (100%) did not achieved complete hematological remission, whereas 3/24 (12.5%) patients without the mutation did not achieve remission. On the other hand 2 out of 4 (50%) patients with NPM-1 mutation had not achieved complete hematological remission and 4/22 (18.18%) patients without mutation did not achieve remission. Finally regarding the interrelation between the two mutations, the two children who had NPM1 mutation and no FLT3-ITD mutation had achieved complete remission on induction therapy whereas the three children who had FLT3-ITD mutation with or without NPM1 mutation did not achieved complete remission.

Conclusion Prevalence of FLT3-ITD and NPM-1 mutations in Iraqi pediatric AML patients is comparable to that recorded worldwide and both mutations were observed in older age children and mainly in male. FLT3-ITD mutation unlike NPM-1 mutation associate with poor response to induction therapy and the adverse effect of FLT3-ITD mutation overcome the favorable effect of NPM-1 mutation when they exist together.

Keywords Pediatric AML, Flt3-ITD mutation, NPM1 mutation, PCR

Introduction

Acute myeloid leukemia (AML) or acute non lymphocytic leukemia (ANLL) is group of marrow-based neoplasm that has clinical similarities but distinct morphologic, immunophenotypic and cytogenetic features, with wide spectrum of molecular genetic abnormalities [(1)]. In Iraq leukemia ranks the 1st cancer among the commonest ten childhood cancers according to Iraqi Cancer Registry 2008 with an incidence of 32.59%. Moreover, 85% of

Iraqi children with myeloid leukemia fall within the age group 5-14 years. AML constitutes 16.35% of all types of leukemia in all age groups [2]. Childhood leukemia rate have more than doubled over the last 15 years especially in the southern of Iraq and its rate are higher than that found in nearby countries, European Union and United States [3]. Generally AML account for about 15% of childhood leukemia and for approximately 80-90% of acute leukemia in adult [4].

In recent years several recurrent molecular markers were identified that allowed further sub classification and prognostic predictions in the vast majority of AML patients especially those with normal karyotype [5]. Of those markers, FLT3 and NPM1 aberrations were the most frequent genetic lesions and they show apparently opposite prognostic significance [6].

Fms-like tyrosine kinase (FLT3) is a cell surface tyrosine kinase [7] with important role in hematopoietic stem/progenitor cell survival and proliferation [8]. Length mutation/internal tandem duplications with the insertion of hundreds of nucleotides in the juxtamembrane domain-coding sequence of FLT3 gene (FLT3-ITM) has been found in approximately 20-25 % of adult [9] with lower prevalence in pediatric AML worldwide which may reach approximately 12% [10].

Nucleophosmin (NPM) is a molecular chaperone that highly express in proliferating cells than quiescent ones and increase in response to mitogenic stimuli [11]. It has a proliferative and growth suppressive role by maintaining genomic stability, modulate the function of other tumor-suppressor transcription factors, regulate the function and stability of various nuclear proteins and promote the biogenesis of the ribosome [12]. NPM can function both as oncogenes and oncosuppressors.

NPM1 mutation result from insertion or combined insertion and deletion in one allele of NPM1. It is found in about 30 % of all adult AML [13] whereas it is much less common in childhood AML where in many study it occur in 8-12% [14,15]. There is a close association between these two markers, so that the incidence of FLT3-ITD mutation is increased in AML with NPM1 mutation, and they are seen twice as often in this group as compared with AML having wild type NPM1 [16].

Methods

This prospective study was conducted on 46 subjects including 30 children with AML and 16 children with benign reactive bone marrow aspirates which served as technical negative control for the mutation. Those patients were attending the Child Welfare Teaching Hospital from January 2011 to October 2011 and they were referred from different governorates of Iraq. This research was approved by the ethical committee at the College of Medicine, Al-Nahrain University Baghdad-Iraq, and informed consents, were obtained from all participants.

Patients were selected randomly in relation to age and sex. All patients were diagnosed as *de novo* AML and 26 out of 30 patients were newly diagnosed, whereas 4 patients were in relapse; all patients presented at diagnosis and were off treatment whether they were newly diagnosed or in relapse. From each patient and control subject peripheral blood sample and bone marrow (BM) aspirate were collected in 2 EDTA tubes. One ml of peripheral blood was used for analysis of hematological parameters by automated hematology analyzer (Sysmex KX-2N) in Al-Kadhimiya Teaching Hospital Laboratories. Of the 1 ml BM aspirate, 0.5 ml was kept in deep freeze (-70 ^{o}C) until the day of DNA analysis and the other 0.5 ml was equally divided into 2 eppendorff tubes, each contain 1 ml triozl reagent, mixed well and kept in deep freeze at -70 C until the day of RNA analysis. Peripheral blood and bone marrow aspirate smears were prepared and stained with leishman stain and special stains Sudan Black B and Periodic Acid Schiff using the standard procedures for staining [17] and were examined by two hematology consultants for diagnosis of AML and their sub-classification according to French-American-British (FAB) classification. Molecular analysis was performed in the Microbiology

Department/Al-Nahrain Medical College. The induction chemotherapy received by the patients consist of doxorubicine (Adriamycin) and cytosine arabinoside (Ara-C), while patients with M3 subtype received ATRA with daunorubicin and prednisolone. The initial response to induction chemotherapy was assessed in each patient after 30 days whether there is complete hematological remission (CR), treatment failure, or early death. Complete remission was defined as apparent recovery of hematopoiesis with < 5% blast cells on aspirate and near normal peripheral blood counts(hemoglobin > 10.0g/dl, neutrophil counts > 1.5 x10^9/l and platelet count > 100x10^9/l) [18].

For detection FT3-ITD Mutation**,** high molecular weight DNA was extracted according to the kit protocol (Promega) following the instruction manual [19]. All samples were analyzed for FLT3 mutation on chromosome 13, exon 11 using conventional PCR. The use of exon 11 specific primers allowed covering the whole juxtamembrane and the first part of tyrosine kinase-1 domain where most of the reported mutations are located [20].

Fifty to 100 ng of DNA(5 µl)was amplified in a 50 µl reaction mixture containing 1.5 mM MgCl, 50 mM KCl, 200 µM for each deoxyribonucleotide triphosphate (dNTP), 2.5 units Taq polymerase, 40 picomol of each primer which have the following sequences (Forward Primer **11F: 5'-CAATTTAGGTATGAAAGCC-3',** Reverse Primer **12 R: 5'-CAAACTCTAAATTTTCTCT-3'**). A positive reaction was assessed in duplicate and a negative control was included in each reaction. PCR amplification was performed using PCR Thermal cycler (Eppendorf Master cycler, France).Amplification process consisted of 40 cycles of 30 sec at 94 ^oC for denaturation. 45 sec. at 50 ^oC for annealing, 1 minute at 72 ^oC for extension and 1 cycle of 7 minutes at 72 ^oC for the final extension. (20) Twenty µl of the PCR product was electrophoresed on 2.5% agarose gel (Promega), using 100 bp DNA ladder (Promega) as molecular weight marker and was stained with ethedium bromide (Promega).

For detection of NPM1 mutation, total RNA was extracted from bone marrow cells using bioZOLTM-G RNA Isolation Kit (bioWORLD-US) following the instruction manual of the kit [21]. To express different types of NPM1 mutations on chromosom 5 exon 12, Single Strand Confirmatory Polymorphisim-Reverse transcriptase-Polymerase Chain Reaction (SSCP-RT- PCR) was used. Approximately 1 µg of RNA and 5 picomoles of each primer **"NPM-F, 5_-ATCATCAACACCAAGATCA and NPM-R, 5_-CATGTCTGACCACCGCTACT 3_** were added to 0.2 ml lyophilized reaction tube (Single step *Accu power*® RocketScript RT/PCR Premix Kit (BiONEER-Korea) and the volume was completed to 50 µl using nuclease free water. For the negative control tube, nuclease free water was added instead of the template RNA, whereas for the positive control tube 1 µg of RNA extracted from OCI-AML3 cell line was added. The PCR reaction conditions adopted by Brown et al study [12] was applied (table 1) and PCR product was visualized by electrophoresis on 3% agarose gel (Promega, US).

Table 1. PCR reaction for NPM1 mutation analysis

Step	Temperature	Time	Cycles
c DNA synthesis	42 ^oC	60 min	1
Inactivation	95 ^oC	5 min	1
Pre-denaturation	94 ^oC	3 min	1
Denaturation	94 ^oC	45 sec	35
Annealing	57 ^oC	1 min	
Extension	72 ^oC	1 min	
Final extension	72 ^oC	10 min	1

The data were analyzed using SPSS program (Statistical Package for Social Sciences) version 16 and Microsoft Office Excel 2007. Numeric data were expressed as mean±SE or SD, frequency was used to express discrete data. Student T-test was used to analyze numeric data while Chi-square was used to analyze discrete data, and Fischer Exact test was used when Chi square test was not valid. P-value was considered significant when it was less than < 0.05.

Results

In this prospective study both patients and control group were age matched ($P > 0.05$). The age of AML patients ranged between 1.5-12 years, with mean of 6.82±3.78 (Mean ± SD) and male to female ratio was 1:1. Whereas the age range of control children was 1-12 years and the mean age was 6.48±4.64 with male to female ratio of 2.2:1 ($P > 0.05$) .The most common FAB subtype in pediatric AML patients included in this study, was M3 (M3 and M3v) which constitute 43.33% of the cases followed by M2 which constitute 36.67% of the cases, lastly M1 and M5 each was detected in 10% of cases.

FLT3-ITD mutation was detected in 3 out 30 pediatric AML patients (10%), by applying conventional PCR on patients and control cases. The amplified DNA product of the wild type (i.e.) unmutated, was approximately 133 bp band whereas the mutated type showed additional band > 133bp (approximately 180 bp) as shown in figure 1. All the control children enrolled in this study showed wild type FLT3 gene and they served as negative control for the mutation.

NPM1 mutation was found in 4/30 cases (13.33%) by applying Single Strand Confirmatory Polymorphisim-Reverse transcriptase-Polymerase Chain Reaction (SSCP-RT- PCR) on extracted RNA. The mutated cases showed hetero duplex formed from mutant allele and wild type allele, both presented as 2 bands, the first band was approximately 550 bp, whereas the second band was approximately 320 bp. Patients negative for NPM1 mutation and control children did not showed hetero duplex formation (i.e.) they showed wild type; The OCI/AML3 cell line which was used as positive control had showed hetero duplex formation on agarose gel electrophoresis (Figure 2).

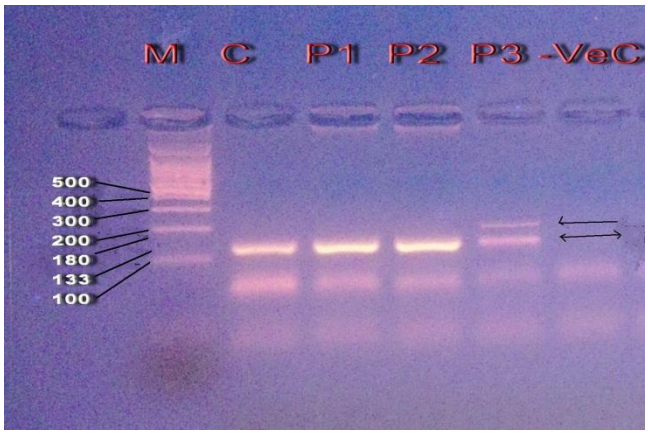

Fig. 1. PCR detected FLT3-ITD mutation. Lane C: Amplified product from healthy control. Lanes P1, P2: amplified product from patients' wild type (app.133 bp, double head arrow). Lane P3: amplified products from patients show extra mutated band (app180 bp arrow) of FLT3-ITD. Lane –VeC: negative control (no template). M: Molecular weight marker (DNA ladder). Electrophoresis was carried in 2.5% agarose gel at (4V/cm) for 60 min.

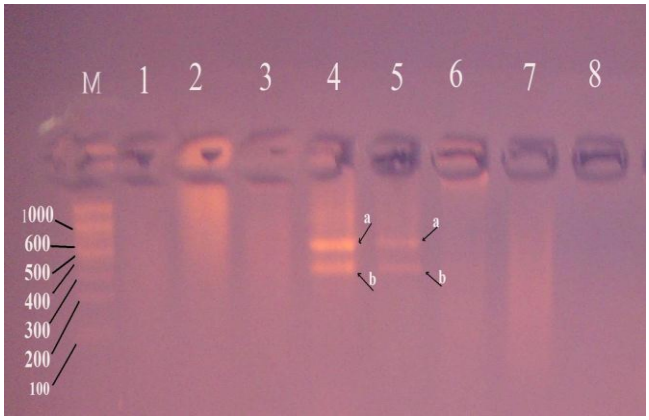

Fig. 2. Detection of NPM1 mutations using Single Strand Confirmatory Polymorphism- RT-PCR in pediatric AML patients. Lanes 1 and 2: control children show absence of hetero duplex formation. Lanes 3, 6, and 7: amplified products from AML children showed absence of hetero duplex formation. Lane 4: positive control OCI/AML3 cell line show hetero duplex formed from mutant and wild type alleles appear as 2 bands (band a app.550 and band b app. 320 bp). Lane 5: mutated AML patients show hetero duplex formation from mutant and wild-type allele of NPM1 gene, arrows a and b. Lanes 8: negative control (no template). M: Molecular weight marker (DNA ladder). Electrophoresis was carried in 3% agarose gel at (4V/cm) for 120 min.

Although equal numbers of male and female were included in the study, the FLT3-ITD mutation was found only in male and NPM1 mutation was predominantly in male M/F ratio 3/1. The mean age of patients was not significantly higher in mutated than non-mutated patients. Regarding FAB classification, the three FLT3-ITD mutated cases were detected only in M3 and M3v, whereas one case carrying NPM1 mutation was detected in M2 and the other three cases were detected in M3 and M3v subtype (Table 2 and 3).

The current study showed that there was no significant correlation between the presence or absence of these two mutations in relation to hematological parameters as shown in table 2 and 3. Although mean peripheral blood and bone marrow malignant cells percent were higher in FLT3 mutated compared to non-mutated patients but both correlations did not reach the level of significance. Also mean peripheral blood and bone marrow malignant cells percent in NPM mutated patients were higher compared to that of non-mutated and this relation was significant in the former but not in the latter. The three FLT3 mutated cases were newly diagnosed and *de novo*-AML cases. To assess the response to induction therapy regardless of the regimen used , the three children with FLT3-ITD mutation (100%) did not achieved complete hematological remission on induction therapy, whereas 4/23 (12.5%) patients without the mutation did not achieved complete hematological remission (Table 2).

Regarding NPM1 mutation, the four mutated patients were newly diagnosed and *de novo*- AML cases. Regardless to the regimen used, 2/ 4 (50%) patients having NPM1 mutation did not achieve complete remission and 4/22 (18.2%) without the mutation did not achieved complete remission (Table 3). Table 4 showed the correlations between the two mutations, it revealed that two patients had both mutation (group 1), two patients had only NPM1 (group 2), one patient had only FLT3-ITD (group 3) and 25 patients did not have any mutations (group 4). Most of these mutations were detected in male (4:1). Moreover 4/5 (80%) of those mutated cases were of M3 subtype. All other hematological parameters were reduced particularly in group 3 whereas peripheral blood and bone marrow malignant cells percent were increased in all AML cases particularly in group 1. The current study revealed that patients harboring FLT3-ITD mutation with or without NPM1mutation, in group 1 and 3 did not achieved response to induction therapy whereas the two patients harboring only NPM1 mutation in group 2 had achieved complete response to induction therapy.

Table 2. Demographic characters and laboratory features of mutated and non-mutated FLT3-ITD pediatric AML patients

Clinical Presentation		FLT3-ITD - ve N = 27	FLT3-ITD +ve N = 3	N	%	*P*-value
Gender	**Male**	12	3	15	50	0.224
	Female	15	0	15	50	
Age (years)		6.72 ± 2.84	9.25 ± 2.07	30	100	0.408
FAB subtype	**M1**	3	0	3	10	
	M2	11	0	11	36.67	
	M3	7	2	9	30	
	M3v	3	1	4	13.33	
	M5	3	0	3	10	
WBC count $X10^9$/L		42.32 ± 10.35	11.55 ± 4.02	30	100	0.039*
Platelet count $X10^9$/L		25.67 ± 5.27	19.00 ± 6.94	30	100	0. 743
Hematocrit %		21.73 ± 1.37	18.00 ± 2.44	30	100	0. 486
Peripheral blood blast %		35.78 ± 5.36	50.50 ± 15.38	30	100	0.491
Bone marrow blast %		56.94 ± 3.80	64 ± 11.42	30	100	0.638
Response*	**Remission**	24	0	24	76.67	0.009*
	Failure	3	3	6	23.33	

* Response to induction therapy

Table 3. Demographic characters and laboratory features of mutated and non-mutated NPM1 mutation pediatric AML

Clinical Presentation		NPM - ve N = 26	NPM +ve N = 4	N	%	P-value
Gender	**Male**	12	3	15	50	0.602
	Female	14	1	15	50	
Age (years)		6.79 ± 0.75	7.00 ± 2	30	100	0.753
FAB subtype	**M1**	3	0	3	10	
	M2	10	1	11	36.67	
	M3	8	1	9	30	
	M3v	2	2	4	13.33	
	M5	3	0	3	10	
WBC count $X10^9$/L		41.35 ± 2.63	27.26 ± 7.79	30	100	0.354
Platelet count $X10^9$/L		24.11 ± 1.14	30.00 ± 12.52	30	100	0. 563
Hematocrit %		21.85 ± 1.80	18.56 ± 1.23	30	100	0. 362
Periphral blood blast %		32.00 ± 1.26	67.00 ± 3.78	30	100	0.037
Bone marrow blast %		56.00 ± 1.96	67.00 ± 3.32	30	100	0.453
Response*	**Remission**	22	2	24	80	0.169
	Failure	4	2	6	20	

* Response to induction the

Table 4: The interrelation between FLT3-ITD and NPM1 mutations in pediatric AML patients

Feature		G1 (NPM+ & FLT3+)	G2 (NPM+ & FLT3-)	G3 (NPM- & FLT3+)	G4 (NPM- & FLT3-)
Number		2	2	1	25
Gender	**Female**	0	1	0	14
	Male	2	1	1	11
Age		11.00±3.00	5.00±2.00	7.50	6.93±0.98
FAB subtype		M3	M2, M3	M3	M1(3), M2(10), M3(9), M5(3)
WBCX10^9/L		16.40±2.50	32.70±2.30	6.70	43.52±4.10
Platelet $X10^9$/L		8.00±3.20	41.00±3.00	30.00	23.75±6.19
Hematocrit (L/L)		21.00±0.22	17.35±0.35	15.00	22.28±1.86
Peripheral blood malignant cells %		73.00±3.00	64.00±4.00	28.00	32.25±6.89
BM malignant cells (%)		78.00±5.00	61.50±6.50	50.00	56.37±5.20
Response*	**Failure**	2	0	1	4

All means were express as mean ± SD

* Response to induction therapy

Discussion

In this study FLT3-ITD mutation was detected in 3 out 30 pediatric AML patients (10%), this result was in agreement with many studies [8,10,22,23], and was in line with Zaker *et al* study from Iran who had reported that the frequency of this mutation in Iranian pediatric AML patients was 7.7% [24]. This study showed that the three patients with FLT3-ITD mutation were male in spite the male:female ratio of the patients included in the study was 1:1, this male predominance was in agreement with many studies and may add an adverse prognostic effect to FLT3-ITD mutation [10,22-25]. Furthermore those patients were older than patients without mutation (p>0.408). Meshinchi *et al* [22] had showed that there was a stepwise increase in the prevalence of FLT3/ITD by age as the prevalence of FLT3/ITD increased from 1.5% in infant AML to 7% for patients aged 1 to 5 years

to nearly 17% in patients aged 10 to 20 years. Such an age-associated increase in prevalence may offer clues to the pathology of FLT3/ITD in AML. FLT3/ITD is considered a cooperating event in the evolution of AML, so that an early molecular event (ex, translocation) may occur in a minor clone leading to maturation arrest. This subpopulation may remain quiescent until such a time when FLT3/ITD is acquired. Such a time-dependent process provides a proliferative advantage and subsequent evolution of AML in the preleukemic clone [22].

FLT3-ITD mutations was detected in M3(M3&M3v) subtype patients, this was in agreement to other studies [10,23-26], however Meshinchi et al [22] and Chang et al [10] had reported that there was no predominance of a particular FAB class in Flt3-ITD + cases. The mean WBC count at the time of diagnosis of those patients with FLT3-ITD mutation was significantly lower than that in patients without mutation, which is expected since FLT3-ITD mutation was detected in M3 and M3v and M3 subtype usually present with lower WBC count, added to this the small sample size [27]. This result differs from that of Meshinchi et al who reported that WBC count at diagnosis was higher in mutated than unmutated cases [22]. Furthermore, the mean malignant cells percent in peripheral blood and bone marrow in patients with FLT3-ITD mutation was non-significantly higher than in patients without this mutation which was in agreement to many studies [22,25]. This was explain by Piacibello *et al* who had propose that FLT3 expression may play a role in the survival or proliferation of leukemic blasts, and that FL (FLT3 Ligand) may induce dose-dependent proliferation of leukemic blasts [28].

Those patients with FLT3-ITD did not achieve remission on induction (0%) as compared to non-mutated cases where 24/30 (80%) had achieved remission, this result was confirmed by other studies [22,23,25] who had stated that the presence of the FLT3/ITD was a significant prognostic factor for induction failure or relapse.

In the current study, NPM1 mutations were found in 4/30 (13.33%) of cases, which was comparable with other studies in which the incidence of NPM1 mutations ranges between 8-12% [12,29-31]. Furthermore the mean age of children harboring NPM1 mutations in this study was higher than those without the mutation (P = 0.753) [12,23], Brown et al had stated that the mutation appears not to occur in children younger than 3 years. On the other hand the incidence of the mutations is high in adult where It ranges between 25.4-41 % as stated by many studies [15,23,31,32]. These epidemiologic data suggest that the risk of acquiring a mutation such as NPMc+ or FLT3/ITD in a myeloid stem/progenitor cell is cumulative and the latency between the acquisition of NPMc or FLT3/ITD and the acquisition of the cooperating mutation(s) for development of AML may be on the order of years. An alternative explanation is that the myeloid stem/progenitor cells in young children are relatively resistant to the acquisition of NPMc+ or FLT3/ITD mutations [12].

In the current study NPM1 mutations were detected mainly in male where male/female ratio was 3/1. Other studies found that there was no significant difference in the prevalence of NPM1 mutations among sexes [29,33]. Whereas, other studies reported that there was a significant presence of NPM1 mutation in female (12, 14).This difference may be due to smaller sample size.

Regarding the frequency of NPM1 mutation within the FAB classification, one of the four mutated children was of M2, two of M3V and one of M3 subtype. Brown et al [12] had stated that NPM1 mutations have been found in all FAB morphologic subtypes of AML but to less extend in M3 and rare in M5. Thus we may propose that the detection of the mutation in M3 and M3v reflect the high no. of M3 and M3v patients (43.33%) included in this study and the small sample size. Regarding the relation of NPM1 mutations to the hematological parameters, children with NPM1 mutations had lower WBC count at diagnosis than that in patients without mutations, (P = 0.354) which was in line with Brown et al (12)who stated that the median white blood cell count at diagnosis did not

significantly differ between NPMc+ and NPMc- cases. The mean platelet count was higher in mutated patients (p = 0.563) which was in line with Rau and Brown et al [34] and Cazzaniga *et al* [29]. Chou *et al* [35], found non-significant difference in the platelet count between mutated and non-mutated patients. Hematocrit % was lower in mutated patients as compared to that in patients without mutation, (P = 0.362) which was expected since three of the mutated cases were of M3 and M3v subtype. This conflicted correlations between mutated and non-mutated patients due to that the exact role of NPMc+ in leukemic transformation is still unknown and more research is needed to clarify how mutated NPM1 promotes leukemogenesis and how it interacts with other mutations, and why it confers a more favorable prognosis [34]. The mean peripheral malignant cell percent was significantly higher in children with mutations than in children with wild type NPM1 (P = 0.037) and the mean BMA malignant cell percent in mutated cases was higher than non-mutated cases (P = 0.453), this result was in agreement with previous studies [12,29,33,34]. The 4 NPM1 mutated pediatric patients were newly diagnosed and *de novo* cases .This result was in agreement with other studies [12,34].

The interrelationship between those two mutations was shown in table 4, Flt3-ITD mutation was detected in 2 out of 4 cases harboring NPM1 mutation , this was similarly observed in many studies [12,31,33,34] which stated that *NPM1* mutations and FLT3 ITD mutations frequently occur together, therefore one might speculate that the two cooperate to cause leukemic transformation. However, a mechanistic link has yet to be established [34,35]. Furthermore both those patients did not achieve early remission on induction therapy which clarifies the adverse effect of FLT-ITD which overcomes the favorable effect of NPM1 mutation [34]. In the current study the two children having NPM1 mutations and no FLT3-ITD mutations had achieved complete remission on induction, this result is supported by Brown *et al* (12) study who had found that within the FLT3/ITD- subgroup, the presence of NPMc+ is associated with a near doubling of 5-year EFS (event-free survival) (from 35-69%), and a greater than 50% increase in5-year OS (overall survival) (from 51-77%) and they had suggested that the NPMc+, FLT3/ITD-negative subset of childhood AML may be prospectively identifiable as a favorable risk group, similar to patients with favorable risk cytogenetics (t(8;21) and inv [16]. The same results were observed by Falini *et al* [31]. Further in the present study , patients who had FLT3-ITD mutation whether they had NPM1 mutation or not , did not achieved remission on induction therapy which clarify the negative prognostic effect of Flt3-ITD mutation within NPM1 + and NPM1- subgroups. This result was supported by study of Rau and Brown (34) Boonthimat *et al* [36] and Brown *et al* [12] who had suggested that there was a possible pathogenic link between these two gene mutations in a way that the FLT3-ITD adverse effect will overcome the favorable effect of NPM1 mutations. Finally within the FLT3/ITD-negative subgroup there was a trend toward improved EFS and 5 years OS, for NPMc positive patients compared with the NPMc negative patients i.e., patients having NPM1c +, had an advantage over those having FLT3-ITD+ or lacking NPM1 [12].

We may conclude that in this pioneer study, the incidence of FLT3-ITD mutations and NPM1 mutations in pediatric AML was 10% and 13.33% respectively in a sample of Iraq patients and both mutations tend to appear in older age group (>3 years). Patients having NPM1 mutation had better response to induction therapy over those harboring FLT3-ITD mutations whether they were NPM1c+ or NPM1 c-.

References

1. Baer MR, Greer JP. Acute myeloid leukaemia in adult. In: Greer JP, Foerster J, Rodgers GM, et al (eds). Wintrobe's Clinical Hematology. 12th ed. Chapter 79. Philadelphia: Lippincott William & Wilkins Publishing; 2009. p. 1843-88.
2. The Iraqi cancer registry 2008: The Commonest Ten Cancers by Site/IRAQ/2008.

3. Hagopian A, Lafta R, Hassan J, et al. Trends in Childhood Leukemia in Basrah, Iraq, 1993-2007. Am J Public Health. 2010 June; 100(6): 1081-7.
4. Appelbaum FE, Gundacker H, Head DR, et al. Age and acute myeloid leukemia. Blood. 2008; 107: 3481-5.
5. Marcucci G, Mrozek K, Bloomfield CD. Molecular heterogenety and prognostic biomarkers in adults with acute myeloid leukemia and normal cytogenetics. Curr Opin Hematol. 2005; 12: 68-75.
6. Lo-Coco F, Cuneo A, Falini B, et al. Prognostic impact of genetic characterization in GIMEMA LAM99P multicenter study for newly diagnosed acute myeloid leukemia. Hematologica. 2008; 93(7): 1017-24.
7. Ksenia B, Ruth S, Tobias M, et al. Mutation in the tyrosine kinase domain of FLT3 defines a new molecular mechanism of acquired drug resistance to PTK inhibitions in FFLT3-ITD transformed hematopoietic cells. Blood. 2004 March; 103(6): 2266-75.
8. Stirewalt DL, Radish JP. The role of FLT3 in hematopoietic malignancies. Nat Rev Cancer. 2003; 3: 650-65.
9. Schittger S, Schoch C, Kern W, et al. Analysis of Flt3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. Blood. 2002; 100: 59-66.
10. Chang P, Kang M, Xiao A, et al. FLT3 mutation incidence and timing of origin in a population case series of pediatric leukemia. BMC Cancer. 2010; 10: 513-9.
11. Pier P, Di F. Playing both sides: nucleophosmin between tumor suppression and oncogenesis. J Cell Biol. 2010; 182(1): 7-9.
12. Brown P, McIntyre E, Rau R, et al. Childhood AML. The incidence and clinical significance of nucleophosmin mutation in childhood AML. Blood. 2007; 110: 979-85.
13. Haferlach C, Mecucci C, Falini B, et al. AML with mutated NPM1 carrying a normal or aberrant karyotype show overlapping biological. Pathological immunophenotypic, and prognostic features. Blood 2009; 114: 3024-32.
14. Hollink IH, Zwaan CM, Thiede C, et al. Favorable prognostic impact of NPM1 gene mutation in childhood acute myeloid leukemia with emphasis on cytogenetically normal AML. Leukemia. 2009; 23: 262-70.
15. Thied C, Creutzig E, Reinhardt D, et al. Different type of NPM1 mutations in children and adults: evidence for an effect of patients' age on the prevalence of the TCTG-tendem duplication in NPM1 exon 12. Leukemia. 2007; 21: 266-7.
16. Wertheim G, Bagg A. Nucleophosmin (NPM1) mutation in acute myeloid leukemia: an ongoing (cytoplasmic) tale of dueling mutations and duality of molecular genetics, testing methodologies. J Mol Diag. 2008 May; 10(3): 198-202.
17. Lewis SM, Barbare BJ. Preparation and staining methods for blood and bone marrow films. In: Lewis SM, Bain BJ, Bates I (eds). Dacie and Lewis practical hematology. 10th ed. Philadelphia: Churchill Livingstone Company; 2006. p. 60-77.
18. Cheson BD, Bennett JM, Kopecky KJ, et al. Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. J Clin Oncol. 2003; 21: 4642-9.
19. Promega Notes. *PCR* Amplification. http//www. Promega .de. (access 10 Oct. 2011)
20. Dina Y, Iman S. Internal Tandem Duplication of FLT3 Gene in Egyptian Pediatric Acute Myeloid Leukemia and Acute Lymphoblastic Leukemia. J Egyp Nat Cancer Inst. 2003 March; 15(1): 17-23.
21. Kadhim ED, Abd Rasak M. High Frequency of Nucleophosmin mutations in thirty two Iraqi adult patients with Acute Myeloid leukemia. Inter J Appl Sci Technol. 2012 May; 2(5): 97.
22. Meshinchi S, Alonzo T, Stirewalt D, et al. Clinical implications of FLT3 mutations in pediatric AML. Blood. 2006 Dec; 108(12): 3654-61.
23. Kadhim ED. Detection of FLT3 and NPM1 mutations in Iraqi AML patients. Thesis submitted to the Council of college of Medicine/Al-Nahrain University in partial fulfillment of the requirement for the degree of PhD in hematology, 2012.
24. Zaker F, Sheykhi M,Mohammadi M, et al. Diagnosis and frequency of FLT3 mutations in pediatric and adult Acute leukemic patients with different subtypes. Inter J Hematol Oncol Stem Cell Res. 2009; 3(4): 27.
25. Yassin D, Sidhom I. Internal Tandem Duplication of FLT3 Gene in Egyptian Pediatric Acute Myeloid Leukemia and Acute Lymphoblastic Leukemia. J Egyp Nat Cancer Inst. 2003 March; 15(1): 17-23.
26. Liang D, Shih L, Hung I, et al. Clinical relevance of internal tandem duplication of the FLT3 gene in childhood acute myeloid leukemia. Cancer 2002; 94(12): 3292-8.
27. McKenna RW, Parkin J, Bloomfield CD, et al. Acute promyelocytic leukaemia: a study of 39 cases with identification of a hyperbasophilic microgranular variant. Br J Haematol. 1982; 50: 201-14.
28. Piacibello W, Fubini L, Sanavio F, et al. Effects of human FLT3 ligand on myeloid leukemia cell growth: heterogeneity in response and synergy with other hematopoietic growth factors. Blood. 1995; 86: 4105-14.
29. Cazzaniga G, Dell"Oro MG, Mecucci C, et al. Nucleophosmin mutations in childhood acute myelogenous leukemia with normal karyotype. Blood. 2005; 106: 1419-22.

30. Pasqualucci L, Liso A, Martelli MP, et al. Mutated nucleophosmin detects clonal multilineage involvement in acute myeloid leukemia: impact on WHO classification. Blood. 2006; 108: 4146-55.
31. Falini B, Martelli M, Bolli N, et al. Acute myeloid leukemia with mutated nucleophosmin (NPM1): is it a distinct entity. Blood. 2011; 117: 1109-20.
32. Suzuki T, Kiyoi H, Ozeki, et al. Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. Blood. 2005; 106: 2854-61.
33. Braoudaki M, Papathanassiou C, Katsibardi K, et al. The frequency of NPM1 mutations in childhood acute myeloid leukemia. J Hematol Oncol. 2010; 3: 41-6.
34. Rau R, Brown P. Nucleophosmin (NPM1) Mutations in Adult and Childhood Acute Myeloid Leukemia: Towards Definition of a New Leukemia Entity. Hematol Oncol. 2009 Dec; 27(4): 171-81.
35. Chou WC, Tang JL, Lin LI, et al. Nucleophosmin mutations in de novo acute myeloid leukemia: The age dependent incidence and stability during disease evolution. Cancer Res 2006; 66: 3310-6.
36. Boonthimat C, Thongnoppakhun W, Auewarakul CU: Nucleophosmin mutation in the Southeast Asian acute myeloid leukemia: eight novel variants, FLT3 coexistence and prognostic impact of NPM1/FLT3 mutations. Hematologica. 2008; 93: 1565-9.

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Finasteride (plus Oral Contraceptive pill) *vs* Metformin in Treatment of Polycystic Ovary Syndrome-Related Infertility: a Prospective Randomized Trial

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Abstract

Background Polycystic ovary syndrome (PCOS) is a common endocrinopathy characterized by oligo-ovulation or anovulation, signs of androgen excess, and multiple small ovarian cysts. PCOS is thought to be one of the leading causes of female subfertility.

Objective To evaluate and compare the effects of finasteride *vs* metformin in treatment of PCOS related infertility.

Methods Seventy seven infertile married women with an age range between 18 and 35 years were studied complaining from infertility due to PCOS. They were divided into group 1 treated with finasteride (5 mg daily concomitantly with an oral contraceptive pill "OCP" continuously for 2 months) and group 2 was treated continuously for 3 months with metformin (500 mg three times daily).

Results The percentage of patients responded to metformin treatment was 35.89%, whereas 26.32% of patients were responded to the treatment with finasteride-OCP combination. There were no significant difference between metformin and finasteride in regard to the mean number of mature follicles (1.21±0.43 *vs* 1.2±0.42) and endometrial thickness (7.26±1.1 *vs* 7.80±2.25 mm) respectively. The pregnancy rate per patient was higher in metformin treated group in comparison to finasteride treated group (60% *vs* 21.42%); however, this difference was insignificant ($P > 0.05$).

Conclusion Finasteride has a good promising effect in the treatment of infertility due to PCOS, as more patients responded to an oral finasteride-OCP combination in comparison to those responded to an oral metformin monotherapy and the difference in the pregnancy rate of the two groups was not significant.

Keywords Finasteride, Metformin, Polycystic ovary syndrome, Infertility

Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders that affects approximately 5-10% of pre-menstrual women [1,2], and it a leading cause of infertility in these women [3,4]. PCOS is a syndrome with unknown etiology, characterized by hyper-androgenism which may be clinical (particularly hirsutism and acne) and/or biochemical (hyperandrogenemia), and chronic anovulation [2,5]. However, PCOS includes a wide spectrum of signs and symptoms (obesity, polycystic ovary), pathology, and laboratory findings. Studies have shown that women with PCOS are frequently insulin resistant and at increased risk of developing glucose intolerance or non-insulin-dependant diabetes mellitus in the third and fourth decades of their life [2,6].

Hyperinsulinemia lead to hyper production of ovarian androgens because insulin like Luteinizing hormone (LH) stimulates directly the ovarian biosynthesis of steroid hormones, in

particular, of ovarian androgens [7]. Furthermore, insulin decreases the sex-hormone-binding globulin (SHBG) production in the liver, thus, further elevating free androgen levels [8-10]. Therefore, both pathways end in the stimulation of ovarian theca cells with elevated ovarian androgen production, resulting in disturbed folliculogenesis, cycle disorders and chronic anovulation [11]. It is therefore probable that women develop PCOS because of a hypersensitivity of their intra-ovarian insulin androgen signaling pathway [12].

Metformin is one of insulin-sensitizing agents, its an oral biguanide used for type 2 diabetes mellitus, is a safe and effective drug that can be used for the treatment of PCOS patients [10,13,14], to induce ovulation in anovulatory PCOS patients [15,16]. In-vitro culture had shown that metformin has a significant inhibitory effect on androgen production by ovarian cells [17,18] also it lowers LH concentration, reduces total testosterone and raises SHBG levels, producing a decrease in the free testosterone index [19,20].

Drugs that block male hormones can protect women with PCOS from developing diabetes, heart attacks, obesity and masculinizing traits such as hirsutism, acne, and large muscles and bones and that progesterone can protect them from uterine cancer [21-23], also lower cholesterol [24] and help the eggs to pop from the ovaries [25,26]. Reduction of ovarian androgen production not only improves ovulation and pregnancy rates, but also reduces spontaneous abortion rates [27].

Finasteride, is a potent 5alpha-reductase (5α-R) inhibitor, has been approved by Food and Drug Administration (FDA) for treatment of androgenetic alopecia in men [28]. The largest application of finasteride consists in treating benign prostate hyperplasia, in women finasteride has been used in some control trials for treatment of hirsutism [29], because of its teratogenicity [30] finasteride should be used a contraceptive. Finasteride is a preferential, competitive inhibitor of the intracellular, 5α-R isoenzyme type II which converts testosterone into dihydrotestosterone (DHT), a more potent androgen. The high loss rate experienced by women with PCOS is partly due to compromised oocyte quality, but may also be due to the compromised uterine perfusion that occurs as a result of elevated androgen levels. Correction of androgen status clearly results in a decrease in the spontaneous abortion rate in these individuals [27].

The intention of the study is to evaluate and compare the effects of finasteride *vs* metformin in treatment of PCOS related infertility.

Methods

Women included in this study had been assessed clinically regarding regularity of the menstrual cycle, body mass index (BMI) [calculated using the equation: [weight (kilograms)/height (meters)^2], duration and type of infertility and presence or absence of hirsutism.

Luteinizing hormone, Follicle stimulating hormone (FSH), LH:FSH ratio, total testosterone, Thyroid stimulating hormone (TSH), prolactin and fasting blood sugar levels were measured at day 2 (early follicular phase) of cycle. Diagnosis of PCOS was based on the revised 2003 consensus on diagnostic criteria and long-term health risks related to PCOS.

All patients enrolled in the study fulfilled the following criteria:

Inclusion criteria: (1) patients who had diagnosed as PCOS in the presence of at least 2 of Rotterdam criteria, based on Rotterdam consensus meeting 2003 [23]. (2) The patients were unable to achieve pregnancy in a period of last 12 months or more despite regular unprotected intercourse. (3) The patients had patent Fallopian tubes proved by hysterosalpingography. (4) The husband infertility evaluation by an urologist doctor revealed no abnormalities in the male side. (5) No history of heart, liver, or kidney disease, and unsuspected pregnancy.

Exclusion criteria: (1) Patient aged more than 35 years. (2) History of recent administration of hormonal therapy. (3) Male factor infertility.

Seventy seven married women in the reproductive age (18-35 years), who had diagnosed to have a PCOS, were included in this prospective study, all were complain infertility due to PCOS. For the amenorrhoeic women among those included in this study, 10 mg dydrogesterone oral tablets daily for 10 days was used to induce withdrawal bleeding. Two months washout period was used to eliminate the effect of any post-treatment in women who had received any treatment before enrolment in this study.

Women included in this study were classified into two groups, 38 women received 500 mg metformin oral tablets three times daily for 3 months and 39 women received 5 mg finasteride oral tablets daily continuously for 2 months, finasteride has been received concomitantly with an OCP consisting of 2 mg cyproterone acetate + 0.035 mg ethinyloestradiol oral tablets, OCP was received daily starting from day 5 of the menses. TVS examination has done at day 12 of the menstrual cycle after 3 months of treatment with metformin, regarding finasteride US has done at day 12 of the third menstrual cycle in which finasteride and OCP have been withdrawn (drug free cycle).

Post treatment the primary outcome measures were the number and size of the growing and mature follicles and endometrial thickness (ET) by monitoring with TVU at day 12 of the menstrual cycle.

Good response was achieved when at least one mature follicle becomes 17 mm in diameter and the patients were advised to have timed intercourse every other day, starting at least 24 hours after the leading follicular diameter reached 17 mm in size.

The secondary outcome measure was the occurrence of pregnancy. Chemical pregnancy was assessed by measurement of ß-HCG (human chorionic gonadotropin) in blood after at least 3 days of miss period and clinical pregnancy by detection of fetal heart beat on sonography after 6-7 weeks of missed period.

Miscarriage rate was determined only in finasteride group because follow up for some women in metformin group had been lost.

Statistical Analysis

SPSS version 17.0 was used for the statistical analysis. ANOVA, chi-square and Fisher exact tests were use when appropriate. P- Values less than 0.05 were considered as statistically significant.

Results

Table 1 shows number of women and their clinical and hormonal characteristics on which the diagnosis of PCOS was determined according to Rotterdam criteria.

Table 2 shows post treatment results with finasteride and metformin including number of women responded to the treatment, means of number and size of mature follicles, number of mature follicles (1 or 2) per women, also value of endometrial thickness and pregnancy rate per patient.

Discussion

Effect of Metformin

Metformin is a very important component of the PCOS treatment [32]. The low percentage of responded patients (26.31%) in the present study disagrees with that found by Palomba *et al* [33] study in which it was (55%) and with that found by Ashrafi *et al* study [34] in which ovulation had occurred in (65.7%) patients after 8 weeks of treatment with metformin. This disagreement may be related to the period of therapy used by the present study which was relatively short or it may be attributed to the presence of clomiphene (CC)-resistance since it had been shown that metformin cause no improvement in insulin resistance in CC-resistant PCOS patients with normal glucose tolerance, and also has no significant effect on ovarian response [35]. Regarding the percentage of patients in whom mono-follicle had developed (80%) and those in whom 2 mature follicles had developed (20%) among patients treated with metformin, up to knowledge studies that deal

with number of mature follicles after treatment with metformin were unavailable.

Table 1. Clinical and hormonal characteristics of the patients pretreatment with finasteride and metformin

Variable	Finasteride N = 39	Metformin N = 38	*P* value
Age (years)	25.10 ± 5.25 (18 - 35)	24.41 ± 4.06 (18 - 35)	> 0.05
Duration of infertility (Years)	2.64 ± 0.44	3.34 ± 0.54	> 0.05
Primary infertility No. (%)	24/36 (66.66)	26/35 (74.28)	> 0.05
Secondary infertility No. (%)	12/36 (33.33)	9/35 (25.71)	> 0.05
Irregular cycle No. (%)	39/39 (100)	36/37 (97.29)	> 0.05
Regular cycle No. (%)	0/39 (0)	1/37 (2.70)	> 0.05
BMI	30.4 ± 5.2	32.2 ± 6.4	> 0.05
Present Hirsutism No. (%)	35/37 (94.6)	29/35 (82.9)	> 0.05
Absent Hirsutism No. (%)	2/37 (5.4)	6/35 (17.1)	> 0.05
Positive US No. (%)	36/38 (94.73)	35/37 (94.59)	> 0.05
Negative US No. (%)	2/38 (5.26)	2/37 (5.40)	> 0.05
LH (mIU/ml)	6.90 ± 4.24	6.63 ± 4.57	> 0.05
FSH (mIU/ml)	5.60 ± 1.43	4.67 ± 1.73	> 0.05
LH/FSH ratio	1.28 ± 0.772	1.52 ± 1.014	> 0.05
Testosterone (ng/ml)	0.99 ± 0.95	0.99 ± 0.95	> 0.05
PRL (ng/ml)	23.38 ± 10.49	19.45 ± 12.33	> 0.05
TSH (mIU/ml)	1.67 ± 0.83	2.46 ± 1.21	> 0.05
FBS (mmol/L)	4.56± 0.4	4.82± 0.9	> 0.05

Table 2. Post treatment results with finasteride and metformin

Variables	Treatment Groups		*P* value
	Finasteride N = 39	Metformin N = 38	
*Responded patients No. (%)	14 (35.89)	10 (26.31)	> 0.05
No. (%) of patients with 1 MF	11 (78.57)	8 (80)	> 0.05
No. (%) of patient with 2 MF	3 (21.42)	2 (20)	> 0.05
Mean No. of mature follicle ± SD	1.21 ± 0.43	1.2 ± 0.42	> 0.05
Mean size of mature follicle ± SD	20.26 ± 3.66	18.75 ± 1.82	> 0.05
ET Mean ± SD (mm)	7.26 ± 1.1	7.80 ± 2.25	> 0.05
Pregnancy Rate / Patient (%)	3/14 (21.42)	6/10 (60.0)	> 0.05

* = Resulted in one or more mature follicles ≥ 17 mm; ET = endometrial thickness

Endometrial thickness measured at day 12 of menstrual cycle after 3 months of treatment with metformin was (7.8±2.25) in the present study and was comparable to (8.2 mm) that found by Sohrabvand *et al* [36] who measured endometrial thickness (ET) at the day of hCG administration in women received letrozole (2.5 mg) after an initial treatment for 6-8 weeks with metformin, while it disagrees with (5.5 mm) found by the same study when CC was combined with metformin instate of letrozole. Up to our knowledge there were no enough studies to which result of ET in the present study could be compared.

In the present study the pregnancy rate per patient in metformin group is in agreement with Heard *et al* and Palomba *et al* studies [17,37] which were (69%) and (69%) respectively, also it goes with a meta-analysis of more than a dozen

randomized clinical trials that found metformin superior to placebo [38].

The high rate of pregnancy in metformin group may be attributed to the small number of patients responded to metformin treatment, or the PCOS was less severe, thus these patients became pregnant despite short term of therapy.

While it disagrees with that found by Tang *et al* and Palomba *et al* studies [39,40] which were (23%) and (20%) respectively, and with that found by Ashrafi *et al* [34] study which was (20%) despite high ovulation rate (65.7%) after 8 weeks of treatment with metformin the disagreement with Ashrafi *et al* may be due to large number of the responded patients (since ovulation rate in Ashrafi *et al* was 65.7% while the percentage of the responded patients (produced at least one mature follicle) in the present study was small (26.31%). Also it disagrees with that found by Palomba *et al* and Ortega *et al* studies [33,41] which were (19%) and (16.7%) respectively over 6 months of treatment with metformin, this disagreement may be attributed to the differences in numbers of total patients and that of the responded patients, as ovulation rate was (55%) in Palomba *et al* [33] study and number of total patients was 18 in Ortega et al. study [41]. Also it disagrees with Legro *et al* study [42] in which pregnancy rate was (8.7%) over 6 months of treatment with metformin.

The high pregnancy rate (60%) of the present study population is consistent with the hypothesis that insulin resistance and/or hyperandrogensim play an important role in the pathogenesis of anovulation in patients with PCOS [17-19,34].

An evidence of decreased insulin sensitivity is seen in both lean (30%) and obese (75%) women [6,43]. It had been confirmed that metformin does not act on BMI but does appear to act on hirsutism and acne and induce the onset of regular cycles [18,38, 44,45].

What have been found by the above studies is in agreement with our study. In the present study the mean of BMI of 4 out of 6 (66.66%) patients who were became pregnant on metformin was 25.68 (range 21.66-27.89), while it was 32.95 for the other 2 (33.33%) patients, this result agrees with other previous studies [18,38,44,45], and this proves the activity of metformin in obese as well as in non obese women which agrees with Ashrafi *et al* [34] who found that BMI and LH level had no significant effect on response to metformin and it's comparable to that of Baillargeon [32] who found that up to 90% of thin women with PCOS ovulated in the six months after initiating metformin treatment.

The response obtained by the present study during the short-moderate term therapy (3 months) with metformin goes with Nestler *et al* [46] who found that by day 35, 34% of women received metformin had ovulated, compared with only 4% of the placebo-treated women, also it goes with Ashrafi *et al* [34] who found that after 8 weeks of metformin monotherapy, ovulation occurred in (65.7%) patients and (20%) patients became pregnant, in regard to the present study 3 pregnant women among those who got pregnancy on metformin they became pregnant after 1.5-2 months of treatment with metformin. Result of the present study in regard to metformin confirms what have been found by Ehrmann [47] who appeared that normal menstrual cycles achieved within 3 months of starting treatment in some groups of patients.

High pregnancy rate (60%) after 3 months of treatment with metformin may be attributed to the reduction in serum insulin level [34]. The correlation between hyperandrogenism and insulin resistance had been recognized in both obese and nonobese anovulatory PCOS women [48,49]. Acien *et al* and Meirow *et al* reported that approximately 10% of non obese PCOS patients could present with insulin resistance [50,51].

Result of the present study agrees with the United Kingdom's National Institute for Health and Clinical Excellence which recommended metformin's usage for PCOS women those whom BMI was above 25 when other therapy had failed [52], while it disagrees with the subsequent randomized control trials [53,54] which in general

not shown the promise suggested by the early observational studies.

Effect of Finasteride

Testosterone is converted into DHT by the enzyme 5α-R. DHT is a more powerful androgen than testosterone as it has a much higher affinity for the androgen receptor [55,56]. Finasteride is an inhibitor of 5a-R by being an aza analog of testosterone, thereby initially binding to 5α-R similarly to testosterone [57]. Up to knowledge the role of finasteride in the treatment of PCOS related infertility had been investigated only by one study done by Tartagni *et al* [58], who add finasteride to conventional protocol of ovarian stimulation with gonadotropin and found that finasteride can improve ovarian follicular growth and ovulation in PCOS women who did not respond to previous stimulation with gonadotropin alone. In regard to finasteride activity in the treatment of PCOS related infertility, result of the present study confirms what had been found by Tartagni *et al* [58].

In comparison to metformin treated group the pregnancy rate in finasteride treated group was low (60% *vs* 21.43%) that may be explained by higher activity of metformin due to its ability to decrease insulin resistant which in turn decrease androgen production, while finasteride is an inhibitor of steroid 5α-R, the enzyme that converts testosterone to the more potent androgen DHT [55,56].

The higher percentage of patients who responded to finasteride comparing to those responded to metformin (35.89% *vs* 26.31%) may be related to the time needed by the mechanism through which metformin decrease androgen production as it first should decrease insulin resistance for which more time may be needed, while finasteride decreases androgenic activity through direct inhibition of 5α-R.

Finasteride has well-documented risk for teratogenicity in male fetuses, and adequate contraception should be used [30]. Thus the lower rate of pregnancy in finasteride group in comparison to that in metformin group (21.43% *vs* 60%), may be related to the presence of OCP which necessarily had received concomitantly with finasteride.

Reduction of ovarian androgen production not only improves ovulation and pregnancy rates, but also reduces spontaneous abortion rates. The high loss rate experienced by women with PCOS is partly due to compromised oocyte quality, but it may also be due to the compromised uterine perfusion that occurs as a result of elevated androgen levels [27] and this may explain why there were no any miscarriage in finasteride group as the correction of androgen status is clearly results in a decrease in the spontaneous abortion rate in PCOS pregnant women [30].

The three pregnancies obtained by finasteride treatment were ended with full term deliveries, this result may be attributed to the reduction in androgen level as found by Ajoss et al. (2002) [27] study which had demonstrated that reduction of ovarian androgen production not only improves ovulation and pregnancy rates, but also reduces spontaneous abortion rates and the high loss rate experienced by women with PCOS is partly due to compromised oocyte quality, but may also be due to the compromised uterine perfusion that occurs as a result of elevated androgen levels.

Up to knowledge at least one miscarriage had occurred among the 6 patients who became pregnant on meformin therapy, the usefulness of metformin in reducing miscarriages and improving pregnancy outcome is in doubt [59]. No occurrence of any miscarriage among the patients who became pregnant on finasteride may be attributed to the higher activity of finasteride in comparison to meformin to improve the compromised uterine perfusion rather than to its activity to improve the compromised oocyte quality which occurs as a result of elevated androgen levels [27].

In conclusion, treatment with finasteride combined with an OCP for 2 months had a good promising effect in the treatment of PCOS related infertility in comparison to metformin as the responded patients were higher in finasteride group and although the rate of

pregnancy was higher in metformin group the difference was not significant ($p > 0.05$).

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References:

1. Polson DW, Worth WJ, Adams J, et al. Polycystic ovarian a common finding in normal woman. Lancet. 1988; 1: 870-2.
2. Franks S. Polycystic ovarian syndrome. N Engl J Med. 1995; 333: 853-61.
3. Goldenberg N, Glueck C. Medical therapy in women with polycystic ovarian syndrome before and during pregnancy and lactation. Minerva Ginecol. 2008; 60(1): 63-75.
4. Boomsma CM, Fauser BC, Macklon NS. Pregnancy complications in women with polycystic ovary syndrome. Semin Reprod Med. 2008; 26(1): 72-84.
5. Dunaif A. Insulin resistance and ovarian dysfunction. The Endocrinologist. 1992; 2: 248-60.
6. Dunaif A, Graf M. Insulin administration alters gonadal steroid metabolism independent of changes in gonadotropin secration in insulin-resistant women with polycystic ovary syndrome. J Clin Invest. 1989; 83: 23-9.
7. Eisenhardt S, Schwarzmann N, Henschel V, et al. Early Effects of Metformin in Women with Polycystic Ovary Syndrome: A Prospective Randomized, Double-Blind, Placebo-Controlled Trial. J Clin Endocrinol Metab. 2006; 91: 946-952.
8. Barbieri Rl, Smith S, Ryan KJ. The role of hyperisulinemia in the pathogenesis of ovarian hyperandrogenism. Fertil Steril. 1988; 50: 197-212.
9. Plymate Sr, Matej La, Jones Re. Inhibition of sex hormone-binding globulin production in human hepatoma (Hep G2) cell line by insulin and prolactin. J Clin Endocrinol Metab. 1988; 67: 460-4.
10. Paquali R, Gambineri A, Biscotti D, et al. Effect of long term treatment with metformin added to hypocaloric diet on body composition, fat distribution, and androgen and insulin levels in abdominally obese women with and without the polycystic ovary syndrome. J Clin Endocrinol Metab. 2000; 85: 2767-2774.
11. Cleemann L, Lauszus FF, Trolle B. Laparoscopic ovarian drilling as first line of treatment in infertile women with polycystic ovary syndrome. Gynecol Endocrinol. 2004; 18: 138-43.
12. Baillargeon J, Nestler J. Polycystic ovary syndrome: a syndrome of ovarian, hypersensitivity to insulin? J Clin Endocrinol Metab. 2006; 91(1): 22-4.
13. Kashyap S, Wells GA, Rosenwaks Z. Insulinsensitizing agents as primary therapy for patients with polycystic ovarian syndrome. Hum Reprod. 2004; 19: 2474-83.
14. Costello MF, Eden JA. A systematic review of the reproductive system effects of metformin in patients with polycystic ovary syndrome. Fertil Steril. 2003; 79: 1-13.
15. Defronzo RA, Barzilai N, Simonson DC. Mechanism of metformin action in obese and lean non-insulin dependent diabetic subjects. J Clin Endocrinol Metab. 1991; 73: 1294-301.
16. Stumvoll M, Nurjhan N, Perriello G, et al. Metabolic effect of metformin in non- insulin dependent diabetes mellitus. N Engl J Med. 1995; 333: 550-4.
17. Heard MJ, PierceA, CarsonSA, et al. Pregnancies following use of metformin for ovulation induction in patients with polycystic ovary syndrome. Fertil Steril. 2002; 77(4): 669-73.
18. Mansfield R, Galea R, Brincat M, et al. Metformin has direct effects on human ovarian steroidogenesis. Fertil Steril. 2003; 79: 956-62.
19. Barbieri RL. Metformin for the treatment of polycystic ovary syndrome. Obstet Gynecol. 2003; 101: 785-93.
20. Nestler JE, Jakubowicz DJ. Decreases in ovarian cytochrome P450C17-alpha activity and serum free testosterone after reduction of insulin secretion in polycystic ovary syndrome. N Engl J Med. 1996; 335: 617-23.
21. Berga SL. The obstetrician-gynecologist's role in the practical management of polycystic ovary syndrome. Am J Obst Gynecol. 1998; 179(6) Part 2: S109-S113.
22. Guzick D. Polycystic ovary syndrome: Symptomatology, pathophysiology, and epidemiology. Am J Obst Gynecol 1998; 179(6) Part 2: S89-S93.
23. Taylor AE. Understanding the underlying metabolic abnormalities of polycystic ovary syndrome and their implications. Am J Obst Gynecol. 1998 Dec; 179(6) Part 2: S94-S100.
24. Diamantikandarakis E, Mitrakou A, Raptis S, et al. The effect of a pure antiandrogen receptor blocker, flutamide, on the lipid profile in the polycystic ovary syndrome. J Clin Endocrinol Metab 1998; 83(8): 2699-705.
25. Falsetti L, Defusco D, Eleftheriou G, et al. Finasteride (5 mg daily) or flutamide (259 mg twice daily) for 6 consecutive months. Treatment of hirsutism by finasteride and flutamide in women with polycystic ovary syndrome. Gynecol Endocrinol. 1997 Aug; 11(4): 251-7.
26. Deleo V, Lanzetta D, Dantona D, Lamarca A, Morgante G. Hormonal effects of flutamide in young women with

polycystic ovary syndrome. J Clin Endocrinol Metab. 1998 Jan; 83(1): 99-102.
27. Ajoss S, Guerriero S, Paoletti AM, et al. The antiandrogenic effect of flutamide improves uterine perfusion in women with polycystic ovary syndrome. Fertil Steril. 2002; 77: 1136-40.
28. Sinclair R: Male pattern androgenic alopecia. BMJ. 1998; 317: 865-9.
29. Cilotti A, Danza G, Serio M. Clinical application of 5alphareductase inhibitors. J Endocrinol Invest. 2001; 24: 199-203.
30. Swiglo BA, Cosma M, Flynn DN, et al. Clinical review: Antiandrogens for the treatment of hirsutism: a systematic review and meta analyses of randomized controlled trials. J Clin Endocrinol Metab. 2008; 93: 1153-60.
31. The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod. 2004; 19: 41.
32. Baillargeon J-P. Oral presentation. The Endocrine Society. San Francisco 2002.
33. Palomba S, Orio F, Nardoo L et al. Metformin Administration versus Laparoscopic Ovarian Diathermy in Clomiphene Citrate-Resistant Women with Polycystic Ovary Syndrome: a Prospective Parallel Randomized Double-Blind Placebo-Controlled Trial. J Clin Endoc Metab. 2004; 89(10):4801-4809.
34. Mahnaz A, Fatemeh Z, Reza BA. Effects of Metformin on Ovulation and Pregnancy Rate in Women with Clomiphene Resistant Poly Cystic Ovary Syndrome. Royal Inst Iranian J Fertil Steril. 2007; 1(1): 39-42.
35. Yarali H, Yildiz BO, Demirol A, et al. Coadministration of metformin during rFSH treatment in patients with clomiphene citrate-resistant polycystic ovarian syndrome: a prospective randomized trial. Hum Reprod. 2002; 17(2): 289-94.
36. Sohrabvand F, Ansari S, Bagheri M. Efficacy of combined metformin-letrozole in comparison with metformin-clomiphene citrate in clomiphene-resistant infertile women with polycystic ovarian disease. Hum Reprod. 2006; 21: 1432-5.
37. Palomba S, Orio F Jr, Falbo A, et al. Prospective parallel randomized, double-blind, double-dummy controlled clinical trial comparing clomiphene citrate and metformin as the first-line treatment for ovulation induction in nonobese anovulatory women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2005; 90: 4068-74.
38. Lord JM, Flight IHK, Norman RJ. Metformin in polycystic ovary syndrome: systematic review and meta-analysis. BMJ. 2003; 327(7421): 951-6.
39. Tang T, Lord JM, Norman RJ, et al. Insulin-sensitising drugs (metformin, rosiglitazone, pioglitazone, D-chiro-inositol) for women with polycystic ovary syndrome, oligo amenorrhoea and subfertility. In: The Cochrane Library, Issue 4. Chichester, UK: John Wiley & Sons Ltd. 2009.
40. Palomba S, Pasquali R, Orio F, et al. Clomiphene citrate, metformin or both as first-step approach in treating anovulatory infertility in patients with polycystic ovary syndrome (PCOS): a systematic review of head-to-head randomized controlled studies and meta-analysis. Clin Endocrinol (Oxf). 2009; 70: 311-21.
41. Ortega-Gonzalez C, Luna S, et al. Responses of serum androgen and insulin resistance to metformin and pioglitazone in obese, insulin-resistant women with polycystic ovary syndrome. Clin Endocrin Metab. 2005; 90(3): 1360-5.
42. Legro RS, Barnhart HX, Schlaff WD. Clomiphene, Metformin, or Both for Infertility in the Polycystic Ovary Syndrome. N Engl J Med. 2007; 356 (6): 551–66.
43. Conway GS, Jacobs HS, Holly JMP, et al. Effects of luteinizing hormone, insulin, insulin-like growth factor-1 and insulin-like growth factor small binding protein-1 in the polycystic ovary syndrome. Clin Endocrinol. 1990; 33: 593-603.
44. Gerard C. The use of metformin in the polycystic ovary syndrome. The Middlesex Hospital, Mortimer Street London W1N 8AA January 2000.
45. Genazzani AD, Battaglia C, Malavasi B, et al. Metformin administration modulates and restores luteinizing hormone spontaneous episodic secretion and ovarian function in non-obese patients with polycystic ovary syndrome. Fertil Steril. 2004; 81: 114-9.
46. Nestler JE, Jakubowicz DJ, Evans WS, et al. Effects of metformin on spontaneous and clomiphene-induced ovulation in the polycystic ovary syndrome. N Engl J Med. 1998; 338: 1876-80.
47. Ehrmann DA. Polycystic ovary syndrome. N Engl J Med 2005; 352(12):1223-1236.
48. The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. Fertil Steril. 2005; 81: 19-25.
49. González F, Rote N, Minium J, et al. Reactive oxygen species-induced oxidative stress in the development of insulin resistance and hyperandrogenism in polycystic ovary syndrome. J Clin Endocrinol Metab. 2006; 91(1): 336-40.
50. Acien P, Querada F, Matallin P, et al. Insulin, androgens, and obesity in women with and without polycystic ovary syndrome: a heterogeneous group of disorders. Fertil Steril. 1999; 72:32-40.
51. Meirow D, Yossepowitch O, Rosler A, et al. Insulin resistant and non-resistant polycystic ovary syndrome represent two clinical and endocrinological subgroups. Hum Reprod. 1995; 10: 1951-6.
52. National Institute for Health and Clinical Excellence. Clinical guideline 11: Fertility: assessment and treatment for people with fertility problems. London 2004.

53. Balen A. Metformin therapy for the management of infertility in women with polycystic ovary syndrome (PDF). Scientific Advisory Committee Opinion Paper 13. Royal College of Obstetricians and Gynaecologists 2008. http://www.rcog.org.uk/files/rcog-corp/uploaded-files/SAC13metformin_inorrevision.pdf. Retrieved 2009-12-13.

54. Leeman L, Acharya U. The use of metformin in the management of polycystic ovary syndrome and associated anovulatory infertility: the current evidence. J Obstet Gynaecol. 2009; 29(6):467-72.

55. Snyder PJ. Androgens. In: Brunton LL, Lazo JS, Parker KL (eds). Goodman and Gilman's - The pharmacological basis of therapeutics. 11th ed. USA: McGraw-Hill Companies, Inc.; 2006. p. 1573-85.

56. George P. The Gonadal Hormones and Inhibitors. In: Katzung BG, Masters SB, Trevor AJ (eds). Basic and Clinical Pharmacology 11th ed. McGraw-Hill; 2009. p. 699-726.

57. Robaire B, Henderson N. Actions of 5α-reductase inhibitors on the epididymis. Mol Cell Endocrinol. 2006; 250(1-2): 190-5.

58. Tartagni M, Cicinelli E, De Pergola G, et al. Effect of finasteride on ovulation induction in nonresponder (hyperandrogenic) polycystic ovary syndrome (PCOS) women. Fertil Steril. 2010; 94(1): 247-9.

59. Mathur R, Alexander CJ, Yano J, et al. Use of metformin in polycystic ovary syndrome. Am J Obstet Gynecol. 2008; 199(6): 596-609.

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Evaluation of Pre-Operative Ultrasound Findings in Predicting Difficulties in Laparoscopic Cholecystectomy for Acute Cholecystitis

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Abstract

Background Pre-operative prediction of difficulties which may occur during laparoscopic cholecystectomy can help in reduction of operative and postoperative complications.

Objectives To study the value of preoperative ultrasound findings for predicting difficulties encountered during laparoscopic cholecystectomy and to assess the usefulness of these findings to identify patients at high risk of conversion from laparoscopic to open cholecystectomy.

Methods A prospective study of 200 patients who underwent laparoscopic cholecystectomy for symptomatic cholelithiasis. Pre-operative abdominal ultrasound was done. The diagnosis of gall stones was made and the presence of ancillary findings was recorded. Five ancillary ultrasound findings were assessed. These included; thickened gall bladder wall more than 4mm, presence of pericholecystic fluid, severely contracted gall bladder, empyma, and gall bladder filled with stones. Ultrasound findings were compared with the operative findings.

Results In 36 patients who had one or more of these findings laparoscopic Cholecystectomy was difficult in 22(61.1%) of them. Thick wall gall bladder > 4mm has the highest sensitivity (69%) and the presence of pericholecystic fluid has the highest specificity (100%) in predicting difficult laparoscopic cholecystectomy and the presence of more than 2 ancillary findings yielded an accuracy rate of (100%). Conversion to open cholecystectomy was needed in 13.9% of these patients. The rates of difficult laparoscopic cholecystectomy and conversion to laparotomy were much lower in those patients who had no ancillary findings (4.3%) and (1.2%) respectively.

Conclusion Preoperative ultrasound findings are of value for predicting difficulties encountered during laparoscopic cholecystectomy which may require conversion to open cholecystectomy.

Keywords Laparoscopic surgery, Cholecystectomy, Ancillary ultrasound findings.

Introduction

Laparoscopic cholecystectomy is now considered the gold standard procedure for symptomatic gallstone disease [(1)] and one of the most frequently performed procedures in surgery. Laparoscopic cholecystectomy has substituted traditional cholecystectomy due to a more comfortable postoperative period than the open approach [(2)]. The increasing experience with laparoscopic cholecystectomy has led to an expansion of the indications for this procedure, a reduction in contraindications of the procedure, and more complex cases being operated laparoscopically. The definition of difficult laparoscopic cholecystectomy is in consistent. The term difficult cholecystectomy refers "to multiple" technical intra-operative difficulties that increase the risk for complications and significantly prolong the operating time [(3,4)]. Although most patients will also benefit from the

laparoscopic approach, difficult cases are at a higher risk for conversion and the resulting complications that may overshadow all advantages of the laparoscopic procedure, making this approach unsafe, uneconomic, inefficient, and hence possibly inferior to traditional open cholecystectomy [5,6]. Reliable predictive factors for difficult procedures and hence conversion of laparoscopic cholecystectomy would be extremely useful in the preparation and planning of management for patients with symptomatic cholelithiasis [7-9].

Methods

This is a prospective study of 200 consecutive patients who underwent laparoscopic cholecystectomy for symptomatic gall bladder stones during the period from October 2005 to March 2007 in Baghdad Teaching Hospital in Baghdad by two surgeons. Exclusion criteria included patients with suspicion of choledocholithiasis. Preoperative workup included a complete history and physical examination and routine laboratory and radiological tests for all patients. Ultrasound examination for all patients was carried out in Baghdad Teaching Hospital by one sonography expert one day before operation, using a high resolution ultrasound machine (3.5MHz, curvilinear probe, G 50 Siemens). The patients fasted for 6-hours before the examination. The initial scan was performed in the right subcostal region during suspended inspiration. Scans were performed both along the long axis and at right angles to the long axis of the gallbladder. When necessary right lateral intercostal scans were performed along the length of the intercostal spaces. A further alternative approach was to lay the patients completely on their left side and to perform anterior sub costal scans. Care was taken to ensure that the entire volume of the organ is examined and that even a tiny calculus not missed. A special care was given to record any ancillary finding including; thick gall bladder wall, contracted gall bladder, presence of pericholecystic fluid, gall bladder filled with gall stones, and finally if the clinical and ultrasound findings would suggest empyma of the gall bladder. The study approved by local research ethics committee. Informed consent was taken from the patients. The procedure of laparoscopic cholecystectomy and the risk of conversion were explained to them, with a special emphasis for those patients with a higher risk of conversion. All patients received 1 gm Cefotaxime i.v. at time of induction of anesthesia and another 2 doses at 8 and 16 hours postoperatively. All operations performed under general anesthesia by standard North American laparoscopic technique [10, 11]. Intra-operative cholangiography was not carried out in any of the procedures. A closed drain was used when indicated. The preoperative ultrasound findings compared with intra-operative findings. The operations which were not converted to open cholecystectomy, graded as difficult or easy operations according to the period between insertion of the ports and clipping of the cystic duct and artery. When this period is less than 30 minutes the operation regarded as easy otherwise it regarded as difficult. The data was collected retrospectively and analyzed to see if the results are statistically significant by applying P-value (if P-value is 5 or less, it will be considered statistically significant) [12].

Results

The study comprised 200 patients. The age ranged between 16 to79 years (mean was 35). One hundred sixty one patients were females and 39 were males. The male to female ratio was (1:4). There was no mortality in the study. All patients had surgically proven gall stones, thus sensitivity, specificity, and accuracy of ultrasound in diagnosis of gall stones were100%. Thirty six patients (18%) had additional ultrasound findings that presumed to be of significance in predicting difficult laparoscopic cholecystectomy and conversion to open cholecystectomy. These ancillary findings are shown in table -1. Laparoscopic cholecystectomy was easy in 164 (82%) patients and difficult in 29 (14.5%) patients, while in 7 (3.5%) patients the

procedure had to be converted to open cholecystectomy.

In patients who didn't have ultrasound ancillary findings, the procedure was easy in 155 (94.5%), difficult in only 7 (4.3%) cases, the reasons of difficulty were; severe adhesions (n=3), morbid obesity (n=2), liver cirrhosis (n=l) and uncontrolled intra-operative bleeding (n=l), while conversion rate was 1.2% i.e. only in two patients and it was because of severe adhesions (Figure 1). Those with no ultrasound findings 162 had Laparoscopic cholecystectomy and 2 ended with open surgery.

Table 1. Ancillary ultrasound findings

Ancillary findings	No. of patients
Thickened gall bladder wall >4mm	26
Presence of pericholecystic fluid	8
Severely contracted bladder	2
Empyema	2
Gall bladder filled with stones	8

The patients may have > one finding

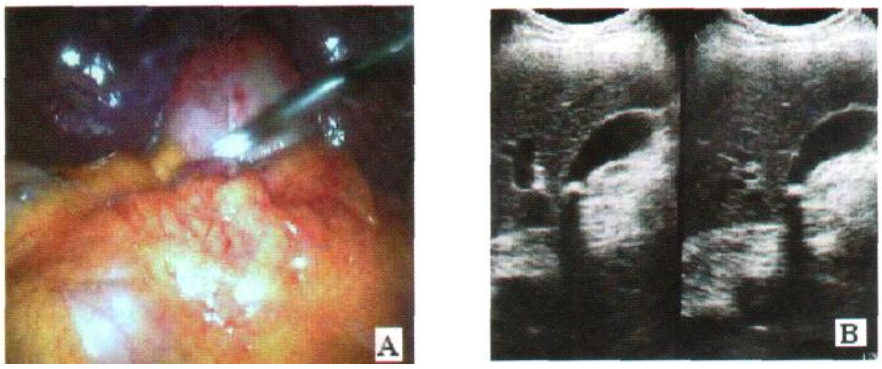

Figure 1. A) Laparoscopic photo of a patient who had severe adhesions. B) The preoperative ultrasound of the same patient showed neither, ancillary findings nor any indication of a difficult procedure.

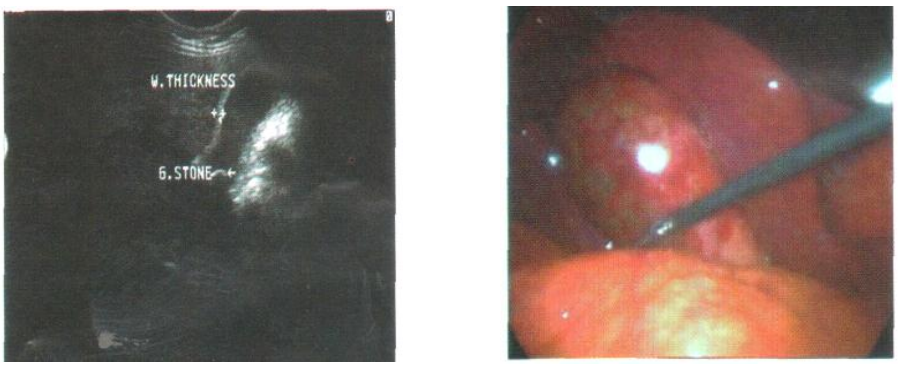

Figure 2. (a, and b.) Ultrasound and laparoscopic photo of a patient who had acute cholecystitis with a thick wall gall bladder

In patients who had ancillary ultrasound findings, the rate of difficult laparoscopic cholecystectomy was 61.1% and conversion rate was 13.9%. These results were much higher and statistically significant when compared with the results of patients who had no ancillary ultrasound findings (P > 0.05) (Table-2).

Table 2. Ultrasound Findings related to difficult laparoscopic cholecystectomy and rate of conversion to open cholecystectomy.

Laparoscopic cholecystectomy	Without ancillary U/S	With ancillary U/S	Total
Easy	155 (94.5%)	9 (25%)	164 (82%)
Difficult	7 (4.3%)	22 (61.1)%	29 (14.5%)
Conversion to open cholecystectomy	2 (1.2%)	5 (13.9%)	7 (3.5%)
Total	164 (82%)	36 (18%)	200 (100%)

Gall bladder wall thickness was an important predicting factor of difficult laparoscopic cholecystectomy. Fifteen of 26 patients with wall thickness greater than 4mm had difficult operation (Figure 2), thus the sensitivity of wall thickness greater than 4mm in predicting difficulty was 69%, and specificity 96.5% (Table 3).

Table 3. Value of Ultrasound findings in predicting difficult Laparoscopic cholecystectomy

Procedure	Ultrasound predictors				
	Wall thickness >4mm	Pericholecystic fluid collection	Contracted GB	GB fluid with stones	Empyema
Easy LC	6	0	0	3	0
Difficult LC	15	3	2	5	1
Conversion to laparotomy	5	5	0	0	1
Sensitivity (%)	69.0	10.3	6.9	17.2	3.4
Specificity (%)	96.5	100.0	100.0	98.2	99.4
PPV	76.9	100.0	100.0	62.5	50.0
NPP	94.8	86.8	86.4	87.5	85.9
Accuracy (%)	92.5	92.5	86.5	86.5	85.5
Total	26	8	2	8	2

GB = gall bladder, PPV = Positive predictive value (%), NPP = Negative predictive value (%)

Presence of fluid in the pericholecystic area resulted in even more difficult laparoscopic procedure (Figure 3). In all of 8 patients with pericholecystic fluid collection the operation was difficult: sensitivity of pericholecystic fluid for surgical difficulties during laparoscopic cholecystectomy was 10.3%, and specificity 100% (Table 3). Other ancillary ultrasound findings apart from gall stones indicating difficult surgical preparation namely contracted gall bladder and empyma were also observed (Table 3). When the gall bladder was filled with stones (n = 8), difficulty was encountered in 5 patients, making its sensitivity 17.2%, it's specificity 98.2% (Table 3).

In patients with more than one ancillary finding by ultrasound, laparoscopic cholecystectomy was never easy i.e. specificity and positive predictive value were 100% (Table 3). Laparoscopic cholecystectomy was converted to laparoscopic cholecystectomy whenever it was deemed necessary to avoid exposing patients to unnecessary risks. Conversion rate was higher when there was more than one ancillary ultrasound findings (Table 3).

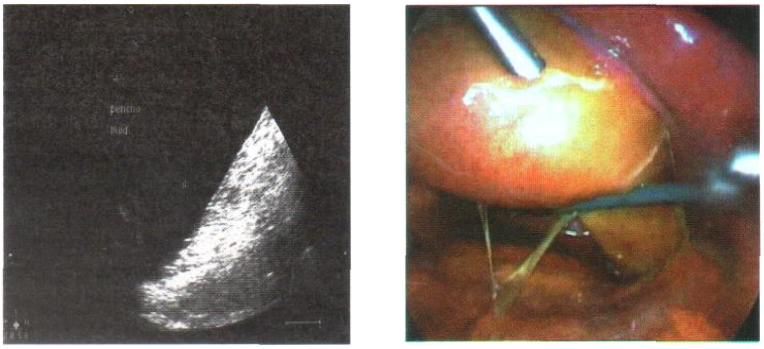

Figure 3. Ultrasound and laparoscopic photo of a patient who had acute cholecystitis with pericholecystic fluid.

Discussion

The first account of gall stones was given in 1420 by a pathologist Antonio Benevieni, in a woman who died with abdominal pain. With the passage of time, first open cholecystectomy and then, laparoscopic cholecystectomy were established as the standard treatment of gall stones [13]. Ultrasound is noninvasive and cost effective, involves no ionizing radiation, and has a reported specificity of 99% for the detection of gall stones [14,15].

In our study its specificity was 100%. It is the cornerstone imaging modality for the diagnosis of gall bladder stones, and it is unlikely to be replaced by other examination methods [16]. It is generally accepted as the modality of first choice for the diagnosis of acute cholecystitis [17,18]. It has relevance for decision taking, and is an indispensable procedure in the emergency setting [19]. In the early years of laparoscopic cholecystectomy, acute cholecystitis was considered a relative contraindication, especially in severe attacks or if the gall bladder wall thickness was more than 4mm [20]. Since then, many reports worldwide documented the safety of the procedure in acute cholecystitis [21] and the operation is recommended as the treatment of choice for acute cholecystitis [22].

The definition of a difficult laparoscopic cholecystectomy is relative and it is linked to the experience of the surgical team [23]. In the present study, difficult laparoscopic cholecystectomy occurred in 14.5% of total surgical procedures. We evaluated preoperative ultrasound's capacity of predicting technical challenges in laparoscopic cholecystectomy. When there were ancillary ultrasound findings, where difficulty was anticipated, the percentage of difficult laparoscopic cholecystectomy increased significantly to 61%.Our findings suggest that patients with thickened gall bladder wall tend to have technical difficulties during laparoscopic cholecystectomy. Similar findings have been reported by many authors [24-26], in fact it was the most sensitive ultrasound findings that predict a difficult laparoscopic cholecystectomy.

Pericholecystic fluid is another predictor of difficult laparoscopic cholecystectomy. In our study eight patients had pericholecystic fluid and the surgicu1 procedure was difficult in all of them, making the presence of pericholecystic fluid more specific than gall bladder thickness in predicting difficult laparoscopic cholecystectomy. When there were more than2 ultrasound findings suggesting acute cholecystitis, the specificity of ultrasound in predicting technical difficulty mounted to 100%. Difficult laparoscopic cholecystectomy needs longer time to finish and the preoperative prediction of long operation when patients are listed for laparoscopic cholecystectomy may have several practical applications. In addition to allowing better planning of the operating sessions, both in terms of service provision and training of junior doctors, it may allow a more

efficient selection of patients for ambulatory laparoscopic cholecystectomy. Moreover, additional anesthetic measures may be taken to minimize postoperative emesis and dizziness [27]. In a large study involving 6,380 patients Kuldip and Ohri reported an overall conversion rate of 0.42% and a rate of 1.86% in difficult cholecystectomies [28], while Livingston and Rege reported conversion rates ranging on average between 5% and 10% in a nationwide study in the USA [29]. Others reported conversion rates of 2.9-10% for elective laparoscopic cholecystectomy and 6-35% for acute cholecystitis [30-34]. In our study the conversion rates were 1.2% in patients without ancillary ultrasound findings, 13.9% in patients with ancillary ultrasound findings, and an overall conversion rate of 3.5%. The quest for predicting the probability of conversion of laparoscopic cholecystectomy in unselected patients with calculus gall bladder disease has been extensive [35,36].

Many authors have reached conclusions that preoperative ultrasound examination is useful in selecting patients who are likely to have difficulty and may require conversion from laparoscopic to open surgery. Thickened gall bladder wall [37,38], and the presence of pericholecystic fluid [39] being the most strong association with conversion, as seen also in our study. In our study, the conversion rate in patients with no ancillary findings was 1.2%, but it was significantly higher (13.9%) when ultrasound showed findings that might predict conversion from laparoscopic to open approach. Conversion rate was higher when there was more than one ancillary finding. Five patients from those with no ancillary ultrasound findings had difficult laparoscopic cholecystectomy and in two of them the procedure was converted to laparotomy. Ultrasound could not predict difficulty, mostly because the reason for difficulty and conversion was severe adhesions around the gall bladder which is a finding the preoperative ultrasound can not show, but rather it is an intraoperative finding [40,41].

On the other hand 25% of our patients with ancillary ultrasound findings, laparoscopic cholecystectomy was easy, this can be explained partially by the fact that some of these procedures were done at later stages of the study i.e. later stage of the learning curve of the surgeon, and partially by the fact there are other factors which may contribute to the degree of difficulty, like age, sex and obesity [42-44].

Conclude the current study that preoperative ultrasound is able to furnish valuable data in predicting laparoscopic cholecystectomy challenges. On the other hand, a relevant number of cases still exist wherein the concordance between the preoperative ultrasound findings and the surgical findings is unsatisfactory. In this group of patients the surgeon cannot safely rely on ultrasound examination alone and factors with a higher predictive value are obtained only during laparoscopic cholecystectomy. The need to convert can only be assessed during an attempt at laparoscopic cholecystectomy.

References

1. Jeremy T, Tan H. Prospective audit of laparoscopic cholecystectomy, experience at a secondary referral center in South Austaralia. ANZ J Surg. 2009; 76: 335-8.
2. Lledo Bueno J, Roig M, Bertomeu C. Preoperative predictive factors of ambulatory laparoscopic cholecystectomy. J A Ambulatory Surg. 2009; 12: 45-9.
3. Capizzi F D, Fogli L, Brulatti M. Conversion Rate in Laparoscopic Cholecystectomy: Evolution from 1993 and Current State. J Laparoendoscopic Adv Surg Tech. 2009; 13(2): 89-91.
4. Singh K, Ohri A. Laparoscopic cholecystectomy - is there a need to convert? J Minim Access Surg. 2009; 1(2): 59-62.
5. Simopoulos C, Botaitis S, Ploychronidis G. The contribution of acute cholecystitis, obesity, and previous abdominal surgery on the outcome of laparoscopic cholecystectomy, Am Surg. 2010; 73: 371-6.
6. Lyass S, Perry Y, Venturero M. Laparoscopic cholecystectomy, what does affect the outcome? A retrospective multifactorial regression analysis. J Surg Endosc. 2010; 14: 661-5.
7. Alpont A, Kum CK, Koh BC. Predictive factors for conversion of laparoscopic cholecystectomy. World J Surg. 2011; 21(6): 629-33.

8. Larvin M, McMahon MG. Elective laparoscopic cholecystectomy, preoperative prediction of duration of surgery. Surg Endosc. 2010; 15: 297-300.
9. Urbano D, Di Nardo R, De Simone P. The role of preoperative investigations in predicting difficult laparoscopic cholecystectomies. Surg Endosc. 2010; 10: 791-3.
10. Kohri S. Difficult laparoscopic cholecystectomy: A large series from north India. Indian J Surg. 2010; 168(4): 35-8.
11. Bellows CF, Berger DH. Crass RA. Management of gall stones. Am Fam Physician. 2010; 72: 638-42.
12. Ahmad M, Cheung R, Keeffe E. Differential diagnosis of gall stone-induced complications. South Med J. 2010; 93: 261-4.
13. Ainsowrth AP, Fallentin EM. Diagnostic imaging modalities for gallstones - where are we heading? Ugeskr Kaeger. 2010; 167(24): 2615-7.
14. Gandolfi L, Torresan F, Solmi L. The role of ultrasound in biliary and pancreatic diseases. Eur J Ultrasound. 2011; 16(3): 141-4.
15. Harvey RT, Miller WT. Acute biliary disease: Initial CT and follow up US versus initial US and follow up CT. Radiology. 2010; 213; 831-6.
16. Hirota M, Takada T, Kawarda Y. Diagnostic criteria and severity assessment of acute cholecystitis: Tokyo Guidelines. J Hepatobilia Pancreat Surg. 2010; 14: 78-82.
17. Kohlberger E. Diagnosis of acute cholecystitis. Ultrasound diagnosis is reliabl. MMW Fortschr Med 2010; 143(13):32-34.
18. Shapiro AJ, Costello C, Harkabus M. Predicting conversion of laparoscopic cholecystectomy for acute cholecystitis. JSLS. 2011; 3(2): 127-30.
19. Wang YC, Yang HR, Chung PK, et al. Urgent laparoscopic cholecystectomy in the management of acute cholecystitis: timing does not influence conversion rate. Surg Endosc. 2011; 20: 806-8.
20. Fabre JM, Fagot H, Domergue J, et al. Laparoscopic cholecystectomy in complicated cholelithiasis. Surg Endosc. 2011: 8:1198-1201.
21. Teixeira JP, Sariva AC, Cabral A. Conversion factors in laparoscopic cholecystectomy for acute cholecystitis. Hepatogastroenterology. 2011; 47 (33): 626-30.
22. Gharaibeh K, Qasaimeh G, Al-Heiss H. Effect of timing of surgery, type of inflammation, and sex on outcome of laparoscopic cholecystectomy for acute cholecystitis. J Lapareoendoscopic Adva Surg Techn 2012; 12(3): 125-8.
23. Ammori BJ, Larvin M. Elective laparoscopic cholecystectomy: preoperative prediction of duration of surgery. Surg Endosc. 2012; 15(3): 297-300.
24. Simopoulos C, Botaitis S. Risk factors for conversion of laparoscopic cholecystectomy to open cholecystectomy. Surg Endosc. 2012; 19(7): 905-9.
25. Cho KS, Baek S, Kang B. Evaluation of preoperative sonography in acute cholecystitis to predict technical difficulties during laparoscopic cholecystectomy. J Clin ultrasound. 2012; 32(3): 115-22.
26. Pinto ME. Relation of pre-operative ecography to laparoscopic cholecystectomy difficulty at the Central Military Hospital, Rev Gastroenterol Peru. 2002; 22(2): 141-51.
27. Tang B, Cuschieri A. Conversions during laparoscopic cholecystectomy: Risk factors and effects on patient outcome. J Gastroint Surg 2012; 10(7):1081-109l.
28. Rattner DW, Ferguson C, Warshaw AL. Factors associated with successful laparoscopic cholecystectomy for acute cholecystitis. Ann Surg. 2010; 217(3): 233-6.
29. Livingston E, Rege R. A nationwide study of conversion from laparoscopic to open laparoscopic surgery. World J. Surg.2008; 30(9): 1698-704.
30. Bingener-Casey J, Richards ML, Strodel WE. Reasons for conversion from laparoscopic to open cholecystectomy: a 10-year review. J Gastrointest Surg. 2012; 6(6): 800-5.
31. Brodsky A, Matter I, Sabo E, et al. Laparoscopic cholecystectomy for acute cholecystitis: can the need for conversion and the probability of complications be predicted? A prospective study. Surg Endosc. 2012; 14(8): 755-60.
32. Jitea N, Burcos T, Boiculescu S. The capacity of preoperative ultrasonography in predicting technical challenges in laparoscopic cholecystectomy. Chirurgia. 2012; 97(3): 239-42.
33. Daradkeh SS, Suwan Z. Preoperative ultrasonography and prediction of technical difficulties during laparoscopic cholecystectomy. World J Surg. 2021; 22(1): 75-7.
34. Santambrogio R, Montorsi M. Technical difficulties and complications during laparoscopic cholecystectomy: predictive use of preoperative ultrasonography. World J Surg. 2011; 20(8): 978-81.
35. Minutolo V, Gagliano G, Rizivillo C. Laparoscopic cholecystectomy (LC): predictive role of preoperative ultrasounds. G Chir. 2011; 26(3): 101-4.
36. Lap P, Aqrawal PN, Malik VP. A difficult laparoscopic cholecystectomy that requires conversion to open procedure can be predicted by preoperative ultrasonography. JSLS. 2012; 6(1): 59-63.
37. Han 1A and El-Tinay OE. Laparoscopic cholecystectomy for acute cholecystitis. Can preoperative factors predict conversion? Saudi Med J 2012; 25(3):299-302.
38. Fried GM, Barkun JS, Sigman H. Factors determining conversion to laparotomy in patients undergoing laparoscopic cholecystectomy. Am J Surg. 2012; 167(1): 35-9.
39. Ishizaki Y, Miwa K. Conversion of elective laparoscopic to open cholecystectomy between 1993 and 2004. B J Surg. 2012; 939(8): 987-91.

40. Rosen M, Brody F. Predictive factors for conversion of laparoscopic cholecystectomy. Am J Surg. 2012; 184(3): 254.
41. Tayeb M, Raza SA, Khan MR. Conversion from laparoscopic to open cholecystectomy: Multivariate analysis of preoperative risk factors. J Postgrad Med. 2008; 51(1): 17-20.
42. Lee CL, Wu CH, Chen TK. Prospective study of abdominal ultrasonography before laparoscopic cholecystectomy. J Clin Gastroenterol. 2012; 16(2): 113-6.
43. Jagdish N, Avinash S. Preoperative prediction of difficult laparoscopic cholecystectomy using clinical and ultrasonographic parameters. Indian J Gastroenterol. 2012; 24: 16-8.
44. Kanaan SA, Murayama KM, Merriam LT. Risk factors for conversion of laparoscopic to open cholecystectomy. Surg Res. 2012; 106(1): 20-4.

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The Effectiveness of Systemic Co-Enzyme Q10 in Vitiligo

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Abstract

Background Vitiligo is the most frequent depigmentation disorder of the skin. None of the therapeutic alternatives is satisfactory.

Objective To evaluate the effectiveness of systemic Co-enzyme Q10 in patients with vitiligo.

Methods Twelve patients received Co-enzyme Q10 75 mg twice daily compared with 12 patients received placebo capsule twice daily orally for 8 weeks in the Department of Dermatology, Al-Kadhimiya Teaching Hospital (November 2011 to march 2012). History of patients was taken and measurement for serum glutathione (S. GSH) (by Elleman methods), malonialdehyde (S. MDA) (by Stocks and Dormandy methods) and VASI score at baseline, 4 and 8 weeks interval.

Results No significant difference in S. GSH was found between Co-enzyme and placebo group after 4 and 8 weeks. High significant decrease in S. MDA occurred after 4 and 8 weeks with significant decrease in VASI were found after 8 weeks.

Conclusion Co-enzyme Q10 may have a role in treatment of vitiligo.

Keywords Co-enzyme Q 10, Vitiligo, Antioxidant.

Introduction

Vitiligo is an acquired pigmentary anomaly of the skin manifested by depigmented white patches surrounded by normal hyperpigmented border [(1)]. It is one of the main cutaneous diseases in which psychological factors are thought to trigger the onset or substantially influence the course [(2)].

Different studies suggest that there is some genetic mechanism involved in the etiology of vitiligo and it is polygenic in nature [(3)] with a positive family history in at least 30% of cases [(4)]. The course of the disease is unpredictable, and is often associated with periods of remission and exacerbation [(5)].

Vitiligo is a multifactorial polygenic disorder with a complex pathogenesis. Several theories have been proposed to explain the loss of epidermal melanocytes in vitiligo; they include autoimmune, cytotoxic, biochemical, oxidant-antioxidant, neural, and viral mechanisms for destruction of epidermal melanocytes [(6)]. The putative association of vitiligo with autoimmune diseases has suggested an immunologic basis for vitiligo [(7)].

The best evidence that vitiligo antibodies play a role in melanocyte destruction is the observation of the disappearance of melanocytes from normal human skin engrafted onto nude mice injected with vitiligo patient sera [(8)]. IL-6 is an important cytokine for skin and is

subject to dysregulation in several human diseases including some with skin manifestations [9].

According to the self-destruction hypothesis initially put forward by Lerner [7], melanocytes in vitiligo have lost an intrinsic protective mechanism that eliminates toxic intermediates or metabolites in the melanogenesis pathway. Several reports provide evidence for increased oxidative stress in the entire epidermis of vitiligo patients [7]. Although greater oxidative stress is observed in the active vitiligo group, this is probably correlated with increased intracellular reactive oxygen species (ROS) production in the tissues of these patients [10]. Excessive free radical generation, leading to lipid peroxidation in vitiligo, may be related to a decrease of superoxide dismutases (SOD) and an increase of xanthine oxidase (XO) activities. In addition, an increased level of malondialdehyde (MDA) can support these findings. Lipid peroxidation in the cellular membrane of melanocytes may play an important role in depigmentation of generalized vitiligo [11], whereas the neural hypothesis [7] was based initially on anecdotal observations suggesting that stress and severe emotional trauma may initiate or precipitate vitiligo.

The conventional treatment for vitiligo include photo chemotherapy (PUVA), phototherapy (UVB), vitamin D3 analogues, topical corticosteroids, topical immunomodulators, excimer laser, and surgery. These treatment options have limited success [12-14], and some present significant risks, including suspected increases in skin cancer risk by PUVA, skin atrophy with corticosteroids, and skin boils with UVB therapy [12-14]; so probable useful therapeutic effects of Co-enzyme Q10 will be evaluated in patients with vitiligo.

Coenzyme Q10 (CoQ10) is a fat-soluble, vitamin-like, ubiquitous compound that functions as an electron carrier in the mitochondrial respiratory chain, as well as serving as an important intracellular antioxidant. CoQ10 protects phospholipids and mitochondrial membrane proteins from peroxidation and protects DNA against the oxidative damage that accompanies lipid peroxidation [15-17]. Coenzyme Q10 is one of the antioxidants found in the skin [18]. Coenzyme Q10 in its reduced form as the hydroquinone (called ubiquinol) is a potent lipophilic antioxidant and is capable of recycling and regenerating other antioxidants such as tocopherol and ascorbate [17,19]. CoQ10 is able to act as an antioxidant against the effects of hydrogen peroxide and UVA in cultured epidermal keratinocytes and UVA in dermal fibroblasts [20].

The aim of this study was to measure the oxidative stress parameters (glutathione and MDA) in patients with vitiligo and to evaluate the effectiveness of systemic Co-enzyme Q10 in them.

Methods

Clinical Study design: This prospective, randomized, single blind and placebo study was done in the Department of Dermatology and Venereology, Al-Kadhimiya Teaching Hospital between November 2011 and March 2012. The total number of patients sharing in this study was 26. Only 24 patients completed the study successfully while 2 patients were unable to do so for unknown reasons. All included subjects have consented to be enrolled in this study and approval of College Council at Al-Nahrain Medical College was taken under order number 1093 in 1/12/2011.

All patients were subjected to detailed examination including the general, physical and mainly the skin examination. The diagnosis was made clinically by dermatologist. For all the patients at the initial visit, baseline characteristics had been made and involve age, sex, medical history, family history and drug allergy.

Participants and Setting: A new onset(less than 2 years), localized small patches vitiligo of both sexes that the affected body surface area of 10-20% and age range 12-58 years were included in this trail.

Along the course of treatment, each patient should satisfy three visits at 0, 4 and 8 weeks. In each visit, the assessment of response of vitiligo

lesion toward treatment was performed by using VASI (Vitiligo Area Scoring Index) calculation which is a quantitative parametric score conceptually derived from the PASI score widely used in psoriasis assessment. The total body VASI is calculated using a formula that includes contributions from all body regions [21].

VASI = All Body Sites [Hand Units] × Residual Depigmentation

A blood sample (5 ml) was obtained to determine Serum Glutathione (S. GSH) (It is based on the reaction of aliphatic compounds with dithio 2-nitrobenzoic acid at ph 8.0 to give p-nitro thiolphenol anion which is highly, colored at 412nm) [22], and S. MDA (its measurement is based on the reaction of thiobarbituric acid with MDA forming a pink- colored adduct that its light absorbance measured at 535 nm) [23].

The patients were allocated into 2 groups and all the patients were given Vaseline and asked to apply it topically two times daily.

Group I: Included 12 patients (8 females and 4 males) were given 75 mg Co-enzyme Q10 (1 soft gel two times daily) orally with food for 8 weeks.

Group II: Included 12 patients 8 females and 4 males were given sucrose as placebo capsule two times daily orally after meal for 8 weeks.

Statistical analyses: The data collected and analyzed using computer facilities of SPSS-18 and Microsoft Excel 2010.

The following measurements and tests were used (1) Mean and standard deviation. (2) Unpaired t-test and was considered statistically significant if the *P* value was less or equal to 0.05 and highly significant if the *P* value was less or equal to 0.001 [24,25].

Results

Table 1 shows the descriptive parameters of all patients in this study. Both the treated groups are comparable with no significant difference in the parameters at baseline (before treatments).

Table 1. Descriptive parameters of all patients with vitiligo

Parameters		Placebo group N = 12		Co-enzyme Q10 group N = 12	
		Frequency	%	Frequency	%
Sex	M/F	4/8	33.3/66.7	4/8	33.3/66.7
Smoking	Yes/No	2/10	16.7/83.3	3/9	25/75
Other skin disease	Yes/No	6/6	50/50	6/6	50/50
Family history	Yes/No	6/6	50/50	6/6	50/50
Drug allergy	Yes/No	0/12	0/100	0/12	0/100

No significant difference in S. GSH was found between Co-enzyme group and placebo group after 4 and 8 weeks, but a highly significant decrease in S. MDA (*P* value ≤ 0.001) in the Co-enzyme group from the placebo group after 4 and 8 weeks (Table 2).

Regarding clinical parameters; no significant difference in VASI between Co-enzyme group and placebo group after 4 weeks while significant decrease in VASI after 8 weeks was found (Table 3).

Discussion

Vitiligo is the most frequent depigmentation disorder of the skin and is cosmetically and psychologically devastating [26]. None of the therapeutic alternatives is fully satisfactory either because its improvement is unpredictable or the treatment is long or because of the side effects and operational difficulty of application of the medication [27].

Table 2. Effect of Co-enzyme Q10 on S. GSH and S. MDA concentration in patient with vitiligo in comparison with placebo-treated group

Parameters	Placebo N = 12	Q10 N = 12	P value
Serum GSH (mmol/l) baseline	1.43±0.22	1.42 ± 0.1	0.8509
Serum GSH (mmol/l) 4wk	1.44±0.23	1.47 ± 0.12	0.7126
Serum GSH (mmol/l) 8wk	1.44±0.23	1.52 ± 0.11	0.3181
Serum MDA (mmol/l) baseline	2.58±0.21	2.51 ± 0.11	0.309
Serum MDA (mmol/l) 4wk	2.58±0.2	1.7 ± 0.13	< 0.0001
Serum MDA (mmol/l) 8wk	2.65±0.24	1.58 ± 0.11	< 0.0001

Table 3. Effects of Co-enzyme Q10 on clinical feature (vasi score) in patients with vitiligo in comparison with placebo-treated patients

Parameters	Placebo N = 12	Q10 N = 12	*P* value
VASI baseline	3.75±2.81	2.33±0.72	0.073
VASI 4wk	3.75±2.81	2.33±0.72	0.073
VASI 8wk	3.88±2.77	2.08±0.9	0.018

No significant difference in S. GSH was found between Co-enzyme group and placebo group after 4 and 8 weeks. In vitiligo, both an imbalance of the intracellular redox status and a significant depletion of enzymatic and non-enzymatic antioxidants feature of vitiligo patients, and an abnormal oxidative stress might be the causes of melanocyte degeneration [28].

High significant decrease in S. MDA between Co-enzyme Q10 group and placebo group after 4 and 8 week was found. This effect was in agreement with the result of Lee et al [29] who showed patients with coronary artery disease on CoQ10 treatment having significantly lower malondialdehyde levels. Co-enzyme Q 10 in its reduced form has long been known to inhibit lipid peroxidation [30]. Co-enzyme Q10 in its reduced form is a potent lipophilic antioxidant and is capable of recycling and regenerating other antioxidants such as tocopherol and ascorbate [31].

The levels of CoQ10 in skin decline with age and UV irradiation [20] and thereby also compromise the skin's antioxidant features, leading to an increased ROS concentration at advanced age. In skin, the epidermis contains a 10-fold higher level of CoQ10 than the dermis [18].

No significant difference in VASI between Co-enzyme group and placebo group after 4 weeks was found, while significant decrease in VASI after 8 weeks occurred.

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References

1. James D, Elston M, and Berger G. Andrews Diseases of the skin clinical dermatology. 11th ed. Philadelphia: Saunders Elsevier; 2011. p. 854.
2. Hywel W. Evidence-based Dermatology. 2nd ed. Oxford: Blackwell publishing; 2008. p. 644.
3. Bhatia PS, Mohan L, Pandey ON, et al. Genetic nature of vitiligo. J Dermatol Sci. 1992; 4: 180-4.
4. Habif P. Clinical Dermatology: A Color Guide to Diagnosis and Therapy. 3rd ed. New York: Mosby; 1996. p. 616.
5. Firooz A, Bouzari N, Fallah N, et al. What patients with vitiligo believe about their condition? Int J Dermatol. 2004; 43: 811-4.
6. Klaus W. Fitzpatrick's Dermatology in General Medicine. 7th ed. McGraw-Hill; 2008. p. 616.

7. Hann SK, Nordlund JJ. Vitiligo. London, Blackwell Science, 2000.
8. Das PK, Van den Wijngaard RM, Wankowicz-Kalinska A, et al. A symbiotic concept of autoimmunity and tumour immunity: Lessons from vitiligo. Trends Immunol. 2001; 22(3): 130-6.
9. Paquet P, Pierard GE: Interleukin-6 and the skin. Int Arch Allergy Immunol. 1996; 109(4): 308-17.
10. Dammak I, Boudaya S, Ben Abdallah F, et al. Antioxidant enzymes and lipid peroxidation at the tissue level in patients with stable and active Vitiligo. Inter J Dermatol. 2009; 48: 476-80.
11. Koca R, Armutcu H, Altinyazar C, et al. Oxidant antioxidant enzymes and lipid peroxidation in generalized Vitiligo. Clin Exper Dermatol. 2004; 29: 406-9.
12. Whitton ME, Ashcroft MD, González U. Interventions for vitiligo [Systematic Review]. Cochrane Database of Systematic Reviews 2007; 3.
13. Grimes PEMD: New insights and new therapies in vitiligo. [Miscellaneous Article]. JAMA 2005; 293(6): 730-5.
14. Forschner T, Buchholtz S, Stockfleth E. Current state of vitiligo therapy-evidence-based analysis of the literature. J der Deutschen Dermatologischen Gesellschaft. 2007; 5(6): 467-75.
15. Crane FL, Sum EE. The essential functions of Coenzyme Q. Clin Invest. 1993; 71: 55-9.
16. Forsmark AP, Ernster L. Evidence for a protective effect of endogenous ubiquinol against oxidative damage to mitochondrial protein and DNA during lipid peroxidation. Mol Aspects Med. 1994; 15: 73-81.
17. Ernster L, Dallner G. Biochemical, physiological and medical aspects of ubiquinone function. Biochim Biophys Acta. 1995; 1271: 195-204.
18. Shindo Y, Witt E, Han D, et al. Enzymic and non-enzymic antioxidants in epidermis and dermis of human skin. J Invest Dermatol. 1994; 102: 122-4.
19. Crane FL. Biochemical functions of coenzyme Q10. J Am Coll Nutr. 2001; 20: 591-8.
20. Hoppe U, Bergemann J, Diembeck W, et al. Coenzyme Q10, a cutaneous antioxidant and energizer. Biofactors. 1999; 9: 371-8.
21. Hamzavi I, Jain H, McLean D, et al. Parametric modeling of narrowband Ultraviolet band B phototherapy. Arch Dermatol. 2004; 140(6): 677-83.
22. Elleman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959; 82(1): 70-7.
23. Stocks J, Dormandy TL. The autoxidation of human red cell lipids induced by hydrogen peroxide. Br J Hematol. 1971; 20: 95-111.
24. Daniel WD. Biostatistics: A foundation for analysis in the health science. 8th ed. New York: John Wiley and Sons Inc., 2005. p. 223.
25. Woolson RF. Statistical methods for the analysis of biomedical data. New York: Wiley; 1987.
26. Eleftheriadou V, Whitton ME, Gawkrodger DJ, et al. Future research into the treatment of vitiligo: where should our priorities lie? Results of the vitiligo priority setting partnership. Br J Dermatol. 2011; 164(3): 530-6.
27. Tamler C, Duque-Estrada B, Oliveira PA, et al. Tacrolimus 0,1% ointment in the treatment of vitiligo: a series of cases. Ann Bras Dermatol. 2011; 86(1): 169-72.
28. Maresca V, Roccella M, Roccella F, et al. Increased sensitivity to peroxidative agents as a possible pathogenic factor of melanocyte damage in vitiligo. J Invest Dermatol. 1997; 109: 310-3.
29. Lee BJ, Huang YC, Chen SJ, et al. Effects of coenzyme Q10 supplementation on inflammatory markers (high sensitivity C-reactive protein, interleukin-6, and homocysteine) in patients with coronary artery disease. Nutrition. 2012 Jul; 28(7-8): 767-72.
30. Forsmak P, Aberg F, Norling B, et al. Inhibition of lipid peroxidation by ubiquinol in submitochondria1 particles in the absence of vitamin E. FEBS Lett. 1991; 285: 39-43.
31. Crane FL. Biochemical functions of Co-enzyme Q10. J Am Coll Nutr. 2001; 20: 591-8.

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The Values of Hyaluronic Acid and as a Marker of Cirrhosis in Children with Chronic Liver Diseases

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Abstract

Background Hyaluronic acid (HA) is removed by the liver via sinusoidal cell adhesion molecules. This is impeded in fibrosis, leading to a rise in serum HA. As a noninvasive marker of fibrosis, HA may obviate the need for liver biopsy.

Objective To evaluate HA as a marker of hepatic fibrosis in unselected children undergoing liver biopsy or ultrasound.

Methods Fifty children aged 2-156 months diagnosed to have different types of chronic liver diseases (CLDs) and thirty healthy children aged 2-156 months were studied as controls were evaluated at the Teaching Hospital and Gastroenterology and Hepatology Center, Medical City, Baghdad, Iraq. The degree of severity of liver infection was assessed by liver biopsy or ultrasound. HA levels were measured using an ELISA.

Results The mean of HA level was 0.61± 0.32 ng/ml in the control group, 3.05± 1.11 ng/ml in patients with significant fibrosis and 1.18±0.86 ng/ml in patients with chronic liver diseases without significant fibrosis. Significant fibrosis was found in 31 out of 50 children with chronic liver disease, 20 of them were classified (METAVIR score) as cirrhotic liver. Seven out of 18 biopsies value of stage 4. Thirteen out of 32 ultrasounds described as having a coarsely textured liver. The sensitivity and specificity of estimated HA values in the diagnosis of liver cirrhosis were 87.1% and 94.74%, respectively.

Conclusion HA is a valid noninvasive predictor of histological fibrosis in children with CLD. It complements the thorough investigations of a child with CLD; however, it cannot at present replace histological examination to identify liver fibrosis. Further evaluation of HA is needed to ascertain the use of serial measurements in the targeted patient groups.

Key word Hyaluronic acid, chronic liver disease, liver fibrosis.

Introduction

A wide spectrum of chronic liver diseases can cause liver fibrosis (LF). Liver biopsy shows these histopathologic changes, determines the degree of fibrosis and is a guide to treatment decisions.

Many children each year require a liver biopsy under anesthesia. Liver biopsies, although generally a safe procedure, can be associated with serious morbidity and mortality, both from the procedure itself and the need for anesthesia in children [1,2]. Serious complications include hemoperitoneum, pneumothorax and bile leak [3] are seen in approximately 1% of liver biopsies [4]. Another important limitation of liver biopsy is inter- and intra observer variation among pathologists [5-8] and therefore, liver biopsies are only carried out if deemed essential.

Histological comparison of liver biopsies is limited by the lack of an ideal hepatic fibrosis

scoring system covering all spectrums of liver disease. The Ishak scoring system has been developed from the Histological Activity Index of Knowdell with modifications allowing for increased knowledge of etiology and histology [9]. Furthermore, the semi quantitative grading systems developed for histopathologic analysis do not reflect linearity of fibrosis deposition or actual matrix content [9,10].

Ultrasonography can provide a non-invasive prediction of liver histology which in moderate and severe steatosis and advanced fibrosis can be both highly sensitive and specific [11]. Ultrasonography is the most common modality used in the diagnosis and staging of hepatic fibrosis [12].

In recent years, interest in identifying and describing LF by using noninvasive surrogate markers has been on the rise. Serum markers of LF offer an attractive, cost effective alternative to liver biopsy for both patients and clinicians. In addition to being substantially less invasive, there are practically no complications, little or no sampling errors and small observer related variability. Moreover, measurements may be performed repeatedly, thus, allowing for a dynamic monitoring of fibrosis [13]. Hyaluronic acid (HA) is a high molecular weight glycosaminoglycan synthesized by mesenchymal cells, circulated by the lymphatic system and widely distributed in connective tissue [14]. It has a half-life of 5 to 6 minutes in plasma and a vital component in producing viscoelasticity in the extracellular matrix; it also lubricates the interstitial tissue. A small percentage of HA is locally metabolized while the greater part of it enters the blood through the lymphatic system and from there goes into the liver where it is immediately metabolized by the hepato-endothelial cells [15, 16].

The aim of the study is to determine the validity of serum HA as a marker of hepatic fibrosis in children with different types of chronic liver disease (CLD), undergoing a liver biopsy or ultrasound assessment.

Methods

This is a case-control study of fifty children with different types of CLDs**,** 18 of them have undergone liver biopsy at Gastroenterology and Hepatology Center, Medical city, Baghdad, Iraq during the period from Sep. 2011 to Feb. 2012. The METAVIR scoring system was applied to stage of LF. The other 32 were subjected to ultrasound evaluation at the same hospital to assess the liver texture as an indicator of significant LF [17,18]. Children with viral hepatitis were excluded from the study. No children were known at the time of sampling to have active juvenile idiopathic arthritis or any joint inflammation.

Conventional laboratory tests of liver function such as alanin aminotransferase (ALT), aspartate aminotransferase (AST), and total serum bilirubin (TSB) were undertaken for patients and controls within 48 hours of taking the samples. Blood samples for hyaluronic acid (HA) were separated and frozen at -20 °C before analysis. HA levels were measured using an enzyme-linked binding protein assay (Kit CUSABIO 2012, China).

Liver biopsies of 18 patients with different types of CLD were done by ultrasound guided technique with spring loaded needle, and then processed in the laboratories of Gastro-enterology and Hepatology Center. An experienced pathologist examined all the liver biopsy specimens during a single sitting. The pathologist was blinded to the results of the HA level and the underlying clinical diagnosis. METAVIR scoring system was applied. No or non significant fibrosis (0-1), significant fibrosis with (2-4). As for the ultrasound evaluated group, a coarse liver texture, especially when associated with irregularity of the liver surface, was regarded as indicator of significant fibrosis [14]. The subjects in this study were divided into three groups:

Group I: Children with different types of chronic liver disease with significant fibrosis.

Group II: Children with unselected chronic liver disease without significant fibrosis.

Group III: Healthy children (control).

All the data were analyzed using the Statistical package for social sciences (SPSS) software (v.17, SPSS, Inc., Chicago, USA). The student t-test was used to compare HA levels and the other biomarkers, in the groups of patients with and without significant fibrosis. Logistic regression analysis was undertaken with the presence and absence of significant fibrosis as the dependent variable and HA levels and standard liver functions tests (serum bilirubin, alanine transferase, and aspartate transaminase) as independent variables. To assess clinical applicability (sensitivity, specify and predictive values) a receiver operator curve was constructed and the area under the curve was calculated.

Results

Significant fibrosis was found in 31 of 50 children with CLDs. Twenty (40%) of them were classified (METAVER score) as cirrhotic liver, eleven (22%) have no cirrhosis and the rest 19 (38%) show no fibrosis (Figure 1). Seven out of 18 biopsies value of stage 4 and 13 of 32 ultrasound assessed group described as having a coarsely textured liver. The mean HA level was 0.61± 0.32 ng/ml in the control group, 3.05± 1.11 ng/ml in patients with significant fibrosis and 1.18 ± 0.86 ng/ml in patients with CLDs without significant fibrosis.

The results of study showed that HA, AST, ALT, and TSB levels were significantly increased in children with fibrosis compared to the control group ($p < 0.0001$; $p < 0.0001$, $p < 0.0001$; $p = 0.0001$, respectively). Similarly, AST/ALT ratio was significantly elevated in children with fibrosis compared to control group ($p = 0.0113$) as shown in table 1.

Table 1. Comparison between control group and patients with significant fibrosis

Parameters	Control Group N = 30 Mean ± SD	Patients with fibrosis N = 31 Mean ± SD	P value
Age (months)	68.8 ± 39.67	80.32±58.85	0.3726
Hyaluronic acid (ng/mL)	0.61 ± 0.32	3.05 ± 1.11	<0.0001
AST (IU/L)	6.47 ± 2.18	53.13 ± 43.96	<0.0001
ALT (IU/L)	7.53 ± 2.49	42.23 ± 32.53	<0.0001
AST/ALT ratio	0.93 ± 0.51	1.41 ± 0.86	0.0113
TSB (mg/dL)	0.62 ± 0.22	9.31 ± 10.79	0.0001

AST: Aspartate aminotransferase, ALT: Alanine transaminase, TSB: total serum bilirubin

As shown in the table 2, mean HA, AST, and ALT levels were higher in children without fibrosis compared to the control group ($p = 0.0108$; $p = 0.0001$; $p = 0.0007$, respectively) whereas, no significant differences in the AST/ALT ratio between the two groups ($p = 0.0834$).

Table (2): Comparison between control group and patients without fibrosis

Parameters	Control Group N = 30 Mean ± SD	Patients without fibrosis N = 19 Mean ± SD	P value
Age (months)	68.8 ± 39.67	60.37 ± 52.19	0.5521
Hyaluronic acid (ng/mL)	0.61 ± 0.32	1.18 ± 0.86	0.0108
AST (IU/L)	6.47 ± 2.18	48.21 ± 37.78	0.0001
ALT (IU/L)	7.53 ± 2.49	40.21 ± 34.76	0.0007
AST/ALT ratio	0.93 ± 0.51	1.45 ± 1.18	0.0834
TSB (mg/dL)	0.62 ± 0.22	7.39 ± 9.84	0.0077

AST: Aspartate aminotransferase, ALT: Alanine transaminase, TSB: total serum bilirubin

Table 3 illustrates comparison between CLDs children with and without fibrosis. The mean HA level was significantly higher in those with fibrosis as compared to those without (*P* < 0.0001) whereas, no significant differences was noticed in AST, ALT, AST/ALT and TSB levels between the two groups.

Table 3. Comparison between patients with and without fibrosis

Parameters	Patients with Fibrosis N = 31 Mean ± SD	Patients without fibrosis N = 19 Mean ± SD	P value
Age (months)	60.37 ± 52.19	80.32±58.85	0.2185
Hyaluronic acid (ng/mL)	1.18 ± 0.86	3.05 ± 1.11	<0.0001
AST (IU/L)	48.21 ± 37.78	53.13 ± 43.96	0.677
ALT (IU/L)	40.21 ± 34.76	42.23 ± 32.53	0.8396
AST/ALT ratio	1.45 ± 1.18	1.41 ± 0.86	0.8925
TSB (mg/dL)	7.39 ± 9.84	9.31 ± 10.79	0.5234

AST: Aspartate aminotransferase, ALT: Alanine transaminase, TSB: total serum bilirubin

The cutoff value of hyaluronic acid was calculated using receiver operating characteristic (ROC) test and it was found to be 1.66 ng/mL (figure 2), the sensitivity and specificity of estimation of HA value in diagnosis of liver cirrhosis were 87.1% and 94.74% respectively, while the positive predictive value of HA in diagnosis of liver cirrhosis was 96.43% and the negative predictive value was 81.82% (tables 4 and 5).

Table 4. Cutoff values, sensitivity and specificity of Hyaluronic acid in all patients groups

Cutoff value	Specificity	Sensitivity	Area under curve	P value
1.66 ng/mL	94.74%	87.1%	0.948	<0.001

Table 5. Positive and negative predictive values for Hyaluronic acid in all patients groups

Predictive values		Cirrhosis		Total
		Positive	Negative	
Hyaluronic acid	Positive test	27	1	28
	Negative test	4	18	22
	Total	31	19	50

+ve predictive value: 96.43%, -ve predictive value: 81.82%

Discussion:

The liver excretes most of circulating HA, while the kidney accounting for approximately 1%. In the liver, HA is cleared from the circulation by binding to CD44 adhesion molecules on sinusoidal endothelial cells, with subsequent transport into the hepatocyte. The CD44 is a transmembrane glycoprotein involved in the interaction between cells and extracellular matrix. Sinusoidal endothelial cells lack a basement membrane and hence, are permeable to molecules moving into hepatocytes. In the presence of fibrosis, the sinusoidal endothelial cells become thickened which is less permeable, so HA clearance is impaired and serum levels rise. Fibrosis also stimulates hepatic mesenchymal cells, so more HA is produced. Serum hyaluronic acid levels are also raised in joint inflammation, due to increased synovial

production. Xie *et al* studied the relationship between HA and procollagen III and collagen type IV and the degree of the liver fibrosis and they reported that HA had the closest correlation with fibrosis [19]. Some studies assessing HA levels have suggested serial measurements are recommended to predict clinical prognosis and correlate HA measurements with other biochemical markers of disease e.g., serum bilirubin and transaminases [20,21].

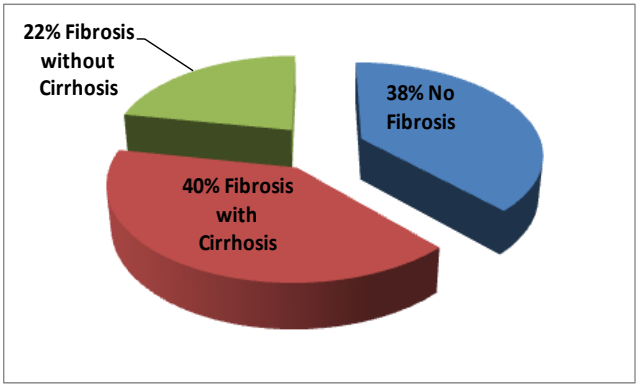

Fig. 1 Results of Liver Biopsy

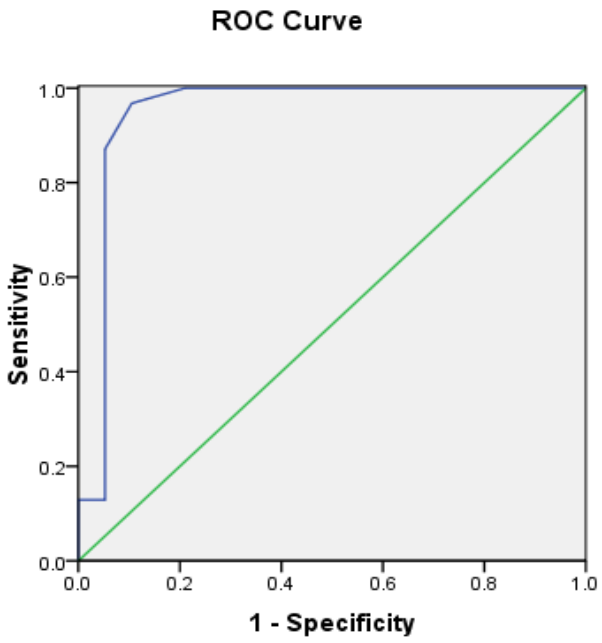

Fig. 2 The receiver operator characteristic (ROC) for sensitivity and specificity of hyaluronic acid test

In children, HA has been studied in specific etiological groups. In biliary atresia, the serum levels of HA measured at diagnosis was found to predict the need for liver transplant within the first five years [22,23]. Comparing serum HA levels with histological fibrosis in children with biliary atresia, Kobyashi *et al* [24] and Hasegawa *et al* [25] showed that significant fibrosis correlated with increased HA levels. In children with cystic fibrosis with biochemical or radiological evidence of liver disease, HA levels were also raised [15].

This study has assessed the validity of HA as a marker of fibrosis in pediatric patients with chronic liver disease, by comparing its level with the gold standard histological diagnosis of fibrosis, or with ultrasound assessment. Thirty one of 50 children with chronic liver disease had significant fibrosis, 20 of them were classified as cirrhotic liver, showed high level of HA.

The patients without significant fibrosis (mild to moderate) were reported to have a mean of HA level 1.18±0.86 ng/ml which is distinguished from those with significant fibrosis (3.05±1.11 ng/ml).

Our findings of the receiver operating characteristic (ROC) study are in agreement with those of the previous studies. Nyberg *et al* [27] have that HA levels are a sensitive tool that can be used to determine the degree of progressive liver injury in patients with primary biliary cirrhosis. Fried *et al* [28] showed that patients who developed liver fibrosis (LF) from chronic veno-occlusive diseases also presented with elevated HA levels. Other similar studies involving patients with hepatitis C also revealed a direct correlation between fibrosis in liver biopsies and elevated HA levels [29,30]. In Iran, Montazeri *et al* [31] reported a relationship between serum hyaluronate and the severity of inflammation and fibrosis in patients with non-HBsAg hepatitis B. Furthermore; the study of Hartley *et al* [32] confirmed these findings in a sample of unselected children undergoing liver biopsy.

References

1. Cohen M, A-Kader H, Lambers D, et al. Complications of percutaneous liver biopsy in children. Gastroenterology. 1992; 102: 629-32.
2. Cohen M, Cameron C, Duncan P. Pediatric anesthesia morbidity and mortality in the perioperative period. Anesth Analg. 1990; 70: 160-7.
3. Piccininno F, Sagnelli E, Pasquale G, et al. Complications following percutaneous liver biopsy. J Hepatol. 1986; 2: 165-73.
4. Terjung B, Lemnitzer I, Dumoulin FL, et al. Bleeding complications after percutaneous liver biopsy. An analysis of risk factors. Digestion. 2003; 67: 138-45.
5. The French METAVIR Cooperative Study Group. Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. Hepatology. 1994; 20: 15-20.
6. Westin J, Lagging LM, WejstalR, Norkrans G, et al. Interobserver study of liver histopathology using the Ishak score in patients with chronic hepatitis C virus infection. Liver. 1999; 19: 183-7.
7. Gronbaek K, Christensen PB, Hamilton-Dutoit S, et al. Interobserver variation in interpretation of serial liver biopsies from patients with chronic hepatitis C. J Viral Hepat. 2002; 9: 443-9.
8. Bravo AA, Sheth SG, Chopra S. Liver biopsy. N Engl J Med. 2001; 344: 495-500.
9. Ishak K, Baptista A, Bianchi L, et al. Histological grading and staging of chronic hepatitis. J Hepatol 1995; 22: 696-9.
10. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology. 1996; 24: 289-93.
11. Joseph AEA, Saverymuttu SH, Al-Sam S, et al. Comparison of liver histology with ultrasonography in assessing diffuse parenchymal liver disease. Lin Radiol 1991; 43: 26-31.
12. Bonekamp S, Kamel I, Solga S, et al. Can imaging modalities diagnose and stage hepatic fibrosis and cirrhosis accurately. J Hepatol 2009 Jan; 50(1): 17-35.
13. Zhou K, Lu LG: Assessment of fibrosis in chronic liver diseases. J Digest Dis. 2009, 10(1): 7-14.
14. George J, Tsutsumi M, Takase S. Expression of hyaluronic acid on N-nitrosodimethylamine induced hepatic fibrosis in rats. Int J Biochem Cell Biol. 2004; 36: 307-19.
15. Wyatt HA, Dhawan A, Cheeseman P, et al. Serum hyaluronic acid concentrations are increased in cystic fibrosis patients with liver disease. Arch Dis Child. 2002; 86(3): 190-3.
16. Ding H, Chen Y, Feng X, et al. Correlation between liver fibrosis stage and serum liver fibrosis markers in patients with chronic hepatitis B. Zhonghua Gan Zang Bing Za Zhi. 2001; 9(2): 78-80.
17. Iacobellis A., Fussili S, Magnia A, et al. Ultrasonographic and biochemical parameters in the noninvasive evaluation of liver fibrosis in hepatitis C virus chronic hepatitis. Alimen Pharmacol Therap. 2005; 22(9): 769-74.
18. Ziol M, Handra-Luca A, Kettaneh A, et al. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. Hepatology. 2005, 41(1): 48-54.
19. Xie S, Yao J, Zheng R, et al. Serum hyaluronic acid, procollagen type III and IV in histological diagnosis of liver fibrosis. Hepatobil Pancreat Dis Int. 2003; 2: 69-72.
20. Leroy V, Monier F, Bottari S, et al. Circulating matrix metalloproteinases 1, 2, 9 and their inhibitors TIMP-1 and TIMP-2 as serum markers of liver fibrosis in patients with chronic hepatitis C: comparison with PIIINP and hyaluronic acid. Am J Gastroenterol. 2004; 99: 271-9.
21. Lindqvist U, Chichibu K, Delpech B. Seven different assays of hyaluronan compared for clinical utility. Clin Chem. 1992; 38: 127-32.
22. Dhawan A, Trivedi P, Cheeseman P, et al. Serum hyaluronic acid as an early prognostic marker in biliary atresia. J Pediatr Surg. 2001; 36: 443-6.
23. Trivedi P, Dhawan A, Risteli J, et al. Prognostic value of serum hyaluronic acid and type I and III procollagenpropeptides in extrahepatic biliary atresia. Pediatr Res. 1995; 38: 568-73.
24. Kobyashi H, Horikoshi K, Yamataka A, et al. Hyaluronic acid: a specific prognostic indicator of hepatic damage in biliary atresia. J Pediatr Surg. 1999; 34: 1791-94.
25. Hasegawa T, Tsasaki S, Kimura T, et al. Measurement of serum hyaluronic acid as a sensitive marker of liver fibrosis in Biliary atresia. J Pediatr Surg. 2000; 35: 1643-6.
26. Hosmer DW, Lemeshow S. Applied Logistic Regression. 2nd ed. USA: John Wiley & Sons Inc; 2000. p. 156-64.
27. Nyberg A, Engstrom-Laurent A, Loof L. Serum hyaluronate in primary biliary cirrhosis--a biochemical marker for progressive liver damage. Hepatology. 1988; 8(1): 142-6.
28. Fried MW, Duncan A, Soroka S, et al. Serum hyaluronic acid in patients with veno-occlusive disease following bone marrow transplantation. Bone Marrow Transplant. 2001; 27(6): 635-9.
29. Halfon P, Bourliere M, Penaranda G, et al. Accuracy of hyaluronic acid level for predicting liver fibrosis stages in patients with hepatitis C virus. Comp Hepatol. 2005; 4: 6.
30. Skripenova S, Trainer TD, Krawitt EL, et al. Variability of grade and stage in simultaneous paired liver biopsies in patients with hepatitis C. J Clin Pathol. 2007; 60(3): 321-4.
31. Montazeri G, Estakhri A, Mohamadnejad M, et al. Serum hyaluronate as a non-invasive marker of hepatic fibrosis and inflammation in HBeAg-negative chronic hepatitis B. BMC Gastroenterol. 2005; 5: 32.
32. Hartley JL, Brown RM, Tybulewicz A, et al. Hyaluronic acid predicts hepatic fibrosis in children with hepatic disease. J Pediatr Gastroenterol Nutr. 2006; 43: 217-21.
33. Pradat P, Alberti A, Poynard T et al. Predictive value of ALT levels for histologic findings in chronic hepatitis C: a European Collaborative Study. Hepatology. 2002; 36: 973-7.

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The Use of Tadalafilin Patients with Chronic Prostatitis/Chronic Pelvic Pain Syndrome

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Abstract

Background The treatment of chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) can be a frustrating challenge to physicians and many drugs had been used with variable results.

Objective To evaluate the safety and the efficacy of adding 5 mg tadalafil for patients with CP/CPPS with the conventional treatment.

Methods Thirty five patients received tamsulosin 0.4 mg capsule once daily, levofloxacin 500mg tablet once daily and indomethacin rectal suppository 100 mg once daily served as control group. Another 35 patients received the alpha blocker, levofloxacin and NSAID as above with tadalafil 5 mg once daily for 1 month period comprised tadalafil group. The NIH Chronic Prostatitis Symptom Index (NIH-CPSI) assessment was completed by each patient at baseline and 4 weeks after the drug therapy to assess the response to treatment. We consider in our study the chronic prostatitis/CPPS or category IIIa or b according to NIH classification system.

Results No significant difference in mean age and baseline score in between groups was found. After one month of starting treatment, it had been found that NIH-CPSI/pain, urinary and quality of life domains were significantly changed from (12.8±1.44, 5.9±1.77 and 8.8±1.82) at baseline to (9.6±1.04, 3.55±0.99 and 3.88±1.31) respectively in group A. In group B also there was a significant reduction in the NIH-CPSI among patients in this group; the baseline NIH-CPSI/pain, urinary and quality of life domains were (13.4±1.66, 5.8±1.85 and 9.3±1.92) and changed to (6.28±0.90, 2.65±0.86 and 2.69±1.43) respectively after treatment. The total NIH-CPSI was 27.5±4.78 and changed to 17.03±3.91 after treatmentin group A and 28.5±4.49 changed to11.62±3.59 in group B.

Conclusion The use of tadalafil in patients of CP/CPPS with conventional treatment for 1 month was safe and has high efficacy in reducing the symptoms for the patients and improving the quality of life.

Keywords Tadalafil, chronic prostatitis, chronic pelvic pain syndrome

Introduction

Prostatitis is the most common diagnosis in urology clinic below 50 year of age and 2-10% of community had prostatitis-like syndrome. The first who described the inflammation of prostate was Legneau in 1815, but it was Verdes, in 1838, who presented the first accurate description of the pathology of the prostatitis. In 20th century, more detailed description of prostatitis done and further bacterialand cytological localization studies of lower urinary tract was carried out [1].

The initial and mainstay treatment of prostatitis in most of the last century was the repetitive prostatic massage. The introduction of antibiotics especially the sulphanilamide in 1930 became the main treatment.The next era of prostatitis management began in the 1960s with Meares and Stamey's description of the four-glass lower urinary tract segmented localization

study.Afterthat new era of treatment started with the introduction of alpha blocker and nonsteroidal anti-inflammatory drugs which then became the standard treatment for the patient of CP/CPPS. The syndrome becomes chronic after 3 months of symptoms. The symptoms wax and wane overtime and the patients become free of pain between attacks. The most common form of prostatitis is the lymphocytic infiltration of prostatic stroma immediately adjacent to prostatic acini, the peripheral zone is mostly affected. Prostatitis had 2 classification systems (traditional and the NIH national institute of health) [1,2].

Phosphodiesterase 5 (PDE5) Inhibitor mediated relaxation of prostatic duct smooth muscle increases washout of prostatic reflux products reducing prostatic inflammation and consequent prostatitis symptoms. The presence of both Nitric Oxide Synthase and PDE5 in human prostatic tissue and the effect of nitric oxide donors and PDE5 inhibitors in vitro indicate PDE5 inhibitors relax prostatic smooth muscle. Significant retrograde urinary flux into prostatic ducts has been described and suggested as the mechanism of chronic prostatitis and it is postulated that PDE5 inhibitors alter prostatic reflux hence prostatitis symptoms [3].

Tadalafil is a selective PDE5 similar to sildenafil and vardenafil. It is administered orally for the treatment of erectile dysfunction (ED). Tadalafil has the longest duration of action (~36 hours) among the current PDE5 inhibitors [4].In patient group we added tadalafil tablet 5mg once daily as the drug of 5PDI with long duration of action that permit us for once daily administration[5].

Table 1.The classification system of prostatitis syndrome[1]

Traditional	National institutes of health	Description
Acute bacterial prostatitis	category I	Acute infection of prostate gland
Chronic bacterial prostatitis	category II	Chronic infection of prostate gland
	Category III chronic pelvic pain syndrome (CPPS)	Chronic genitourinary pain in the absence of uropathogenic bacteria localized to the prostate gland employing standard methodology
Nonbacterial prostatitis	Category IIIA (Inflammatory CPPS)	Significant No. of WBCs in expressed prostatic secretions, post-prostatic massage urine sediment (VB3), or semen
Prostatodynia	Category IIIB (non-Inflammatory CPPS)	Insignificant No. of WBCs in expressed prostatic secretions, post-prostatic massages urine sediment (VB3), or semen.
	Asymptomatic inflammatory prostatitis	WBSs (&/or bacteria) in expressed prostatic secretions, post-prostatic massage urine sediment (VB3), semen or histologic specimens of prostate gland.

In this study we include category IIIa and b as they had the same clinical features and course of the disease [6,7].

Method

The study was carried out from the January 2012 to September 2012 by participation of 70 patients from the Baghdad Medical city/Urology OutpatientClinic and divided into 2 groups; group A as control and group B as tadalafil group, and the duration of treatment was for 1 month to both groups provided that a written questionnaire was given to both groups pre and post study for assessment of treatment responsecalled NIH chronic prostatitis symptoms index(NIH-CPSI).The NIH Chronic Prostatitis Symptom Index (NIH-CPSI)assessment was completed by each patient at baseline and 4 weeks after the drug therapy.

Group A (35) patients received treatment of alpha blocker (tamsulosin 0.4 mg capsule once daily), levofloxacin 500mg tablet once daily and NSAID (indomethacin rectal suppository 100 mg once daily) and Group B received the alpha blocker, levofloxacin andNSAID as above with tadalafil 5 mg once daily for 1 month period after documentation of chronic prostatitis and performing 4 glasses test as following:

Patient voided the first 10 ml in 1st glass (VB1) then voided the next 30 ml in the 2nd glass (VB2) which represent the midstream urine afterthat prostatic massage was performed and the prostatic secretion was collected in another glass which represented (EPS) and finally let the patient void after prostatic massage and collect it in a separate glass (VB3) the result of microscopic examination of these 4 glasses represent the localization study of inflammatory process. The three urine specimens were centrifuged for 5 minutes and the sediment examined under high power for leukocytes (including aggregates of leukocytes), macrophages, oval fat bodies, erythrocytes, bacteria, and fungal hyphae. A wet mount of a drop of EPS can be examined under a coverslip in a similar manner and the 4 samples sent for histopathological examination [8].

We considered in our study the chronic prostatitis/CPPS or category IIIa or b according to NIH classification system.The inclusion criteria were patients from age 20 to 49 year old with prostatitis like symptoms that documented by history, clinical examination and 4glass test and persistence of symptoms more than 3 months that classified as category IIIa or b.

The exclusion criteria were patients who had acute and chronic bacterial prostatitis, age more than 50 years old to exclude the cases of benign prostatic hyperplasia, sexually transmitted disease documented infection, any patient who had urethral catheter, prostate surgery, urethral stricture or peptic ulcer, and patients with ischaemic heart disease on nitrate.

By using SPSS (statistical package for social sciences) software for windows version 20, all data of both groups were entered and analyzed using appropriate statistical tests and procedures.

Descriptive statistics for baseline characteristics were presented as mean ± standard deviation (SD).

Multiple tables then had been conducted and comparative statistics were performed using students' paired (t) test to assess the significance of reduction in NIH-CPSI before and one month after treatment within each group.Independent two sample students' (t) test was used to assess the difference in between group regarding the NIH-CPSI. The level of significance of ≤ 0.05 was assumed. Finally all data and results were presented in tables and or graphs.

Results

The overall mean age of studied population was (34.7 ± 5.2) years with a range of (20-49) years. For group A, the mean age was (34.1 ± 4.8) years while for group B was (33.6 ± 3.9) years. No significant difference in mean age of both groups had been found, $P > 0.05$ (Table 2).

The baseline NIH-CPSI/ pain domain was (12.8 ± 1.44) in group A and (13.4 ± 1.66) among group B, The baseline NIH-CPSI/urinary domain was (5.9 ± 1.77) in group A and (5.8 ± 1.85) among group B. The baseline NIH-CPSI/ quality of life domain was (8.8 ± 1.82) in group A and (9.3 ± 1.92) among group B. No significant difference in baseline score in between groups, $P > 0.05$. The baseline total NIH-CPSI was (27.5 ± 4.78) in group A and (28.5 ± 4.49) among group B.

After one month of starting treatment, it had been found that NIH-CPSI/ pain, urinary and quality of life domains were significantly changed from (12.8 ± 1.44, 5.9 ± 1.77 and 8.8 ± 1.82) at baseline to (9.6 ± 1.04, 3.55 ± 0.99 and 3.88 ± 1.31) respectively in group A, $P < 0.05$.In group B also there was a significant reduction in the NIH-CPSI among patients in this group; the baseline NIH-CPSI/pain, urinary and quality of life domains were (13.4 ± 1.66, 5.8 ± 1.85 and 9.3 ± 1.92) and changed to (6.28 ± 0.90, 2.65 ± 0.86 and 2.69 ± 1.43) respectively after treatment, $P < 0.05$. The total NIH-CPSI was (27.5 ± 4.78) and changed to (17.03 ± 3.91) after

treatment, in group A and (28.5 ± 4.49) changed to (11.62 ± 3.59) in group B, $P < 0.05$ (Table 3).
On comparing the mean reduction in between studied group it had been significantly found that despite both groups get areduction in the NIH-CPSI but the reduction among groupB was much higher than that in group A, $P < 0.05$ (Figure 1).

Table 2. Baseline patients' characteristics

Variable	Group A	Group B
Age (years)	34.1 ± 4.8	33.6 ± 3.9*
Baseline NIH-CPSI/ pain domain	12.8 ± 1.44	13.4 ± 1.66*
Baseline NIH-CPSI/ urinary symptoms domain	5.9 ± 1.77	5.8 ± 1.85*
Baseline NIH-CPSI/ Quality of life	8.8 ± 1.82	9.3 ± 1.92*
Baseline NIH-CPSI/ Total	27.5 ± 4.78	28.5 ± 4.49*

*P value > 0.05

Table 3. Comparison of NIH-CPSI before and onemonth after treatment in both groups

Patient Group	NIH/CPSI pain domain		
	Before treatment	1 month after treatment	Mean reduction
Group A Group B	12.8 ± 1.44 13.4 ± 1.66	9.6 ± 1.04* 6.28± 0.9*	3.2 ± 1.28 7.12 ± 1.14
NIH-CPSI/ urinary symptoms domain			
Group A Group B	5.9 ± 1.77 5.8 ± 1.85	3.55 ± 0.99* 2.65 ± 0.86*	2.35 ± 1.36 3.15 ± 1.47
NIH-CPSI/ Quality of life			
Group A Group B	8.8 ± 1.82 9.3 ± 1.92	3.88 ± 1.31* 2.69 ± 1.43*	5.49 ± 1.52 6.61 ± 149
NIH-CPSI/ Total			
Group A Group B	27.5 ± 4.78 28.5 ± 4.49	17.03 ± 3.91* 11.62 ± 3.59*	10.47 ± 4.07 16.9 ± 4.32

*P value <0.05 compared with before treatment

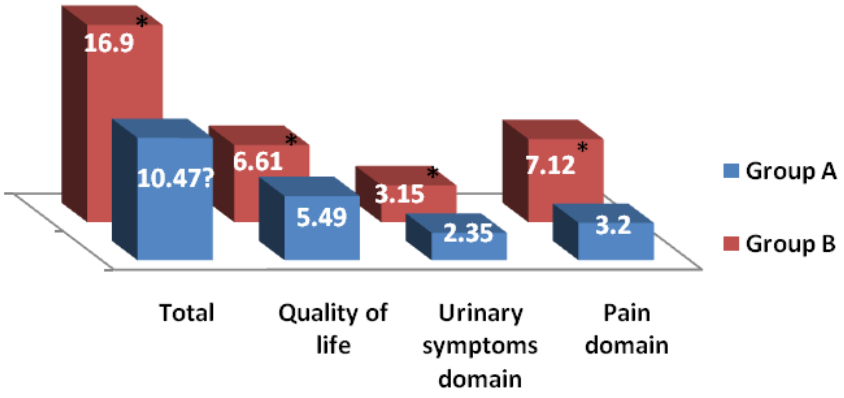

Figure 1. Comparison of mean reduction in NIH-CPSI one month after treatment in both groups (P < 0.05)

Although side-effects, such as headache, dyspepsia, myalgia and Back pain occurred more in patients who were given tadalafil (P< 0.05), no significant side-effects was detected so as to require exclusion of a patient from the study, and medical intervention was not performed in any of the patients because of side-effects (Table4).

Table 4. Side-effects between the two groups

The adverse effect	Group AN (%)	Group BN (%)
Headache	5 (14.2)	6 (17.1)
Flushing	0	2 (5.7)
Dyspepsia	3 (8.6)	5 (14.2)
Abnormal ejaculation	10 (28.6)	10 (28.6)
Myalgia	0	5 (14.2)
Back pain	0	3 (10)
Dizziness	6 (17.1)	7 (20)
Limb pain	0	2 (5.7)
Nasal congestion	2 (5.7)	3 (8.6)
Nausea	2 (5.7)	3 (8.6)
Diarrhea	1 (2.8)	2 (5.7)
Serious adverse events	0	0

Discussion

Over decades CP/CPPS was adifficult disease to treat that start with repetitive prostatic massage that permits frequent prostatic evacuation and decrease the pain for the patients [(1)], after that with introduction of antibiotics and subsequent addition of NSAID became the main treatment for this group of patients [(9)], which then overcome by the revolution of alpha blocker especially the highly selective group [(10-12)]. The latest fashion for treating patients with CP/CPPS is the combination therapy of agents termed the "three As" antibiotics, nonsteroidal anti-inflammatory drugs and α_1-blockers [(13)].

In our study, tadalafil in asmall dosewas introduced in addition to the conventional combination therapy "three As" to assess its safety and efficacy in improving the patients symptoms.

The efficacy of tadalafil in relieving the patients' symptoms may be due to PDE5 inhibitor mediated relaxation of prostatic duct smooth muscle which increases washout of prostatic reflux products reducing prostatic inflammation and consequent prostatitis symptoms [(3)].

This drug relieves lower urinary tract symptoms in patients with CP/CPPS because the PDE5 inhibition leads to smooth muscle relaxation in the bladder neck and prostate. This in turn permits increased urine flow and decreased urinary retention. How this agent relieves the pain associated with CP/CPPS is less clear, however it may be due to increased frequency of the sexual activity that will relieve the congestion of prostate and decrease the pain which is the main complain of our patients.

Mehik *et al* stated that alfuzocin improves the chronic prostatitis pain symptoms alone [(14)], while Nickel *et al* [(15)] use levofloxacin as the antibiotic of choice for chronic prostatitis symptoms improvement. Yoshimura *et al* [(16)] stated that levofloxacin had arole in CP/CPPS as it had immunomodulatory action on cytokine production by human peripheral blood mononuclear cells. Jeong *et al* [(17)] use both levofloxacin and doxazocin alone and in combination, the symptoms improvement was greater in combination group.

In our patient group we noticed that the addition of tadalafil to the combination therapy of NSAID, antibiotic, alpha blocker achieveda marked improvement in patient symptoms especially the pain over the control group who use the NSAID, antibiotic and alpha blocker only. Caldwell *et al* [(18)] also stated that acombination therapy better than monotherapy for treatment

of CP/CPPS although the PDE5 inhibitor was not included in their study. Similar results to our study were reported by Park *et al* who showed the use of tadalafil in addition to levoflxacin achieved significant symptomatic improvement in young and middle aged patients with CP/CPPS [19].

The addition of tadalafil to the conventional combination therapy of CP/CPPS was highly effective in improving the patients symptoms as reflected by significant improvement in the NIH-CPSI and was safe because no significant side-effects was detected so as to require exclusion of a patient from the study, and medical intervention was not performed in any of the patients because of side-effects.

Conclusion

The use of tadalafil in patients of CP/CPPS with conventional treatment for 1 month is safe and has high efficacy in reducing the patientsymptoms.

References

1. Nickel JC. Infection and inflammation of prostate. In: Wein AJ, Kavoussi LR, Novick AC, et al (eds). Campbell's Urology, Vol. 1, 10th ed. Philadelphia: Saunders Elsevier; 2011. p. 342-52
2. Collins MM, Stafford RS, O'Leary MP, et al. How common is prostatitis? J Urol. 1998; 159: 1224-8.
3. Grimsley SJS, Khan MH, Jones GE. Mechanism of Phosphodiesterase 5 inhibitor relief of prostatitis symptoms. Med Hypotheses. 2007; 69: 25-6.
4. Burnett AL. Evaluation and management of erectile dysfunction. In: Wein AJ, Kavoussi LR, Novick AC, et al (eds). Campbell's Urology, Vol. 1, 10thed. Philadelphia: Saunders Elsevier; 2011. p. 721-48.
5. Drugs for erectile dysfunction. British National Formulary (BNF), No.63, 2012 March, Ch.7, Sec.7.4.5.
6. Krieger JN, Nyberg L Jr, Nickel JC. NIH consensus definition and classification of prostatitis. JAMA. 1999; 282: 236-7.
7. McNaughton-Collins M, MacDonald R, Wilt TJ. Diagnosis and treatment of chronic abacterial prostatitis. Ann Intern Med. 2000; 133: 367-81.
8. De la Rosette JJ, Hubregtse MR, Meuleman EJ, et al. Diagnosis and treatment of 409 patients with prostatitis syndromes. Urology 1993; 41: 301-7.
9. Nickel JC, Pontari M, Moon, et al. Rofecoxib Prostatitis Investigator Team. A randomized, placebo controlled, multicenter study to evaluate the safety and efficacy of rofecoxib in the treatment of chronic nonbacterial prostatitis. J Urol. 2003; 169: 1401-5.
10. Nickel JC, Krieger JN, McNaughton-Collins M, et al. Chronic Prostatitis Collaborative Research Network. Alfuzosin and symptoms of chronic prostatitis-chronic pelvic pain syndrome. N Engl J Med. 2008; 359: 2663-73.
11. Nickel JC, Narayan P, McKay J, et al. Treatment of chronic prostatitis/chronic pelvic pain syndrome with tamsulosin. J Urol. 2004; 171: 1594-7.
12. Cheah PY, Liong ML, Yuen KH, et al. Terazosin therapy for chronic prostatitis/chronic pelvic pain syndrome. J Urol. 2003; 169: 592-6.
13. Cho IR. The present and future of prostatitis. Korean J Urol. 2008; 49: 475-89.
14. Mehik A, Alas P, Nickel JC, et al. Alfuzosin treatment for chronic prostatitis/chronic pelvic pain syndrome. Urology. 2003; 62: 425-9.
15. Nickel JC, Downey J, Clark J, et al. Levofloxacin for chronic prostatitis/chronic pelvic pain syndrome in men. Urology. 2003; 62: 614-7.
16. Yoshimura T, Kurita C, Usami E, et al. Immunomodulatory action of levofloxacin on cytokine production by human peripheral blood mononuclear cells. Chemotherapy. 1996; 42: 459-64.
17. Jeong CW, Lim DJ, Son H, et al. Treatment for chronic prostatitis/chronic pelvic pain syndrome: levofloxacin, doxazosin and their combination. Urol Int. 2008; 80: 157-61.
18. Caldwell DM, Ades AE, Higgins JP. Simultaneous comparison of multiple treatments. BMJ. 2005; 331: 897-900.
19. Park PHJ, Park PNC. The efficacy of tadalafil for chronic prostatitis/chronic pelvic pain syndrome in youngand middle aged patients. Eur Urol. 2012; 11: e280.

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Bell's Palsy: Evaluation of Clinical Response to Medical Treatment

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Abstract

Back ground Corticosteroid and antiviral agents are widely used to treat the acute phase of Bell's palsy but their effectiveness is still uncertain.

Objective This study aimed to compare the theraputic effect of Acyclovir and steroid versus steroid alone, in combanation with physiotherapy, in patients with Bell's palsy.

Methods This interventional study was conducted in Al-Yarmok Teaching Hospital and Al-Kadhimiya Teaching Hospital, during the period from July 2007 to July 2008. It involved (58) patients, who were divided into two groups: Group A; 28 patients, treated by steroid for 10 days. Group B; 30 patients, treated by steroid and acyclovir for 10 days. Physiotherapy for one month for both groups was followed and reassessment according to House-Brackmann grading system was done after completion of each therapy.

Results The recovery of patients treated with steroid and Acyclovir was (66.6%), while the patients treated with steroid alone was (46.4%), however, the diffrence was statistically insignificant. After one month physiotherapy the responses were (76.7% and 53.5%) for patients in both groups respectively.

Conclusion The addition of Acyclovir therapy in Bell's palsy has not found to be benefitial.

Keywords Bell's palsy, Corticosteroid, Antiviral agents, Physiotherapy.

Introduction

Bell's palsy is a dysfunction of the seventh cranial nerve that results in the paralysis of the facial muscles on the affected side of the face [(1)]. It has an annual incidence of 11 to 40 cases per 100000 population [(2)]. Male and female, are equally affected, although the incidence was found to be higher in pregnant women (45 cases per 100000) [(3)].

Many patients recover without intervention; however, up to 30% have poor recovery of facial muscle control and experience facial disfigurement, psychological trauma, and facial pain [(2)]. Two main types of pharmacological treatment have been used to improve outcomes from Bell's palsy: steroids and antivirals [(4)]. Recent guidelines from the American Academy of Neurology suggest that acyclovir combined with prednisone is "possibly effective" for Bell's palsy [(5)]. Two recent placebo-controlled trials demonstrated full recovery in a higher percentage of patients treated with an antiviral drug in combination with prednisolone than with prednisolone alone [(6)]. The rationale for these treatments is based on the presumed pathophysiology of Bell's palsy, namely inflammation and viral infection [(2)]. In 2001 a report of the quality standards subcommittee of the American Academy of Neurology mentioned

that the available evidence suggests that steroids are probably effective and acyclovir (combined with prednisone) is possibly effective in improving facial functional outcomes and a well-designed studies of the effectiveness of treatments for Bell's palsy are still needed [7].

The objective of this study is to determine whether steroids plus antivirals provide a better degree of facial recovery in a sample of Iraqi patients with Bell's palsy than steroids alone.

Methods

This study was performed in Al-Yarmouk Teaching Hospital and Al-Kadhimiya Teaching Hospital (2 of the main taching hospitals in the capital Baghdad, Iraq) from July 2007 till July 2008.

A randomized controlled trial was done on 58 patients attended the consultant of neurology complaining from symptoms suggestive of Bell's palsy. Patients consent to participate in the study was taken verbally from the patient.

Detailed history was taken from each patient and all patients were clinically examined of seventh cranial nerve in addition to the systemic examination. Then the patients were graded according to House–Brackmann grading system [8]. House–Brackmann grading system started with grade I, normal facial in all areas and ended in grade VI with total paralysis. All were sent for routine laboratory investigations including: Complete blood picture, ESR, blood sugar, metabolic screen, and connective tissue screen, to identify or exclude any associated illness which may interfere directly or by its treatment with the outcome of Bell's palsy.

Then the patients were randomly assigned to two groups:

Group (A): Included 28 patients, who received medical treatment in the form of Prednisolone tablets, 60 mg/day in divided doses, for 5 days, tapered gradually over the next 5 days. Then those patients were reevaluated clinically according to House-Brackmann grading system for Bell's palsy.

Group (B): Included 30 patients, who were put on medical treatment in the form of oral Prednisolone tablets, 60 mg/day in divided doses, with Acyclovir tablets, 400 mg, five times per day, for 10 days. Then these patients were reevaluated clinically according to House-Brackmann grading system for Bell's palsy.

After the medical treatment the patients in both groups were referred for physiotherapy in a physiotherapeutic department. Again they were reevaluated clinically after 4 weeks of physiotherapy.

The physiotherapy included:

1. Electrical Stimulation:

The electrical stimulation was done using a special electrical machine. It is usually started after the first week of medical treatment. One electrode is put on a finger of the hand and the other electrode was used as a stimulator for the facial muscles supplied by VII cranial nerve on selected points for few seconds on each point.

The usual voltage of the current is 100 µV, while the intensity of the current ranged from 0 - 10, according to the patient's sensitivity to the current. Each setting lasted from 10 - 15 minutes. The number of sessions was depending on the response of the patient (from one week – one month, or more).

2. Facial Massage:

Facial massage was used after electrical stimulation either daily or on alternate day using the following techniques:

a. Stroking: By applying powder on the face and massaging of the face by the hands of the physiotherapist. Rubbing started from the angle of the jaw to the angle of the mouth, and then to the temporal area. This rubbing was repeated with the same manner for at least 10 minutes.
b. Percussion: By tapping the muscles of facial expression by fingers, for 10 minutes.
c. Instruct the patients to do certain maneuvers at home, such; blowing, snuffing the nose, and rapid closing and opening of the eye, for 10 times daily or more in front of the mirror. Also all the patients were instructed to use antibiotic eye ointment daily.

Best clinical response was considered as clinical improvement and full recovery from the condition.

Data analysis was performed using descriptive statistics, Chi square, and student's t-test for proportions whatever applicable. The statistical package of social sciences (SPSS) version 15 was used for data input and analysis. P values of less than 0.05 were considered as statistically significant.

Results

The fifty eight patients participated in this study were between (18-47 years) of age, thirty seven of them were females making male:female ratio about 1:1.8. According to House-Brackmann score the patients were assessed before starting the treatment and found to be between grade 4 (G4) and grade 6 (G6) [as moderately sever to total paralysis]. Their distribution into the two treatment groups (A and B) are shown in table 1.

Table 1. Distribution of patients according to House-Brackmann's grading system for Bell's palsy in both groups before starting treatment.

Grade	Group A		Group B	
	No.	%	No.	%
G4	6	21.42	7	23.33
G5	14	50	15	50
G6	8	28.57	8	26.6
Total	28	100	30	100

$X^2 = 0.04$ $P = 0.98$

After 10-days medical treatment reassessment revealed that the best clinical response occur in 13 patients out of the 28 (46.4%) for patients treated with Prednisolone, and in total 20 patients out of 30 (66.7%) had clinical improvement to treatment with steroids and acyclovir, however the difference in the proportions were insignificant *P*= 0.12 (table 2).

Table 2. Distribution of patients improved by treatment according to House-Brackmann's grading system for Bell's palsy in both groups after using drugs alone.

Grade	Group A		Group B		Significance*
	No./total	%	No./total	%	
G4	4/6	66.67	5/7	71.43	*P* = 0.85
G5	7/14	50	12/15	80	*P* = 0.09
G6	2/8	25	3/8	37.5	*P* = 0.59
Total	13/28	46.43	20/30	66.67	*P* = 0.12

*Student's t-test for proportion

Patients in both treatment groups underwent physiotherapeutic treatment at hospital and at home for one month, then reassessed clinically according to House-Brackmann grading, there was an additional number of patients who got clinical improvement within each grade but with no statistically significant difference between both treatment groups (table 3).

Table 3. Distribution of patients improved by treatment according to House-Brackmann grading system for Bell's palsy in both groups after using drugs and physiotherapy

Grade	Group A		Group B		Significance*
	No./total	%	No./total	%	
G4	5/6	83.33	6/7	85.71	*P* = 0.91
G5	8/14	57.14	13/15	86.67	*P* = 0.08
G6	2/8	25	4/8	50	*P* = 0.30
Total	15/28	53.57	23/30	76.67	*P* = 0.06

* Student's t-test for proportion, #OR= 0.35 (95% C.I.=0.1-1.24)

Discussion

This study involved (58 patients) had onset of Bell's palsy of (3-5 days) duration, with male:female ratio of 1:1.76 that differs from many studies which reported equal male:female ratio of Bell's palsy or slightly in males more than females [9, 10, 11].

Recovery rate of the 28 patients treated with steroid alone was (46.4%), and this was less than the recovery rate (61.8%) of the 34 patients reported by Shahidullah *et al* [12], (65.2%) of the 210 patients reported by Engstorm *et al* [10], (74.5%) of the 47 patients reported by Yeo *et al* [9], (74.8%) of the 107 patients reported by Hato *et al* [6], and the recovery rate (83.0%) of the 130 patients reported by Sullivan *et al* [11] who were treated by steroid alone.

On the other hand, recovery rate of the 30 patients treated with steroid and acyclovir was (66.7%), and this was close to the recovery rate (65.0%) of the 206 patients reported by Engstorm *et al* [10], but less than the recovery rate (79.9%) of the 124 patients reported by Sullivan *et al* [11], (81.8%) of the 44 patients reported by Yeo *et al* [9], (82.5%) of the 114 patients reported by Hato *et al* [6], and the recovery rate (94.1%) of the 34 patients reported by Shahidullah *et al* [12] who were treated by steroid and antiviral therapy.

Despite the addition of physiotherapy in the treatment regimen treating Bell's palsy with antivirals plus corticosteroid may lead to slightly higher recovery rates (66.67%) compared to treating with prednisone alone (46.43%), but this does not reach statistical significance. This goes with the conclusion of many studies [6,9,10,11,] and 4 meta analyses who found that antivirals did not provide an added benefit in achieving recovery compared with steroids alone in patients with Bell's palsy [2,13,14,15]. This result may explained by the nerve palsy is due to the effect of the edema of the inflammatory reaction of the infection rather than the infection itself.

In conclusion: in spite of the small sample size of this study but it can be concluded that adding of Acyclovir in patients with Bell's palsy, had no significant improvements, as compared to those treated by steroid alone.

References

1. Oskoyi DS, Abedi A. Survey of Enviromental factors in incidence of Bell's palsy in Ardabil. Res J Biol Sci. 2008; 3 (1): 18-20.
2. Quant EC, Jeste SS, Muni RH, et al. The benefits of steroids versus steroids plus antivirals for treatment of Bell's palsy: a meta-analysis. BMJ. 2009; 39: b3354.
3. Holland NJ, Weiner M. Recent developments in Bell's palsy. BMJ. 2004; 329: 553-7.
4. Gilden DH. Clinical practice. Bell's palsy. N Engl J Med. 2004; 351: 1323-31.
5. Grogan PM, Gronseth GS. Practice parameter: Steroids, acyclovir, and surgery for Bell's palsy (an evidence-based review): report of the Quality Standards Subcommittee of the American Academy of Neurology. Neurology. 2001; 56: 830-6.
6. Hato N, Yamada H, Kohno H, et al. Valacyclovir and prednisolone treatment for Bell's palsy: a multicenter, randomized, placebo-controlled study. Otol Neurotol 2007; 28: 408-13.
7. Grogan PM, Gronseth GS. Practice parameter: steroids, acyclovir, and surgery for Bell's palsy (an evidence-based review) report of the quality standards subcommittee of the American Academy of Neurology. Neurology. 2001; 56; 830-836.
8. House JW, Brackmann DE. Facial nerve grading system. Otolaryngol Head Neck Surg. 1985; 93: 146-7.
9. Yeo SG, Lee YC, Park DC, et al. Acyclovir plus steroid vs

steroid alone in treatment of Bell's palsy. Am J Otolaryngol. 2008; 29(3): 163-6.

10. EngstromM, Berg T, Stjernquist-Desatnik A, et al. Prednisolone and valaciclovir in Bell's palsy: a randomised, double-blind, placebo-controlled, multicentre trial. Lancet Neurol. 2008; 7: 993-1000.

11. Sullivan FM, Swan IR, Donnan PT, et al. Early treatment with prednisolone or acyclovir in Bell's palsy. N Engl J Med. 2007; 357: 1598-607.

12. Shahidullah M, Haque A, Islam MR, et al. Comparative study between combination of famciclovir and prednisolone with prednisolone alone in acute Bell's palsy. Mymensingh Med J. 2011 Oct; 20(4): 605-13. [Abstract].

13. Numthavaj P, Thakkinstian A, Dejthevaporn C, et al. Corticosteroid and antiviral therapy for Bell's palsy: A network meta-analysis. BMC Neurology. 2011; 11: 1-10.

14. de Almeida JR, Al Khabori M, Guyatt GH, et al. Combined corticosteroid and antiviral treatment for Bell palsy: a systematic review and meta-analysis. JAMA. 2009; 302(9): 1003-4. [Abstract]

15. Goudakos JK, Markou KD. Corticosteroids vs corticosteroids plus antiviral agents in the treatment of Bell palsy: a systematic review and meta-analysis. Arch Otolaryngol Head Neck Surg. 2009; 135(6): 558-64. [Abstract]

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A Study of Serum Magnesium and Calcium Levels in Missed Abortion

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Abstract

Background Pregnancy is considered as a physiological stress, normal static metabolism of a woman is changed into dynamic anabolism, calcium (Ca) is the first most abundant cation in the human body, whereas magnesium (Mg) is the fourth most abundant one, role of calcium and magnesium in enzymatic activity of cell to release energy is well established.

Objective To assess the relation of serum magnesium and calcium levels in cases of missed abortion.

Methods Eighty two pregnant women at their 1st and 2nd trimester of pregnancy (before 24 completed weeks of pregnancy), 42 of them with missed abortion compared with 40 normal pregnancies served as a control group. Calcium analysis done using manual colorimetric method while magnesium analysis was done by magnesium calmogite method at the hospital laboratories.

Results Serum calcium was found to be insignificantly altered while serum magnesium was found to be significantly reduced in cases of missed abortion compared with normal pregnancy. Serum Ca/Mg ratio was found to be significantly elevated in cases of missed abortion compared with normal controls.

Conclusion Estimation of serum magnesium and Ca/Mg ratio in selected pregnancies can be valuable parameters for predicting missed abortion.

Keywords Missed abortion, Serum Calcium, Serum Magnesium.

Introduction

Miscarriage is the spontaneous end of a pregnancy at a stage where the embryo or foetus is incapable of surviving generally defined at or prior to 20 weeks of gestation, it is the most common complication of early pregnancy [(1)]. A missed miscarriage is a type of abortion in which the foetus dies but the fetal tissue is not expelled by the woman's body and remains there until it is removed by a doctor [(2)].

The exact cause of a missed miscarriage is unknown. However, about half of all early miscarriages occur due to a genetic problem within the ova or sperm. In addition, other factors such as immune system problems and serious infections can increase the risk of miscarriage. The chance of having a miscarriage also increases with age. About one percent of all pregnancies end in a missed miscarriage [(3,4)].

Excellent nutrition is one of the primary cornerstones of maintaining a healthy pregnancy, and there is no better time to start than in the first trimester. A highly varied diet is the most important aspect of maintaining excellent health during pregnancy, but the three most important nutrients during the first trimester are folic acid, vitamin B-6 and calcium [(5)]. Calcium (Ca) is required for muscle

contraction, blood vessel expansion and contraction, secretion of hormones and enzymes, and transmitting impulses throughout the nervous system [6]. Magnesium (Mg) is needed for more than 300 biochemical reactions in the body. It helps to maintain normal muscle and nerve function, supports a healthy immune system, regulates blood sugar levels, promotes normal blood pressure, and is known to be involved in energy metabolism and protein synthesis [7,8].

The aim of the study is to assess the relation of serum magnesium and calcium levels in cases of missed abortion.

Methods

This case control study was conducted at Al-Elwiya maternity teaching hospital in Baghdad from Jan. 2010 to Jul. 2010. The study protocol was approved by the Obstetrics and Gynecology Committee of the Arab Board for Medical Specialization and the authority of Al-Elwiya Teaching Hospital.

Eighty two women in their 1st and 2nd trimester of pregnancy (before 24 completed weeks of gestation) were included in the study, forty two of them with proved missed abortion and 40 with normal pregnancy served as control group. The patients were selected during their visit to the outpatient clinic during the study period after submission to inclusion and exclusion criteria.

The inclusion criteria included all singleton pregnancies before 24 completed weeks of gestation, age between 18-35 years, parity not more than 3 and abortions not more than 2.

The exclusion criteria included all pregnancies with lethal congenital abnormalities; any associated maternal medical diseases that may lead to fetal compromise and pregnant women receiving medications for chronic illnesses like diabetes mellitus, thyroid disease and parathyroid disease, also cases of multiple pregnancies, patient receiving calcium and/or magnesium during current pregnancy and currently lactating women.

A detailed history was taken from each woman included her gestational age (which was established according to the last regular menstrual period (LMP) and early pregnancy ultrasonography), fetal viability was established by 2 consecutive ultrasound examinations, detailed history of a current pregnancy and previous pregnancy outcomes with any obstetrical or medical complications or early pregnancy disorders. The information recorded on special form designed for the study.

Blood samples were taken from each patient in both study and control groups, serum isolated and sent for calcium analysis by manual colorimetric method, and magnesium analysis by magnesium calmogite method.

Statistical analysis

Data were collected and arranged in tables and then subjected to analysis using descriptive statistics (No. and %) and inferential statistics (unpaired t-test). *P* value less than 0.05 was considered statistically significant and CI not containing zero also was considered significant.

Results

All participants were comparable in regard to their age and gestational age of pregnancy (Table 1).

Table 1. Age and GA (in weeks) of both study and control groups respectively

	Study group	Control group	*P* value
Frequency	42	40	-
Age (years)	26.45 ± 5.62	26.4 ± 5.09	0.965
GA (weeks)	11.33	12.03	0.374

There was no statistical difference between study and control groups in the mean of serum calcium level. A significantly lower magnesium level was noticed in women with missed abortion compared with normal controls (Table 2).

Table 2. Serum calcium and magnesium level of both study and control group respectively

Serum level (mg/dl)	Study group Mean ± SD	Control group Mean ± SD	*P* value
Calcium	8.074 ± 0.682	8.072 ± 0.598	0.99
Magnesium	1.597 ± 0.162	1.682 ± 0.140	0.012

The study also showed a statistically significant difference between study and control groups regarding mean of Ca/Mg ratio, there was significantly higher Ca/Mg ratio noticed in women with missed abortion compared to normal controls (Table 3).

Table 3. The mean and SD for Ca/Mg ratio of both study and control groups respectively

Ca/mg ratio	Study group	Control group	P-value
Frequency	42	40	
Mean ± SD	5.116 ± 0.698	4.834 ± 0.521	0.041

Discussion

The loss of pregnancy is always distressing to the mother irrespective of its timing. Abortion has serious consequence with appreciable risk of maternal mortality and long term morbidity [9].

Pregnancy is considered as a physiological stress, normal static metabolism of women is changed into dynamic anabolism, calcium is the first most abundant cation in the human body, whereas magnesium is the fourth most abundant one, role of calcium and magnesium in enzymatic activity of cell to release energy is well established [9].

We selected eighty two pregnant women at their 1st and 2nd trimester of pregnancy, the participants were singletons, not currently lactating, their parity not more than three (to exclude exhaustion of maternal stores of trace elements), also no more than two abortions to exclude the other common causes of recurrent abortions like congenital abnormalities of the foetus, incompetent cervix and congenital abnormalities of the uterus.

Nearly half of the sample served as a study group (pregnant with proved missed abortion) and another half served as a control group (normal pregnancy) of approximately the same gestational age in weeks.

In order to focus on the difference in serum Ca and Mg levels and Ca/Mg ratio in the study cases and comparing the results with those of normal pregnancy, we excluded the statistical difference of gestational age in weeks, patient's age, parity and number of abortions.

No statistical significance was found between the two groups in regard to serum Ca level (Table 2), indicating that there is no direct relation of Ca level with missed abortion. This result is comparable with the study of Han et al.[10] who concluded that calcium level did not show statistical significance in most cases. While serum Mg level showed statistical significance between the study groups (Mg deficiency in cases of missed abortion compared with normal controls, as shown in table 2). These results are comparable with studies of Borella et al [11] who found that 25% of patients with abortion have hypomagnesemia.

The same thing is seen with Ca/Mg ratio which shows statistically significant difference between the study groups (Ca/Mg ratio increased in cases of missed abortion compared with normal controls). This goes with the results obtained by

Borella [11] who reported an increased Ca/Mg ratio in patients with abortions, but not when patient had a successful continuation of pregnancy.

We find that our study does not go with the results of Cilensec *et al* [12] where whole blood and serum magnesium levels were determined in 66 healthy pregnant women and in 68 women with imminent, incipient, or incomplete abortions. Serum magnesium levels were equally depressed in abortion and in normal pregnancy, but magnesium concentrations per 100 ml erythrocytes were significantly higher in abortion patients. This finding is probably due to the increased release of immature erythrocytes with high magnesium concentrations to compensate for blood losses [12].

We think that trace elements and minerals are of value in continuation of normal pregnancy; this is confirmed by Barrington *et al* who suggested that low selenium levels were found in a significant number of women with single miscarriage [13].

Moreover, group comparison performed by analysis of variance [ANOVA] followed by dunnett test indicated substantially lower plasma concentration of copper in pathological conditions diagnosed during first trimester of pregnancy (spontaneous abortion, threatened abortion, missed abortion and blighted ovum) but not in the 2nd trimester [14].

Many studies show strongly positive balance of magnesium retention during pregnancy i.e. the formation of new tissues (maternal and fetal) during pregnancy requires higher magnesium intake than that of the normal non-pregnant women of comparable age [15].

In conclusion, there is a significant relation among magnesium deficiency, Ca/Mg ratio and missed abortion while there is no significant relation noticed between calcium deficiency and missed abortion.

References

1. Petrozza CJ. Early pregnancy loss, e medicine. The Daily Telegraph. 2007 July; 22: 14-6.
2. Arck PC, Rucke M, Rose M, et al. Early risk factors for miscarriage. Reprod Biomed online. 2008 Jul; 17(1): 101-13.
3. Christopher FC, Gertie FM. Physiological changes associated with pregnancy, pregnancy encyclopedia. Britanica. 2010; E.B.online 2:6.
4. Baker PN, Johnson J, Jones G, et al. Obstetrics by ten teachers.18th ed. Edward Arnold Ltd; 2006. p. 60.
5. Juniper AR. The healthiest foods for early pregnancy. Digital J. 2009 Mar; 4: 2.
6. Jonathan C. Dietary reference intakes for calcium, phosphorus, magnesium, vitamin D and fluoride. Health Dis Cond Apr. 2011; 12: 123-6.
7. Wester PO. Magnesium. A M J Obstet Gynecol. 1987; 45: 1305-12.
8. Saris NE, Mervala E, Karppanen H, et al. Magnesium: an update on physiological, clinical, and analytical aspects. Clinica Chem Acta. 2000; 294: 1-26.
9. Kishorkumar H, Sinha SK, Kumudini J. A study of serum magnesium level in different types of abortions. Int J Gynecol Obstet. 2009; 92: 16.
10. Han MH, PoLau L, DaZe S, et al. Hair and serum calcium, iron, copper and zinc levels during normal pregnancy at 3rd trimester. Biolog Trace Element Res. 2008; 69(2): 111-120.
11. Borella P, Szilagyi A, Than G, et al. Maternal plasma concentrations of magnesium, calcium, zinc and copper in normal and pathological pregnancies. Sci Total Environ. 1990 Dec; 99(1-2): 67-76.
12. Cilensec M, Mende HE, Simon V. Behaviour of serum magnesium level during abortion. Zentralbl Gynakol. 1975; 97(19): 1176-8.
13. Barrington JW, Indsay P, James D, et al. Selenium deficiency and miscarriage: A possible link? Br J Obstet Gynaecol. 1996; 103: 130-2.
14. Hilal OZ, Sinan M, Duru S, et al. Instant effect of induced abortion on serum ceruloplasmin activity, copper and zinc levels. Arch Gynaecol Obstet. 2005; 240(1): 21-5.
15. Milder SS. Early roots of cardiovascular, skeletal and renal abnormalities. Magnesium deficiency in the pathogenesis of disease. New York J, 1980 Aug; 12: 465-7.

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Endometrial Changes in Women on Tamoxifen for Breast Cancer

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Abstract

Background Tamoxifen is a selective estrogen receptor modulator (SERM) that is widely used in the treatment of patients with breast cancer and for chemoprophylaxis in high risk women. Tamoxifen results in a spectrum of endometrial changes due to it's estrogenic effect on the endometrium.

Objectives To evaluate the extent of endometrial pathologies that might develop in postmenopausal breast cancer patients following treatment with tamoxifen.

Methods Sixty postmenapausal with breast carcinoma women were involved in this study, Thirty women were receiving 20-40 mg of tamoxifen daily for a period of 6 to 60 months constitutes the study group, and a control group included 30 postmenopausal breast carcinoma patients who were not receiving tamoxifen. Transvaginal sonography was performed for the measurements of the endometrial thickness and the presence of endometrial pathology. All the patients underwent endometrial sampling and the curetting were sent for histopathological examination.

Results There was statistically significant increase in the frequency of endometrial pathology in those on tamoxifen;there was 11 endometrial pathologies in the case group,while the control group was associated with only 3 pathologies(p=0.015). There was significant difference in endometrial thickness between case group (0.73+0.32 mm) and the control group (0.5+0.16mm) with p value 0.002. Only patients with endometrial thickness of more than 5mm were associated with pathologies, 7 (38.8%) of the endometrial biopsies revealed normal endometrium, whereas, 11(61.1%) had endometrial pathology like hyperplasia, endometrial polyp or carcinoma. The rate of endometrial pathologies considerably increase with increasing duration of treatment.

Conclusion The long term use of tamoxifen as adjuvant therapy for carcinoma breast is associated with increase frequency of endometrial pathology. Endometrial thickness is increased in such patients and is related to the duration of tamoxifen use.

Key Words Tamoxifen, Endomatrial pathology, Breast cancer.

Introduction

Tamoxifen, A nonsteroidal antiestrogen, was first approved by the Food and Drug Administration for the treatment of patients with breast cancer in 1978. Large clinical trials involving over 75,000 patients have demonstrated an improved recurrence-free and overall survival benefit in both pre- and postmenopausal women [(1)]. Long-term adjuvant tamoxifen is the endocrine treatment of choice for selected patients with breast cancer, and there are currently large-scale trials continuing to evaluate its role as a chemopreventative agent in healthy women at risk for breast cancer. One of the most significant complications of long-term tamoxifen use is the possible development of endometrial cancer. Although tamoxifen is believed to exert its main effect by blocking the binding of estrogen to the estrogen receptor (ER), it exhibits a wide range of biologic

effects that may account for its activity in ER-negative tumors as well as some of its unwanted side effects. These include inhibition of calmodulin, stimulation of transforming growth factor beta secretion, induction of apoptosis, interaction with P-glycoprotein, inhibition of protein kinase and phospholipase C, and stimulation of phosphoinositide kinase activity [(2)].

Although primarily an antiestrogen, tamoxifen may also exhibit some mild estrogenic effects. After an initial report by Killackey et al [(3)] which suggested a possible link between tamoxifen use and the development of endometrial carcinoma in three patients. The National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14 [(4)] performed a randomized trial of tamoxifen versus placebo in women with ER-positive breast cancer confined to the breast with negative axillary nodes. Results from this trial of 4,063 patients revealed a 7.5-fold increase in the risk of developing endometrial cancer in the tamoxifen-treated group. The average annual hazard rate for endometrial cancer was 0.2 per 1,000 women in the placebo group and 1.6 per 1,000 women in the randomized tamoxifen-treated group.

Indications for tamoxifen use have broadened to include long-term adjuvant therapy as well as preventative therapy for selected high-risk women. Consequently, a large number of women, including healthy, young patients with no history of cancer, will be subjected to the long-term effects of tamoxifen. In the NSABP *P*-1 trial, 13,338 women at increased risk for breast cancer were randomly assigned to tamoxifen versus placebo for 5 years [(5)]. Tamoxifen reduced the risk of both invasive and noninvasive breast cancer by 49% and 50%, respectively. The authors reported 33 cases of endometrial cancers in the tamoxifen-treated group compared with 14 in the control group (relative risk = 2.53). The cancers in the tamoxifen-treated group occurred predominantly in women over the age of 50. These cancers were diagnosed at an early stage and, therefore, did not result in any deaths as a result of endometrial cancer. This study investigates the association between tamoxifen uses in breast cancer patients and associated uterine pathology.

Methods

This case control study was conducted from the beginning of March 2010 to the end of July 2011. Postmenopausal women with breast cancer who attended the oncology outpatient clinic at al-kadhmiya teaching hospital for regular control for their breast cancer were asked to participate in this study.

All patients were treated by primary breast surgery, Adjuvant radiotherapy and/or chemotherapy were included in the therapeutic plan, according to current guidelines of oncology department at Al-Kadhmiya Teaching Hospital.

Postmenopausal status was defined as more than 12 months amenorrhea.

In total, 60 women with an intact uterus and without gynecologic symptoms (bleeding or discharge) took part in the current study.

Thirty women were receiving 20-40 mg of tamoxifen daily for a period of 6 to 60months constitutes the case group, and a control group included 30 postmenopausal breast carcinoma patients who were not receiving tamoxifen.

Patient approval was obtained from each patient after the nature of the study was fully explained.

None of the women in either group used hormone replacement therapy at the time of the investigation.

Age, body mass index, the years since menopause, history of hypertension, diabetes and smoking were recorded.

Transvaginal ultrasound

Transvaginal ultrasonographic measurements of the endometrial thickness was performed by senior sonographist using Siemens Versa (Germany) ultrasound machine with 6.3 MHz transducer as follows: Patients consent was obtained and following explanation of the technique, the women asked to empty her bladder and were placed in the dorsal lithotomy position, a small amount of gel is applied to transducer tip, and the tip and the shaft of the

probe covered with condom, apply a small amount of lubricant gel to allow easy insertion of the probe.

The Measurements of the endometrial thickness were performed in the thickest part in the longitudinal plane. The measurement included both endometrial layers. When the endometrial layers were separated by intracavity fluid, both layers were measured and the sum was recorded.

All the patients underwent endometrial sampling using Novak curette size 3mm without anesthesia and the curetting was sent for histopathological examination done in the pathology department in Al-Kadhimiya hospital. Slides were reviewed by senior pathologist.

Endometrial pathology was defined by the presence of one or more of the following histologic findings: proliferative endometrium, simple hyperplasia, complex hyperplasia with or without atypia, endometrial polyp, or endometrial carcinoma. The endometrium was considered as negative if no finding, other than atrophic endometrium, was diagnosed.

Statistical analysis

Data were analyzed using SPS version 16 & Microsoft office Excel 2007. Numeric data were presented as means±SD and nominal data were presented as number and percents. Numeric data were analysied using T test or ANOVA while nominal data were analysed using Chi-Square. P value <0.05 was considered siginificant.

Results

60 patients with carcinoma of breast were included in this study. 30 patients were on tamoxifen constitue the study group and 30 patients were without tamoxifen use constitue the control group.

The medium age of the on tamoxifen group was 57.17±5.71 and of the without treatment was 54.80±4.92.

Mean BMI for the study group was 30.73±3.09, and 29.40±4.19 for the control group. Mean parity was 3.40±2.12 and 4.20±3.02 respectively. Patients in study group were older, with higher BMI, but with lesser parity than the control group. The median duration of tamoxifen use was 3.31 years(range: 4.83-1.79).

The mean time since breast cancer diagnosis in months in study group was 48±17.57 and in control group was 32.73±13.00, the difference was statistically significant with longer duration in study group. The study group was associated with 11 endometrial pathologies while the control group was associated with only 3 pathologies. The p value was (0.015) so it was statistically significant. There was significant difference in endometrial thickness between study group (7.3+3.2 mm) and the control group (5.0+1.6mm) with p value 0.002. as shown in table 1. There is no difference in co morbid conditions(hypertention, DM) between the two groups.

Table 1. Comparison Between Patients On Tamoxifen Treatment And Those With No Tamoxifen Treatment

Characteristic	Patients on tamoxifen N = 30	Patients with no tamoxifen N = 30
Age (years)	57.17 ± 5.71	54.8 ± 4.92
BMI (Kg/m2)	30.73 ± 3.09	29.4 ± 4.19
Parity	3.4 ± 2.12	4.2 ± 3.02
Age at menopause (years)	48.4 ± 2.56	47.63 ± 2.32
Smoking	6	6
DM	8	7
Hypertension	8	5
Time since breast cancer diagnosis (months)	48.0 ± 17.57	32.73 ± 13.0***
Endometrial pathology	11	3*
Endometrial thickness (mm)	7.3 ± 3.2	5.0 ± 1.6**

* = P 0.05, ** = P 0.005, *** = P 0.001

Table 2: Relation of Endometrial Thickness and Frequency of Endometrial Pathology in Study Group

Endometrial thickness	No. of patients	%
< 5 mm	5	16%
5 mm	7	23.3%
> 5 mm	18	60%
P = 0.003		

In the study group, 5 patients (16%) showed endometrial thickness of less than 5 mm, while 7 (23%) showed endometrial thicknessof 5 mm, 18 patients (60%) with endometrial thickness more than 5 mm. The mean endometrial thickness was 0.73 mm.

There is statistically significant relation between endometrial thickness and endometrial pathology (p=0.003).as shown in table 2.

Only patients with endometrial thickness of more than 5mm was associated with pathologies, 7 (38.8%) of the endometrial biopsies revealed normal endometrium, whereas, 11(61.1%) had endometrial pathology like hyperplasia, endometrial polyp or carcinoma. As endometrial thickness increased, the incidence of abnormal finding was increased. Endometrial pathology was present in three cases in the control group and all of them were in those with endometrial thickness of more than 5mm, as shown in table 3.

Table 3. Relation of endometrial thickness and frequency of endometrial pathology in control group.

Endometrial thickness	No. of patients	Percent %	endometrial pathology
< 5 mm	13	43.3	0
5 mm	8	26.6	0
> 5 mm	9	30	3
P = 0.02			

There were four cases of simple hyperplasia ,two cases of Complex atypical hyperplasia, two cases of benign endometrial polyp and three cases with endometrial carcinoma in the study group. As shown in table 4.

There were only two cases of simple hyperplasia and one case with polyp in the control group. There was no endometrial carcinoma detected in control group. As shown in table 4, the incidence of total endometrial abnormalities in the tamoxifen group was greater than that in the control group ($P < 0.015$)

The three cases of endometrial carcinoma developed in those on tamoxifen were well differenciated stage I endometrioid adenocarcinoma.

Table 4. Type of pathologies of the endometrium for study and control groups.

Endometrial pathology	Tamoxifen		Total	P value
	Yes	No		
Negative	19	27	46	0.015
Simple hyperplasia	4	2	6	0.671
Complex atypial hyperplasia	2	0	2	0.492
Polyp	2	1	3	1.000
CA	3	0	3	0.237
Total	30	30	60	

There was significant relation between duration of treatment and endometrial thickness, after 3 years of treatment there is marked rise in number of patient with thickened endometrium. As shown in table 5.

Table 5. Relation between duration of use of tamoxifen and endometrial thickness

Duration of use	≤ 5 mm thickness	> 5 mm thickness
6 months – 1 year	2	1
1 year – 2 years	3	0
2 years – 3 years	4	3
3 years – 4 years	0	3
> 4 years	3	11
P = 0.036		

Table 6. The correlation between duration of tamoxifen exposure and the abnormalities detected in the study group

H/P diagnosis	≤1 year exposure N = 3	>1 year exposure N = 27	Odds ratio	95% CI	P value
Negative	2	17	1.17	(0.094-14.68)	1.000
(atrophic)	1	10	0.98	(0.77-1.25)	1.000
Positive	0	4	1.17	(1.00-1.37)	1.000
simple	0	2	1.08	(0.97-1.20)	1.000
complex hyperplasia polyp	1	1	0.1	(0.01-0.64)	0.18
endometrial carcinoma	0	3	1.12	(0.98-1.28)	1.000

Table 6 describes the distribution of endometrial pathologies with relation to duration of tamoxifen treatment, 3 (10%) patient were on tamoxifen for a duration of one year or less, 27(90 %) used it for a duration more than one year. The rate of endometrial pathologies considerably increase with increasing duration of treatment.

Three patient were on tamoxifen therapy (40 mg \day). One of them had carcinoma and the other have complex hyperplasia and one of normal endometrium. Twenty seven patients was on tamoxifen 20 mg \day, two had cancer, two with polyp, one with complex hyperplasia and four with simple hyperplasia, as shown in table 7.

Table 7. Comparison between different doses of tamoxifen regarding endometrial pathology in the study group

Dose	Negative	Positive pathology	Total
20	18	9	27
40	1	2	3
Total	19	1	30
P = 0.548			

Discussion

The oestrogen-receptor antagonist tamoxifen is effective in premenopausal, perimenopausal, and postmenopausal women with oestrogen-receptor positive breast, including hyperplasia, polyps, cancer, and uterine sarcoma reported; Tamoxifen users have a two fold to seven fold increased risk of endometrial cancer, and the risk seems to be highest after long-term use (2-3/1000 women per year, during or after Tamoxifen therapy) [6,7].

In our study there was statistical significant difference in number of pathologies between tamoxifen treated and control groups (p=0.015). Our findings are consistent with those of Cohen et al. They found a high rate of pathological endometrial changes among asymptomatic, postmenopausal patients who have been treated with tamoxifen for breast cancer, compared with non-treated patients. Twenty-two (29%) of the 77 postmenopausal women tested had positive histological findings in the endometrial biopsy [8].

Mcgonigle *et al* failed to detect a single case of endometrial hyperplasia after tamoxifen therapy, in this study patients with thickened endometrium and significant endometrial pathologies prior to tamoxifen therapy were excluded. As such, the effects on the endometrium detected after tamoxifen therapy were unlikely to be preexisting but rather a true effect of tamoxifen. At baseline, 80% of patients had atrophic endometrium and 9% proliferative endometrium compared with 61% and 26% at 1 year, respectively. No cases of endometrial hyperplasia or adenocarcinoma were detected. Findings observed at 6 months persisted through 5 years of follow-up. They conclude that tamoxifen exerts a weak estrogenic effect on the vagina and uterus in pre-screened

postmenopausal women without preexisting endometrial pathology [9].

We found a significant increase in endometrial thickness in tamoxifen treated group than in the control, and a highly significant positive correlation between the duration of tamoxifen treatment and endometrial thickness (P=0.036), and we found abnormal endometrial biopsy in 36.6% (p 0.015) of women treated with tamoxifen and a significant relation between endometrial thickness and risk of endometrial pathology (p=0.003). Women with long-term use of tamoxifen (four or more years) were more likely to develop uterine pathology than nonusers.

The time-dependent nature ofthe development of endometrial pathology by women treated with tamoxifen (shown in our study) is in accordance with the report by Decensi et al that tamoxifen at 20 mg per day exerts a time-dependent proliferative effect on the endometrium [10].

Kochar *et al* noticed a significant relation between endometrial thickness and duration of tamoxifen treatment (P=0.025) as in the present study and a significant linear relationship between the symptomatic status and duration of tamoxifen use (P = < 0.01), and a significant linear relationship between endometrial thickness and duration of tamoxifen use [11].

The British Tamoxifen Second Cancer Study Group showed that the odds of endometrial cancer associated with tamoxifen use increased significantly with increasing duration of use up to 10 years (P= 0.001) [12].

Fishman *et al* found that endometrial thickness increased with increasing duration of tamoxifen use at a rate of 0.75 mm/yr. The mean endometrial thickness after 5 years of tamoxifen use was 12 mm (range 6 to 21 mm). After discontinuation of tamoxifen treatment the endometrium decreased by 1.27 mm/yr [13].

MIGNOTTE *et a*l results clearly support the notion that tamoxifen increases the risk for endometrial cancer, with a significant crude overall risk, mainly dependent on the duration of treatment (relative risk 1.5 per year of treatment) irrespective of the daily dose. estimates for the RRs for endometrial cancer associated with the duration of treatment at this dose 20 mg were particularly high after 3 years of treatment [14].

Gerber *et al*, reported that mean endometrial thickness in tamoxifen- treated patients ranged from 7.2 ± 8.5 to 12.1 ± 12.4 mm, compared with 1.5 ± 4.3 to 5.4 ± 2.7 mm in controls [15].

Nahari *et al* were unable to find a significant effect of the duration of tamoxifen exposure on the endometrial thickness, whereas other investigators have reported such a correlation [16].

Ozsner *et al* have shown that tamoxifen use increases the risk of endometrial cancer and premalignant change. They also noticed significant relation between endometrial thickness and duration of tamoxifen treatment (*P*=0.025) as in our study [17].

Bernstein *et al* in a case control study concluded that endometrial cancer associated with tamoxifen use and the risk increased with the duration of tamoxifen use [6].

Leeuwen *et al* emphasized that there was a significant increasing risk of endometrial carcinoman with duration of tamoxifen use, and also with cumulative dose [7].

Cohen I et al, reported that endometrial pathologies are associated with high cumulative doses of tamoxifen administered to postmenopausal breast cancer patients. Women who received 20 mg of tamoxifen daily developed endometrial pathologies after longer periods of treatment compared to those who were treated with 40 mg of tamoxifen daily [8].

This is not consistent with our result since we fail to demonstrate any association between dose of tamoxifen and endometrial pathologies, perhaps because of small size of our sample.

The National Surgical Adjuvant Breast and Bowel Project study suggested that the incidence of uterine malignancies is increased in women taking tamoxifen [4].

Magriples, et al. reviewed 53 patients diagnosed with breast cancer who subsequently developed uterine malignancy and found that 67% of

tamoxifen users developed a uterine cancer of high risk histologic type as compared to 24% of tamoxifen non-users, p=0.03 [18].

The Stockholm Trial showed a continued divergence of the cumulative incidence curves of endometrial cancer for the tamoxifen treated and control groups even several years after cessation of tamoxifen treatment [19].

Katase et al concluded that tamoxifen does not appear to increase subsequent endometrial carcinoma in patients with primary breast carcinoma who underwent annual screening for gynaecologic cancer [20].

Long-term tamoxifen exposure, obesity, and prior estrogen replacement therapy older menopausal age, and longer duration of breast disease may increase the risk of tamoxifen-associated endometrial pathology.

In our study we found no difference in age, BMI, parity, age at menopause, DM and HT co morbidity in both treatment and control group .

We found no difference in these variables between patient with pathology after tamoxifen treatment and patient without pathology,Therefore, it was impossible to predict which of these women would have developed pathological endometrial changes.

Bland et al find There were no significant differences in age, BMI, or medical co-morbidities or other demographic variables that they identified between tamoxifen users and non-users [21].

Mandana et al. find that, patient-related risk factors for endometrial cancer including age, history of unopposed estrogen use, and comorbid conditions such as obesity, hypertension, and diabetes were similar between the tamoxifen treated and non tamoxifen groups [22].

Cohen *et al* could find no risk factors nor any high risk subgroup among the women in their study There was only a statistical tendency of lower mean age or those patients with positive histological findings when compared to those with negative findings (P = 0.0510) [8].

Patients should be told of the small risk of endometrial cancer (even after stopping the use of the drug), and encouraged to report relevant symptoms early. They can, however, be reassured that the clinical benefits of treatment far outweigh the risks.

Women using tamoxifen should seek prompt medical attention and prompt investigation required if abnormal vaginal bleeding including menstrual irregularities, vaginal discharge, and pelvic pain or pressure in those receiving (or who have received) tamoxifen.

Seoud et al concluded that the value of routine screening for endometrial pathology in patients on tamoxifen is controversial. They found that all patients who developed an abnormal endometrium had abnormal vaginal bleeding [23].

Peters-Engl et al demonstrated that clinical benefits of tamoxifen greatly outweigh the risk. They recommended annual follow up of patients on tamoxifen [24].

The etiology of endometrial cancer in tamoxifen-treated patients has been theorized to be related to estrogenic effects on the endometrium, and randomized studies have been undertaken to determine if oral or intrauterine progestins can reduce the negative effects of tamoxifen on the uterus [25,26].

Depending on the specific clinical situation, recent data suggest equal or superior efficacy of selective estrogen receptor modulators and new antiestrogens compared with tamoxifen for women with breast cancer [27,28].

Despite the introduction of these newer hormonal therapies, tamoxifen remains the standard initial adjuvant therapy for women with hormone receptor–positive breast cancer and is still the most common hormonal therapy used for breast cancer patients. The clinician must keep in mind, however, that tamoxifen is highly effective in reducing recurrence and deaths from breast cancer, and the risk to the patient of developing endometrial cancer while taking this drug is no worse than that of unopposed estrogen administration.

The most recent American College of Obstetricians and Gynecologists guideline for tamoxifen use and endometrial cancer risk has several recommendations for postmenopausal

women:Routine endometrial screening is not recommended for women taking tamoxifen because of the costs incurred and risk of unnecessary further investigation. Instead, women should be educated regarding the symptoms of endometrial cancer and instructed to consult their doctor if they developany spotting or postmenopausal bleeding. If a woman develops endometrial hyperplasia, the use of tamoxifen should be re-assessed [29].

The present study has shown that long term use of tamoxifen as adjuvant therapy for carcinoma of the breast is associated with increased frequency of endometrial pathology. Endometrial thickness is increased in such patients and is related to the duration of tamoxifen use. All patients on long term tamoxifen should be annually screened for endometrial pathology.

References

1. Early Breast Cancer Trialists' Collaborative Group: Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy: 133 randomized clinical trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Lancet. 1992; 339: 1-15.
2. Friedman ZY. Recent advances in understanding the molecular mechanisms of tamoxifen action. Cancer Invest. 1998; 16: 391-6.
3. Killackey MA, Hakes TB, Pierce VK. Endometrial adenocarcinoma in breast cancer patients receiving antiestrogens. Cancer Treat Rep. 1985; 69: 237-8.
4. Fisher B, Costantino JP, Redmond CK, et al. Endometrial cancer in tamoxifen-treated breast cancer patients: Findings from the National Surgical Adjuvant Breast and Bowel Project (NSABP) B-14. J Natl Cancer Inst. 1994; 86: 527-37.
5. Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: Report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst. 1998; 90: 1371-88.
6. Bernstein L, Deapen D, Cerhan JR. Tamoxifen therapy for breast cancer and endometrial cancer risk. J Nat Cancer Inst. 1999; 91: 1654-62.
7. Van Leeuwen FE, Bergman L, Benraadt J. Does risk of endometrial Cancer increase with longer duration of tamoxifen use, Eur J Cancer. 1998; 34: 44-5.
8. Cohen L, Rosen DJD, Shapira J, et al. Endometrial changes in postmenopausal women treated with tamoxifen for breast. Br J Obstet Gyn. 1993 June; 100; 567-70.
9. Mcgonigle KF, Smith DD, Marx HF, et al; Uterine effects of tamoxifen: a prospective study 2006. Int J Gynecol Cancer 2006; 16: 814-820.
10. Decensi A, Fontana V, Bruno S, et al. Effect of tamoxifen on endometrial proliferation. J Clin Pathol 1996; 14: 434-440.
11. Kochar S, Arora P, Chattopadhyay AB. Tamoxifen therapy for breast cancer and endometrial pathology. MJAFI 2005; 61: 313-315.
12. Swerdlow AJ, Jones ME. Tamoxifen treatment for breast cancer and risk of endometrial cancer: A case control study. J Natl Cancer Inst. 2005; 97: 375-84.
13. Fishman M, Mona B, Sheiner E, et al. Changes in the sonographic appearance of the uterus after discontinuation of tamoxifen therapy. J Ultrasound Med. 2006; 25: 469-73.
14. Mignotte H, Lasset C, Bonadona V, et al. Iatrogenic Risks Of Endometrial Carcinoma After Treatment For breast cancer in a large French case-control study. Int J Cancer. 1998: 76: 325-330.
15. Gerber B, Krause A, Muller H, et al. Effects of adjuvant tamoxifen on the endometrium in postmenopausal women with breast cancer: A prospective long-term study using transvaginal ultrasound. J Clin Oncol. 2000; 18: 3464-70.
16. Nahari C, Tepper R, Beyth Y, et al. Long-term transvaginal ultrasonographic endometrial follow-up in postmenopausal breast cancer patients with tamoxifen treatment. Gynecol Oncol. 1999; 74: 222-6.
17. Ozsener S, Itil I, Dikmen Y. Endometrial pathology of 104 postmenopausal breast cancer patients treated with tamoxifen. Eur J Gynaecol Oncol. 1998; 19(6): 580-3.
18. Magriples U, Naftolin F, Schwartz PE, et al. High-grade endometrial carcinoma in tamoxifen-treated breast cancer patients. J Clin Oncol. 1993; 11(3): 485-90.
19. Rutqvist LE, Johansson H, Signomklao T, et al. Adjuvant tamoxifen therapy for early stage breast cancer and second primary malignancies. J Natl Cancer Inst. 1995; 87: 645-51.
20. Katase K, Sugiyama Y, Hasumi K, et al. The incidence of subsequent endometrial carcinoma with tamoxifen use in patients with primary breast carcinoma. Cancer. 1998; 82(9): 1698-703.
21. Bland AE, Calingaert B, Secord AA, et al. Relationship between tamoxifen use and high risk endometrial cancer histologic types. Gynecol Oncol. 2009; 112: 150-4.
22. Saadat M, Truong PT, Kader HA, et al. Outcomes in patients with primary breast cancer and a subsequent diagnosis of endometrial cancer comparison of cohorts treated with and without tamoxifen. Am Cancer Soc. 2007; 110: 31-7.
23. Seoud M, Shamseddine A, Khalil A, et al. Tamoxifen and endometrial pathologies: a prospective study. Gynaecol Oncol. 1999; 75(1): 15-9.

24. Peters-Engl C, Frank W, Danmayr E, et al. Association between endometrial cancer and tamoxifen treatment of breast cancer. Breast Cancer Res Treat. 1999; 54(3): 255-60.

25. Gardner FJ, Konje JC, Abrams KR, et al. Endometrial protection from tamoxifen-stimulated changes by levonorgestrel-related intra-uterine system: a randomized controlled trial. Lancet. 2000; 356: 1711-7.

26. South West Oncology group: S9630-Phase III Intergroup: a randomized comparison of medroxyprogesterone acetate (MPA) and observation for prevention of endometrial pathology in postmenopausal breast cancer patients treated with tamoxifen.

27. Winer EP, Hudis C, Burstein HJ, et al. American Society of Clinical Oncology technology assessment on the use of aromatase inhibitors as adjuvant therapy for women with hormone receptor-positive breast cancer: status report 2002. J Clin Oncol. 2002; 20: 3317-27.

28. Winer EP, Hudis C, Burstein HJ, et al. American Society of Clinical Oncology technology assessment working group update: use of aromatase inhibitors in the adjuvant setting. J Clin Oncol. 2003; 21: 2597-9.

29. The American College of Obstetricians and Gynecologists guidelines: Tamoxifen and uterine cancer. Obstet Gynecol. 2006; 107: 1475-8.

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