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NLRP3 Inflammasome: A Promising Theranostic Target in Inflammatory Diseases

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Abstract

Currently, the evaluation and treatment of chronic inflammatory diseases relies on specific clinical signs accompanied with single or multiple marker(s) for that disease. Despite that, these diseases share a common innate immune mechanism involved upregulation of "Inflammasome" that have been proved in its implication in the pathologic mechanism (s) and direct irreversible conversion of pro-interleukin 1β and pro-interleukin 18 into active IL-1b and active IL-18 contributing in disease progression and tissue damage, suggesting a common theranostic target to reduce inflammatory mechanism.

Keywords NLRP3, inflammasome, theranostic marker

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List of abbreviation: PAMP: Pathogen associated molecular patterns; DAMP: Damage associated molecular pattern; NLRP3: Nucleotide binding oligomerization domain-like protein 3; ASC: Apoptosis-associated speck-like protein; TLR: toll like receptor; NF-kB: Nuclear factor kappa B.

Theranostics is a term refers to examination of a biomarker molecule as a target for both diagnostic and therapeutic purposes allowing integration between in vitro diagnostics and reducing delay of treatment for optimum patients care [(1)]. The main concept of immune system is defending against pathogen associated molecular pattern (PAMPs) as external pathogens and damage associated molecular pattern (DAMPs) as internal threats like uric acid and heat shock proteins mediated by certain pattern recognition receptors (PRRs) whom responsible for generation of inflammation and other effector mechanisms in diverse conditions [(2)]. The Nucleotide-binding oligomerization domain-like protein 3 (NLRP3) is the best characterized molecule belongs to a complex called "Inflammasome" in addition to apoptosis-associated speck-like protein (ASC) and procaspase-1 resulting in irreversible immune activity through both pro-inflammatory cytokines (IL1β and IL18) [(3,4)].

NLRP3 inflammasome presents in inflammatory immune cells like: dendritic cells, macrophages and monocytes [(5)]. The activation process of the NLRP3 inflammasome appears to occur in two steps involves a priming or initiating signal in which, many PAMPs or DAMPs are recognized by toll like receptors (TLRs), leading to activation of nuclear factor kappa B (NF-κB)-mediated transcription of inflammasome proteins, including inactive NLRP3, proIL-1β, and proIL- 18 [(5)]. Followed by the oligomerization of NLRP3 and subsequent assembly of NLRP3, ASC, and procaspase-1 into inflammasome complex allowing its autocleavage and activation. The activated caspase-1 enzyme in turn cleaves upregulated

inactive proinflammatory cytokines: IL-1 and IL18 in to active IL-1β and IL-18 and secreting them into extracellular compartment resulting in induction of proinflammatory and proapoptotic pathways depending on type of threatening patterns [5,6]. In fact, the assembly of NLRP3 inflammasome and its activation happens downstream to many stimuli induced by various PAMPs or DAMPs showing its importance in playing an essential role various disease processes [7].

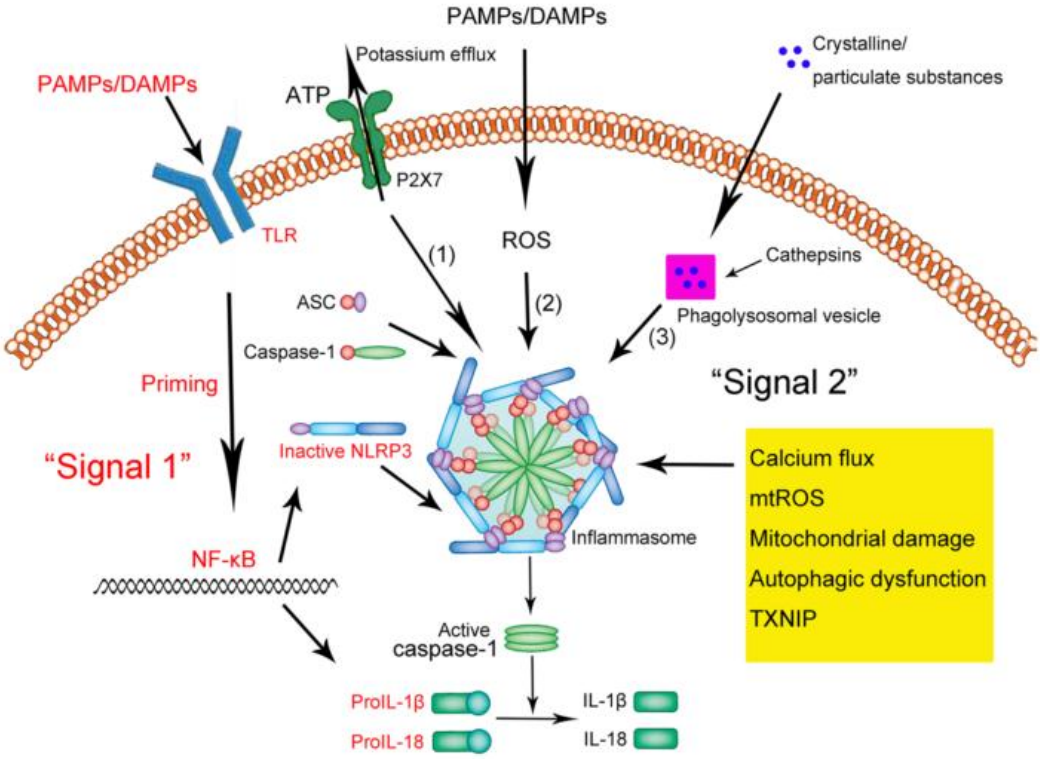

Figure 1: Illustration of the NLRP3 inflammasome activation [8]

The inappropriate activation of NLRP3 inflammasome can contribute in the initiation and progression of various diseases like: metabolic disorder and metabolic syndrome [9], atherosclerotic plaque in both atherosclerotic patients and animal model [10,11], NLRP3 inflammasome proteins up-regulated in patients with type 1 diabetes [12], IgA nephropathy [13], multiple sclerosis [14] cancer [15] and other autoimmune diseases.

Consistent with the associations of NLRP3 inflammasome activation in these diseases, inhibiting the NLRP3 inflammasome may reduce their pathogenesis [3,8,16]. By another mean, targeting NLRP3 inflammasome will affect downstream signals preventing secretions of active proinflammatory processes. As well as an anti-infective [16]. Until recently, researchers found a selective direct inhibitor MCC950 specifically inhibited activation of NLRP3 that later on Novartis company funded Inflazome as a trade mark for this molecule [17], (CY-09) [18] and telmisartan [19].

In conclusion, all these evidences rising the suggestion for targeting NLRP3 inflammasome as a theranostic marker for inflammatory disease. Its suggested as a specific and sensitive biomarker superior than CRP, or other proinflammatory markers because its expressed downstream to real threat recognition by innate immune receptors and

provide upstream signaling for activation and production of active proinflammatory cytokines (IL-1B and IL18) mediating inflammation and apoptotic signaling. In another hand, NLRP3 inflammasome as a valid target for treating inflammatory diseases.

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Prevalence and Diagnosis of Genital Herpes by Immunological and Molecular Study

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Abstract

Background Genital herpes simplex infection is a viral infection caused by the herpes simplex virus (HSV) type 1 or 2. This disease transmitted during close skin or mucus membranes contact with an infected person who is shedding the virus. Infections that are commonly spread by sex, especially vaginal intercourse, anal sex and oral sex.

Objective For detection of HSV by immunological and molecular methods.

Methods Two hundred (200) samples were collected from females attending the Gynecology Outpatient Department in the Al-Imamein Al-Kadhimein Medical City and Baghdad Teaching Hospital during the period from May 2014 to April 2015. Based on availability of full clinical information about each patient, high vaginal swabs were taken from females at different ages (15-54 years) representing patients group complaining of abnormal vaginal discharge with or without other symptoms. The Statistical Analysis System- SAS program was used to study the effect of difference factors in study parameters. Chi-square test was used to significant comparison between percentages in this study.

Results Each of the vaginal swabs collected were examined, was preserved at -20 °C for DNA extracts were analyzed. In RT-PCR, the rate of infection was in women with HSV, those with age group (25-34) years and (35-44) years were 50%.

Conclusion HSV infections were detected in genital tract infection in women; molecular methods are considered the gold standard for diagnosis, given the excellent sensitivities, specificities, rapid and accurate laboratory diagnosis of HSV.

Keywords Genital herpes, diagnosis, immunological, molecular study

Citation Ali MK, Hathal HD, Almoayed HA. Prevalence and diagnosis of genital herpes by immunological and molecular study. Iraqi JMS. 2018; Vol. 16(1): 4-7. doi: 10.22578/IJMS.16.1.2

List of abbreviations: ELISA = Enzyme Linked Immuonosorbent Assay, HSV= Herpes simplex virus, RT-PCR = Real-time polymerase chain reaction, STPs = Sexually transmitted pathogens

Introduction

Herpes simplex is an enveloped DNA virus (150-200 nm in diameter) belonging to the alpha-herpesviridae. Based on antigenic, biochemical and biological differences it can be divided into two serotypes, HSV-1 and HSV-2. Man is the only known natural host and source of the virus. The types of disease seen in patients depend on route of infection and individual host factors. Infection is relatively common, with seroprevalence approaching 80% for HSV-1 and 20% for HSV-2 in adult populations; however, prevalence can be much higher in certain demographics or in undeveloped countries [(1-3)]. An important property of both viruses is the ability to establish latency following initial infection, leading to lifelong carriage [(4,5)]. While there is currently no cure for latent infection, effective therapy exists for alleviating

symptoms, shortening the duration of severe outbreaks, and treating some of the more life-threatening manifestations. Effectiveness of therapy for severe acute HSV infections hinges on rapid administration of appropriate antivirals. This creates the need to establish a prompt diagnosis and necessitates HSV diagnostic testing that is both rapid and sensitive [6,7]. Testing must also be highly specific, since clinical manifestations of HSV are relatively nonspecific and overlap other potentially severe infections. Finally, while many tests are designed for use on mucocutaneous or skin lesions. There is often a need to test patients without such lesions. Physicians may need to establish a serologic diagnosis or detect nucleic acid. Therefore, effective testing should be applicable to a variety of clinical specimens [8,9].

The objective of this study was detection of HSV by immunological and molecular methods.

Methods

Two hundred (200) samples were collected from females attending the Gynecology outpatient department in the Imamein Al-Kadhimein Medical City and Baghdad Teaching Hospital during the period from May 2014 to April 2015. Based on availability of full clinical information about each patient, high vaginal swabs were taken from females at different ages (15-54 years) representing patients group complaining of abnormal vaginal discharge with or without other symptoms, questionnaire was applied.

Five ml of venous blood sample was collected from each woman, the serum was collected into another sterile tube and was kept in deep freeze at -20 °C for diagnosis of herpes virus antibodies. Each of the vaginal swabs collected was examined, what are the remaining was preserved at -20 °C for DNA extraction and analyzed with the real-time polymerase chain reaction (RT-PCR).

This research underwent to the terms of ethical considerations and in accordance with the form prepared for this purpose by the committee of ethical standards in the Collage of Medicine, University of Al-Nahrain.

Identification of Herpes simplex virus

Enzyme-linked Immunosorbent Assay (ELISA tests)

Detection of Herpes Simplex virus 1, 2 IgGby (ELISA; (NovaLisa™, Germany).

These tests were done using human diagnostic ELISA kits, Germany, for the qualitative determination of human antibodies of the IgG against herpes simplex virus in serum or plasma.

Molecular Study

Singleiplex RT-PCR kit (Sacace ™ Biotecnologies) for the direct, qualitative detection of herpes simplex virus. The principle of (DNA extraction Nano drop, Agarose electrophoresis, and RT-PCR) preparation sample, and interpretation of the results were as same as those in herpes simplex virus determination method.

Results

HSV-2

Only 1/200 samples were positive by ELISA test. This test assay showed (1) case of herpes simplex virus (IgG) Ab.

PCR amplification was performed using RT-PCR. The detection of appropriate channels was used as follow: channel (FAM) for detection HSV2, channel (CY3) for internal control of DNA (ICD). Used for no evidence of inhibition of the amplification in any of the samples, with the internal control of the RT-PCR samples as shown in Figure (1).

Discussion

Only one of the 200 samples was positive by ELISA test. This test assay showed one case of herpes simplex virus (IgG) Ab.

The classical technique can be replaced by ELISA for its simplicity and the limited laboratory tools requirements and also the use of immunological methods has increased lately, the ELISA method chosen due to the reality that, it is rapid, and reliable and particularly is

useful for the rapid investigation of a large number of blood samples in laboratories [10].

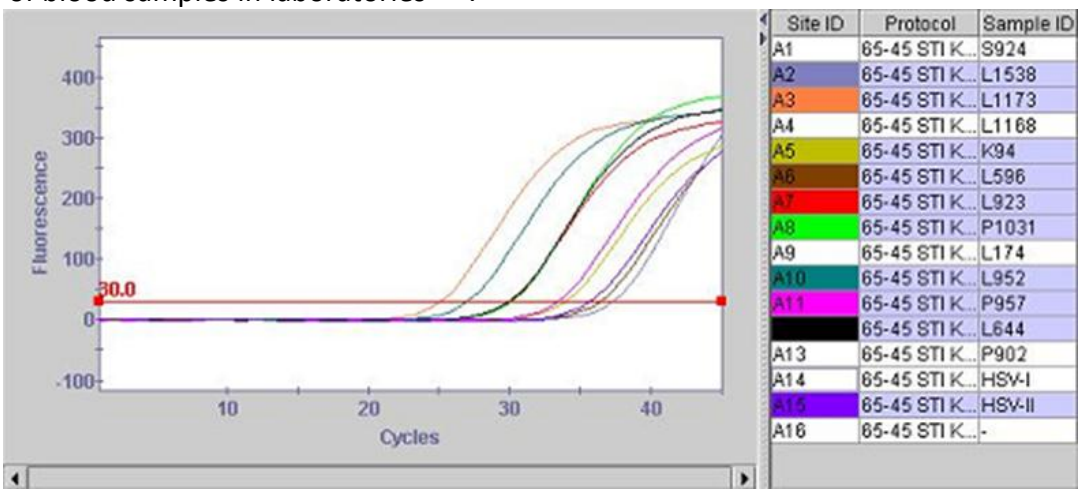

Figure 1. Showing that from 200 samples, only 2 (1%) cases of Herpes simplex virus infection

This result was lower than the percentage in other neighboring countries; Syria (52%) [11], KSA (27.1%) [11], Qatar (26.3%) [12], Iran (43.75%) [13], Turkey (63.1%) [14] and with other countries such as Indonesia (9.9%) [15], Tanzania (20.7%) [16], Australia (30%) [17], USA (22%) [2], Canada (17.3%) [19] and in Iraq (Waset), (6.60%) [20].

The HSV -2 IgG seroepidemiology varies among different countries, and between groups of individuals depending on the demographic and clinical characteristics of the population.

The low infectious rate of HSV2 in symptomatic patients may be due to large number of co infection with other causative agent that produce genital tract infections, this study agrees with other studies [21-24].

Among 200 samples, only 2(1%) cases of Herpes simplex virus 2 infection were detected by RT-PCR.

PCR is more accurate to detect the STPs. Molecular methods are considered the gold standard for diagnosis, given the excellent sensitivities and specificities in diagnosis, because it allowed to distinguish between STPs. However, new molecular methods such as PCR, qualitative real time PCR for rapid detection of HSV2 in comparison to serological methods have been used.

Moreover, monitoring of DNA level of a pathogen in body fluids can reveal the status of the disease, its response to medication, and its resistance patterns [24,25].

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Authors Contribution:

Ali: conducted the sampling, isolation, and staining, the molecular work and writing the manuscript. Dr. Al-Marsome: drafting the article and revising it critically for important intellectual content. Dr. Almoayed: Selection of samples and patients.

Conflict of interest

The authors declare no conflict of interest

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Genotyping of *Listeria Monocytogenes* in Iraqi Women with Spontaneous Abortion Using Pulse Field Gel Electrophoresis

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Abstract

Background *Listeria monocytogenes* (*L. monocytogenes*) is a Gram-positive, facultative intracellular bacterial pathogen that can cause a severe invasive disease (listeriosis) mainly in pregnant women. Based on genetic content, *L. monocytogenes* can be divided into 3 lineages I, II and III. Several molecular methods have been developed to assist in the characterization of *L. monocytogenes*, macrorestriction analysis by pulse-field gel electrophoresis (PFGE) is one of the most used methods for the genotyping of *L. monocytogenes*.

Objective To determine the predominant genotype of *L. monocytogenes* isolated from clinical cases in a group of aborted Iraqi women.

Methods A study was designed and included 15 clinical isolates of *L. monocytogenes* and one isolate from locally made cheese. The PFGE protocol was performed as described by Graves and Swaminathan. Briefly, bacterial suspensions adjusted to optical density of 1.3 at 610 nm were embedded in 1.2% Sea Kem Gold agarose plugs. The lysed, washed five times and a 2 mm thick piece was cut, equilibrated and digested with 200 µl of AscI enzyme master mix at 37 °C for 2 h. The macrorestriction fragments were separated by electrophoresis on a CHEF-DRIIBIO-RAD. Images were analyzed with the software Bio Numerics Gel Compar II version 6.6.11 (Applied Maths).

Results All *L. monocytogenes* isolates displayed AscI digestion profiles after incubation with the enzyme, about 6-12 majorfragments of 30 to 675 kb were obtained, sixteen isolates have been used in this study, however, 11 different AscI profiles were encountered, the distribution of the isolates according to the restriction profiles showed that two profiles were the most predominant A, A2 with a percentage of similarity of 100%.

Conclusion *L. monocytogenes* genotyping showed profiles that can be well used in tracing down infection source and outbreak of this bacterium. PFGE represents a great discrimination and reproducibility method for molecular sub-typing of *L. monocytogenes* and it is considered the gold standard.

Keywords *Listeria monocytogenes*, genotyping, placenta, aborted women, pulse field gel electrophoresis

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List of abbreviations: CHEF = Contour-clamp homogeneous electric field, PFGE = Pulse field gel electrophoresis, RFLP = Restriction Fragment Length Polymorphism, UPGMA = Unweighted-pair group method with arithmetic averages

Introduction

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive intercellular, rod shaped, nonspore-forming, motile, facultative anaerobic, bacterium. It causes a severe invasive disease [(1)].

Listeriosis during pregnancy may lead to intrauterine infection, which may result in severe complications like preterm labor, spontaneous abortion, and stillbirth and or infection of the neonate which may result in high morbidity and mortality rate [(2)]. Of several molecular methods currently available like

polymerase chain reaction (PCR), amplified fragment length polymorphism detection, DNA sequencing and others, macrorestriction analysis by pulse-field gel electrophoresis (PFGE) is one of the most used methods for the subtyping of *L. monocytogenes*. The using of restriction endonuclease AscI, as advised by Pulse Net USA, has shown excellent discrimination for *L. monocytogenes* and the technique is shown to be reproducible. PFGE is considered to be the international standard for subtyping [3].

PFGE is a form of RFLP (Restriction Fragment Length Polymorphism) typing in which, bacterial genome is digested with a rare cutting restriction enzymes that cut the bacterial genomic DNA infrequently and therefore generate a small number of DNA fragments between (10-20 bands); these fragments are of a large sizes, from 20 kb to 10,000 kb, and are separated using specific electrophoresis techniques [4] for *L. monocytogenes*, the enzymes are AscI or ApaI, these enzymes may generate between 6 to 12 and 14 to 17 fragments respectively separated through the PFGE. Combinations of the two profiles generated by the enzymes are used to characterize L. monocytogenes strains [5].

Genetic comparisons among isolates carry out by using the differences in the restriction profiles. Computer-based analysis is very simple and enables rapid comparison on strains. Currently, PFGE represent the "gold standard" of the molecular typing techniques for foodborne pathogenic bacteria such as *Salmonella, E. coli, Yersinia, Vibrio* and *Listeria* [6].

The aim of this study and the benefit of it is to assess the extent of *L. monocytogenes* in causation of human spontaneous abortion in a sample of Iraqi women, and to determine the genetic subtypes of listeria monocytogenes associated with abortion.

Methods

A cross-sectional study was designed that included 250 placental tissues obtained from aborted women (which their acceptance was taken before sample collection) attended Al-Imamein AL-Kadhimein Teaching Hospital in Baghdad during the period from June 2014 to November 2015. Out of total number of the placentas samples, only fifteen isolates of *L. monocytogenes* were identified. The isolation and identification of *L. monocytogenes* were performed according to Collee et al. 1996 and McFaddin, 2000 [7,8].

PFGE of *L. monocytogenes*

The laboratory Protocol for molecular subtyping of *L. monocytogenes* by PFGE includes the following:

The Culture growth: An isolated colony from a pure culture of *L. monocytogenes* was streaked onto nutrient agar (NA) plates and the plates were incubated at 37 °C for 14-18 h.

Pulse field gel electrophoresis: The PFGE protocol was performed as described by Graves and Swaminathan (2001) [6]. Briefly, bacterial suspensions adjusted to optical density of 1.3 at 610 nm were embedded in 1.2% SeaKem Gold agarose plugs. The lysed, washed five times and a 2 mm thick piece was cut, equilibrated and digested with 200 µl of AscI enzyme master mix at 37 °C for 2 h. The macrorestriction fragments were separated by electrophoresis on a CHEF-DR II BIO-RAD (USA) in a 1% Pulse Field Certified Agarose gels (Ultrapure DNA grade agarose) at Initial switch time: 4.0 s; Final switch time: 40.0 s; Voltage: 6 V; Included Angle: 120° Run time: 18-19 hours.

Staining and Documentation of an Agarose gel: After electrophoresis run was over, gel was removed and stained with ethidium bromide for 20-30 min in covered container. The use of restriction endonucleases AscI, is advised by Pulse Net USA, and has shown excellent discrimination for *L. monocytogenes*. Images were analyzed using Gel Compare II evaluation software version 6.6.11 from Applied Maths.

The similarity between fingerprints was determined using number of different bands coefficient with a 1% tolerance between band positions. The cluster analysis and generation of Dendrogram was performed using

Unweighted-pair group method with arithmetic averages (UPGMA) with a similarity score value of 100% as the cutoff.

Results

All isolates of Listeria monocytogenes were digested with AscI restriction endonuclease...GG▼CGCGCC... .
...CCGCGC▲GG...
Only one was isolated from local made cheese, which presented in lane number 1 (Figure 1). Isolates of *L. monocytogenes* were typed using PFGE. Analysis of the AscI restriction profiles by automated cluster analysis was done using number of different bands as a similarity coefficient and tolerance: 1 %.

All the isolates displayed AscI digestion profiles after incubation with the enzyme (Figure 1), about 6-12 major fragments of 30 to 675 kb were obtained following the digestion using AscI.
Sixteen isolates have been used in this study, fifteen represent the clinical isolates and the

Eleven PFGE types and two subtypes were distinguished with the restriction endonuclease AscI through visual inspection, using Unweighted-pair group method with arithmetic averages (UPGMA) with a similarity score value of 100% as the cutoff.

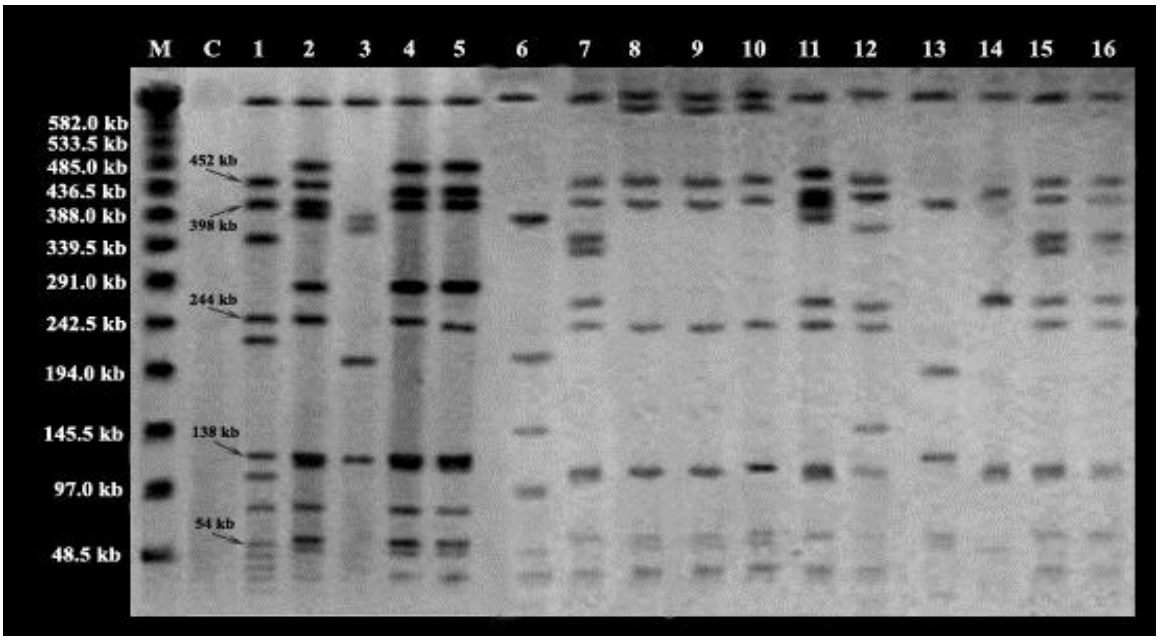

Figure 1. Genomic DNA restriction patterns of *Listeria monocytogenes* isolates, performed by PFGE after digestion with AscI

Cluster analysis of the AscI restriction digest profiles showed that PFGE subtype A and subtype A2 were predominant with a percentage of similarity of 100% for both followed by type A3 with a percentage of similarity of 99% then A5 and B with a percentage of similarity of 98%, D and A of 97.5%, A4 and A5 of 97%, A1 of 94.5%, F and E of 93.5%, I was of 93%, and G was presented with a percentage of similarity of 90%. Cluster analysis of L. monocytogenes isolates and the PFGE types assigned by visual inspection and by automated cluster analysis are shown in (Figure 2).

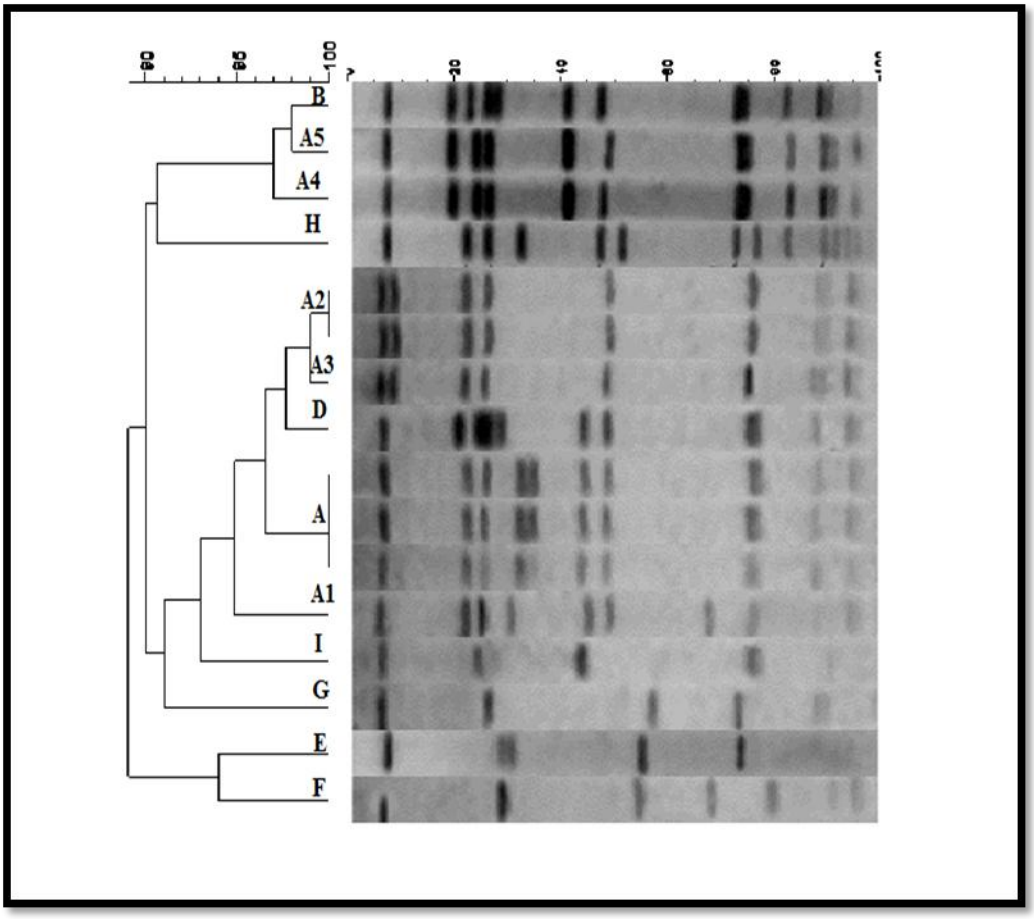

Figure 2. Dendrogram representing PFGE types assigned by Automated Cluster Analysis (ACA) of the AscI restriction digest profiles of *Listeria monocytogenes* isolates

Discussion

The large number of different PFGE types (sixteen isolates yield eleven PFGE types) may indicate that a variety of genetically distinct strains cause listeriosis in Iraq, however, this high number of PFGE types is in line with several published data for clinical cases [9,10], this variation in the number of PFGE types obtained in different studies may be due to that the clinical strains used in these studies not corresponded to sporadic cases; the choice of the rare cut endonucleases; and the analysis protocol used in the study. However, the current work was done using the Pulse Net method described by Graves and Swaminathan [6].

The cluster analysis results showed that there was two predominant subtypes A and A2, which include three isolates and two respectively, those isolates were genetically indistinguishable from each other and also genetically related to other subtypes and or PFGE types with a percentage of similarity range from 99% to 89%, however those with high similarity may differ in a single band only while the lower similarity percentage was associated with isolates differ in more than one band.

Although PFGE profiles give a considerable degree of strain diversity in *L. monocytogenes* populations, the number of bands differences required for discrimination between strains is controversial [11]. Strains differing by more than one band are clearly distinct but some strains from geographically distinct sources may exhibit few or no differences in DNA profile and by the Turnover criteria could be considered epidemiologically related [12]. According to

Autio et al. (2002) [13], the isolation of indistinguishable PFGE types may mislead the investigation towards establishing the vehicle or the source of infection. However, many food products may carry genetically identical strains without any cross-contamination or common infection sources. Therefore, a suspected epidemic infection may consist of two or more independent sporadic cases.

In conclusion, human listeriosis in Iraq involves a great variety of genetically different isolates. However, in this study, indistinguishable isolates were isolated from more than one patient, suggesting the existence of more than one episodes of listeriosis. In all of the listeriosis episodes, the food vehicle was not determined, because of a lack of epidemiological evidence.

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Authors Contribution:

Qassim collected the cases, performed the bacterial tests and analyzed the results. Dr. AL Attraqchi helped in the study design and supervising the work. Dr. Khatab participated collection of cases and performed the histopathological examination.

Conflict of interest

The authors declare no conflict of interest.

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Motor Evoked Potential in Patients with Parkinson's Disease: A Transcranial Magnetic Stimulation Study

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Abstract

Background Parkinson's disease (PD) is a neurodegenerative condition of the central nervous system, which is accompanied by the impairment of the cortico-subcortical excitation and inhibition systems. It is characterized by motor and non-motor symptoms, having both hypokinetic and hyperkinetic features.

Objective To investigate the integrity of the central motor pathways by studying the motor evoked potential (MEP) latencies, amplitudes and central conduction time (CMCT) of the median nerve in patients with PD as compared to healthy controls.

Methods Twenty-five patients with documented PD were studied; with a mean age of (63.16±5.49 years) as compared to 25 age and sex matched apparently healthy controls. All subjects were instructed about the examination and informed consent was provided. Transcranial magnetic stimulation TMS-MEP study of the right median nerve was done. Cortical and cervical latencies and amplitudes of the MEP study were determined. The responses were recorded with both relaxed and slightly contracted target muscle. CMCT calculation was done by subtraction of the latency of peripheral segment of the motor pathway (spinal motor root to muscle) from that of the entire motor pathway (motor cortex to muscle) or by calculation of the CMCT with the F-wave method.

Results The means of the cortical latencies of PD patients during relaxation and facilitation states were lower than controls; and the differences were significant for both (P=0.03 and 0.02; respectively). In both relaxed and facilitation states, the means of CMCT in PD patients were lower than in control and the difference was significant during contraction (P=0.02), and near statistical significance during relaxation (P=0.08). CMCT calculations by the estimation of F wave and distal motor latency (DML) were equivocal between relaxation and facilitation states. Nevertheless, the differences were not statistically significant (P=0.45; P=0.62; respectively). The means of the MEP amplitude of PD patients were lower than controls (4.21±1.94 versus 4.28± 1.84 mV; respectively). Nevertheless, the differences were not significant (P=0.89).

Conclusion Single-pulse TMS is a valuable study to investigate central motor dysfunction in PD. CMCT measurement of the median nerve or any nerve in the upper limb is a potential marker for the evaluation of the severity of PD; especially in the facilitated state.

Keywords Parkinson's disease, TMS, MEP, CL, CMCT

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List of abbreviations: ABP= Abductor pollicis brevis, CLC = Cortical latency during contracted state, CLR = cortical latency during relaxed state, CMCT = Central motor conduction time, CMCT-C = Central motor conduction time during contracted states, CMCT-F = Central motor conduction time with F wave, CMCT-R = Central motor conduction time during relaxed states, DML = Distal motor latency, Fmin. = Minimal F-wave latency, MEP = Motor evoked potential, PD = Parkinson's disease, PMCT = Peripheral motor conduction time, SNc= Substantia nigra pars compacta, STN= Subthalamic nucleus, TMCT = Total motor conduction time, TMS = Transcranial magnetic stimulation

Introduction

Parkinson's disease (PD) is a neurodegenerative condition of the central nervous system (CNS), which is accompanied by the impairment of the cortico-subcortical excitation and inhibition systems, hence belonging to the involuntary movement diseases [(1)]. Figures suggest that there are 7-10 million people worldwide who have been diagnosed with PD. Men are 1.5 times more likely than women to develop the disease [(2)]. The core pathology results from the degeneration of dopaminergic neurons in the

substantia nigra (pars compacta - SNc); where the fibers to the putamen (part of the striatum) are most severely affected. When 80% of dopamine is being depleted, deficiency in the motor neuron circuitry manifest in the cardinal symptoms of the disease, which include tremor, rigidity, bradykinesia, and postural instability [3]. Following degeneration of the SNc dopaminergic neurons projecting to the striatum, several biochemical and electrophysiologic changes occur that cause a characteristic increase in firing rate of the internal globus pallidus (GPi) and the subthalamic nucleus (STN). The consequence of this is inhibition of the thalamo-cortical and brainstem motor systems; reversed by administration of dopaminergic agents [4].

Transcranial magnetic stimulation (TMS) can be regarded as a transcranial electrodeless electric stimulation by electromagnetic induction, because electrical charges flow into an excitable cell membrane, initiating an action potential [5]. TMS made it possible for the first time to study the function of the human motor cortex noninvasively and almost painlessly. TMS is now routinely used whenever an objective evaluation of the motor system is required [6]; offering a noninvasive and safe approach of stimulating the human motor cortex, and assessing the integrity of the central motor pathways [7].

Of all the different TMS-MEP parameters, the latency of the MEP is generally regarded as the most reliable and useful. If combined with a measure of the peripheral motor conduction time (PMCT), a calculation of the central motor conduction time (CMCT) is possible. In routine clinical practice, this is the most important MEP parameter for evaluation of pyramidal tract function [8]. TMS has a series of clinical application in many movement disorders like PD, multiple system atrophy, progressive supranuclear palsy, essential tremor, Huntington's chorea and restless leg syndrome [9]. When TMS directed on different levels of the motor system, it will give data about the excitability of the motor cortex, the functional integrity of intracortical neurons, the conduction along corticospinal, corticonuclear, and callosal fibers, as well as the function of nerve roots and peripheral motor path [10].

The aim of the current study was to investigate the integrity of the central motor pathways by studying the MEP of the median nerve and analysis of the cortical latency and CMCT both in relaxed and contracted abductor pollicis brevis (APB) muscle as well as MEP amplitude in patients with PD as compared to healthy controls.

Methods

Twenty-five patients with documented PD were included in the study; with a mean age of (56.6±5.6) years as compared to twenty-five age and sex matched apparently healthy controls. All subjects were instructed about the examination and informed consent for participation was provided. The study was approved by the Institute Review Board of the College of Medicine/ Al-Nahrain University.

Each participant will be subjected to TMS-MEP study of the right median nerve, conventional sensory and motor nerve conduction studies to exclude peripheral neuropathy.

The patients with one or more of the following exclusion criteria were eliminated; atypical parkinsonism, secondary parkinsonism, parkinsonism related to other neurodegenerative disease, cerebral and/or medullar pathology, untreated or refractory epilepsy, deep brain stimulation, cardiac pacemaker, prior history of head injury, cranial surgery, stroke, bullet or any implanted electrical biomedical device.

Magnetic stimulation was performed using the (Micromed, 8-channel elecromyograph) EMG /EP machine and Magstim 200 stimulator with the large stimulating coils (type 9784, UK). Subjects were lying down comfortably in a supine position on the couch to guarantee the easy access to the subject's head and they should be seated comfortably to guarantee the easy access to their spine while stimulating them. The subject should be relaxed with eyes

open. In order to minimize variability in threshold and EP amplitude, it is advised to ask the subject to perform simple mathematical calculations (such as serially adding 7 to 5 or subtracting 9 from 100) [(11)]. MEPs were recorded from the right (APB) muscle by surface electrodes placed on the belly and tendon of the muscle. Stimuli were delivered as single shocks at least 5-10 seconds apart. The electrophysiolgical setting was: high pass filer = 30 Hz, low pass filter = 3000 Hz, time base = 100 ms, and gain = 1 mV/Division [(12)].

Cortical stimulation of the upper limbs was done by placing the coil tangential to skull, over the vertex in mid-sagittal plane (Cz, the intersection of the nasion-inion and tragus-tragus lines) flat on top of the head. The coil edge with maximum magnetic field strength under the middle coil windings thus overlies the motor cortex region for the hand and arm. The direction of the current was clockwise for stimulation of left cortex with the side A of the stimulating coils facing upward. Stimulation was started with about 50% of maximal stimulator output and then increased until a response with maximum amplitude was registered. To facilitate the response, patients were asked to perform a slight contraction of the target muscle during cortical stimulation. At least three reproducible cortical responses were recorded in order to minimize the variability of the amplitude and latency of the cortical magnetic stimulation [(10)].

For magnetic stimulation of the cervical root, the center of the coil was placed over 7th cervical spinal process with the subject in the sitting position for the commonly studied hand muscles [(13)]. The circular coil is usually placed in the midline or slightly lateral to this (up to 2 cm) toward the site under investigation. The coil may also be placed lower at the T3 level at ~2 cm laterally, thus placing the C8/T1 nerve roots under the upper quadrant of the coil for optimal APB muscle recordings. When using a monophasic stimulator, the current direction is less important for nerve root stimulation compared to cortical stimulation, but a direction of the induced current from medial to lateral has been suggested for both upper and lower extremities, i.e. clockwise orientation of the coil current (looking from behind) for the right side and vice versa for the left [(12)].

Cortical latency of the TMS-MEP study was determined with activation of target muscle as the shortest interval between time of stimulation and onset of first negative wave of MEP. Recording was both during relaxation and slight muscle contraction [(10)]. The CMCT, which is the latency difference between the MEPs induced by stimulation of the motor cortex and those evoked by spinal (motor root) stimulation. It is calculated by the: Subtraction of the latency of peripheral segment of the motor pathway (spinal motor root to muscle) from that of the entire motor pathway (motor cortex to muscle) so CMCT (ms) = TMCT – PMCT; or by calculation of the CMCT with the F-wave method so

$$\text{CMCT (ms)} = \text{TMCT} - \frac{(Fmin. + DML) - 1}{2}$$

Where

CMCT = central motor conduction time,

TMCT = total motor conduction time (cortico-muscular latency),

Fmin. = minimal F-wave latency,

DML = distal motor latency,

PMCT = peripheral motor conduction time (spinal motor root latency) (Wassermann et al. 2008) [(10)].

Statistical analysis was done using Student's t-test for continuous parameters and Chi-square test for categorical parameters. A p-value ≤0.05 was considered significant (Daniel and Cross, 2013).

Results

Fifty subjects were enrolled in this study; the mean age of those with documented PD (n=25) was (63.16±5.49 years); comprised (22) males and (3) female as compared to that of 25 healthy controls (65.12 ±7.26 years); comprised (23) males and (2) females. There was no significant difference regarding the mean ages and gender between the two studied groups, (Table1).

Table 1. Demographic characteristics of the studied patients with Parkinson's disease and control groups

Parameters		Patients (n=25) mean±SD	Control (n=25) mean±SD
Sex	Male	22	23
	Female	3	2
Age (yr)*	Mean±SD	63.16±5.49	65.12±7.26
	Range	47-87	49-76

* No significant difference between patients and controls using unpaired T-test (p value = 0.29)

Regarding the cortical-latency of the TMS-MEP of the right median nerve was calculated both during relaxed and contracted (facilitated) states. The means of the cortical-latencies of PD patients during relaxation and facilitation states were lower than controls (22.06±2.24 versus 24.12±4.08 and 19.28±2.63 versus 21.44±3.72 ms; respectively); and the differences were significant (P=0.03 and 0.02; respectively), (Table 2).

As in table 2, CMCT was estimated by subtracting the peripheral MCT obtained by cervical spinal roots stimulation from the total MCT obtained by cortical stimulation both during relaxation and facilitation states. In both relaxed and facilitation groups, the means of CMCT in PD patients were lower than in control groups (7.90±1.66 against 8.69±1.41, and 5.28±1.84 against 6.58±1.85 ms; respectively), and the difference was significant during contraction (P=0.02), and near statistical significance during relaxation (P=0.08), (Table 2).

On the other hand, CMCT calculations by the estimation of F wave and DML were lower in PD patients than their counterparts in the control group for the contracted state; while they were higher during relaxation (5.34±2.29 versus 5.78±1.71 and 8.00±2.1 versus 7.71±1.98 ms; respectively). Nevertheless, the differences were not statistically significant (P=0.45; P=0.62; respectively), (Table 2).

The amplitude of the studied TMS-MEP of the right median nerve was calculated. The means of the MEP amplitude of PD patients were lower than controls (4.21±1.94 versus 4.28± 1.84 mV; respectively). Nevertheless, the differences were not significant (P=0.89), (Table 2). Figures (1) and (2) shows Right median nerve TMS-MEP parameters both after cortical and peripheral stimulation, recorded from patient with PD and a healthy control; respectively.

Table 2. Comparison of TMS-MEP study parameters of the right median nerve between patients with Parkinson's disease and control group

Parameters	Patients (n=25) mean±SD	Control (n=25) mean±SD	*P* value (unpaired t-test)
CLR (ms)	22.06±2.24	24.12±4.08	0.03
CLC (ms)	19.28±2.63	21.44±3.72	0.02
CMCT-R (ms)	7.90±1.66	8.69±1.41	0.08
CMCT-C (ms)	5.28±1.84	6.58±1.85	0.02
CMCT-F-R (ms)	8.00±2.10	7.71±1.98	0.62
CMCT-F-C (ms)	5.34±2.29	5.78±1.71	0.45
MEP Amplitude (mV)	4.21±1.94	4.28±1.84	0.89

CLR= cortical latency during relaxed state, CLC = cortical latency during contracted state, CMCT-R= central motor conduction time during relaxed states, CMCT-C = central motor conduction time during contracted states, CMCT-F = central motor conduction time with F wave, MEP = motor evoked potential

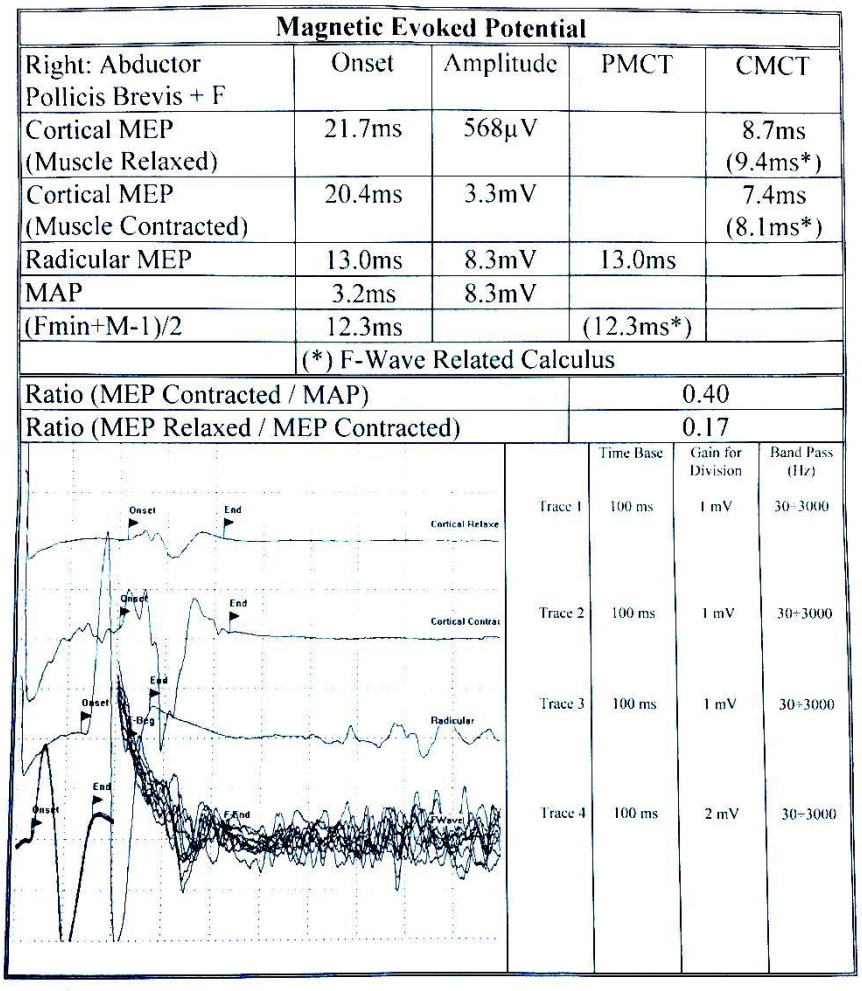

Figure 1. Right median nerve TMS-MEP parameters both after cortical and peripheral stimulation, recorded from patient with Parkinson's disease

Magnetic Evoked Potential				
Right: Abductor Pollicis Brevis + F	Onset	Amplitude	PMCT	CMCT
Cortical MEP (Muscle Relaxed)	21.7ms	568µV		8.7ms (9.4ms*)
Cortical MEP (Muscle Contracted)	20.4ms	3.3mV		7.4ms (8.1ms*)
Radicular MEP	13.0ms	8.3mV	13.0ms	
MAP	3.2ms	8.3mV		
(Fmin+M-1)/2	12.3ms		(12.3ms*)	
	(*) F-Wave Related Calculus			
Ratio (MEP Contracted / MAP)			0.40	
Ratio (MEP Relaxed / MEP Contracted)			0.17	

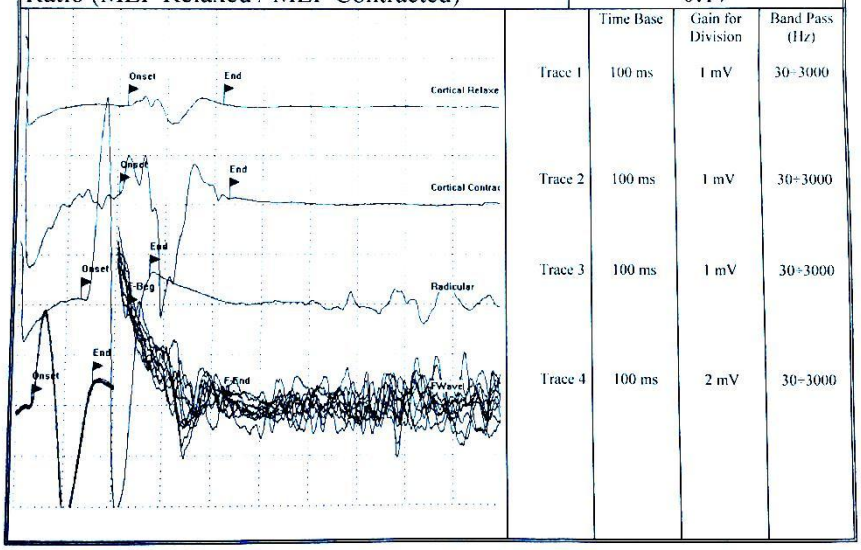

Figure 2. Right median nerve TMS-MEP parameters both after cortical and peripheral stimulation, recorded from a healthy control subject

Discussion

The TMS is a noninvasive and painless method to stimulate the human brain; it has provided substantial new pathophysiological insights that can be very helpful in providing objective information about the severity of the disease. In the current study, cortical latency of the TMS-MEP study of the right upper limb was calculated both during relaxed and contracted (facilitated) states. Results of the present study showed significantly decreased CLs during both states when compared to controls. This is in agreement with findings of some researchers [14,15]; but disagree with others [16,17]. Rossini and co-workers in 1999 stated that in patients with PD the cortico-motoneuron conduction was normal [18].

Popa and colleagues stated that their results supported those of previous studies, and that their normal (insignificantly different from controls) values of the MEP latencies and CMCTs in PD patients could be attributed to the fact that the pyramidal tracts are not impaired in pure PD [1].

It is recommended to measure the CMCT while the target muscle contracts at 5% to 20% of its maximum strength, because the MEP size saturates for stronger contractions [19]. Results of the CMCT calculations in the current study which were recorded both in relaxed and facilitation states were lower than those of the control group and the differences were statistically significant for the contracted state and near statistical significance for the relaxed

state (P=0.08). This finding is going in accordance with the result of Kandler et al. 1990 and Choi et al. 1999 [20,21]. On the other hand, calculation of CMCT through the estimation of F wave and DML displayed controversial CMCT data; which were lower than their counterparts in the control group for the contracted state; but, higher than the control for the relaxed state. Nevertheless, the differences were not statistically significant.

The reports on TMS have heterogeneous findings. Kandler et al. in 1990 have observed a decrease in CMCT, Choi et al. in 1999 [20,21] showed that CMCT was shorter in PD patients compared with normal subjects (P<0.05). However, others did not find any difference in CMCT in patients as compared to controls [16]. There are only a few studies evaluating the relationship between disease severity, duration of disease and predominant type of PD (akinetic rigid versus tremor dominant) with TMS parameters. Some have reported a decrease in CMCT in patients with predominant rigidity and bradykinesia [20], but others found no difference in any of the TMS parameters, including CMCT in the two broad clinical categories of 'tremor dominant PD' and 'akinetic rigid PD' [16].

MEP amplitude reflects the global excitability of cortical interneurons, corticospinal neurons and spinal motoneurons [22]. Results of the present study showed decreased mean amplitude of MEP as compared to healthy controls but with insignificant changes. Several workers have reported increased MEP amplitude at rest in PD patients; which was proposed to be related to an imbalance towards disinhibition in the motor pathway [23]. The occurrence of peripheral tremor leads to permanent stimulation of the cerebral cortex. Dopamine secretion diminution is accompanied by intracortical inhibitory mechanism alteration; hence an excessive response to single-pulse stimulation in PD, the consequence of which is the increase of the MEP amplitude [24]. However, cortical excitability disturbance is not univocal and unlike healthy subjects, in PD, MEP amplitude increases very little or even decreases further to facilitation [1]. Hence, insignificant results of the MEP-amplitude between patients with PD and controls could be most likely expected.

In Conclusion, single-pulse TMS is a valuable study to investigate central motor dysfunction in PD. CL and CMCT measurements of the median nerve or any nerve in the upper limb are potential markers for the evaluation of the severity of PD; especially in the facilitated state. Results of the MEP amplitude are conflicting and require further investigation.

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Authors Contribution

Dr. Ahmed performed the major neurophysiological assessment (TMS-MEP of the Rt. Median N) as well as the conventional sensory and motor nerve conduction study and part of the writing of the manuscript. Dr. Al-Hashimi participated in the neurophysiological assessment of the patients and controls, analyzing the results and writing the major part of manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Pattern and Determinants of Psychogeriatric Disorders Among the Attendees of Old Age Psychiatric Unit, Baghdad, Iraq

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Abstract

Background Mental disorders in old age are frequent.

Objective To determine the diagnostic pattern of mental disorders in elderly patients aged ≥ 60 years, attending the Old Age Psychiatry Unit, Ibn-Rushed Psychiatric Teaching Hospital, Baghdad, Iraq.

Methods A retrospective study to all attendees to the Old Age Psychiatry Unit between January 2009 and November 2011 was carried out. Data collected included diagnoses, comorbid disorders, treatment received, and socio demographic characteristics.

Results Analysis of 907 patients was done; the mean age 68 ± 6.3 years, 67.5% age range 60 – 69 years, 70% married, 50% without income (unemployed and housewives), 52% illiterate, and 98.5% live with their families. Depression was 46.9%, schizophrenia 23.2%, and 20.7% dementia. 48% of clients had comorbid illness. All patients had at least one pharmacological medication. Diagnoses high statistical significant association with gender (P=0.000), marital status (P=0.001), occupation (P=0.000), and education level (P=0.000).

Conclusion Mental disorders in old age are frequent. Many old age people were with limited access to mental health services. Mental health services must be designed to meet the needs of older people at all points of the mental health continuum.

Keywords Pattern, determinants, elderly, psychiatry, Iraq

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List of abbreviations: CVA = Cerebrovascular accident, OCD = Obsessive compulsive disorder, OAPU = Old age psychiatry unit. SAMHSA = substance and mental health services of America

Introduction

Ageing is an inevitable developmental phenomenon bringing along a number of changes in the physical, psychological, hormonal and the social conditions. To define ageing in terms of the biology; referring to "the regular changes that occur in mature genetically representative organism living under reprehensive environmental conditions as they advance in chronological age" [(1)]. Population ageing is a global phenomenon affecting both developed and developing countries with several implications [(2)]. The number of older population of both developed and developing countries has considerably increased in the 20th century [(3)]. The elderly population is growing faster than the total population throughout the world [(4)]. Currently, the number of people aged 60 and over is more than 800 million. Projections indicate that this figure will increase to over two billion in 2050. People aged 60 can now expect to survive an

additional 18.5 to 21.6 years [5]. The majority of older people live in low- and middle-income countries, and some of the fastest rates of ageing are occurring in these areas [6,7]. It is estimated that between 5% and 17% of the older adults suffers from a mental disorder [8, 9]. Factors such as poverty, social isolation, loss of independence, loneliness and losses of different kinds, can affect mental health and general health. Older adults are more likely to experience events such as bereavements or physical disability that affect emotional well-being and can result in poorer mental health. [10,11]. Many researchers have divided old age into three categories; Early old age or young old age, which extended from age 60 to age 69. Old age or advanced old age, this begins at the age 70 and ends at age 79. From the age 80 and the above is considered older old age. The disintegrating system of joint family, rapid industrialization and urbanization and changing social values have together caused serious problem for the aged. They are treated like an unavoidable burden if they ceased to remain productive members. Occupational problems of aging are generally accepted fact that the lack of employment security of older [1]. Many older people with a mental disorder also have a comorbid or co-existing physical illness or disability. Specialist geriatric psychiatry services include assessment, treatment, rehabilitation and clinical liaison services provided by one or more members of a multidisciplinary team. In Iraq, a mental health services for older people is presented at the old age psychiatry unit (OAPU), Ibn-Rushed psychiatric teaching hospital, Baghdad. OAPU was established at December, 2008. Multidisciplinary team was trained in geriatric psychiatry services at Michigan, USA, through Iraq-SAMHSA initiative, first cohort (June-July) 2008.

This study aimed to determine the pattern of diagnostic disorders seen in elderly patients aged 60 years and above attending OAPU, Ibn-Rushed Psychiatric Teaching Hospital, Baghdad, Iraq and also to determine and explore the factors affected management process.

Methods

Design and setting

This is a retrospective study with analytic component. It was conducted in the OAPU, Ibn-Rushed Psychiatric Teaching Hospital, Baghdad, Iraq. The data collection was done during the period from January 1st, 2009 to November 1st 2011. Ibn-Rushed Psychiatric Teaching Hospital is one of the major mental hospitals in Iraq, with adult and child outpatient psychiatric clinic, it deals with acute cases, with about 70 beds for short stay admission, serving patients from all over Iraq. Record system of the hospital was renewed after year 2005; paper file system and appointment card system were founded. Electronic record computerized system was established during 2009-2010. OAPU has its own separated records. All paper files were converted into computerized system. Appointment card system was updated.

Study population and sampling

All elderly clients aged 60 years and above attending at the OAPU regularly, both sexes were included.

Sample size

Sample size is represented by the total number of clients who attended the OAPU during January, 2009 – November, 2011.

Inclusion criteria

All elderly aged ≥ 60 years, of both gender, have regular visits, and with complete record.

Exclusion criteria

All clients with incomplete data were excluded from the study.

Data collection tools

Information extracted from the case notes file system were entered into information list. The information included sociodemographic variables, main clinical features at presentation, diagnoses, co-morbid physical disorders, type of treatment given to the patients.

Definition of variables

The independent variables were socio-demographics, age, gender, marital status, level of education, occupation, admission times, and comorbid condition.

Ethical Issues

Names were kept anonymous and all paper files and electronic records were kept with full privacy.

Statistical analysis

Data extracted were analyzed using SPSS 17 software for windows.

Results

Demographic data

The total number of patients referred to the hospital during the 3 years was 4534. Clients age 60 and above, visited the OPAU were 931. Only 24 clients had incomplete record and were excluded from the study. Clients who complete records with regular visits and follow up were 907 (493 male and 414 female), and this accounted for about 20% of the total referral for the three years under study. Their mean age was 68 ± 6.3 years. Majority of the clients (67.5%) were within the age range 60 – 69 years. about 70% were married. Clients without income (unemployed and housewives) were about 50%. Clients had no formal education were 52%. About 98.5% live with their families (Table 1).

Table 1. Distribution of the study group by sociodemographic characteristics

Parameter		Sex				Total	
		Male		Female		No.	%
		No.	%	No.	%		
Age group	60 - 69 yrs	367	59.97	245	40.03	612	67.5
	70 - 79 yrs	100	40.82	145	59.18	245	27.01
	80 - 89 yrs	24	54.54	20	45.46	44	4.85
	90 - 99 yrs	2	33.33	4	66.67	6	0.66
Marital status	Single	21	50	21	50	42	4.6
	Married	414	65.1	222	34.9	636	70.12
	Widowed	45	22.73	153	77.27	198	21.8
	Divorced	13	41.94	18	58.06	31	3.4
Occupation	Unemployed	108	82.44	23	17.56	131	14.4
	Employed	47	73.43	17	26.57	64	7.05
	Retired	280	85.89	46	14.11	326	35.9
	house-wife	3	0.93	323	99.07	326	35.9
	free works	55	91.67	5	8.33	60	6.6
Education level	Illiterate	169	35.89	302	64.11	471	52.0
	Primary	80	59.25	55	40.75	135	14.8
	Intermediate	57	82.6	12	17.4	69	7.6
	Secondary	104	88.13	14	11.87	118	13.0
	Institute & college	77	71.97	30	28.03	107	11.8
	Postgraduate	6	85.7	1	14.3	7	0.8
Living circumstances	Live with family	486	54.4	407	45.6	893	98.5
	Live alone	6	85.7	1	14.3	7	0.77
	Live in geriatric house	1	14.3	6	85.7	7	0.77
Total		493	54.4	414	45.6	907	100

Diagnoses

Depression, schizophrenia, and dementia were the three most common diagnoses, 46.9%, 23.2% and 20.7% respectively. Conditions such as generalized anxiety disorders, substance abuse, Parkinsonism, bipolar disorder, obsessive compulsive disorder, and epilepsy accounted for another 9.3% (Table 2).

Table 2. Frequency and percentage of medical diagnoses among the study group

Diagnosis	Frequency	%
Depression	425	46.9
Schizophrenia	210	23.2
Dementia	188	20.7
Parkinsonism	28	3.1
Anxiety	22	2.4
Substance abuse	15	1.7
Bipolar disorder	9	1.0
OCD	2	0.2
Epilepsy	8	0.9
Total	907	100.0

The Nature of co-morbid physical illness

Forty eight percent of the clients reported history of co-morbid physical illnesses. hypertension, diabetes mellitus, and gastrointestinal disease accounted for about 55% of reported disease history (Table 3).

Mode of treatment

All (100%) of the clients received pharmacotherapy as the main treatment. Majority, 64.1%, was with two drugs medication (Table 4).

Significance

A cross classification of clients with 9 different diagnosis by socio-demographic explored high statistical significant association with sex (P=0.000), marital status (P=0.001), occupation (P=0.000), and education level (P=0.000) (Table 5).

Table 3. Frequency and percentage of medical history among the study group

Comorbidity	Frequency	%
No Comorbidity	465	51.3
Hypertension	242	26.7
Diabetes Mellitus	152	16.8
Gastrointestinal disorders	97	10.7
Renal system	36	4.0
Ischemic heart disease	30	3.3
Cerebral vascular accidents	26	2.9
Rheumatology	15	1.7
Hearing	13	1.4
Vision	8	0.9
Respiratory	5	0.6
Hyperlipidemia	5	0.6

Table 4. Frequency and percentage of drug medication

Medications	Frequency	%
One drug medication	69	7.6
Two drug medications	581	64.1
Three drug medications	253	27.9
Four drug medications	4	0.4
Total	907	100.0

Table 5. Distribution of the study group and correlation of medical diagnoses with the socio-demographic characteristics

		Diagnosis									Total		
		Dementia	Depression	Schizophrenia	Anxiety	Substance abuse	Parkinsonism	Bipolar	OCD	Epilepsy	No.	%	P value
Age Group	60 - 69 yrs	129	264	158	14	11	20	9	1	6	612	67.5	**0.444**
	70 - 79 yrs	53	129	43	8	3	6	0	1	2	245	27.	
	80 - 89 yrs	6	27	8	0	1	2	0	0	0	44	4.9	
	90 - 99 yrs	0	5	1	0	0	0	0	0	0	6	0.6	
Sex	Male	115	218	98	17	15	21	5	1	3	493	54.4	**0.000**
	Female	73	207	112	5	0	7	4	1	5	414	45.6	
Occupation	Unemployed	38	52	25	1	2	12	1	0	0	131	14.5	**0.000**
	Employed	6	36	20	1	0	0	0	0	1	64	7.1	
	Retired	90	137	62	15	5	9	5	1	2	326	35.9	
	house-wife	51	166	90	4	0	6	3	1	5	326	35.9	
	free works	3	34	13	1	8	1	0	0	0	60	6.6	
Marital Status	Single	4	15	17	0	1	3	0	0	2	42	4.6	**0.001**
	Married	138	292	137	21	13	22	6	2	5	636	70.2	
	Widowed	45	104	40	1	1	3	3	0	1	198	21.8	
	Divorced	1	14	16	0	0	0	0	0	0	31	3.4	
Education Level	Illiterate	78	248	103	7	6	18	3	2	6	471	52.0	**0.000**
	Primary	22	71	30	2	1	7	1	0	1	135	14.8	
	Intermediate	15	31	16	4	1	1	1	0	0	69	7.6	
	Secondary	61	24	25	1	6	1	0	0	0	118	13.0	
	Institute & college	12	45	35	8	1	1	4	0	1	107	11.8	
	Postgraduate	0	6	1	0	0	0	0	0	0	7	0.8	
Total		188	425	210	22	15	28	9	2	8	907	100.0	

Discussion

Old age psychiatric unit attendees accounted for about 20% of the total referral of the hospital. This number appears small and may not represent the true mental health status of the older population. Some of the possible reasons suggested for the reluctance of older adults to seek and continue with mental health care include physical frailty, transportation difficulties, isolation, stigma, and patient provider preferences. Current violent atmosphere, feeling unsecured, and terror car and belts explosions were affecting the total number of attendees. There is therefore the need for the proper integration of mental health into the primary health care system of this country and the training of mental health care providers at that level of care to identify, provide basic intervention measures and refer when necessary, elderly patients with mental health problems to the tertiary level of health care system. If this is done access to mental

health care will be increased for the elderly patients in the community. This study sample is higher number of attendees than the studies carried out in different country [12-15], and lower than one study (2010) [16]. Current study showed that depression (46.9%), schizophrenia (23.2%), and dementia (20.7%), similar to study done in Accra, Ghana (1997) that found depression (51.4%) higher than dementia (31.5%) [12]. Beside comorbid organic syndromes, personal history of depression, death of spouse, health related factors and anxiety disorders show significant associations with incidence of depression [17]. Other studies showed dementia more frequent than other diagnosis [13,18]. Chronic organic mental disorders, dementias, are the main reason for the necessity of geriatric care units [19]. Dementia is defined as a syndrome of acquired impairment of memory and other cognitive functions secondary to structural brain damage. The clinical interface of depression and dementia is a rich and complex topic [19]. This study found that dementia is less frequent than depression and schizophrenia, which is less than expected. Possible explanations for the small number would include a lower cut off age of 60 years used as the inclusion criteria in this study which could have increased the number of clients with other diagnoses to be included in the study. This is lower than the cut off age of 65 years used in previous studies. Advances in medicine are permitting many people to live to the age of sixty or more, with the result that the proportion of the world community over the age of 60 years is rapidly increasing [20]. Thus the practice in the past as reported by Prince [21], where less attention was given to dementia because it was considered to be a relatively uncommon condition. Depressive syndromes are frequent in old age [22,23] and especially frequent are minor forms of depression like dysthymia, or subsyndromal depression. Although late-life depression is a chronic and disabling illness, there is a common misconception that it is a normal feature of aging. Depression at old age is therefore under-recognized and severely under-treated, especially in very old age with high somatic comorbidity [24]. Poor physical health has long been recognized to be one of the most important risk factors for depression in older adults. Generalized anxiety disorder possibly a pre-stage of depressive illness in many old patients, is very frequently found in medical institutions. In The Longitudinal Aging Study Amsterdam [25], which was based on a random sample of 3107 older adults, the overall prevalence of anxiety disorders was estimated at 10.2 %. Generalized anxiety disorder was the most common disorder (7.3%), followed by phobic disorders (3.1%), whereas panic disorder (1.0%) and obsessive compulsive disorder (0.6%) were rare. Vulnerability factors such as female sex, lower education level, traumatic experiences, stresses commonly experienced by older adults (recent losses of family members and chronic somatic illness), and a smaller size of the social network appeared to be associated with anxiety disorders. This study founded statistical significant association of old age attendees diagnoses and presence of walking aids like wheel chairs or walking sticks(P=0.000), that explored getting disability as going older. Wandera et al. (2014) founded disability was associated with advancement in age [2].

In conclusion, old age is a growing category with some biopsychosocial privacy. Mental disorders in old age are frequent. Mental health of old age needs proper assessment and early detection. Many old age people were with limited access to mental health services due to physical frailty, transportation difficulties, isolation, stigma, and patient provider preferences. Mental health services must be designed to meet the needs of older people at all points of the mental health continuum. This study recognizes the need for the proper integration of mental health into primary health care system of the country and the need to train mental health care providers at that level of care to identify and provide basic intervention measures and refer when

necessary, to the tertiary level of care. Mental health promotion should be embedded in all policies, programs, and services for all older adults (including those with mental illness) and their caregivers, and encompass anti stigma strategies, public awareness, education, and training. Older adults, caregivers, service providers and the public should be informed about the importance of early identification of symptoms of mental illness, prevention strategies and the hope for recovery and well-being. Transformation of a mental health service system must include training, education and support for caregivers and health care providers to increase their capacity to respond to the mental health needs of seniors.

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Authors Contribution:

Dr. Al Abbudi: Consultant Psychiatrist, data collection, data entry, electronic record system, data analysis, and the writer of this paper. Dr. Ezzat: Social worker, data collection, data entry, and file record system.

Conflict of interest

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Frequency of Hepatitis C Virus Genotypes /Subtypes Association with Response to Therapy in a Sample of HCV Infected Iraqi Patients

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Abstract

Background Hepatitis C virus (HCV) is an important human pathogen affecting 120-170 million individuals in the world. Identification of the causative virus genotype is of a significance to both clinical practices and predict the likelihood to therapy response.

Objective To determine the distribution of HCV genotypes/subtypes and its association with response to therapy among newly diagnosed HCV patients.

Methods Fifty patients with confirmed anti-HCV antibodies were included in this study for HCV genotyping in association with response to therapy. Blood samples from patients were subjected to RNA extraction and reverse transcription step; viral load of HCV was measured by polymerase chain reaction (PCR) at time zero, 3 months and 6 months of dual therapy. Response to therapy was measured as a decrease in viral load (2 log or more) and was described as: good (median log is zero after 6 months of therapy), moderate (median log declines more than 2 log but not zero after 6 months of therapy), poor (median log does not decline or decline less than 2 log after 6 months of therapy).

Results Two genotypes of HCV were detected, genotype 4 was the predominant (27/50, 54%) followed by genotype 1 (23/50, 46%). For HCV subtypes, subtype 1a was of highest percentage (28%) followed by 4e (24%), 1b (18%), 4a (14%), 4b (12%), and 4e (4%).The results revealed a significant association between HCV subtypes, but not genotypes, with response to therapy. HCV subtype 1a followed by 4a showed the highest rate of response 85.7% and 71.4%, respectively, while interestingly HCV subtype 4d showed no response and 1b showed poor response 11.11%.

Conclusion HCV subtypes of great importance in predicting success to HCV therapy and it is believed this would affect the newly emerging directly acting drugs as well.

Keywords HCV, genotypes, response to therapy

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List of abbreviations:c DNA = Complementary DNA, CHC = Chronic hepatitis C, ELISA = Enzyme linked immunosorbant assay, HCC = Hepatocellular carcinoma, HCV = Hepatitis C virus, HIV = Human immunodeficiency virus, IC = Internal control, NR = Non-responder, R = Responder, SVR = Sustained virological response

Introduction

Hepatitis C virus (HCV) is an important public health problem worldwide that causes acute and chronic liver diseases like cancer. Approximately 80% of subjects with acute hepatitis C progress into a chronic disease [(1)]. Chronic hepatitis C virus (CHC) infection is an important cause for developing (10-20%) cirrhosis and hepatocellular carcinoma (HCC) that often results in liver failure and thus liver transplantation [(2)]. HCV is divided into six major genotypes and more than 80 subtypes [(3,4)].Identification of the causative virus genotype is of significance to

both clinical practices and epidemiological studies. Regarding the clinical practices, once the genotype is identified, the result of the treatment and determination of its duration are facilitated as the genotype is the strongest predictor of the sustained viral response (SVR), which is defined as undetectable HCV RNA after six months from completion of treatment [5]. Recent data strongly indicate that HCV genotype is the key determinant of response to interferon-alpha (IFN-α) based treatment regimens[6,7].Genotype should be determined in all HCV-infected persons prior to treatment in order to predict the likelihood of treatment response [8,9]. Patients with genotypes 1 and 4 generally exhibit a poorer response to IFN-based therapy than those with genotypes 2 and 3. HCV genotype 5 appears to be an easily treatable virus, with response rates compatible with those of genotypes 2 and 3 therapy [7,10].Treatment response in genotype 6 HCV patients may be at an intermediate level between that observed in genotype 1 and genotypes 2/3. The optimal duration of treatment for HCV genotype 6 is unclear and currently under investigation [7,10].

This study relates HCV genotypes/subtypes with response to therapy. This will provide basis for better predictability of treatment success in HCV patients in this area.

Methods

Patients and sampling:

This study was carried out from March 2015 to November 2015 on diagnosed HCV infected patients referred to Gastroenterology and Hepatology Center at Baghdad governorate; however, diagnosed cases of HCV infection included mostly chronic HCV infection. For each patient, 5 ml of blood were collected at three occasions, before treatment, after three months, and after six months of treatment.

First of all, potential patients were screened by enzyme linked immunosorbant assay (ELISA) 3rdgeneration assay. Plasma samples from only those showed seropositive anti-HCV antibodies were included in this study. Accordingly, 50patients were included. The recruited cases were subjected for measuring HCV RNA load at time zero, 3 months and 6 months of therapy. In addition, cDNA of HCV RNA was subjected for genotyping. Ethical approval for interview, collecting information from records and taking specimens from patients was obtained.

Detection of serum anti-HCV antibodies

The initial screening for anti-HCV antibodies was carried out by a third generation ELISA. The procedure and result interpretations were done according to the manufacturer instructions HCV ELISA test kit 3rd generation (plasmatic, UK). The absorbance of the solution was read at 450 nm by the ELISA reader. The mean absorbance of the negative control (NCx) was calculated. The cut-off value = 0.120+NCx; specimens with absorbance values less than the cut-off value were considered to be negative, specimens with absorbance values greater than the cut-off value were considered to be positive.

Viral RNA extraction for viral load and viral genotyping

QIAamp viral RNA Minikit (Cat.No 52906. Qiagen, Germany) was used for HCV-RNA extraction from plasma samples according to the manufacturer guidelines. Briefly, the sample was first lyzed under denaturating condition to inactivate RNase and for isolation of intact viral RNA. Buffering solutions were used to provide optimum binding of RNA to the QIA amp membrane. Sample is loaded onto the QIA amp Mini spin column. The RNA binds to the membrane and contaminants are efficiently washed in two steps using two different wash buffers. High- quality RNA is eluted in a special RNase- free buffer ready for use.

Viral load detection

Twenty µl of extracted HCV-RNA were used for Real-time qPCR amplification step using HCV virus-RGRT-PCR (Ref No.4518233. Qiagen, Germany). The protocol of technique was according to the manufacturer guidelines. Briefly, the Hepatitis C virus RGRT-PCR kit consists of Master A and B containing reagents,

enzymes for the reverse transcription and specific amplification of a 240 bp region of the HCV genome and for the direct detection of the specific amplicon in fluorescence channel cycling Green of the Rotor- Q-Gene. In addition, the protocol pursued included a second amplification target to identify possible PCR inhibition, namely internal control (IC) in fluorescence channel cycling orange of the Rotor-Q-Gene. External positive controls were allowing the determination of the amount of viral RNA.

Rotor-Q -gene thermo cycler (Qiagen, Germany) was used and run by the following program shown in table 1.

Table 1. Thermo cycling program of real-time qPCR for measurement of HCV RNA load

Temperature(°C)	Time	Number of cycle
50	30 min	1
95	15 min	2
95	30 sec	
50	60 sec	50
72	30 sec	

The standard curve was constructed by adding 20µl of serial dilutions of HCV standard RNA from 10 IU/µL to10000 IU/µL. Standard curve was also used for calculating PCR run efficiency which was above 96%. Internal control was added through extraction steps, PCR water grade used as negative control. After the run is finished, the data was analyzed via signal fluorescence. The HCV load in patients; blood was calculated as follows:

$$\text{HCV RNA (IU/ml)} = \frac{Result\ (IU/\mu l) \times Elution\ volume\ (\mu l)}{Sample\ volume\ (ml)}$$

Reverse transcription and HCV cDNA genotyping

The protocols used were according to the manufacturer guidelines. The amplification step was achieved by using HCV Real time-PCR 2.0 Kit AC 032/24 (NLM, Italy). Up to 10 µl of amplified PCR products were used to hybridize with universal specific probe immobilized on nitrocellulose strip through many steps using Gene GEN-C 2.0 AC004/24 (NLM, Italy).The reverse hybridization step is based on reverse-hybridization principle. Briefly, biotinylated amplicons generated by RT-PCR of the 5-UTR and Core regions of HCV RNA, were hybridized to specific probes that are bound to nitrocellulose strip, biotinylated hybrids were then detected using streptavidin bound to alkaline phosphatase; amplicons not complement were washed out. Then substrate reacted with the streptavidin-alkaline phosphatase complex forming a purple precipitate and coloring banding pattern on the strip. Genotype specific bands were developed on the strip and the resultant profile was analyzed using interpretation table came with the kit.

Statistical analysis

Data of this study were analyzed using SPSS software version 23. Descriptive statistics were done in terms of frequencies and percentages. Values of viral load of HCV were shown to be nonparametric; therefore, median ± confidence interval rather than mean was used. Qualitative assessment of response to therapy was grouped into responders (R) and non-responders (NR) patients based on two log reduction cutoff of viral load. Mann Whitney test was used for measuring P values for medians. P values less than 0.05 were considered significant.

Results

Population of the study

For age of patients, it was shown that age groups 41-50 and then31-40 years showed the highest percentages of HCV infection as 78% of hepatitis patients were of age older than 31 and younger than 50 years old, hepatitis C virus patients were shown to be of equal sex ratio (1:1).The control group was confirmed to be sex and age matched, moreover hepatitis patients were shown to be mainly non-smokers (smokers rate 18%) and non-alcoholics (alcoholic rate 8%) with no predilection for being diabetic (12%), thalassemic (6%), or had history of blood transfusion (14%).

Nineteen out of 50 HCV patients were presented with liver fibrosis. Stage 3 of fibrosis was of highest occurrence (36.8%) then stages 2 (26.3%), 4 (21.1%), and 1 (15.8%). This finding indicates that more than half (57.9%) of HCV patients with fibrosis were with advanced stage of liver fibrosis (stages 3 and 4).

Percentage of HCV genotypes and subtypes among Iraqi patients

The study was conducted on 50 HCV seropositive patients for both sexes; the result showed that two genotypes of HCV were detected: HCV genotype 4 in 27/50(54%) followed by HCV genotype 1 in 23/50(46%).The percentage of the most dominant HCV subtypes1a, 4e, and 1b, were 28%, 24%, and 18%, respectively as shown in figure 1 and 2, table 2.

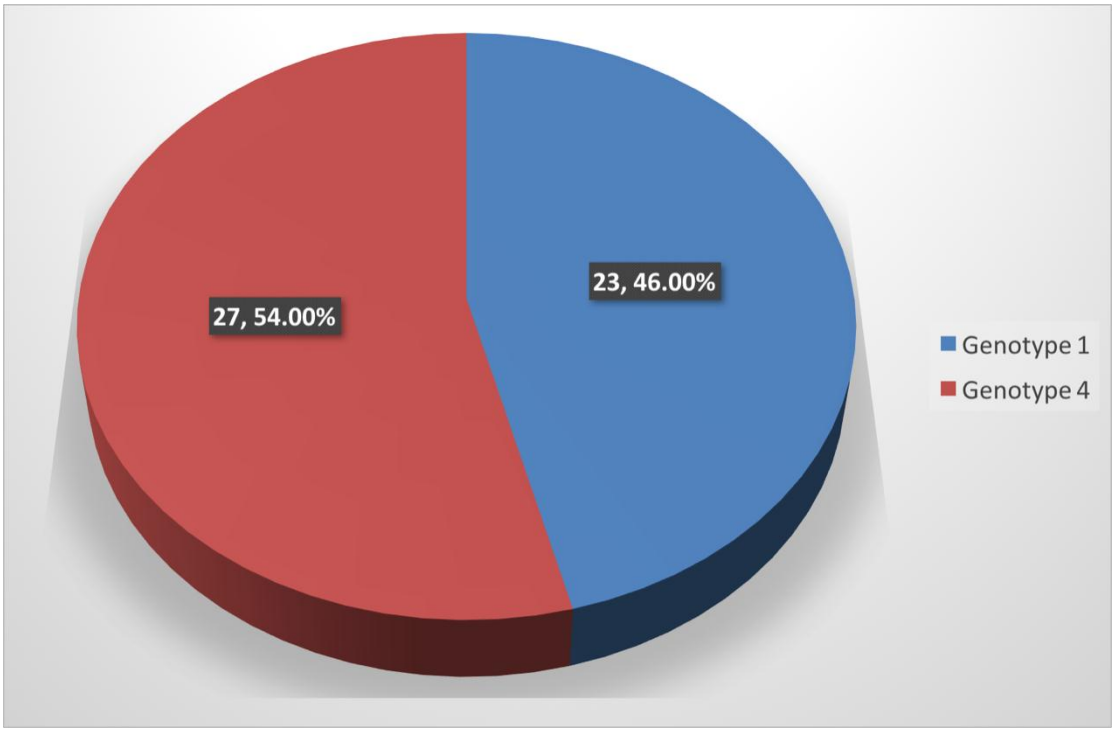

Figure 1. The percentage of HCV genotypes in a sample of Iraqi HCV patients

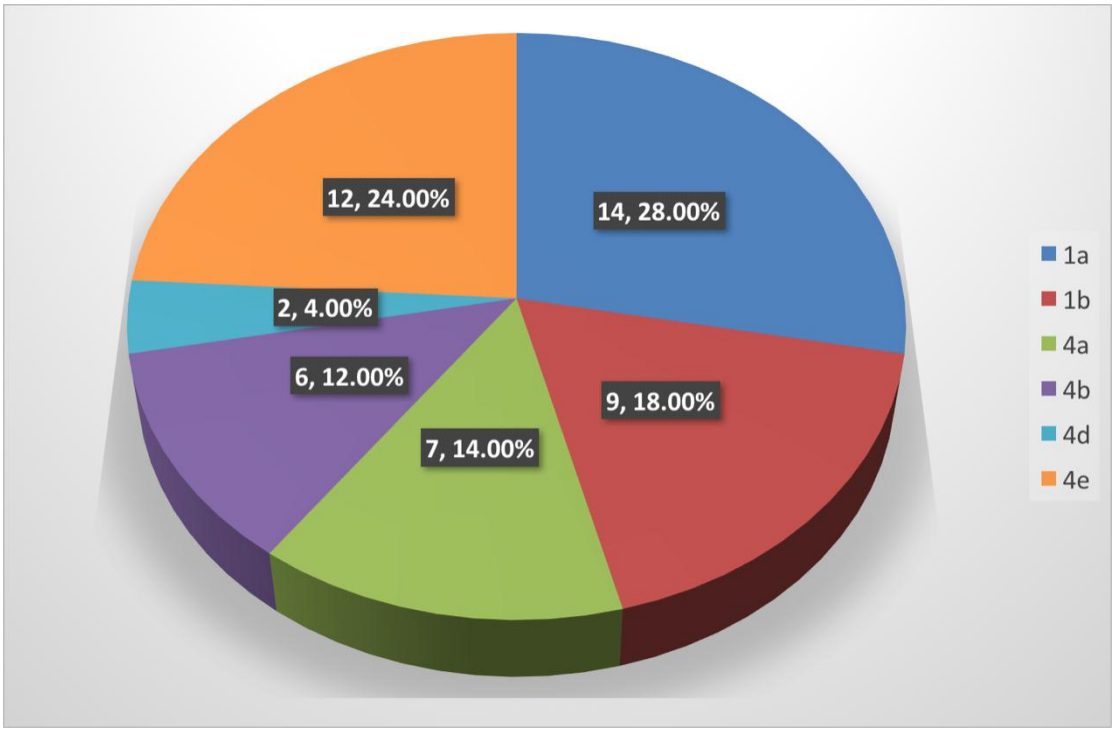

Figure 2. The percentage of HCV subtypes in a sample of Iraqi HCV patients

Table 2. (A) Frequency of HCV genotypes and (B) subtypes in a sample of Iraqi patients.

		Frequency	%
A. Genotype	1	23	46.0
	4	27	54.0
	Total	50	100
B. Subtype	1a	14	28.0
	1b	9	18.0
	4a	7	14.0
	4b	6	12.0
	4d	2	4.0
	4e	12	24.0
	Total	50	100

Association between Hepatitis C virus genotypes and the demographic characteristics of hepatitis C virus patients

This study revealed significant association between HCV genotypes and sex of patients ($P<0.05$), while no significant association was seen between HCV genotypes and age, body mass index, smoking, and alcohol intake ($P>0.05$). Female patients were shown to be infected with HCV genotype 1 (56%) more than genotype 4 (44%) while male patients were infected more with genotype 4 (64%) than genotype 1 (36%) as shown in table 3.

Table 3. Association between Hepatitis C virus genotypes and the demographic characteristics of hepatitis C virus patients

Parameter		HCV genotype		Total Frequency (%)	p value
		Genotype 1 Frequency (%)	Genotype 4 Frequency (%)		
Gender	Female	14 (56.0)	11 (44.0)	25 (100)	0.035
	Male	9 (36.0)	16 (64.0)	25 (100)	
Age groups (years)	31-40	11 (61.1)	7 (38.9)	18 (100)	0.156
	41-50	8 (38.1)	13 (61.9)	21 (100)	
	51-60	3 (37.5)	5 (62.5)	8 (100)	
	>60	1 (33.3)	2 (66.7)	3 (100)	
BMI (kg/m^2)	<20	1 (50.0)	1 (50.0)	2 (100)	0.517
	20-25	12 (40.0)	18 (60.0)	30 (100)	
	25-30	9 (56.2)	7 (43.8)	16 (100)	
	>30	1 (50.0)	1 (50.0)	2 (100)	
Smoking	No	19 (46.3)	22 (53.7)	41 (100)	0.547
	yes	4 (44.4)	5 (55.6)	9 (100)	
Alcohol intake	No	20 (43.5)	26 (56.5)	46 (100)	0.089
	yes	3 (75.0)	1 (25.0)	4 (100)	
Others	No	19 (46.3)	22 (53.7)	41 (100)	0.231
	DM	1 (16.7)	5 (83.3)	6 (100)	
	Thalassemia	3(100)	0 (0)	3 (100)	
Employment status	Employee	11 (47.8)	12 (52.2)	23 (100)	0.976
	Unemployed	12 (44.4)	15 (55.6)	27 (100)	
Total		23 (46.0)	27 (54.0)	50 (100)	

Association between Hepatitis C virus genotypes/subtypes and stage of liver fibrosis

It was shown no significant association between stage of liver fibrosis and HCV genotypes (P> 0.05). However, when taking HCV subtypes into account, it is found that liver fibrosis was associated significantly with the HCV subtypes (P<0.05) in that HCV subtypes 1b, 4d, and 4e showed predilection to advanced (3 and 4) stages of liver fibrosis while the percentage of advanced stages of liver fibrosis in subtypes 1a, 4a, and 4b showed either mild predilection to early stages or equal preference to early and advanced stages of fibrosis as shown in table 4.

Response to therapy in respect to HCV genotypes/subtypes

No significant difference in response to therapy, in terms of logarithmic reduction of viral load, was found between viral genotype 1 and 4 (P>0.05). Both genotypes showed good response to dual therapy and the response was seen within the first 3 months of therapy. However, both genotypes 1 and 4 reached median log 1 after 6 months of therapy as shown in table 5.

Table 4. Association between stage of fibrosis and HCV subtypes

Subtype		Fibrosis				Total
		1	2	3	4	
1a	Count	2	1	2	0	5
	% within subtype	40.0	20.0	40.0	0.0	100
	% within Fibrosis	50.0	33.3	50.0	0.0	26.3
1b	Count	0	0	1	1	2
	% within subtype	0.0	0.0	50.0	50.0	100
	% within Fibrosis	0.0	0.0	20.0	20.0	10.5
4a	Count	0	2	2	0	4
	% within subtype	0.0	50.0	50.0	0.0	100
	% within Fibrosis	0.0	40.0	28.6	0.0	21.1
4b	Count	0	1	0	0	1
	% within subtype	0.00	100	0.0	0.0	100
	% within Fibrosis	0.00	25.0	0.0	0.0	5.3
4d	Count	0	0	0	1	1
	% within subtype	0.0	0.0	0.0	100	100
	% within Fibrosis	0.0	0.0	0.0	14.3	5.3
4e	Count	1	1	2	2	6
	% within subtype	16.7	16.7	33.3	33.3	100
	% within Fibrosis	20.0	20.0	28.6	66.7	31.6
Total	Count	3	5	7	4	19
	% within subtype	15.8	26.3	36.8	21.1	100
	% within Fibrosis	100	100	100	100	100
p value				0.003		

Table 5. Quantitative Association between patients' response totherapy and hepatitis C virus genotypes

Viral load		Genotype 1	Genotype 4
HCV viral load before treatment (IU /ml blood)	Median	3.00E+05	6.00E+05
	25-75 CI	(4.E+04-1.E+06)	(1.E+05-2.E+06)
HCV viral load 3 months after treatment (IU /ml blood)	Median	7.00E+02	1.00E+03
	25-75 CI	(0.E+00-3.E+05)	(0.E+00-1.E+05)
HCV viral load 6 months after treatment (IU /ml blood)	Median	0.00E+01	0.00E+01
	25-75 CI	(0.E+00-3.E+02)	(0.E+00-4.E+04)
Baseline-after 3 months		<0.001	<0.001
Baseline-after 6 months		<0.001	<0.001
3 - 6 months		1	0.943

As an attempt to illustrate deeply the association of dual therapy response by patients with different HCV viral subtypes, it was shown that all HCV subtypes in this study, except subtype 4d, responded in some way to dual therapy and the response was excellent in subtypes 1a, 4a, and 4b while the response to dual therapy was less remarkable in 1b and 4e, as summarized in table 6.

Table 6. Quantitative association between patients' response to therapy and hepatitis C virus subtypes

Viral load		HCV subtypes					
		1a	1b	4a	4b	4d	4e
HCV viral load before treatment (IU /ml blood)	Median	**3.00E+05**	**9.00E+05**	**1.00E+06**	**2.00E+05**	**2.00E+06**	**1.00E+06**
	25-75 CI	(3.E+04-4.E+05)	(2.E+05-1.E+06)	(2.E+05-2.E+06)	(3.E+04-2.E+05)	(6.E+05-3.E+06)	(3.E+05-2.E+06)
HCV viral load 3 months after treatment (IU /ml blood)	Median	**2.00E+01**	**7.00E+03**	**1.00E+03**	**2.00E+02**	**1.00E+06**	**6.00E+03**
	25-75 CI	(0.E+00-3.E+04)	(0.E+00-8.E+05)	(0.E+00-2.E+05	(0.E+00-4.E+03)	(1.E+05-2.E+06)	(0.E+00-7.E+04)
HCV viral load 6 months after treatment (IU /ml blood)	Median	**0.00E+00**	**4.00E+02**	**0.00E+00**	**0.00E+00**	**9.00E+05**	**0.00E+01**
	25-75 CI	(0.E+00-3.E+02)	(0.E+00-0.E+00)	(0.E+00-0.E+00)	(0.E+00-4.E+04)	(9.E+04-2.E+06)	(0.E+00-2.E+04)
Baseline-after 3 months		0.084	<0.001	0.032	0.017	0.945	<0.001
Baseline-after 6 months		<0.001	<0.001	<0.001	<0.001	0.899	<0.001
3 - 6 months		1	1	0.439	0.129	0.932	0.324
Description of response*		Good	moderate	good	Good	poor	moderate

*Description of response: good (median log is zero after 6 months of therapy), moderate (median log declines more than 2 log but not zero after 6 months of therapy), poor (median log does not decline or decline less than 2 log after 6 months of therapy

In the qualitative assessment of association, patients were classified into responders (R) and non-responders (NR). Subtype 1a showed the highest percentage of responders to dual therapy, 85.71%, followed by 4a 71.43%, and 4b 66.67%. On the other hand, HCV subtype 4d followed by 1b showed the poorest percentage of response,0% and11.11% respectively, while 4e subtype of good response but much less evident than 1a, 4a, and 4b as shown in table 7.

Discussion

Viral genotypes and subtypes of HCV are considered as markers for morbidity and mortality [(11)], clinical status, pathogenesis and outcome of disease [(12)]. Moreover, HCV genotypes/subtypes can be used for elucidating the possible mode of transmission, assessing the duration and benefit of antiviral therapy and future development of vaccine [(13)]. The present study showed that only HCV genotype 4 and 1 were detected in 54%, 46%, respectively of HCV-RNA positive patients. The rate of HCV genotypes found in this study is similar to these reported from different Middle East countries like Saudi Arabia and Lebanon where genotype 4 is the most common [(14)]. However, the results of this study are close, but not similar to that shown by previous studies conducted in Iraq; A study conducted in 2012 [(15)], found that genotype 1 is slightly more common than genotype 4 in that genotype 4 was 41.38%while genotype 1 was 48% of cases. Another study in Iraq, Al-Kubaisy et al, [(16)] reported that genotype 1 was 49% and genotype 4 was 35.4% of thalassemic Iraqi children. The above difference in genotype/subtype patterns in Iraq may be

attributed to that HCV genotype 4 might become more resistant to therapy and became under selective pressure of therapy especially the most resistant subtypes to therapy found in this study were 4d and 4e which might explain the increasing percentage of HCV genotype 4 over 1 over time in Iraq.

Table 7. Qualitative Association between patients' response totherapy and hepatitis C virus subtypes

		Subtype						Total
		1a	1b	4a	4b	4d	4e	
Responsiveness	R	12	1	5	4	0	7	29
		85.7%	11.1%	71.4%	66.7%	0.0%	58.3%	58.0%
	NR	2	8	2	2	2	5	21
		14.3%	88.9%	28.6%	33.3%	100%	41.7%	42.0%
Total		14	9	7	6	2	12	50
Description of response*		good	poor	good	good	No response	good	
p value		<0.001						

* Description of response. Good (R>NR), poor (NR>R), no response (all patients are NR).

Concerning HCV subtypes, in general, the current study findings indicated that 1a subtype was of the highest percentage, 28%, followed by 4e (24%), 1b (18%), 4a (14%), 4b (12%), and 4d (4%). This is the contrary to the previous study conducted on 492 histologically proven chronic HCV cases recruited from all region of Saudi Arabia between 1999 and 2002; they found that HCV subtype 4c/d are the major subtypes observed in the genotype 4 and as follows: 4a (0.5%), 4b (2.2%), 4c/d (19.5%), 4e (3.7%), and 4h (5.7%), while they found the rate of subtypes within HCV genotype 1 to be 1a (5.1%) and 1b (16.1 %)[17].

The results of the current study exhibited a remarkable lowering in the median of HCV viral load in blood of the studied patients in response to therapy for 3 and 6 months in both HCV genotypes 1 and 4 without any significant difference between HCV genotype 1 and 4. These findings are consistent with a recent study performed at Gastroenterology and Hepatology teaching hospital in Baghdad from 2011-2012 on 90 HCV antibody positive patients which found that the patients infected with HCV genotypes 1 and 4 exhibited similar early virological response in 93.3%of patients underwent dual therapy [18]. In another study conducted in Baghdad 2011; investigators found similar results by interpreting end treatment virological response to HCV genotypes 1 and 4 [19]. Regarding studies abroad, several studies found the same [20-22]. On the other hand, there are some other reports showed inverse outcome where response to therapy was different with different genotypes of HCV, some revealed better response to therapy in HCV genotype 4 [23,24].

Concerning HCV-1 subtypes, the findings of this study coincide with that of another study [25] which revealed dual antiviral therapy is more effective against HCV subtype 1a than subtype 1b (55% vs 43%), respectively. Another study confirmed this finding, showing that HCV subtype 1b is associated with more severe liver disease, not because it is a more aggressive form of HCV but because it reflects a longer duration of infection [26].

Regarding HCV-4 subtypes, the results of this study are pioneering in Iraq; this study showed that rate of response to therapy was lowest in subtypes 4d, then 1b and 4e while subtypes 1a, 4a and 4b showed best response to therapy.

This finding is in agreement with another international study [27] which found that patients infected with HCV -4a subtype respond significantly better to combination therapy than HCV-4d subtype, 4a achieved 77% while 4d achieved 52% of successful therapy in term of SVR. Another study in France [28] reported a poor response to 4d group of 10 HIV-positive patients, who were acutely infected with HCV. Also, another study on French patients infected with HCV-4, where subtype -4a had significantly higher rate of SVR (58%) than subtype 4d (43%) [29].

Taken together, HCV genotypes and subtypes showed remarkable association with response to HCV dual therapy, sex of patients, and stage of liver fibrosis. This highlights the importance of extending the genotyping tests of HCV infections to the level of subtypes within HCV genotypes1 and 4 in Iraqi population to help predict success of treatment and likelihood of development of advanced stages of liver fibrosis.

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Authors Contribution:

Dr. Abdulamir made the drafting of the article and revising it critically for important intellectual content; Dr. Al-Khalidi did clinical examination and diagnosis; Dr. Alwaysi helped in provided the samples and Abdulhassan collected blood samples, did the laboratory analyses and preparation of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Mesenchymal Cell Death in Mouse Limb Bud After the Onset of Primary Myogenesis

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Abstract

Background The vertebrate limb bud develops as an outgrowth of mesoderm, which forms all their elements (muscles, nerves, vessels, bone, cartilage, and tendon). Myogenic precursor cells are seen at E11.5 mouse embryo, when the first nerve fascicles begin to enter the limb. The first signs of musculature masses are seen at E12.5 in both fore and hind limb buds. Apoptosis or programmed cell death is essential in the development of the limbs. In vertebrate, the developing limb morphogenesis depends on the appropriate spatial and temporal balance between cell death and cell proliferation.

Objective To perform comprehensive analysis of the proximo-distal pattern of cell death, evaluated by (TUNEL test) in cross sections of mouse limbs during prenatal development after onset of primary myogenesis.

Methods Fifteen pregnant female mice (Musmusculus) were divided into three groups according to the days of pregnancy into day (14, 16 and 19), only two embryos were taken from each mouse. All the limb buds were involved in this study. Paraffin embedded histological cross-sections of the limb buds were prepared, histological staining (using H&E stain) and TUNEL test labeling were done. Assessment of the number of apoptotic cells in the limb bud mesenchyme was done by counting these cells.

Results The H&E stained sections of the limb buds showed less amounts of mesenchymal tissues in older embryos (day 19). The TUNEL stain showed active apoptotic changes at proximal parts of the limb buds at gestational day 19, while the distal parts of the limbs buds showed active apoptotic changes at the early days (day 14). The evaluation of TUNEL test reaction in the proximal regions showed statistical significant increase of apoptotic cells in day 19 compared to day 14 ($p = 0.001$ for both). The mean number of apoptotic cells in the proximal regions were statistically significant ($p = 0.001$) between day 16 and day 19. While the mean number of apoptotic cells of distal regions of the limb buds was higher at day 14 compared to that of day 16 and day 19. These differences between day 14 and day 16 were statistically significant and between day 16 and day 19 while statistically non-significant between day 14 and day 19. Comparison of mean number of apoptotic cells between proximal and distal regions in all the three groups showed a statistically significant higher mean number of apoptotic cells in the distal regions compared to proximal region ($p = 0.001$). The mean number of the apoptotic cells in both regions (proximal and distal) of the limb buds revealed statistically significant differences between day 16 and day 19 ($p = 0.001$).

Conclusion Apoptosis was higher in all parts of the developing limbs during day 19, and that could be associated with degenerative changes occurring at the apical ectodermal ridge. Moreover, apoptosis was higher in the distal part of the limb bud and this may be due to more differentiation of the distal parts than in the proximal part of the limb bud.

Keywords Development, limb bud, mouse, embryo, TUNEL, apoptosis, mesenchyme

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List of abbreviations: AER = Apical ectodermal ridge, H&E = Hematoxylin and eosin

Introduction

Vertebrate limbs develop from undifferentiated homogenous mesenchymal cells that are surrounded by the ectoderm [(1)]. The limb patterning includes three spatial axes; (proximal to distal, anterior to posterior, and dorsal to ventral axes [(2)].

Morphogenesis of the upper and lower limbs is similar; their outer shape is formed when

mesenchymal cells in the buds start to condense. The layer of ectoderm at the distal border of the limb is thickened to form the apical ectodermal ridge which induces limb bud formation and elongation [3].

In mouse, the first sign of the limb development recorded during the E9.5 and E10. At E11.5, the long bones of the limbs develop in proximodistal direction and by the E14.5 the most distal phalanges are formed [4].

The limb buds develop as an outgrowth of mesoderm which form all their elements (muscles, nerves, vessels, bone, cartilage, and tendon) with a supervision of the apical ectodermal ridge (AER), which is formed at the junction between the ventral and dorsal ectoderm [5].

During development, the digits of limbs become separated due to interdigital apoptosis as well as differential growth of the digital tips [6].

The ossification of the limb elements shows different stages which start by the stage of pre-cartilaginous condensations in the presumptive bones, then displaying the proliferating chondrocytes. The chondrogenic precursor cells condense in the center of the limb buds to form chondrogenesis center, in addition for tendons and muscles, which condense in the rims of bud. Myogenic precursor cells are seen at E11.5 mouse embryo, when the first nerve fascicles begin to enter the limb. The first signs of musculature masses were seen at E12.5 in both fore and hind limb buds [4].

The cell death (apoptosis) is important to balance the cell proliferation during development [7]. The apoptosis is the major cell death pathway for removing unwanted in a clean or silent manner during embryonic development [8]. Apoptosis as a programmed cell death is essential in the development of the limbs [9]. The programmed cell death take place in the developing limb in areas of the undifferentiated mesoderm in connection with the establishment of the pre-chondrogenic condensations of the skeleton, in addition cell death is also observed during the formation of the joints [10]. The areas of apoptosis are recoded with the formation of the shape and skeleton of the limb, it shows a significance difference between species members [11].

The (TUNEL) is a detecting technology for DNA fragmentation by labeling the terminal end of nucleic acids. It uses to recognize DNA fragmentation of apoptotic signaling cascades [12]. The DNA fragmentation might occur after the release of enzymes from cytoplasmic membrane [13].

This study had been done to perform comprehensive analysis of the proximodistal pattern of cell death evaluated by (TUNEL test) in cross sections of mouse limbs during prenatal development after onset of primary myogensis.

Methods

Fifteen pregnant female mice (Musmusculus), aged about 10-12 weeks, weighing between 25-30 g, were divided into three group according to the days of pregnancy into day (14,16 and 19), only two embryos were taken randomly from each mouse. All the limb buds were involved in this study (Table1).

Limb buds were separated according to their age in containers in order to be fixed with formalin 10%, followed by fixation, dehydration, clearing, paraffin embedding and sectioning dewaxing and hydration were done 14. Serial sections of 5-6 µm thickness were cut using the electrical microtome.

The TUNEL-based detection kit was provided by abcam® with kit code ab66108. The fluorescin-labeled DNA was observed with a fluorescent microscope.

Statistical evaluation of the number of apoptotic cells in the limb bud mesenchyme that were showed in a bright yellow fluorescent color was done depending on the counting these cells.

Table 1. Arrangement of Samples groups according to the days of Pregnancy

Days of pregnancy	No. of female mice in each day	No. of embryos that were taken randomly	No. of limb bud that were taken from embryo
14	5	10	40
16	5	10	40
19	5	10	40
Total	15	30	120

Results

The TUNEL test of the limb buds at the variable stages of development showed three different fluorescent colors:

- Yellow color representing the apoptotic cells (positive color).
- Orange color for the non-apoptotic cells (negative color).
- Green color, also a negative color.

The Abcam's in situ Direct DNA Fragmentation Assay Kit used provided two components including positive and negative control cells for conveniently detecting DNA fragmentation by fluorescence microscopy.

The TUNEL test applied in this study showed evaluation of apoptosis ratio in the limb buds.

The variable regions of mesenchymal tissue of the proximal and distal limb bud were evaluated by counting the number of the cells undergo apoptosis. The counting of the positive fluorescent cells in the mesenchymal tissue of the limb buds illustrated the bright yellow colored apoptotic cells (Figures 1, 2, 3, 4).

Evaluations of apoptotic cells in proximal regions of limb bud

Day 14 and Day 16

The counting of the fluorescent cells in the proximal regions of limb bud showed a mean value at day 14 was (90.1±2.7). While that at day 16 was (95.0±4.7) (Table 2); t-test revealed a non-significant difference between the mean value in the proximal regions at day 14 and day 16.

Day 16 and Day 19

The mean value in proximal regions of limb buds at day 16 was (95.0±4.7). While that at day 19 was (180.0±8.3) (Table 3); t-test revealed significant higher values of apoptotic cell in day 19 compared to day 16 ($p \leq 0.05$).

Day 14 and Day 19

The mean value of apoptotic cell in proximal regions of limb bud at day 14 was (90.1±2.7). While that at day 19 was (180.0±8.3) (Table 4); t-test revealed a significant increase of apoptotic cell in day 19 compared to day 14 ($p \leq 0.05$).

Evaluations of apoptotic cells in distal regions of limb buds

Day 14 and Day 16

The mean value of apoptotic cell in distal regions at day 14 was (279.0±10.07). While that at day16 was (203.0±10.56) (Table 5); t-test revealed a significant increase of apoptotic cell in day 14 compared to day 16 ($p \leq 0.05$).

Day 16 and Day 19:

The mean value of apoptotic cell in distal regions at day 16 was (203.0± 10.56). While that at day 19 was (257.0±14.06) (Table 6); t-test showed a significant increase of apoptotic cells in day 19 compared to day16 ($p \leq 0.05$).

Day 14 and Day 19

The mean value in distal regions at day 14 was (279.0± 10.07). While that day 19 was (257.0±14.06) (Table 7); t-test in distal regions

revealed a non-significant variability between the mean value at day 14 and day 19.

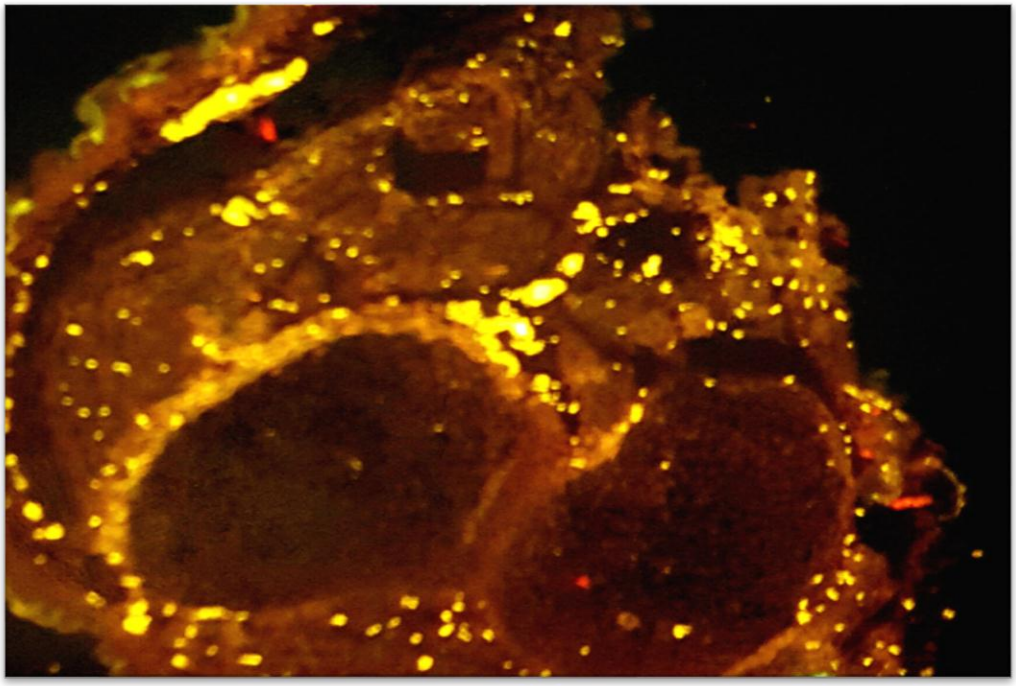

Figure 1. Cross section of the proximal regions at day 16 showing bright yellow color of apoptotic foci TUNEL test (100X)

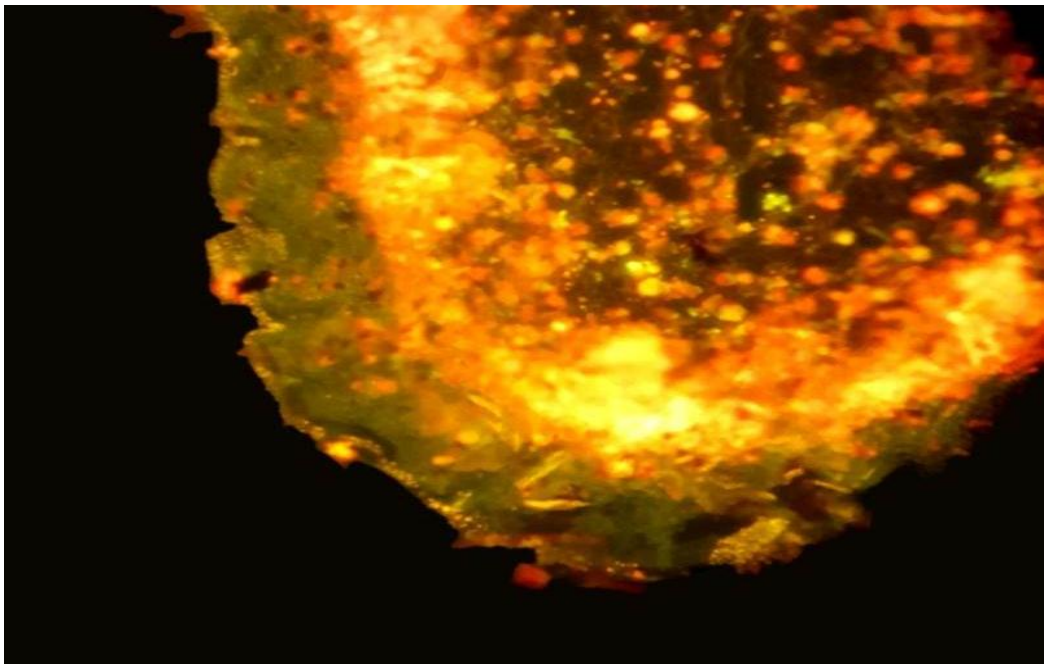

Figure 2. Cross section of the proximal regions at day 19 showing bright yellow color of apoptotic foci TUNEL test (400X)

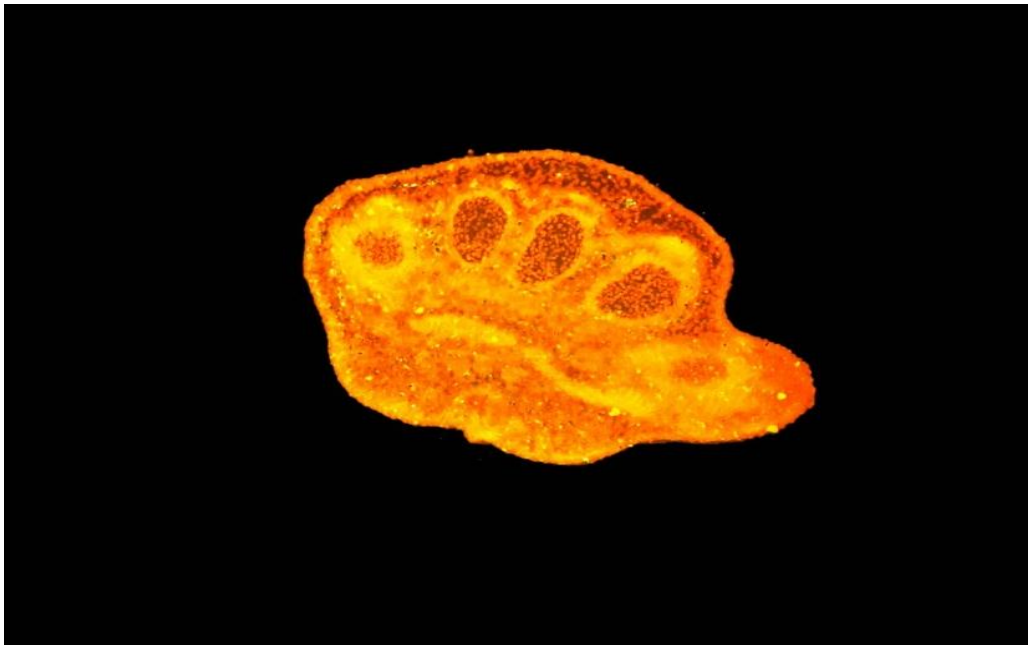

Figure 3. Cross section of the distal regions at day 14 showing bright yellow color of apoptotic foci TUNEL test (100X)

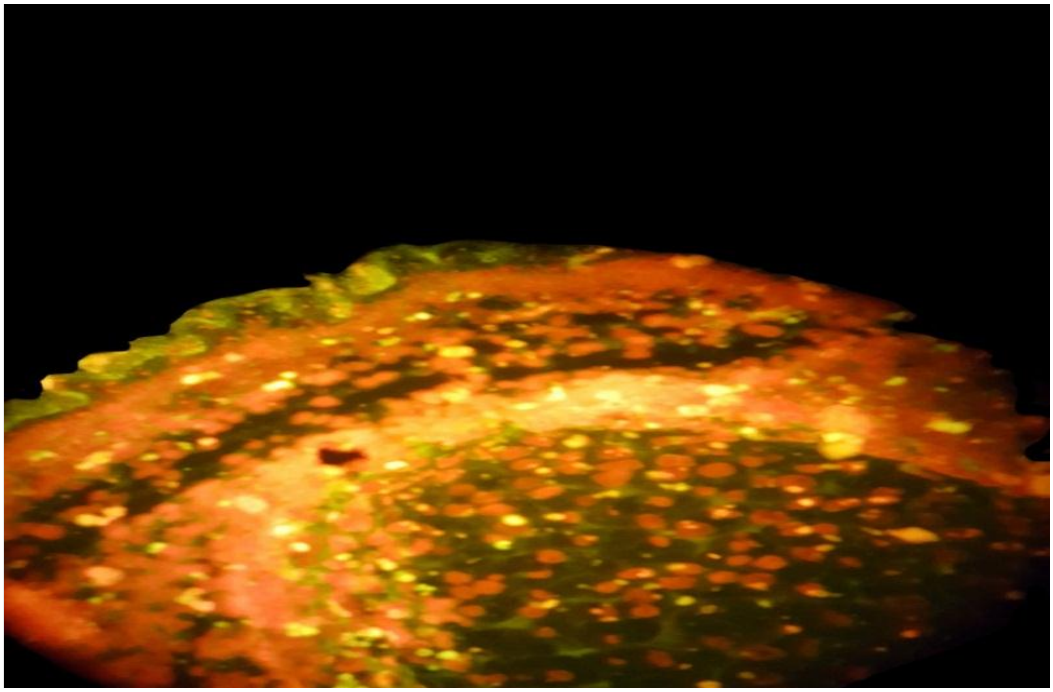

Figure 4. Cross section of the distal regions at day 16 showing bright yellow color of apoptotic foci TUNEL test (400X)

Table 2. Mean number of apoptotic cells revealed in proximal regions in E14 and E16 by TUNEL test

Age	Mean± SE	P value
Day 14	90.1±2.7	0.2
Day 16	95.0±4.7	

Table 3. Mean number of apoptotic cells revealed in proximal regions in E16 and E19 by TUNEL test

Age	Mean± SE	P value
Day 16	95.0±4.7	0.001
Day 19	180.0±8.3	

Table 4. Mean number of apoptotic cells revealed in proximal regions in E14 and E19 by TUNEL test

Age	Mean± SE	P value
Day 14	90.1±2.7	0.001
Day 16	180.0±8.3	

Table 5. Mean number of apoptotic cells revealed in distal regions in E14 and E16 by TUNEL test

Age	Mean± SE	P value
Day 14	279.0±10.07	0.004
Day 16	203.0±10.56	

Table 6. Mean number of apoptotic cells revealed in distal regions in E16 and E19 by TUNEL test

Age	Mean± SE	P value
Day 16	203.0±10.56	0.004
Day 19	257.0±14.06	

Table 7. Mean number of apoptotic cells revealed in distal regions in E14 and E16 by TUNEL test

Age	Mean± SE	P value
Day 14	279.0±10.07	0.2
Day 19	257.0±14.06	

Comparison of mean number of apoptotic cells between proximal and distal limb bud

The mean value of apoptotic cell in proximal regions at days E14, E16 and E19 were (180.0 ±16.5). While that in distal region at days E14, E16 and E19 were (246.3±7.83) (Table 8). T-test showed significantly higher mean values of the apoptotic cells in the distal regions compared to proximal region ($p \leq 0.05$).

Table 8. Mean number of apoptotic cells revealed in proximal regions and distal regions by TUNEL Test

Region	Mean± SE	P value
Proximal	121.7±6.5	0.001
Distal	246.3±7.83	

The mean number of apoptotic cells in both regions of limb buds

Day 14 and Day 16

The mean value of apoptotic cells at day 16 was (149.0 ±15.3). While that at day E14 was (184.5 ±15.9) (Table 9); t-test showed a non-significant variability was in the mean value of the counted cells during day 14 and 16.

Day 19 and Day 16

The mean value of apoptotic cells at day 19 was (305.5±11.8). While that at day 16 was (149.0 ±15.3) (Table 10); t-test showed significantly higher mean values of the apoptotic cells in the day 19 compared to day 16 ($p \leq 0.05$).

Day 14 and Day 19

The mean value of apoptotic cells at day 19 was (305.5±11.8). While that at day 14 was (184.5±15.9) (Table 11); t-test revealed a significant value of apoptotic cell which was higher in day 19 compared to day 14, ($p \leq 0.05$).

Table 9. Mean number of apoptotic cells revealed in both regions at E14 and E16 by TUNEL test

Age	Mean± SE	P value
Day 14	184.5±15.9	0.2
Day 16	149.0 ±15.3	

Table 10. Mean number of apoptotic cells revealed in both regions at E16 and E19 by TUNEL test

Age	Mean± SE	P value
Day 16	149.0 ±15.3	0.001
Day 19	218.5 ±11.8	

Table 11. Mean number of apoptotic cells revealed in both regions at E14 and E19 by TUNEL test

Age	Mean± SE	P value
Day 14	184.5±15.9	0.001
Day 19	218.5 ±11.8	

Discussion

It was reported that the developing vertebrate limb morphogenesis depends on the appropriate spatial and temporal balance between cell death and cell proliferation [15].

The results of this study indicate that the proximal parts of the limb bud are more actively involved with apoptotic changes at gestational day 19. The pattern of apoptosis in the proximal parts of the limb buds at the earlier developmental stages was found to be steady. The distal parts of the limbs showed active apoptotic changes at the earlies day 14.

The myogenic differentiation was reported to be associated with cell death to eliminate excess cells around the developing muscles [16]. However, the evaluation of proximodistal sequential localization of the phenomenon of apoptosis was not described.

The localization of cell death along the arthogonal axes in relation with the organization of the skeletal pattern of the limb described in this study was also reported in a study on early avian limb, the avian limbs showed regional variability with an anterior and posterior areas of cell death [11].

The results of this study do not agree with Milaire and Roze in 1983 [17] who mention that no regional variability in different zones of early developing limb of mammals. This disagreement could be related to the more accurate enlightenment of apoptosis by TUNEL technique used in this study, or it may be related the different chronological development of the limb bud as this study evaluate more later developmental stages.

The more massive apoptosis in the distal regions found in this study during the gestational day 14 was supported by the Hurle et al in 1996 [18] who reported massive programed cell death at the distal mesoderm that serve in phenotyping of digits.

Many physiological triggering signals for apoptosis were reported in the developing limb buds including the expression of BMP-2, BMP-4 and BMP-7 [19].

Also, previous studies support the coexistence of different cell death effectors in association with apoptosis of the mesodermal tissues of the developing limb buds [20-22].

The evaluation of the programmed cell death in both the upper and lower limbs buds was based on the assumption reported in the literatures that apoptosis in the upper and the lower limb buds provides a valuable model that mold the limb bud tissues to present the morphological and functional specialization in different vertebrated including ducks, turtles, bats, chickens, humans, and lizards [23,24].

The programmed cell death detected by the TUNEL stain in this study after the onset of primary myogenesis after day 14 was higher in all parts of the developing limbs during day [19]. This result is supportive to the finding of, casps-3 and -9 began was found to be detected from 13.5 gestational day and become more strengthened during the later period of embryogenesis suggesting that the Casp family starts to be expressed in accordance with onset of myogenesis [25].

It was concluded that signaling (as fibroblast growth factor) from the most distal part of the limb buds (the apical ectodermal ridge) at different developmental stages regulates cellular growth located at the most proximal parts. This signaling was essential to ensure normal skeletal elements, and perhaps other limb tissues [26]. Also, it was reported that the mesenchymal region underlying the distal ectoderm was found to show massive cell death after removal of the apical ectodermal

ridge [27,28]. This finding is supportive to the results of this study, the later stage involved in this study (day 19) is associated with degenerative changed of the apical ectodermal ridge [29] and this may the underlying factor leading to massive cell death at this developmental stage as cell death might be due to an indirect effect of loss of signaling associated with absence of the apical ectodermal ridge in later developmental stages.

The results of this study evaluated the reactivity of TUNEL test labeling apoptotic cell in mesenchymal tissues seen in the transverse sections of limb around the various tissue components. This approach was established after the reports that ensure uncertainty whether the labeling of dying cells could be a progenitor of chondrocytes or other cell types in the limb [30].

The higher apoptotic activity at the distal regions of the developing limb was determined in all the three developmental stages involved in this study. This result was in agreement with the with previously suggested limb patterning of the progress zone model [31], which had been the most important model of limb proximal–distal patterning since decades. This model postulated that limb progenitors in a 'progress zone' in the limb bud distal tip become progressively 'distalized'. As cells proliferate but progress zone size remains constant, cells must be continually exiting the progress zone. Once they leave, they cease distalizing. Therefore, cells exiting early form proximal skeletal elements whereas those exiting late form distal elements. Signaling from the apical ectodermal ridge was thought to be the accommodating factors that allow progress zone cells to continue distalizing. Therefore, the proximal regions of the limb buds showing active apoptosis in this study as a phenomenon associated with early maturation of the proximal musculoskeletal elements, the late distal increase of labeled apoptotic cells found in this study is related to maturation of the distal musculoskeletal elements of the limbs. This interpretation was supported by the establishment reported previously that distal region is specified only after proximal [32].

The methodology of t-test statistical analysis has been used in this study to compare variables associated with the subsequent chronology of mesenchymal cell apoptosis, this scheme of statistical analysis provides simplicity of interpretation and ease for calculation in the manner that inaugurate with the aims of the study [33].

This study concluded that the active apoptosis during myogenesis showed proximodistal pattern regulating limb morphogenesis.

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Authors Contribution:

Al-Musawi: Performing the laboratory research work. Dr. Mubarak: Performing the production and interpretation of the results.

Conflict of interest

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Interleukin-4 Single Nucleotide Polymorphism C-590T Polymorphisms in Relation to Asthma

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Abstract

Background Single nucleotide polymorphisms in the promoter regions of genes encoding for some interleukins may associate with occurrence of asthma.

Objective To investigate the association of single nucleotide polymorphisms of interleukin-4 (IL-4) (C-590T) and asthma.

Methods Forty-five patients with asthma and 40 apparently healthy subjects (represent the control group) were enrolled in this study. Blood samples were collected from both patients and controls. DNA was extracted from blood samples and gene fragments corresponding to IL-4 C-590T were amplified with specific primers using conventional PCR technique.

Results The heterozygote genotypes of IL-4 C-590T (CT) showed significant association with asthma (OR = 3.922, 95% CI= 1.153-13.339, P = 0.028).

Conclusion These results suggest the significance of IL-4 C-590T polymorphism as a risk factor for asthma.

Keywords Asthma, interleukin-4, polymorphism

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List of abbreviations: DNA = deoxyribonucleic acid, IL = Interleukin, PCR= Polymerase chain reaction, SNPs = Single nucleotide polymorphisms

Introduction

The precise causes of asthma are not yet clear, but genetic-environmental interaction is probably responsible for much of the variation in the prevalence rate of this disease. Environmental factors associated with asthma can be easily recognized and avoided. Furthermore, the vast majority of these factors do not exert their effect unless chronic genetic predisposing factors are present [(1)].

Therefore, investigating the genetic factors associated with asthma could be the cornerstone for defining the most susceptible individuals. Interleukin-4 (IL-4) is one of the most important cytokines in the regulation of allergic response. This cytokine is responsible for immunoglobulin iso-type switching towards immunoglobulin IgE expression [(2)]. Furthermore, it acts as a growth factor for mast cells and is the key signal for the development of Th2 from CD+4 cells [(3)]. All these activities, and may be others, make this cytokine one of the main player in the initiation of asthma. Any genetic alterations that cause an increase in

the amount and/or activity of IL-4 in expected to affect asthma. Many single nucleotide polymorphisms (SNPs) in the promoter region of this IL-4 gene were found to be associated with different diseases [4,5]. Among these SNPs is C-590T, which is associated with higher promoter activity and affects the production of IL-4 [6].

This study aimed to assess the impact of different variants of IL-4 C-590T on the susceptibility to asthma in a sample of Iraqi patients.

Methods

A total of 45 patients with asthma (age range 16-48 years, mean = 34.61±4.11 years, 18 males and 27 females) who were attending Al Zahra'a Consultative Centre for Allergy and Asthma and the Consultative Clinic for Chest and Radiology during the period from January to April, 2015 were enrolled in this study. In addition, 40 apparently healthy individual age and sex- matched with patients group were included in this study as control group.

Blood Samples, DNA Extraction and Gene Amplification

From each subject, 5 ml of blood was drawn from vein puncture in an EDTA tube. DNA was extracted from these samples using ready kit (ZymoBead™ Genomic DNA Kit, USA). For polymerase chain reaction (PCR) amplification of IL-4 gene, the primer set was forward primer: 5'-TAAACTTGGGAGAACATGGT-3' and reverse primer 5'-TGGGGAAAGATAGAGTAATA-3' with 195 bp fragment length amplicon.

PCR protocol

An initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 50 sec, 53 °C for 50 sec and 72 °C for 1 min. The final extension was achieved at 72°C for 7 min.

PCR products from patients and controls were directly sequenced using Big Dye Terminator method/ Sandor Life Sciences Pvt. Ltd /India. The obtained sequences were aligned with normal sequence from GenBank and examined for the presence of SNPs.

Statistical analysis

The Statistical Package for the Social sciences (SPSS, version 14) was used for statistical analysis. Continuous variables were expressed as mean ± standard deviation (SD). Risk association between the genotypes and asthma susceptibility was estimated by the calculation the adjusted odds ratio (OR) and 95% confidence intervals (CI) using binary logistic regression. Chi square was used for testing the deviation from Hardy-Weinberg equilibrium as well as for comparing between categorical variables. A p-value < 0.05 was considered statistically significant.

Results

Table 1 shows the demographic data of the study population. The only demographic factor, which had significant association with asthma is family history. Among asthma patients there was 48.89% who had one or more first relative with asthma compared to only 5% among controls who had such relative (P<0.001).

Genotyping

Gel electrophoresis of PCR product for IL-4 genes are shown in figure 1.

IL-4 C-590T polymorphism appeared in three genotypes which were CC, CT and TT (figure 2). The distribution of these genotypes was within Hardy Weinberg Equilibrium. The frequencies of these genotypes in asthma patients were 64.44%, 28.89% and 6.67%, respectively, while they were 87.5%, 10% and 2.5%, respectively among controls with significant difference for the heterozygous genotype (OR=3.922, 95%CI=1.153-13.339, P=0.028) as shown in table 2.

At allelic level, asthma patients had more frequent allele T than controls (21.11% vs 7.5%) with significant difference as shown in table 2 (OR=3.30, 95%CI= 1.246-8.740, P= 0.016).

Table 1. Demographic data of asthma patients and controls

Risk Factors		Cases N=45	Control N=40	P-value
Mean age in years (SD)		34.61 (4.11)	36.68 (4.08)	0.229
Family history	No	23 (51.11 %)	38 (95.0%)	< 0.001
	Yes	22 (48.89%)	2 (5.0%)	
Sex	Male	18 (40.0%)	18 (45.0%)	0.403
	Female	27 (60.0%)	22 (55.0%)	
Smoking	Never	37 (82.22%)	38 (95.0%)	0.090
	Smoker (ex/current)	8 (17.78%)	2 (5.0%)	
Dwelling	Urban	29 (64.44%)	27 (67.5%)	0.082
	Rural	16(35.56%)	13 (32.5%)	

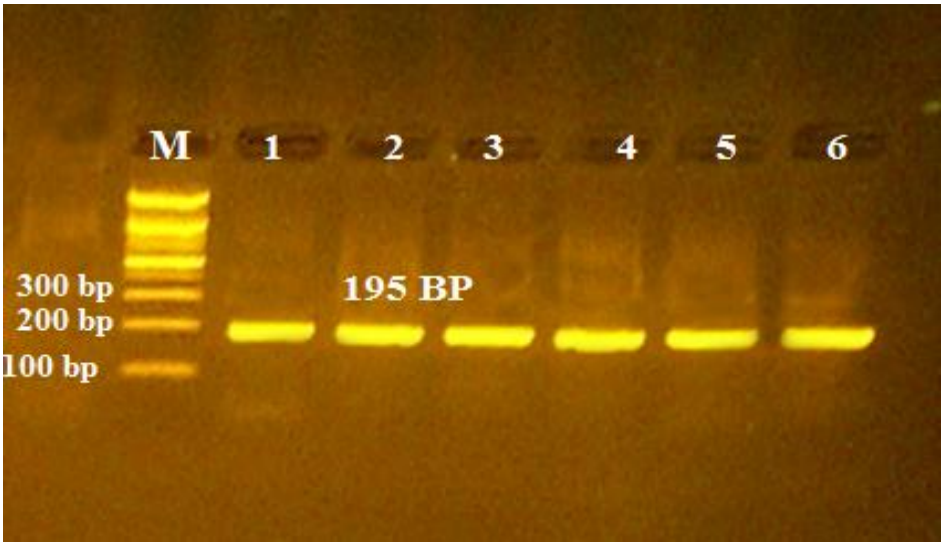

Figure 1. Gel electrophoresis for IL-4 PCR products visualized under UV light after staining with ethidium bromide. M: 100 bp DNA marker; lane 1-6: positive amplification of the gene from DNA extracted from blood samples of asthma patients and controls. The size of PCR product is 195 bp

Table 2. Genotypes and allele frequencies of the SNPs C-590T

Variables		Cases N=45	Control N=40	P-value	OR (95% CI)
C-590T Genotypes	CC	29(64.44%)	35 (87.5%)	0.061	1.0
	CT	13(28.89%)	4 (10%)	0.028	3.9 (1.153-13.339)
	TT	3 (6.67%)	1 (2.5%)	0.276	3.6 (0.357-36.698)
Allele	C	71 (78.89%)	74 (92.5%)	0.016	1.0
	T	19 (21.11%)	6 (7.5%)		3.3 (1.246-8.740)

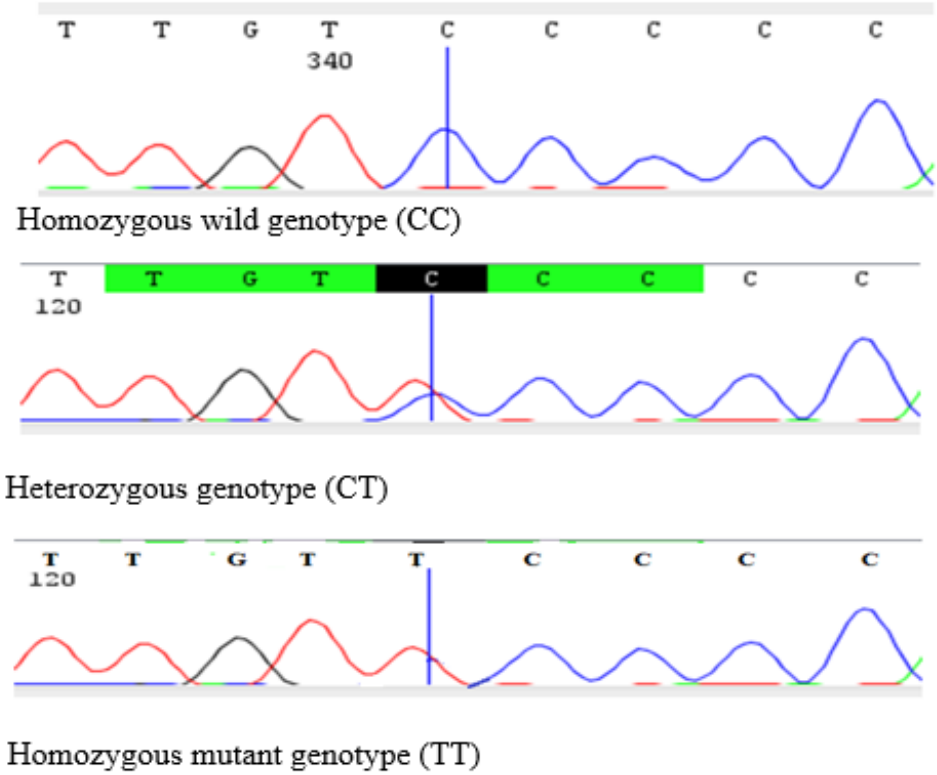

Figure 2. Different pattern of IL-4 C-590T polymorphism

Discussion

A significant association between the heterozygote genotype (CT) and the susceptibility to asthma was shown in this study. This implies that CT carriers are at 3.92 folds risk to develop asthma compared to CC carriers. This result was further confirmed at allelic level, where the mutant allele (T) was more frequent among asthma patients than controls with significant difference. Significant association of this SNP with asthma was recorded worldwide such as in Japan [7], Taiwan [8], Algeria [9], Germany [10], west Siberia [2], Macedonia [11], China [12] and Iran [13]. However non-significant association was also previously reported in United Kingdom [14], China [15] and Brazil [16]. These conflicting results may be related to differences in ethnical and racial origin of the study population. Promoter is a region of DNA where the transcription of a particular gene is initiated. Transcription factors bind to this region and enable RNA polymerase to be situated in an orientation that allows the transcription to begin. Thus, polymorphisms in promoter are expected to influence gene expression although this region is not coding for protein. Interleukin-4 and IL-13 are considered as the main factors that regulate allergic response through their effect on isotype switching of immunoglobulin to IgE in B-lymphocyte [17]. Furthermore, this cytokine (IL-4) acts as a growth factor for most cells and as a main signal for the differentiation of CD4+ to Th2 [18]. All these activities are associated with allergic phenotype. Therefore, it is reasonable to postulate that increased production of this cytokine, for whatever cause, will predispose to allergy. The SNP IL-4 C-590T is located very close to binding site (-603 to -588) of the nuclear factor of activated T-cells -1 (NFAT-1). This factor is one of the most important factors in IL-4 transcriptions [19]. The substitution of cytosine to thymine in the position - 589 was shown to increase the accessibility of NFAT-1 dimer to this site. Thus, more IL-4 is expected to be produced [9].

Practical studies have supported this hypothesis through the association of T allele with two main phenotypes of asthma with mutant allele (T). The first phenotype is elevated serum levels of IL-4. The second phenotype which a direct result of the first one is the increased serum levels of IgE [14].

On the other hand, there was no statistically significant difference in the frequency of TT genotypes between asthmatic patients and controls in the current study. This may be explained by the relatively small sample size and the possible presence of linkage disequilibrium with other polymorphisms in the same gene. In this regard, Smolnikova et al. reported that this SNP links with other polymorphisms in 3' un-translated region (3'UTR) of IL-4 gene, which may affect the asthma phenotype of the patient [2].

Taken together, these data strongly indicate that allele T of IL-4 C-590Tpolymorphism could be considered as risk factors for asthma in Iraqi patients.

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Authors Contribution:

Gaidan: sample collection processing and working. Dr. Abbas: Design of the work, data interpretation, drafting and critical revision of the article. Dr. Hassan: Sample processing. Dr. Hashim: Samples collection.

Conflict of interest

The authors declare no conflict of interest.

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Corneal Endotheliopathy in Pseudoexfoliation Syndrome

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Abstract

Background Pseudoexfoliation syndrome is a common disorder with a wide range of ophthalmic presentation and risks. Corneal endotheliopathy is one of these presentations.

Objective To evaluate corneal endothelial cell morphology, density and function in eyes with pseudoexfoliation syndrome.

Methods 120 eyes of sixty patients with clinically evident unilateral pseudoexfoliation were examined with non-contact specular microscopy (SP-3000P) Topcon Corporation. Central corneal thickness (T), cell density (CD), and percentage of cell hexagonality (HEX%) were measured and the values of the affected eyes were compared to those of the fellow normal eyes. Corneas with CD less than 2000/mm2 or HEX% <50% were considered as at-risk corneas for decompensation. The pseudoexfoliated eyes were subdivided into three groups according to the density of the pseudoexfoliated material (those with deposits on the lens capsule alone, on the pupil margin alone or on both) and the morphometric values of endothelial cells in each group were studied. Statistical analysis was performed using a 2-tailed Student t-test and the Chi square test, P value <0.05 was considered significant.

Results Significant increase in central corneal thickness (500.25±28.95 micron vs 493.18±28.59 micron) and reduction in endothelial cell density (2307.5±272.3 vs 2480.2±289.9 cell/mm^2) with a non-significant decrease in cell hexagonality (51.78±8.9 vs 53.48±6.2 %) was noticed in eyes affected by pseudoexfoliation syndrome as compared to the contralateral normal eyes. The changes were noticed more when the severity of the condition increases as reflected by the density of the pseudoexfoliated material. At risk corneas were found more frequently in eyes with pseudoexfoliation based on both endothelial cell density and cell hexagonality.

Conclusion Pseudoexfoliation syndrome is a cause of corneal endotheliopathy and a risk for corneal decompensation. More endothelial cell changes are found in eyes with advanced pseudoexfoliation. Extra care and more meticulous handling is required while operating upon eyes with pseudoexfoliation to reduce the risk for corneal decompensation.

Keywords Pseudoexfoliation, corneal endothelium, endotheliopathy, decompensation.

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List of abbreviations: CD = cell density, HEX% = Percentage of cell hexagonality, IOP= intraocular pressure, PEX = Pseudoexfoliation, T = Corneal thickness

Introduction

The pseudoexfoliation syndrome is a wide spread multisystem age related degenerative disorder of extracellular matrix, which was described as far back as 1917. In the eye a fibrilar substance, the nature of which is still not known, accumulates not only upon and within the lens capsule but also in association with the internal limiting membrane of the iris and ciliary body, upon

their epithelial surface and around the blood vessels of the anterior uvea [1,2].

Pseudoexfoliation affects 0.2-38% of general population at different areas around the world without sex predilection. The condition is of relevance to all comprehensive ophthalmologist because of its wide range of pathological manifestation including phacopathy, zonulopathy, cyclopathy, iridopathy, trabeculopathy and corneal endotheliopathy [3-5].

The wide spectrum of ocular affection can result in a corresponding wide range of clinical problems and surgical complications such as glaucoma, phacodonesis, early corneal decompensation, iris rigidity and fibrosis with poor pupillary dilation together with blood-aqueous barrier disruption and pseudouveitis because of the affection of the iridial small and large blood vessels secondary to the deposition of pseudoexfoliative material. Pseudoexfoliation syndrome is a risk factor for vitreous loss during cataract extraction. Zonular instability, capsular fragility and poor quality of mydriasis account for the higher incidence of such complication [6-8].

Local in situ production and deposition of pseudoexfoliative material is found focally in the corneal endothelium in some of the affected eyes. This explains why endothelial cell loss and decompensation take place resulting in a form of keratopathy different from that seen in conventional cases of corneal guttata [1].

A stable normal corneal endothelium has cells of relatively uniform size and shape, additionally, adequate cell density is essential to maintain corneal function. The corneal thickness measured by pachymetry is an important indicator of the corneal edema and endothelial function [9-13].

Any pathological effect on the cornea is best illustrated by discussing changes in the endothelial morphometric parameters and corneal thickness [14]. Polymegathism (increased variation in individual corneal endothelial cell areas) and pleomorphism (a decrease in the hexagonal corneal endothelial cells with concomitant increase in number of cells with more than or fewer than six sides) increase significantly in response to some ocular pathologies and may be a sign of endothelial stress [15].

The aim of this study is to demonstrate and describe the corneal endothelium morphometric changes in eyes with pseudoexfoliation syndrome.

Methods

Study design and patients' selection

This prospective case control study was conducted on (60) patients with clinically evident unilateral pseudoexfoliation syndrome attending the ophthalmology clinic at Kufa University teaching hospital for variable ocular complaints.

The patients were eligible for the study if they have clear corneas with no evident ocular disease apart from cataract of variable density and they all shared the fact of negative past history for ocular trauma, surgery or contact lens wear. Patients with glaucoma, ocular hypertension, keratic precipitates, guttata, corneal dystrophies, corneal edema or leukomas were excluded from the study. Intraocular pressure was checked for every eye using the Goldmann applanation tonometer. All participants signed a consent form before their inclusion in this study.

Workup

Both eyes were assessed for each patient referring to the pseudoexfoliative eye as diseased eye (Group A) and the normal eye as control (Group B). Patients were considered to have pseudoexfoliation when biomicroscopic examination of the anterior segment under pupil dilation showed the characteristic grayish white pseudoexfoliative material on the anterior capsule of the lens or pupillary margin. Group A eyes were further subdivided according to the severity of the condition: eyes with deposit on the lens capsule only, on the pupil margin only or on both. Eyes with deposition on both the lens capsule and the

pupillary margin were considered to be more severely affected than eyes that show the deposit on either site alone.

The following corneal measurements were taken for each eye in the two groups by a masked single experienced technician using the automatic noncontact specular microscopy (SP-3000P, Topcon Corporation, Tokyo, Japan):

- Central corneal thickness (T) in micrometer.
- Endothelial cell density (CD), cell count per square millimeter area.
- Percent of cell hexagonality (HEX%), the percentage of cells that have six sides in the best captured images with 60 cells or more.

At risk corneas were defined as those with hexagonal cell percent (HEX%) less than 50% or those with endothelial cell density (CD) less than 2000/mm^2 [9,16,17].

Statistics

Corneal morphometric measurements were tabulated for the two groups of eyes (A and B). The mean and standard deviation were calculated and the statistical analysis was carried on using the paired differences analysis by the student`s t-test to compare eyes in groups A and B.

The frequency of the "at risk corneas" was determined in each group and the Chi square test was used for statistical analysis.

In group A eyes, and after dividing the eyes into three subgroups depending on the site of deposit of the pseudoexfoliative material, the effect of the disease severity on the three corneal morphometric parameters was studied applying the student`s t-test.

The SPSS software v.17 was used for the statistical analyses and a P value less than 0.05 was considered significant.

Results

In this series of the 60 cases with unilateral pseudoexfoliation, we found:

- Male to female (M:F) ratio to be (1.2:1), 58% of the patients were males and 48% were females.
- Mean age of the patients was (63 yr) with a range of (42-84 yr). Mean intraocular pressure (IOP) in group A (eyes with pseudoexfoliation) was 18 mmHg and 16 mmHg in group B (normal eyes).

Table 1 summarizes the above mentioned general criteria of the studied subjects.

Mean central corneal thickness (T) in microns, cell density (CD) per square millimeter and hexagonal cells percentage (HEX %) in eyes of group A and B are shown in table 2.

Figure 1 shows the corneal morphometric parameters in the two groups.

Table 3 displays the statistical analysis of the paired differences for these parameters according to the student t-test. In group A, 21 eyes out of the 60 eyes (35%) have hexagonal cell percent less than 50% compared to 15 eyes in group B (25%). Cell density less than 2000/mm2 was found in 12 eyes of group A (20%) and in 3 eyes of group B (5%).

Table 1. General criteria of the studied patients

No. of patients	60
Male to Female ratio	1.2:1
Mean age of the patients	63 years
Age range	42-84 year
No. of eyes with pseudoexfoliation (group A)	60
No. of normal eyes (group B)	60
Mean IOP in group A	18 mmHg
Mean IOP in group B	16 mmHg

Table 2. Specular microscopy findings in diseased and normal eyes

Group	Specular microscopy findings		
	T (µm)	CD (cell/mm²)	HEX (%)
Diseased eyes (group A)	500.25±28.95	2307.5±372.38	51.78±8.98
Normal eyes (group B)	493.18±28.59*	2480.23±289.91*	53.48±6.27

Mean of corneal thickness (T), cell density (CD), Hexagonal cells percent (HEX), *=significant P value (<0.05)

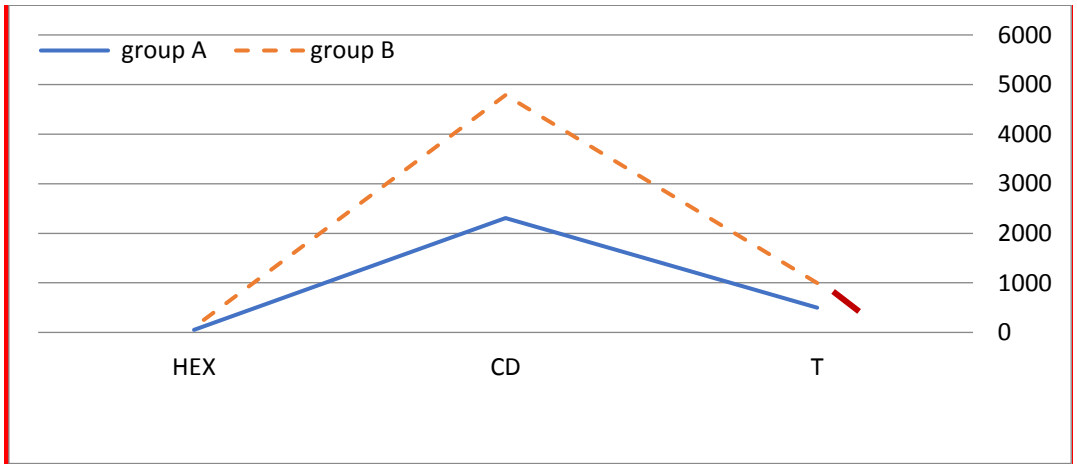

Figure 1. Comparing Specular microscopy findings in diseased (group A) and normal eyes (group B). Mean of corneal thickness (T), cell density (CD), hexagonal cells percent (HEX)

Table 3. Comparison of thickness, cell density and hexagonal cells percentage in group A and group P by paired ttest

		Paired differences (group A-group B)					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% CI Interval of the Difference				
					Lower	Upper			
Pair 1	Corneal thickness	7.07	24.45	3.17	0.75	13.38	2.239	59	0.029 *
Pair 2	Cell density	-172.67	546.82	70.59	-313.92	-31.41	-2.446	59	0.017 *
Pair 3	Hexagonal cells %	-1.70	13.47	1.74	-5.18	1.78	-0.977	59	0.332

*=significant P value (<0.05)

Tables 4 and 5 show the number of "at risk corneas" in the two groups. The P value was calculated according to the chi square test. The appearance of pseudoexfoliative material in group A eyes was as follows:

- 15 eyes show the material on the pupilary margin only-P (25%)
- 8 eyes had it on the anterior lens capsule only-C (13%)
- 37 eyes had it on both the pupil and the lens capsule-P&C (62%)

Table 4. Number of eyes with at risk corneas for decompensation in group A and B based on hexagonal cell percent

Group	HEX% <50%	HEX%≥50%	Total
A	21	39	60
B	15	45	60
Total	36	84	120

X2 =1.42, P value >0.05

Table 5. Number of eyes with at risk cornea for decompensation in group A and B based on endothelial cell density

Group	CD <2000/mm^2	CD ≥2000/mm^2	Total
A	12	48	60
B	3	57	60
Total	15	105	120

X2=6.17, P value<0.05

Figure 2 shows the distribution of the affected eyes according to the site of the deposits.

Mean of central corneal thickness (T) in microns, cell density (CD) per square millimeter, hexagonal cell percent (HEX%) in eyes with pseudoexfoliation material present on pupil only were (487, 2418, 52%) respectively. In eyes with pseudoexfoliation material on capsule only the findings were (492, 2455, 55%) and in eyes with pseudoexfoliation material on both the pupil and lens capsule they were (507, 2237, 51%) respectively (Table 6).

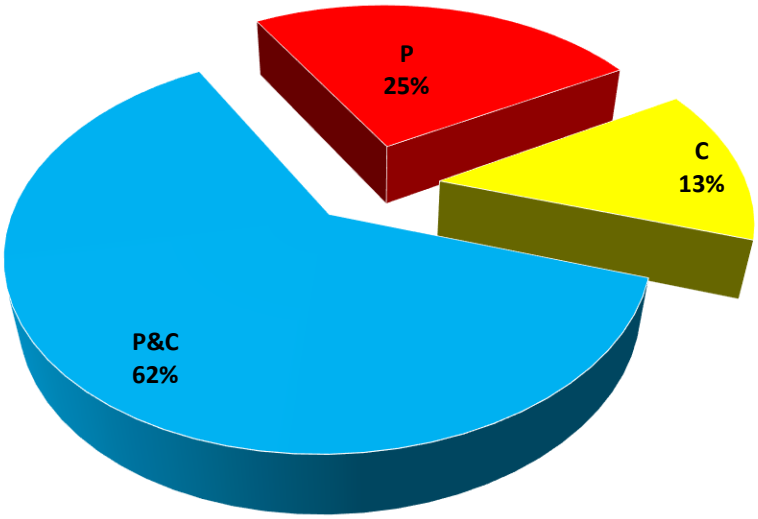

Figure 2. Distribution of eyes with pseudoexfoliation according to the site of deposit (P= on the pupil margin only, C= on the lens capsule only, P&C=on both the pupil and lens capsule)

Table 6. Corneal thickness (T), endothelial cell density (CD) & hexagonal cell percentage (HEX%) in eyes with different severity of pseudoexfoliation (PEX)

Parameter	T (µm) mean±SD	CD (cell/mm^2) mean±SD	HEX (%) mean±SD
PEX on pupil only	488±13.86	2403.73±196.38	51±6.8
PEX on lens capsule only	492.25±19.81	2455±219.25	55±5.63
PEX on both the pupil &lens capsule	506.5±35.05 *	2236.8±448.29 *	51.49±10.5

(*) P<0.05

Discussion

Pseudoexfoliation syndrome is a systemic disorder that probably results from multifocal abnormal metabolic processes of unknown etiology leading to primary cell changes and is characterized by accumulation of fibrilar material containing basement membrane components in the anterior segment of the eye and other organ systems [1-3].

It is a frequent clinical feature seen in patients with cataract because its prevalence increases with age. In patients with pseudoexfoliation, insufficient mydriasis, in addition to zonulopathy, was determined to be the most significant risk factor for zonular breaks or rupture of the posterior lens capsule with consequent vitreous loss during extra capsular cataract extraction [4-6]. Pseudoexfoliation syndrome is also known to present with corneal endotheliopathy.7 Thus, the risk for decompensation whether spontaneous or following intraocular surgery should always be thought of in eyes with this disease.

In this study, the important morphological aspects of corneal endothelial cells in patients with unilateral pseudoexfoliation were evaluated. Studying unilaterally affected eyes and comparing with the contralateral normal eyes may give more impressive information than comparing normal eyes with diseased eyes of different subjects. Such strategy minimizes the effects of all other variables in the studied subjects that might alter the results. Upon comparing the findings of the affected eyes with those of the contralateral normal eyes the following points were observed:

1- Significant reduction in endothelial cell density in eyes with pseudoexfoliation.
2- Significant increase in central corneal thickness in eyes with pseudoexfoliation.
3- The deviations from normal values increase as the density or severity of pseudoexfoliation increases in the affected eyes.
4- No significant changes were found in the shape of corneal endothelial cells in diseased eyes. Hexagonal cells percent did not vary significantly between groups.
5- At risk corneas were found more frequently in the diseased eyes than the normal eyes. Statistical analysis showed that the difference is significant if the judgment is based on endothelial cell density and non-significant if we consider the hexagonal cell percent.

The above findings reinforce the fact that pseudoexfoliation is a cause of corneal endotheliopathy and is a risk factor for corneal decompensation either spontaneously or in response to minor endothelial stress or surgical trauma.

The mean endothelial cell density difference between the diseased and normal eyes in this study was 127.66 cells/ mm^2, which is less than the 300-500 cells/mm^2 differences reported in the literature [18-21]. However, this difference is statistically significant with a P value of 0.017. Similarly, the 7.066 micron difference in corneal thickness between the two groups is statistically significant with a P value of 0.029 compared to the 4 micron insignificant difference reported by other researchers [22].

Many published studies demonstrated pseudoexfoliation corneal endotheliopathy but none of them was performed on unilaterally affected patients similar to current study. However, it is valuable to summarize the results of some of these studies. Table 7 summarizes the corneal endothelial morphometric parameters found in eyes with pseudoexfoliation syndrome as compared to normal control eyes in relevant published studies.

Table 7. Summary of comparable studies on corneal endothelial morphometric parameters in eyes with or without pseudoexfoliation

Author	Population	No. of eyes		Cell density/mm^2			HEX%		
		PEX	Normal	Eyes with PEX	Normal eyes	P value	Eyes with PEX	Normal eyes	P value
Current study	Iraq	60	60	2307.5± 372.38	2480.23 ±289.9	0.017	51.78± 8.98	53.48± 6.27	0.332
Quiroga et al [17]	Paraguay	61	453	2315±49 .13	2482±20 .63	0.002	56.1± 1.06	57.9± 0.4	0.123
Ostern & Drolsum [23]	Norway	46	101	2024± 371	2144± 365	0.07	46.4± 10.1	46.3± 10.9	0.99
Wali et al [16]	Oman	126	-	2465.86 ±506.68	-		34.63± 11.92	-	

The morphological and functional parameters of central corneal endothelial cells display more deviation as the density or severity of pseudoexfoliation increases, this fact was concluded by comparing the specular microscopical findings of the diseased eyes after grouping them into three groups depending on the severity of clinically visible pseudoexfoliation material. Eyes with pseudoexfoliative material deposits on both the lens capsule and the pupil represent a more advanced presentation than those with deposits on the lens capsule alone or on the pupil alone. The more severely affected eyes have a statistically significant lower cell density and thicker corneas (P<0.05) but their lower percent of hexagonal cells was not significant (P>0.05). Wali et al studied the effect of disease severity on corneal endothelial morphometric values by comparing eyes with pseudoexfoliation without glaucoma and those with pseudoexfoliation and glaucoma. They considered glaucoma as a reflection of an advanced stage of pseudoexfoliation. Eyes with pseudoexfoliation and glaucoma showed lower endothelial cell densities and hexagonal cell percent than non-glaucomatous eyes but the findings they reported were not significant [16]. Ostern and Drolsum reported lower cell densities and percents of hexagonality in eyes with glaucoma when compared to eyes without glaucoma in a group of pseudoexfoliated eyes. Their finding was statistically non-significant [22].

Corneas at risk for decompensation are those with tendency to decompensate when subjected to stress or trauma. Endothelial cell density less than 2000/mm^2 or hexagonal cell percent <50% are features of at risk corneas [9,16]. Corneas with such parameters were found more frequently in the pseudoexfoliated eyes than the normal eyes of our series with a statistically significant difference (P<0.05) based on endothelial cell density but the difference was non-significant on the basis of reduction in hexagonal cell percent where the P value was >0.05 (Tables 4 and 5). Corneas of pseudoexfoliated eyes are expected to be at risk of decompensation or compromise of their endothelial cell functions and the most

probable cause for such deviation might be attributed to the decrease in cell count or density rather than deviation in the cell shape.

These observations suggest that the corneal endothelial changes represent a consistent finding in eyes affected with pseudoexfoliation. Based on clinical and microscopic evidence, a specific corneal endotheliopathy that may be more susceptible to the effects of surgery was postulated, which is distinguishable from other forms of corneal edema. Such evidence and clinical findings point strongly to the fact that in patients with pseudoexfoliative keratopathy only moderate rises of IOP or minor intraoperative trauma might lead to a relatively early occurring diffuse corneal decompensation.

In conclusion, pseudoexfoliation is a cause for morphological changes in the corneal endothelium with a decrease in cell density that can lead to corneal decompensation. Corneal thickness is increased in pseudoexfoliative eyes with no significant pleomorphism. Denser affection by pseudoexfoliation, reflected by wider deposition of pseudoexfoliative material, shows more evident endotheliopathy.

Carful assessment for the eyes of elderly patients looking for pseudoexfoliation while preparing for intraocular surgery is recommended with special attention and care to be paid with meticulous handling during the surgery to protect the corneal endothelium. Viscoelastic substances use and avoiding instrumental touch to the endothelium are to be emphasized on to decrease the risk of corneal decompensation.

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Authors Contribution

Dr. Neamah collected the data and interpreted the results with dr. Kareem who finalized the writing of the paper.

Conflict of interest

The authors have no conflict of interest to declare.

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Frequency of Type 2 Diabetes in Young Age Groups in Northern Iraq

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Abstract

Background Type 2 diabetes (T2D) is frequently encountered among younger ages during last decades in both developed and developing countries largely contributed to the increasing degree and prevalence of obesity in such ages.

Objective To determine the frequency of T2D in patients younger than 40 years at Northern Iraq.

Methods Retrospectively a total of 9331 patients were studied consisted of 3471 males and 5860 females with diabetes mellitus (DM) at two settings in Northern Iraq in a period from January 2009 – January 2015. Demographic measurements and clinical evaluation were performed for all patients. The diagnosis of DM and its types was depended on the clinical background and confirmed by plasma glucose level measurement. The data from all patients were assessed and statistically analyzed.

Results T2D contributed by 8704 (93.3%) of total number of study sample. The mean values for body weight and body mass index for T2D were higher than those of T1D patients (78.0±14.2, and 30.93±5.42 vs. 56.1±22.6 and 23.72±6.89) respectively. The female to male ratio in T2D was approximately 1.73:1.00. Out of 8704 patients with T2D, almost 2134 (24.52%) patients were ≤ 39 years of age.

Conclusion Type 2 diabetes appears to be seen more frequently in younger age groups in Northern Iraqi society in parallel to increased rate of obesity particularly in adolescent and children.

Keywords Diabetes in young, obesity and diabetes, type 2 diabetes.

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List of abbreviations: ANOVA = Analysis of variance, BMI = Body mass index, DM =Diabetes mellitus, IDF = International Diabetes Federation, MODY = Maturity Onset Diabetes of Young, SPSS = Statistical package for Social Science, T1D = Type 1 diabetes, T2D = Type 2 diabetes

Introduction

The global pandemic of diabetes mellitus (DM), which principally involves type 2 diabetes (T2D) is a well-recognized by World Health Organization (WHO) and affects the majority of adults in developed countries such as in North America, Japan and Europe [(1)]. The greatest increase in the prevalence of DM is however expected to occur rapidly in developing and low or middle-income countries in Asia and Africa toward the years 2030 and 2035, probably following the people's tendency for urbanization, changing a dietary habit and increasing sedentary lifestyle patterns [(1-3)].

According to International Diabetes Federation (IDF), it is estimated that 382 million people have diabetes in 2013; this figure is expected to reach 592 million by 2035 [(4)]. The estimated global prevalence of DM is 8.3% while its prevalence in North America and the Caribbean

is (11%), the Middle East and North Africa (9.2%), and Western Pacific (8.6%) [5].

With exception of some countries in Gulf region and Egypt, the exact prevalence of DM in Arab countries including Iraq is lacking [5]. This is perhaps due to unavailability of large clinical and epidemiological studies linked to this disorder.

In general, T2D is more common than type 1, making up to 90% of DM cases and traditionally considered a disease of adults aged 40 years or older [1,6], however, in the last 2-3 decades, T2D has been frequently encountered among children and adolescents [7- 9].

The risk of T2D is clearly linked to an increasing degree and prevalence of obesity in children and adolescents in many populations [10,11]. Overweight and obesity are obviously driving the global diabetes epidemics and if no global strategies are planned to fight and prevent obesity, the number of overweight people is projected to increase from 1.3 billion in 2005 to nearly 2.0 billion by 2030 [11,12].

There are significant economic consequences of diabetes mellitus on patients and their families as well as on country's health systems. This is particularly true in regard to offering the health-care facilities for young adults and children who are living in developing countries. Worldwide diabetes mellitus caused 4.6 million deaths in 2011, and health-care expenditure attributed to DM was estimated to be at least US$465 billion, or 11% of total health-care expenditure [1,13,14].

Compared to older age groups, there are paucity of large-scale population-based studies focusing on youth with T2D and the majority of such data come from developed countries, particularly North America and Japan, with a distinct lack of information from many regions in the world, particularly from Africa and South America [5, 15-17].

For best of our knowledge, the current study is the first largest clinical study at our settings, aiming to determine the frequency of T2D in a population younger than 40 years in Iraqi society at the northern area.

Methods

In a period from January 2009 – January 2015, we retrospectively analyzed data of 9331 patients with DM collected from two settings at Northern Iraq. The bulk of cases were from Duhok Centre for Diabetes - Duhok city in Duhok Governorate and a smaller number were from outpatient clinics at Ibn Sena Teaching Hospital - Mosul city in Nineveh Governorate as well as scattered cases from private clinics. Duhok Centre for Diabetes is a well-recognized diabetic referral center at Northern Iraq that offers all necessary outpatient services for diabetic patients. Those who need tertiary care in the hospital are directly referred to medical wards in hospitals with which such settings are affiliated.

Data collection

The detailed and comprehensive reviews of personal, demographic characters and clinical data particularly those related to gender, body weight, height, body mass index (BMI), features of diabetes or its complications, family history of DM, duration of DM and age at the onset, were registered and collected for all patients in study group. The current and previous laboratory tests and details of medications are also taken into consideration.

All patients were subjected to complete hematological and biochemical tests in the study settings. The initial and subsequent fasting plasma glucose measurements in every visit, as well as a 3-monthly glycated hemoglobin (HbA1c) results, were recorded. The patients were also screened for the presence of chronic diabetic complications (peripheral neuropathy, cardiovascular, renal and eye complications, etc.). A schedule for follow-up was performed for all patients as well as an “Electronic Data-Base” using Excel Microsoft Program 2010 was kept for future reference.

The diabetes diagnostic criteria in this study were based on the presence of symptoms suggestive of DM and/or positive history of DM confirmed by plasma glucose level measurement according to American Diabetes Association current criteria for diagnosis of DM

which are: A fasting venous plasma glucose ≥ 7.0 mmol/l (126 mg/dl), or 2-hour plasma glucose ≥ 11.1 mmol/l (200 mg/dl) and/or HBA1c ≥ 6.5 [18]. T2D in patients younger than 40 years in the current study were differentiated from those with type 1 on the basis of history, physical examination findings including weight and BMI calculation as well as laboratory tests and response to therapy (insulin / oral hypoglycemic). We defined the younger age groups in the present study as those patients with an early-onset T2D in age groups < 40 years and include: young adults (20 – 40 years), adolescents (13-19 years) and children (<13 years) [19].

Patients with BMI >25% - 29.9 kg/m^2 were considered over-weighted, whilst those with BMI ≥ 30 kg/m2 were considered obese [20].

A unique inherited form of DM in young patients which is traditionally named a maturity onset diabetes of young (MODY) was not specifically searched for [21].

The mean age of onset, the duration of disease, sex distribution, BMI, and body weight for T1D and T2D were compared. Furthermore, the age group distribution and the age of presentation above and below 40 years in T2D were calculated. The relation between BMI, body weight and age groups below and above 40 years in T2D were studied. The relationship between BMI, gender, and types of DM was also studied.

Statistical analysis

Data has been processed and analyzed using software of statistical package for Social Science (SPSS) version 20 for windows. All variables were expressed as a number and percent and compared. The mean value ± standard deviation (SD) was calculated for age variable of the patient in a year. Independent t-test for two means, one-way ANOVA test and Chi-square test were used in the statistical analysis of the various data. A p-value ≤0.05 was regarded as the limit of statistical significant.

Results

Among 9331 patients, 3471 were males and 5860 were females. T2D contributed by 8704 (93.3%) while the remaining 627(6.7%) patients were T1D.

The displayed data in table 1, which are relating to personal characteristics of the study population indicate that the mean age ± SD (years) at the onset was 47.31 ± 10.92 for T2D versus 20.26 ± 10.31 for T1D and duration of symptoms (years) at the time of referring was 8.13 ± 6.13 for T2D vs. 8.58 ± 7.14 years in T1D. BMI (kg/m^2) for T2D was 30.93 ± 5.42 vs. 23.72±6.89 for T1D. This was statistically significant (p=0.0001). Male patients constituted 36.67% in T2D vs. 44.50% in T1D, while 63.33% of patients in T2D vs. 55.50% in T1D were female, in other words, the ratio of male to female was 3192:5512 (~1:1.73) in T2D and 279:348 (~1:1.3) in T1D.

As it is clear from table 2, the average body weight (kg) was 78.9 ± 16.0 for male and 75.1 ± 15.7 for female patients (p=0.0001). The average weight in T2D was 78.0±14.2 and 56.1 ± 22.6 for T1D (p= 0.0001). The differences were statistically significant. The age distribution of patients with T2D is demonstrated in table 3 as follow: about a quarter, 2134 (24.52%) out of 8704 patients were ≤ 39 years, and just slightly more than three-quarters 6570 (75.48%) out of 8704 patients were ≥40 years of age and 35 (0.41%) patients were ≤19 years while 1062 (12.20%) were ≥60 years.

The relationship between BMI, body weight and age groups below and above 40 years in the study group was clarified in table 4. There was a significant difference in weight and BMI between age group 0-19 years and other groups (p=0.0001) while no such differences were found between other groups whether below or above 40 years when compared with each other.

In table 5: 7764 (about 90%) out of 8704 patients with T2D were having BMI ≥ 25kg/m^2 and just about a quarter (24.52%) of these patients were aged < 40 years. The remaining 940 (10%) out of 8704 patients having BMI ≤ 25 kg/m^2 and about 30% of them were aged < 40 years. The average BMI was higher in female than in male patients with type 2 DM (table 6): 32.12 ± 5.66 vs. 28.86 ± 4.25. This was statistically significant (p- value 0.0001).

Table 1. Personal characteristics of the study population [n = 9331]

Parameters	Type 1 diabetes Mean ± SD [range]	Type 2 diabetes Mean ± SD [range]
No.	627	8704
Age at onset (years)	20.26 10.31 [0.0; 49.0]	47.31 10.92 [6.0; 102]
Duration of DM (years)	8.58 ± 7.14 [0.0; 40.0]	8.13 ± 6.13 [0.0; 45.0]
BMI (Kg/m^2)	23.72 ± 6.89 [15.620; 54.62]	30.93 ± 5.42 [15.36; 68.73]
Gender	No. (%)	No. (%)
Male	279 (44.50)	3192 (36.67)
Female	348 (55.50)	5512 (63.33)

Table 2. The relationship between body weight and type of diabetes in the study sample

	Body weight (Kg) Mean ± SD		P-value *
Gender	Male [n = 3471] 78.9 ± 16.0	Female [n = 5860] 75.1 ± 15.7	0.0001
Type of DM	Type 1 56.1 ± 22.6	Type 2 78.0 ± 14.2	0.0001

* Independent t-test for two means was used

Table 3. Age distribution in type 2 diabetes patients [n = 8704]

Age groups (years)	Count	%
0-9	4	0.05
10-19	31	0.36
20-29	257	2.95
30-39	1842	21.16
40-49	2885	33.15
50-59	2623	30.14
60+	1062	12.20
Total	8704	100.00

Table 4. The body weight, BMI in different age groups in type 2 diabetes

Age groups (years)	Count (n=8704)	%	Body weight (Kg) Mean ± SD	BMI (Kg/m^2) Mean ± SD
0-19	35	0.40	57.34 ± 19.20	23.15 ± 5.09
20-29	257	2.95	78.68 ± 15.45	30.08 ± 5.34
30-39	1842	21.16	79.33 ± 14.62	30.71 ± 5.55
≥ 40	6570	75.48	77.75 ± 13.88	31.06 ± 5.35
P-value *	---	---	0.0001	0.0001

* One-way ANOVA test was used

Table 5. The relationship between BMI and age groups in T2D

Age groups (years)	BMI (Kg/m^2) BMI < 25.00 No. (%)	BMI ≥ 25.00 No. (%)	Total No. (%)	P-value *
0-19	22 (2.34)	13 (0.17)	35 (0.40)	0.0001
20-29	39 (4.15)	218 (2.81)	257 (2.95)	0.0001
30-39	214 (22.77)	1628 (20.97)	1842 (21.16)	0.0001
≥ 40	665 (70.74)	5905 (76.06)	6570 (75.48)	0.0001
Total	940 (100)	7764 (100)	8704 (100)	---

* Chi-square test was used. P-values were highly significant

Table 6. The relationship between gender and BMI in T2D

Gender	Count (n=8704)	BMI (Kg/m^2) Mean ± SD	P- value*
Male	3192	28.86 ± 4.25	0.0001
Female	5512	32.12 ± 5.66	

*Independent t-test for two means was used

Discussion

T2D was diagnosed in > 93% of the studied population in the current study. This observation is consistent with 2013 WHO report about diabetes [(1)].

T2D once thought to be a disease of adulthood, has been increasingly recognized in early age groups [(9)]. While still, the bulk of patients with new onset T2D in the present study is within age groups of 40-60 years, however nearly a quarter of such patients are falling below 40 years and clustered mainly at age group (30-39). The US National Diabetes Statistics 2011 found that the rate of new cases among Asian/Pacific Islander Americans and Americans Indian youth in the age group 10 - 19 years was greater for T2D than for T1D [(22)]. It is interesting to note that in our settings, the figure for new onset T2D in patients younger than 40 years was exceeding that of T1D (77% vs. 23%), but the yield of this proportion between the types of DM probably will be changed if above comparison was done for patients younger than 30 or 20 years, as more patients with T1D were fall within such ranges, anyhow this point, in particular, was not our main objective. Furthermore, the majority

of patients with T2D were obese or overweighed including those younger than 40 years and body weight as well as body mass index in younger age groups with T2D, particularly those in age groups of 20-40 years were significantly higher than that of T1D. No such differences in body weight and BMI were found between different age groups of T2D whether above or below 40 years except for age group (0 –19) years which contributed only for less than 1% of total T2D.

Approximately 63% of patients with T2D in the present study were in ages (40-59) year, a fact is in consisting of key message information released in IDF Diabetic Atlas in 2014 [4]. Despite the fact that the patients with T2D are eventually gathering in older age groups but new onset T2D in age groups above 60 years of the present study was contributed to not more than 12%. In contrary some studies, however, observed the higher occurrence of T2D in older age groups [23,24]. These observations undoubtedly reflect an increased frequency and severity of obesity in younger age groups in the present study as a result of dietary, lifestyle changes and urbanization that involved Iraqi society too.

Obesity is a strong environmental factor, which is directly linked to the development of T2D particularly in those who have a clear family history of DM [25-27].

A study concluded that the Asians, develop T2D at younger ages and even at lower degrees of obesity compared with western populations [28], this is another point of concern as our societies are potentially sharing the same characters.

The possible role of chronic stress and multiple conflict situations that Iraqi people have had suffered from for years can't be ignored as risk factors for DM and obesity, the mechanism by which this phenomenon could happen is still unclear but some investigators related it to de-synchronization of the temporal pattern of leptin and triglyceride release and dysregulation of the hypothalamic-pituitary-adrenal axis that leads to changes in glucocorticoids and ACTH serum levels [29,30].

The mechanism and pathogenesis of T2D in younger age groups are not so much differ from those in older age groups, that is to say, it is mainly due to increase obesity-induced insulin resistance and inadequate β cell insulin secretion [31,32].

The present study showed a pronounced female:male predominance in T2D (63% vs. 37%) with BMI in females significantly higher than males (p=0.0001). A similar finding was present in the study of Lasky et al. in Uganda [33], while some studies found equality in the prevalence of T2DM between men and women in most populations in western countries with some evidence of male predominance in others [34].

In addition to genetic factor(s) for obesity and T2D [35], and lacking of healthy dietary habit that involves both genders; we believe that one of forgetting reason behind a female predominance in T2D in our society is probably related to more sedentary life for women in Iraqi society especially who are living in Urban areas, as most of them are not involving in active working outside their homes in contrast to women in western societies who are sharing actively with their men partners for family financial income. Repeated pregnancies in women in Iraqi society may be an additional factor for obesity and DM [36,37]; thereby the women in our society are putting themselves at greater risk for obesity and T2D early in life.

We are very concerned about the increased prevalence of T2D in a younger age group in Iraqi society, not only because of its economic impact but also because of its probable association with increased morbidities and mortalities early in life especially those related to cardiovascular in such population.

The current study concluded that until recently, T2D in Iraq has been viewed as a disease of older adults, but as shown by this study, T2D appears to be seen more frequently in younger age groups in Iraqi society and this is probably a reflection of dramatic changes in lifestyle and dietary habits as part of modern globalization and industrialization that also affected the Iraqi

society and led to increased rate of obesity particularly in adolescent and children.

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Authors Contribution

Nearly, all authors are contributed equally in this research. Dr. Alhabbo contributed his work for preparation of statistic, methodology and result sections. The data collection was largely performed by Dr. Khalaf, while Dr. Saeed contributed his work for writing the introduction, discussion sections and selection of the required references. All authors were shared their ideas in final revision of the article.

Conflict of interest

The authors disclose that, there is no any financial and personal relationships with others (people, organizations or institution).

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Combining Strain Elastography Findings with Ultrasound BIRADS System to Discriminate between Benign and Malignant Solid Breast Masses

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Abstract

Background Elastography has been attracting attention as a new non-invasive diagnostic tool with the potential to improve breast masses characterization.

Objective To assess the value of incorporating strain elastography into the ultrasound Breast Imaging Reporting and Data System (BIRADS) System to differentiate benign from malignant breast masses.

Methods Fifty-six women with 61 solid breast masses were enrolled in this study. Ultrasound was performed and the mass was given an US BIRADS category. Elastographic examination was performed and each lesion was assigned an Elasticity Score (ES) according to the Tsukuba scoring system. Strain Ratios (SRs) were calculated from a tumor adjusted Region of Interest (ROI) and a reference ROI in the fatty tissue. The US BIRADS was modified according to the elasticity criteria. Sensitivity, specificity, area under the curve (AUC) and cutoff values were calculated for US BIRADS, ES, SR and the modified BIRADS method using (ROC) curve analysis.

Results The final results were based on 61 masses, 25 benign and 36 malignant. The sensitivity and specificity were respectively (97% and 80%) for US BIRADS, (86.1% and 84%) for ES, (94.44% and 84%) for SR, and (97% and 84%) for the modified BIRADS.

Conclusion Combining elastography with conventional ultrasound yielded better diagnostic performance with improved specificity.

Keywords Elastography, breast masses, elasticity, strain ratio, modified BIRADS

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List of abbreviations: ACR = American College of Radiology, AUC = Area under curve, B-mode = Brightness mode, BIRADS = Breast imaging reporting and data system, ES = Elasticity score, FNAC = Fine needle aspiration cytology, FOV = Field of view, ROC = Receiver operating characteristic, ROI =Region of interest, SR = Strain ratio, US = Ultrasound

Introduction

Elastography is a recently introduced ultrasound technology [(1)] with the potential to visually and objectively assess the elastic properties of tissues previously assessed roughly by physical palpation [(2-4)].

It has the potential to differentiate benign from malignant lesions [(5)] as cancerous tissue becomes harder due to cell proliferation and angiogenesis [(1)]. Two main elastographic technologies used in clinical practice are strain elastography and shear wave imaging which differ by the method utilized to displace the

tissue and the imaging system, which quantify the magnitude of displacement and converts it into a color-coded map termed elastogram [6].

One of the earliest applications of elastgraphy in medical field is in breast lesion characterization. It is considered a useful adjunct to the standard B mode imaging. Initial clinical trials showed that strain elastography has the potential to improve breast masses characterization [6].

The method most widely used for classifying the elastographic images is the Tsukuba scoring system proposed by Dr. Ueno et al [7-9], which uses a five-point scale to classify elastograms. This system has been shown to have a diagnostic performance comparable to the US BIRADS system in evaluating breast lesions for malignant properties [10,11].

Elastography has been added to the ACR ultrasound BIRADS lexicon 2013 [12] but its exact role has not been defined. Because elastography cannot be used in isolation for breast lesion evaluation and other ultrasound parameters should be taken into consideration, several methods were proposed to combine the elastographic criteria of breast masses with the US BIRADS to better assess the lesion for malignant potential, thereby decreasing the false negative biopsies [13-15]. An Italian study has used only elastographic parameters to elucidate a scaling system for breast lesion characterization [16]; however, only few studies incorporated both elasticity score and strain ratio measurement to modify the US BIRADS [17,18].

This work was done to evaluate the clinical usefulness of a proposed method of modifying the B-mode dependent US BIRADS making use of the elastographic parameters of breast lesions namely: elasticity scoring (ES) and strain ratio (SR).

Methods

Patients and Data Collection

This analytic cross-sectional study was conducted in the breast clinic at the Oncology Teaching Hospital, Baghdad, Iraq from April 2014 to April 2015, a period during which, 62 patients with 68 solid breast masses were examined.

The patients included in the study were the females presented for the first time with solid breast mass visible on B-mode ultrasound that is either palpable or incidentally discovered by other imaging modalities. Follow up was obtained for some masses (No.=15) with benign ultrasound and elasticity features for whom FNAC was done as the pathological diagnosis (pathological analysis detailed later).

Excluded from the study patients with previous surgical intervention, patients with BIRADS 0 masses, patients who received chemotherapy and patients with benign looking masses who refused FNAC in the first place.

Out of all patients who were examined (No. =62), 6 patients were excluded as no follow up data were obtained. Thus, the final data analysis was based on 61 solid breast masses obtained from 56 women. Informed verbal consent was obtained from each patient.

Examination Technique

Conventional B-mode ultrasound was performed with the patient in the supine position then elastographic examination was done using high end ultrasound system (GE healthcare, Voluson E6) with high frequency linear probe (10-14 MHz). For elastographic examination, the field of view (FOV) was set so that the lesion is not at the periphery and does not exceed 1/2 of the FOV with the inclusion of adequate surrounding normal breast tissue, subcutaneous fat and the pectoral muscles where feasible.

The elastographic examination was accomplished by applying very light touch with the transducer perpendicular to the skin and to the lesion, a minimum of 5-6 compression release cycles were applied trying not to have lateral movement.

Measurements and Image Interpretation

On B-mode examination the location of each lesion was labeled including the side, the O'clock face, the distance from the nipple and the depth of the lesion. The lesion was assigned an ultrasound BIRADS category by the joint

decision of 2 radiologists. The maximum dimension of the lesion in the B-mode image was measured. Each lesion was assigned an elasticity score according to the Tsukuba elasticity score system. Measuring the strain ratio was achieved by placing 2 circles of approximately equal diameter, one in the subcutaneous fat adjacent to the lesion (reference) and the other in the lesion (ROI) and the diameter was calibrated so that to include the lesion without extending into the adjacent tissues.

Elastography Based BIRADS Modification

Our proposed system for modifying US BIRADS (figure 1) was applied to BIRADS 3 and BIRADS 4 lesions based on the cut off points for elasticity score (>3) and strain ratio (>3.2) derived from the ROC curve analysis.

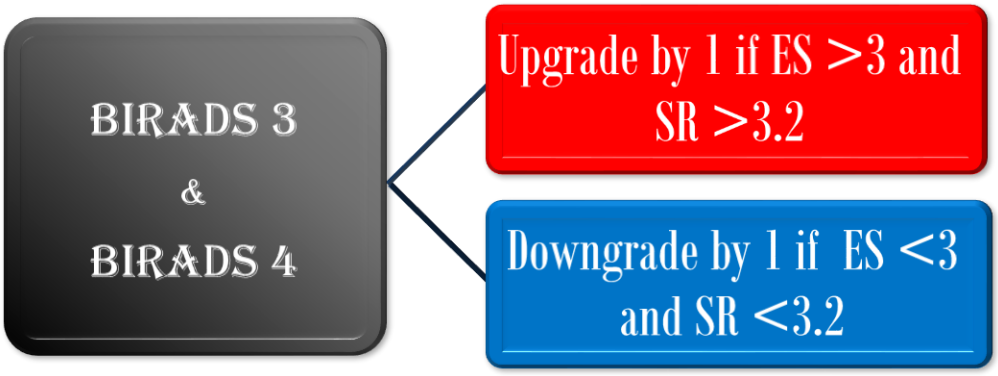

Figure 1. A schematic drawing illustrating the BIRADS modification method

Pathological Analysis

Our final diagnosis was based on pathological analysis of breast mass samples obtained with fine needle aspiration cytology (FNAC) (No. =15), needle biopsy (No. =10), excision biopsy (No. =16), or radical surgery (No. =20)

FNAC was considered the standard only for masses with benign ultrasonographic and elastographic appearances with concordant FNAC results, for those patients 3 months follow up with ultrasound was performed which showed no interval change in size.

For lesions with discordant ultrasonographic and elastographic diagnoses or for whom the FNAC results were suspicious biopsy was performed to confirm the diagnosis with the exception of 3 BIRADS 3 lesions, which had elasticity score suggestive of malignancy (score 4) but they had calcification with benign ultrasound features consistent with calcified fibroadenoma, in addition, in 2 of these masses mammograms were available which confirmed internal calcification, so we depended on the FNAC results plus follow up and no biopsy was performed. A net of 25 benign and 36 malignant breast masses were enrolled in the study.

Statistical Analysis

Data collection and descriptive statistics including the graph design were accomplished via Microsoft excel 2010. Cross tables were built using Statistical Package for Social Sciences (SPSS) statistics for windows, version 22.0.

Comparing the mean SR for benign and malignant masses was performed using independent student test (t-test).

The diagnostic performance of the parameters incorporated in the study was assessed by using receiver operating characteristic (ROC) curve analysis based on De Long et al method with calculation of the sensitivity and specificity in addition to cut off point calculation based on Youden index. The same De Long method was used for pairwise comparison between different

ROC curves. The above mentioned statistical tests (t-test, ROC curve analysis, ROC curves comparison) were performed using MedCalc for Windows, version 14.12.0 (MedCalc Software, Ostend, Belgium). For all tests, a P value of less than 0.05 was considered to indicate a statistically significant difference.

Results

The patients' population consisted of 56 women with 61 solid breast masses, mean age of the patients was 44.7 years; age range 20-65 years. Of these women, 42 presented with palpable breast mass while 14 women had their masses discovered incidentally on imaging performed for other indications. The mean diameter for the masses was 1.65 cm, range 0.4-3 cm, mean diameter for benign masses was 1.47 cm, and mean diameter for malignant masses was 1.79 cm.

Ultrasound BIRADS

Twenty-one of the masses included in the study were classified as BIRADS 3, 26 as BIRADS 4 and 14 as BIRADS 5. Of the masses given BIRADS 5 category, all were malignant; BIRADS 3 masses had one mass, which proved to be malignant, and 21 masses given BIRADS 4 were malignant. The diagnostic performance of the US BIRADS was evaluated by ROC curve analysis; the area under the curve (AUC) was 0.925. US BIRADS had sensitivity and specificity of 97 % and 80 % respectively considering scores 4 and 5 malignant and scores 1, 2, and 3 benign.

Elasticity Score

Out of the four masses given an ES of 1, none was malignant. On the other hand, 87.5% (14 out of 16) of score 2 masses that were benign. Six masses were given an ES of 3 (3 were benign and 3 malignant), 80% (16 out of 20) of masses given Tsukuba score 4 were malignant while all fifteen masses which were given a score of 5 were malignant (figures 2).

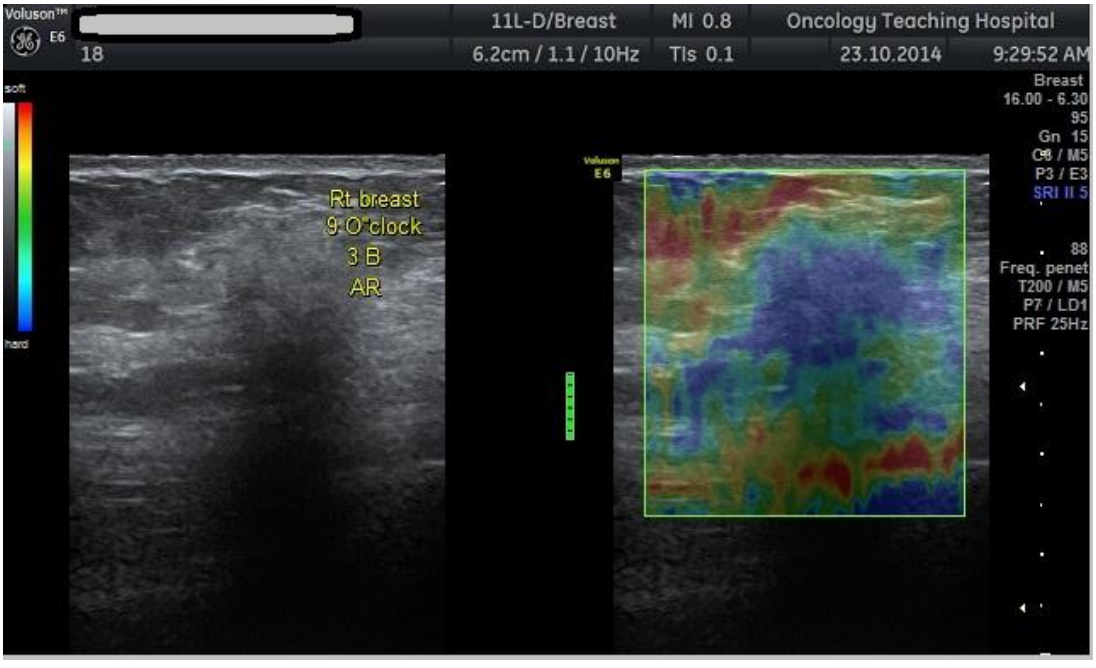

Figure 2. Malignant mass with elasticity score of 5. The tumor margins are indistinct on B-mode; on the contrary elastogram better depicts the tumor margins

Of the masses given an elasticity score 4, 20% (4 out of 20) were benign. likewise, 12.5% (2 out of 16) of masses with a score of 2 were malignant. The overall diagnostic performance of elasticity score was evaluated using (ROC) curve; with a cutoff point (> 3) ES had a sensitivity and specificity of 86.1% and 84% respectively.

Strain Ratio

The distribution of masses according to SR is shown in figure 3. Thirteen out of twenty-five (52%) of benign masses with strain ratio less than 2.1. On the other hand, 19 out of 36 (52.7%) of malignant masses had strain ratios ≥ 5.1 (Figure 4).

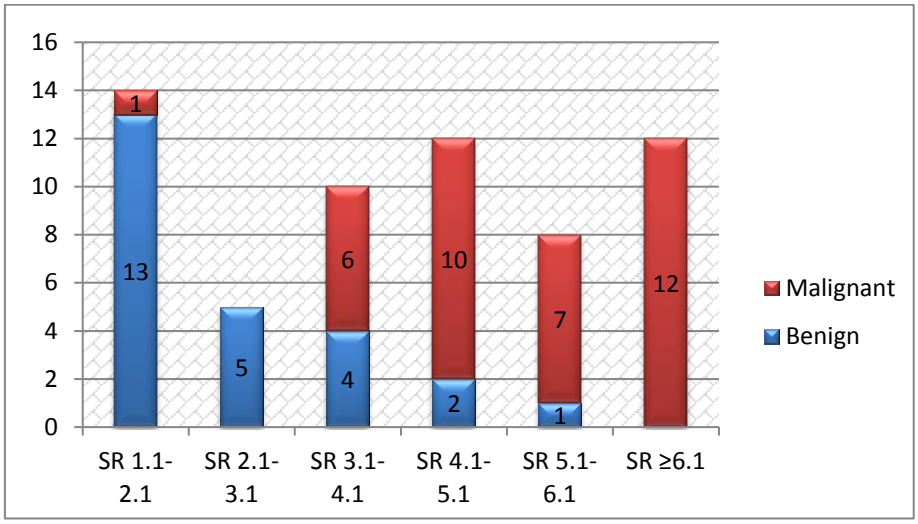

Figure 3. Distribution of strain ratios in benign and malignant masses

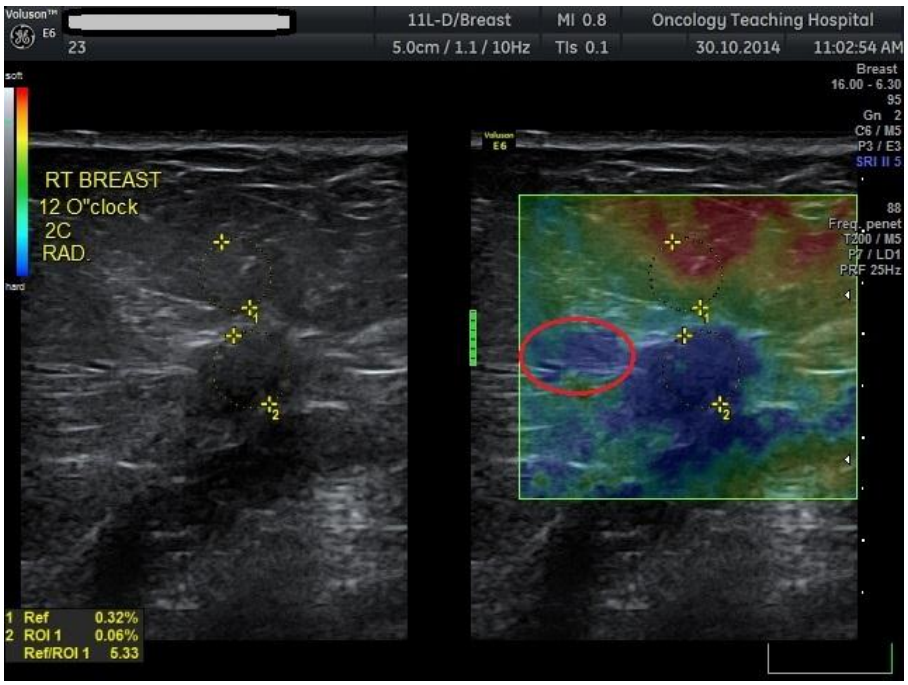

Figure 4. Malignant mass with ES 4 and SR of 5.33. There is a band of stiffness (circle) extending from the main tumor not well depicted at B-mode which could possibly represents intraductal extension

The mean strain ratio for all masses included in the study was 4.3, mean SR for benign masses 2.4, mean SR for malignant masses was 5.6. Independent sample t-test was used to evaluate whether the difference between the mean SR for benign and malignant masses was statistically significant. The result of the test showed significant difference between the two means (P value<0.0001)

The overall diagnostic performance of Strain Ratio measurement was evaluated with ROC curve, AUC was 0.936 with a cut off value (>3.2), SR had sensitivity and specificity of 94.44% and 84% respectively. Using comparison between ROC curves, there was no significant difference between the diagnostic performance of ES and SR (P value =0.56).

Comparing the diagnostic performance of ES and SR with the ultrasound BIRADS showed no statistically significant difference with a P value of 0.8299 and 0.7990 respectively.

Moreover, no statistically significant difference between diagnostic performance of ES and SR.

Assessing the Proposed Modified BIRADS

Thirteen masses categorized as BIRADS 3 have been downgraded to BIRADS 2 and were benign. Likewise, four masses categorized as BIRADS 4 have been downgraded into BIRADS 3 and proved to be benign (Table 1).

Table 1. Modified BIRADS vs. BIRADS

Mass	BIRADS	Modified BIRADS				Total
		Category 2	Category 3	Category 4	Category 5	
Benign	Category 3	13*	4	3*		20
	Category 4	0	4*	1		5
Malignant	Category 3		1	0	0	1
	Category 4		0	1	20*	21

On the other hand, three BIRADS category 3 masses have been upgraded into BIRADS 4 and they were benign. Eighteen BIRADS category 4 masses have been upgraded to BIRADS 5 and proved malignant.

The diagnostic performance of the modified BIRADS was excellent as assessed by the ROC curve analysis with sensitivity 97%, specificity 84% and AUC =0.984 when a cut-off point of >3 was considered.

Comparing the diagnostic performance of the modified BIRADS with the other diagnostic parameters used in this study showed significant difference between the modified BIRADS and standard BIRADS (P=0.0251) and between modified BIRADS and ES (P=0.0068). On the other hand, no statistically significant difference was found between the modified BIRADS and SR diagnostic performance (P=0.0884).

Discussion

This study showed good elastography diagnostic performance, comparable to the US BIRADS, the currently widespread system for breast masses characterization. Elastography, although showed lower sensitivity than BIRADS in the study, had higher specificity with the potential to reduce the rate of negative biopsies. The proposed method for integrating elastography in the US BIRADS system showed better overall diagnostic performance as compared to US BIRADS alone.

Elasticity Score

Tsukuba elasticity scoring is useful for differentiating benign from malignant breast lesions. Two of the 5 false negative masses had ES of 2 and 3 had an ES of 3. Three masses had a maximum diameter exceeding 2 cm and one was deeply located within the breast, which

could reflect the difficulty in obtaining good elastographic images in large and in deeply located masses. This is in concordance with the study by Ciurea et al., [19] which showed difficulty in obtaining good quality elastographic images in large and in deep masses near the chest wall. There has been difficulty in categorizing masses with mixed strain color pattern (blue and green) as Tsukuba score 2 masses are defined as masses in which, there is mixed green and blue colors with no reference as to the predominant color pattern. Tsukuba score 4 pattern as described originally should have no strain over the whole lesion meaning the lesion appears blue all over. In this work we encountered three lesions with mixed color pattern but predominance of blue color was noted, we have given them as score 2. Two of these masses were malignant and one was fibroadenoma with calcification.

Of the 20 masses which had an ES 4, four were benign. These masses had calcification, which could account for the lack of strain in these lesions. In our work, we noticed better definition of lesion margins at elastography for some malignant masses which had indistinct margins with acoustic shadowing (Figure 2). Also, in some cases a band of stiffness was noted extending from the lesion in a course likened to the ductal anatomy which could represent intraductal extension (Figure 4). The results for Tsukuba elasticity score is consistent with the original work done by Itoh et al. [7] who first described this scoring system. They found a sensitivity of 86.5 % and a specificity of 89.8 %, values which are comparable to ours, using the same cut off value (>3) for benign versus malignant lesions differentiation. In their study all lesions with elasticity score of 1 were benign. Several other studies [14,20-23] showed improved specificity for elasticity scoring compared to the US BIRADS.

Strain Ratio

Strain ratio measurement showed better diagnostic performance than ES with a sensitivity of 94.4% and specificity of 84 %. Two masses had false negative results. One mass had SR of 1.9 far less than our calculated cut off point of 3.2. This mass had a maximum dimension of 2.5 cm, which could affect the quality of the elastogram obtained. The other false negative mass had a SR of 3.2 (our cut off value). It was located deep within the breast which can potentially affect the quality of elastogram obtained. Four benign masses had SR above our cut off value. Two of them were calcified and one had a maximum dimension of 2.3. The fourth mass was located in a predominantly fatty breast which could account for the relatively high strain ratio as compared to fat.

Zhi et al. [24], in concordance with our results, concluded that strain ratio provides a more reliable diagnostic performance in comparison to Tsukuba scoring system for elastography with sensitivity and specificity 92.4% and 91.1% respectively with a cutoff point of 3.05.

Farrok et al. [22] concluded that strain ratio would help increase the specificity of elastography. In their study, they did not report the cut off value for discriminating benign from malignant masses.

Proposed Modified BIRADS

In our proposed system for BIRADS modification we used elasticity score and strain ratio criteria to re-categorize BIRADS 3 and 4 masses. Our method for modifying the US BIRADS according to the elasticity score and strain ratio measurements showed improved specificity as compared to the US BIRADS alone (84% vs. 80%) with no reduction in sensitivity (97%). In the current study, 18 out of 21 masses with BIRADS 3 category were downgraded to BIRADS 2 and all proved benign (Figure 5) our masses classified as BIRADS 4 were downgraded into BIRADS 3 and all were benign. On the other hand, three benign masses originally categorized as BIRADS 3 were upgraded into BIRADS 4 which is a drawback for our modified system. BIRADS 4 masses, which has been upgraded into BIRADS 5 (No. =18), all proved malignant. Albeit the increase in specificity in

the modified system is not huge, the clinical implications would be of paramount importance. Downgrading BIRADS 3 lesion to BIRADS 2 means less patient anxiety. In addition, no further follow up is needed decreasing the cost burden to the patient and to the health institutions. In a similar fashion downgrading benign BIRADS 4 masses into BIRADS 3 would be even more beneficial as this would spare the patient the unnecessary biopsy reflecting the core value of introducing elastography in breast imaging algorithm.

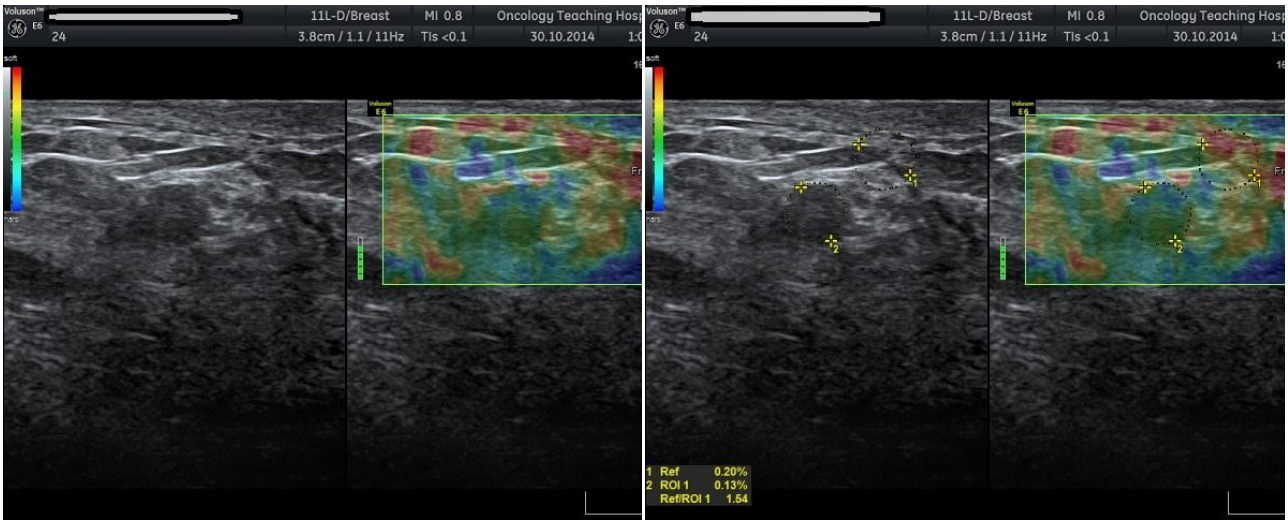

Figure 5. BIRADS 3 mass downgraded to BIRADS 2 (ES =1, SR =1.54)

Limitations

One of the limitation encountered in this study is the overrepresentation of malignant lesions, which is actually related to the fact that our cases were examined in the Breast Clinic at the Oncology Teaching Hospital, which is considered a tertiary center so we are, by virtue of the place, more likely to encounter malignant masses than in a usual everyday practice. Another limitation is the fact that not all masses were confirmed histopathologically as we were obliged to follow the policies and procedures adopted at the place we were working at which, state that a mass with benign findings at ultrasound would be followed to confirm its benign nature with no role for invasive measures in such masses.

The current study results concluded that using strain elastography, there is significant difference between benign and malignant solid breast masses, however; overlap still exists. Strain ratio measurement had better diagnostic performance than elasticity score. By incorporating elasticity assessment in the US BIRADS categorization increased specificity was noted which may reduce the rate of unnecessary biopsy. Applying our modified BIRADS system would also change the clinical course of BIRADS 3 lesions with profound clinical impacts both on the patient and the health institutions and potential reduction in the number of BIRADS 3 lesions requiring follow up.

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Authors Contribution

All authors contributed equally to the design of the study and data analysis. Data collection, statistical analysis and writing the manuscript

was done by Dr. Nee'ma. Dr. Tawfeeq and Dr. Khalel have thoroughly reviewed the article and contributed to the writing of the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Histopathological Characteristics of Pleomorphic Adenoma; A Retrospective Analysis of 120 Cases

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Abstract

Background The salivary gland pleomorphic adenoma (PA) is a benign epithelial neoplasm, histologically characterized by a great diversity of morphological aspects. It is the most common neoplasm of salivary gland origin.

Objective Studying the histopathological characteristics of PA with special attention to the various morphological features of the epithelial cells and stromal components of this neoplasm.

Methods Hematoxylin and Eosin (H&E) stained tissue sections of 120 cases of PA were reviewed. The tumors were classified according to their histological subtypes as described by Seifert *et al*. The epithelial components were analyzed considering the type of cells and the morphological pattern. The stromal components were analyzed according to the presence of myxoid, hyaline, chondroid or calcified tissue.

Results This study revealed that most of the tumors were located in the parotid gland (44%). Myxoid or stroma-rich was the most frequent histological subtype (43%). Plasmacytoid cells were the most commonly seen epithelial component (100%), followed by cuboidal cells in (80%) of the cases. Trabecular pattern was the predominant epithelial morphological pattern (90 %), and the myxoid component was the most frequent stromal component (80%).

Conclusion PA of the salivary glands demonstrates a wide variety of cells, stromal components, and morphological characteristics. Since it is the most frequent salivary gland neoplasm that can resemble other salivary gland tumors, the knowledge about these variations is essential for a correct diagnosis.

Keywords Histopathology, pleomorphic adenoma, salivary gland neoplasms

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List of abbreviations: H&E = Hematoxylin and eosin, PA = Pleomorphic adenoma

Introduction

Pleomorphic adenoma (PA) is a benign mixed tumor, and it is the most common salivary gland tumor. It occurs predominantly in females at a premenopausal age [(1)]. PA represents 60% to 73% of the parotid gland tumors, and 40% to 60% of the submandibular and minor salivary glands tumors [(2)].

These tumors consist of excretory ductoacinar units and associated myoepithelial cells, which are believed to originate from totipotential stem cells. This may explain the morphologic diversity in parotid gland neoplasms [(3)]. They are characterized by a biphasic growth of both epithelial cells and myoepithelial cells that are

arranged with various morphological patterns and subtypes[4].

Histologically, PA is characterized by a great diversity of morphological aspects. Its structural pleomorphism is given both by the epithelial component, as a result of the cytological differentiations and the growing patterns, and by the stromal component because of its rich morphological and quantitative diversity [5].

Epithelial cells typically form duct-like structures associated with nonductal cells presenting variable shapes and forms. The stromal element demonstrates varying degrees of myxoid, hyaline, cartilaginous, or osseous differentiation [2]. Among these morphological aspects, one aspect is usually predominant in variable proportion [6]. The pathognomonic stromal feature of pleomorphic adenoma is the presence of chondro-myxoid stroma.

PA usually presents as a slow progressing asymptomatic, parotid gland swelling without facial nerve involvement [7]. Up to 10% of cases show malignant transformation and features predictive of malignant change include advancing age, massive tumor size, a long duration of the mass, occurrence in the submandibular salivary gland, and hyalinized connective tissue [8].

The best treatment option is a wide local excision with good safety margins and follow-up for few years.It had been suggested that focal infiltrations of the tumor capsule of the pleomorphic adenoma could be left behind if the lesion was simply enucleated. Moreover, multicentricity of pleomorphic adenoma has been recorded in up to 11% of the examined tissue specimens [9]. Currently, it is generally accepted that these histopathological features of pleomorphic adenoma explain tumor recurrence after simple enucleation. Therefore, it is well recognized that the best surgical treatment option for a pleomorphic adenoma of the superficial parotid gland is a wide local excision with good safety margins and follow-up for few years. A better option is a lateral lobectomy [7]. Adopting these procedures, the recurrence rate has declined to a figure less than2.5% in recent years.Nevertheless, there is still a debate about the optimal treatment option of parotid pleomorphic adenoma. Some authors have propagated a local dissection or a subtotal superficial parotidectomy as an alternative to superficial parotidectomy[10].

In this study, we describe the histopathological characteristics of 120 cases of PA with special reference to the morphology of the epithelial cells and stromal components.

Methods

This retrospective study included 120 formalin-fixed, paraffin embedded biopsy specimens of PA obtained from the archives of the Department of Pathology AL KindiHospital and the Department of Oral Pathology, College of Dentistry, Baghdad University.

The ethical approval was obtained from the authority of these medical institute to facilitate this work and obtaining the materials from histopathological archives.

The clinicopathological information regarding age, gender, lesion sites, clinical presentation, in addition to any other pertinent information were obtained from the case sheets presented with the surgical specimen. Sections of 5µm thickness were cut from each tissue specimen. All H&E-stained tissue sections were reviewed by two Pathology specialists to confirm the diagnosis.

Data regarding age, site distribution and histological subtypes in regard to different salivary glands, various cellular types of the epithelial component in addition to the morphological pattern of epithelial and stromal components of PAs, were considered.

The tumors were classified as myxoid or stroma-rich, cellular or cell-rich and classic (balanced amount of epithelial and stromal components) as described by Sergi *et al*. [7]. The epithelial components were analyzed taking into consideration the types of cells (plasmacytoid, spindle, clear, squamous, basaloid, cubic, oncocytoid and mucous cells) and the morphological pattern (trabecular, ductal, cystic and solid). The stromal

components were analyzed according to the presence of myxoid, hyaline, chondroid or calcified tissue.

The statistical data were analyzed using SPSS software (version 21). P value < 0.005 was considered.

Results

The study sample consisted of 120 pleomorphic adenomas of the salivary glands, of which 72 cases were females (60%), and 48cases were males (40%), with a M/F ratio of 1/1.2. The age range was 15-70 years, with a mean age of 38 years. The majority of the tumors were found within the (30-40) age group (Table1).

Table1. Incidence of PA in different age groups

Age groups(yrs)	No.	%
10-20	8	6.6
21-30	28	23.3
31-40	41	34.16
41-50	19	15.83
51-60	15	12.5
61-70	9	7.5

All patients were presented with a unilateral lesion, with slightly dominant left side occurrence (63 on the left, 57 on the right). The majority of the cases were located in the parotid gland 53 cases (44%), the second most common location was the minor salivary glands 38 cases (32%), distributed as follows; the palate (15cases), the oral mucosa (13cases) and the lower lip (10 cases).Followed by 28 cases in the submandibular gland (24%). No cases were recorded in the sublingual salivary gland (Table 2).

Table 2. Site distribution of 120 cases of PA

Location	No.	%
Parotid	53	44
Minor salivary glands	38	32
Submandibular salivary gland	28	24
Total	120	100

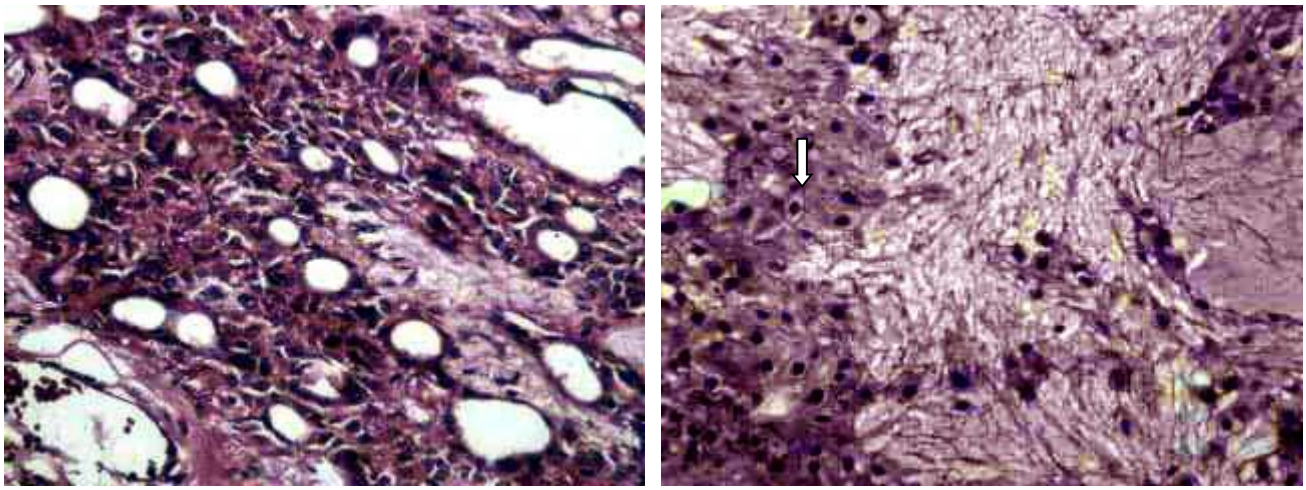
The study revealed that myxoid or stroma - rich is the most frequent histological subtype found in the examined specimens (43%), followed by the cellular or stroma-poor and classic subtypes, in (38%) and (19%) of the tumors respectively (Table 3).

Plasmacytoid cells were the most commonly presented epithelial component, as they were present in all of the examined cases (100%)(Figure 1). Spindle cells were the second most common cell type presented in 90 cases (75%), followed by cuboidal cells in (63%) of the cases.The other components presented in a descending manner as follows: basaloid cells (60%), squamous and clear cell types (32% & 29 %) respectively. On the other hand, oncocytoidand mucous cells were the least found epithelial components, they are

considered as occasional findings, (2% and 1%) respectively (Table-4).

Table 3. The histological classification of 120 cases of PA

Histological classification	No.	%
Stroma-rich	52	43.3
Stroma-poor	45	37.5
Classic	23	19.1
Total	120	100



A **B**

Figure1. A: PA with predominant plasmacytoid cell(arrow head) and ductal morphological pattern (H&E,4X), B: PA plasmacytoid epithelial cell(arrow) (H&E 10 X)

Table 4. Various epithelial cell types present in the studied sample of PAs

Histological cell types	No.	%
Plasmacytoid	120	100
Spindle cell	90	75
Cuboidal cell	76	63
Basaloid cell	72	60
Squamous cell	32	26.6
Clear cell	29	24.1
Oncocytic cell	2	1.6
mucous cells	1	0.8

Regarding the morphological patterns of the epithelial component, trabeculae formation was found in 90% of the cases, the next common morphological pattern was ductal formation it was found in 78% of the cases (Figure 2), followed by solid and cystic formation, found in 42% and 23% of the cases respectively.

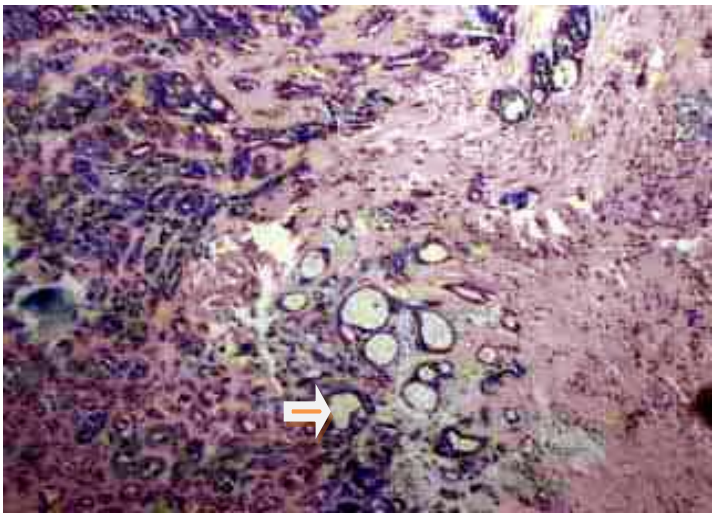


Figure2. PA shows trabeculae(arrow head) and ductal morphological patternarrow (H&EX4)

Concerning the stromal components, myxoid component was the commonly seen component (Figure 3), it was found in (80%) of the cases, followed by chondroid (Figure 4) and hyaline components (Figure 5), they were found in (54% and 48%) of the cases. On the other hand, calcified component was considered as an occasional finding; it was found in 7 cases only, as shown in (Table5).

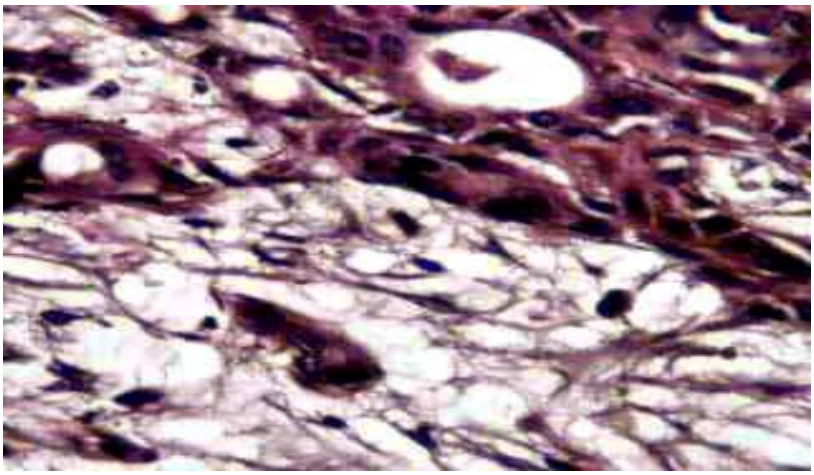


Figure3. PA showing myxoid background (H&E 20X)

Table 5. The morphological pattern of the epithelial and the stromal components of the study sample

Morphological pattern				Stromal components			
Trabeculae No. (%)	Ductal No. (%)	Solid No.(%)	Cystic No.(%)	Myxoid No.(%)	Chondroid No.(%)	Hyaline No.(%)	Calcified No.(%)
108(90)	91(76)	50(42)	28(23)	96(80)	65(54)	57(48)	7(6)

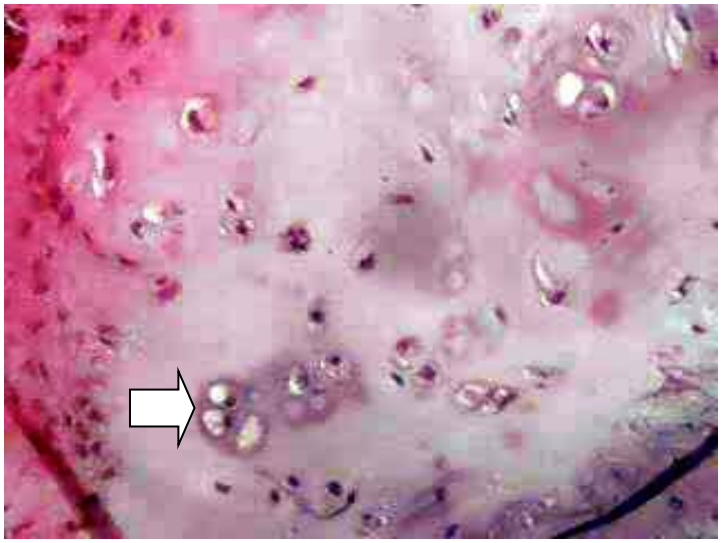


Figure4. PA showing chondroid background (H&E 10X)

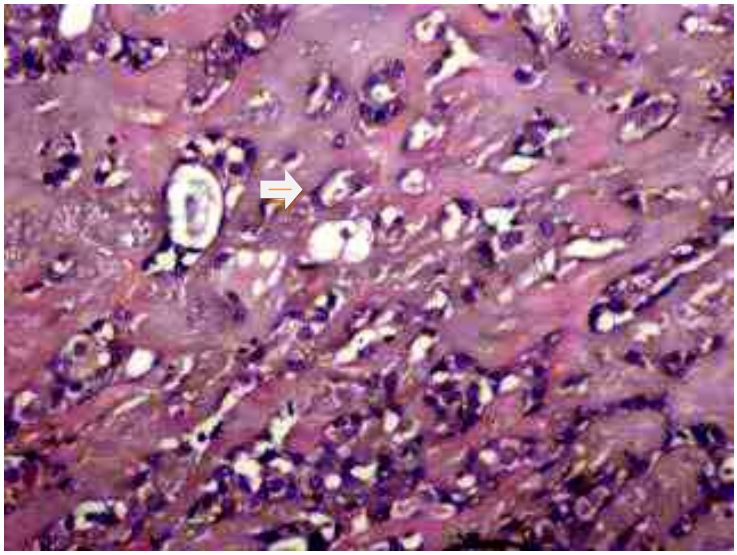


Figure5. PA shows hyaline background(H&E10X)

Discussion

PA is regarded as a slow-growing benign tumor that can affect all the salivary gland groups. Most commonly arising in the parotid gland, less frequently affecting the minor salivary glands and the submandibular glands, and occasionally affecting the sublingual group of salivary glands [11].

The present study showed that PA was more frequent in the parotid gland than the other salivary glands. Females were more frequently affected than males and the peak incidence was in the third-fifth decades of life, similar findings were reported in several previous related studies [12-15].

Inspite of the presence of a large variety of histological elements, the diagnosis of PA depends mainly on the presence of epithelial and mesenchymal like tissue as a major diagnostic feature. The relative proportion of these two elements has been used to subclassify PA into stoma – rich, cellular rich and classic types, however, this classification

doesn't imply any therapeutic or diagnostic importance [16].

In this study, stroma-rich subtype corresponded to (43%) of the cases, cell-rich (38%) and classic type (19%), interestingly, these results are close to those reported by Stennert *et al.* [17] and Paris *et al*. [18].

The current study revealed plasmacytoid cells predominance followed by cuboidal cells and spindle cells predominance respectively. Similar findings were reported by Ito *et al*, 2009 [19]. They explained the predominance of these cells over the other types that these cells appear to be in transition from one form to the other. Moreover, oncocytic and mucoid cells were the least presented cell types and were considered occasional findings, this again comes in accordance to the findings of Ito *et al,* 2009 and Takeda *et al*. [19,20].

Concerning the stromal components, in the present work myxoid component was the commonly seen area, it was found in (80%) of the cases, followed by chondroid & hyaline components respectively. On the other hand, calcified components were considered as occasional findings, as they were found in only 7 cases. These findings agree with those recorded by Ito *et al.*[19] who explained the presence of hyalinization to be related to an aggressive behavior or malignant transformation of PA. In summary, the present study demonstrated a wide variety of cells, stromal components and, morphological characteristics present in PA of the salivary glands. Since PA is the most frequent salivary gland neoplasm and can resemble other salivary gland tumors, the knowledge about these variation is essential for a correct diagnosis. However, these histological and morphological variations have no therapeutic or prognostic value.

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Authors Contribution

Dr.Sarkis and Dr.Al-Drobie: Selection, sectioning and processing of the studytissue specimen, pathological microscopic examination of the tissue section. Dr.Majeed: Data collection regarding clinico-pathological information, article editing regarding writing,organization and clinical information. Dr.Al-Marzooq: Data collection, clinical information, references, statistical analysis.

Conflict of interest

The authors have no conflict of interest to declare.

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Post-operative Hypocalcemia among Ongoing Patients After Total and Subtotal Thyroidectomy

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Abstract

Background Hypocalcemia is a major post-operative complication of total thyroidectomy, causing severe symptoms and increasing hospitalization time. The primary cause is secondary hypoparathyroidism following damage to or devascularization of one or more parathyroid gland during surgery.

Objective To identify the occurrence rate of post-operative hypocalcemia as an indicator of parathyroid gland function and its relation to the type of the surgical procedure of thyroidectomy whether it's a subtotal or total thyroidectomy.

Methods One hundred and ninety patients with total and subtotal thyroidectomy were selected in this study (144 females and 46 males). The patients were divided into 2 groups, group 1 (95 patients) represent the patients with total thyroidectomy, and group 2 (95 patients) represent the patients with subtotal thyroidectomy. Serum calcium and parathyroid hormone were done pre-operatively and post-operatively for all patients with a follow-up for serum calcium for 6 months.

Results Of the total number serum calcium levels decreased from pre-operative levels in 156 patients (82%), but still within normal range (2.1-2.6 mmol/L). The overall incidence of transient hypocalcemia was 22 % (42 patients), 35 patients belong to group 1 and 7 patients to group 2, and that of permanent hypocalcemia (hypocalcemia persisted at the 6 months assessment) was 2.6 % (5 patients), 4 patients belong to group 1 and one patient to group 2. Most of the patients with hypocalcemia were asymptomatic 19.4% (n=37) and did not require calcium supplementation. Symptomatic hypocalcemia occurred in 5.2 % (n =10) patients. It was found that the overall incidence of hypocalcemia after thyroidectomy was 24.7 % (22% transient and 2.6% permanent).

Conclusion It could be concluded that, insuring the integrity of parathyroid glands is important to avoid post-thyroidectomy hypocalcemia. If incidental removal or devascularization of the parathyroid glands is noted, parathyroid auto-transplantation should be done.

Keywords Post-operative hypocalcemia, total thyroidectomy, subtotal thyroidectomy.

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List of abbreviations: MNG= Multi-nodular goiter, pg= pico gram, PTH = Parathyroid hormone, TNG = Toxic-nodular goiter

Introduction

Calcium regulation is essential for normal cell function in the human being. It plays an important role in neural transmission, membrane permeability, skeletal structure, and blood coagulation.

Approximately 99% of calcium is found in bone, and less than 1% is found in extracellular fluid. It is this extra-cellular calcium that is critical for normal physiological function.

Transitory hypocalcemia is one of the most common complication following thyroid surgery. The reported incidence ranges from 16.5 to 71% [1,2].

Permanent hypocalcemia is less frequent following thyroidectomy, occurring in 1.5-1.8% [2].

The cause of post-surgery hypocalcemia is inadequate secretion of parathyroid hormone (PTH) by the parathyroid glands, results from direct injury to, devascularization of, or accidental removal of the parathyroid glands [3]. Other possible causes are hemodilution, hypoalbuminemia, and changes in peripheral sensitivity to parathyroid hormone [4].

Theodore Kocher recognized tetany as the main complication after thyroid surgery as early as 1883. Tetany was believed to be caused by hypothyroidism until Moussu (1898) could treated this condition with a parathyroid gland extract [5].

Depending on the degree of parathyroid gland damage, hypocalcemia may be temporary lasting for few days to few months, or permanent, necessating lifelong oral calcium and vitamin D supplementation.

The immediate manifestations of hypocalcemia secondary to hypoparathyroidism are mostly neuromuscular symptoms including muscle cramps, tingling, circumoral and peripheral paresthesia, carpopedal spasm or tetany, seizures [6].

Permanent symptomatic hypocalcemia is extremely distressing, it prolongs the hospital stay and it increases the cost of treatment, and causes substantial impact on patient's health [7].

Many studies have attempted to identify risk factors that will predict those patients who will develop post-thyroidectomy hypocalcemia thereby preventing significant morbidity and mortality.

In the last few decades few authors have examined the influence of the type of the surgical procedure of thyroidectomy on the incidence of post-operative hypocalcaemia [8,9].

The aim of current study is to evaluate the occurrence rate of post-operative hypocalcemia as an indicator of parathyroid gland function and its relation to the type of the surgical procedure of thyroidectomy whether it's a subtotal or total thyroidectomy.

Methods

This observational prospective study was done on patients undergone surgery for benign and malignant thyroid diseases between 1st of January 2015 and 1st of December 2016 at Al-kindy Teaching Hospital by well-trained general surgeons.

During the study period, 190 consecutive patients undergone primary thyroid surgery were prospectively underwent analysis regarding post-operative parathyroid function. Of these, 144 (75.8%) were females and 46 (24.2%) were males with a female to male ratio of 3:1, median patients age was 42 years (range 24-65).

Informed consent to participate in this study were obtained and the study was approved by the Ethics Committee of Al-Kindy Teaching Hospital.

The following demographic data collected included age, gender, co-morbid conditions were defined. Other variables recorded included length of stay (LOS), and signs or symptoms of hypocalcemia, indication for surgery, histopathological results, presence or absence of thyroiditis.

Initial work up included a thorough clinical examination by specialized surgical team, biochemical analysis of thyroid hormones. Ultrasonography study of the thyroid gland and neck in general was done for all patients. Fine needle aspiration cytology was done for all patients with solitary nodules and those with nodules showing suspicious features of malignancy on clinical examination or ultrasound study. CT-scanning was done when there are clinical evidences of retrosternal extension.

Thyrotoxicosis was controlled preoperatively. Assessment of vocal cords mobility by indirect laryngoscopy prior to operation was done. Surgery was performed by well-trained surgical team under general anesthesia.

For analysis the patients were divided into two groups: group 1 patients who underwent total

thyroidectomy, and group 2 patients who underwent subtotal thyroidectomy

Exclusion criterions were: (1) completion thyroidectomy following hemithyroidectomy, (2) concomitant radical/modified radical lymph node dissection, (3) patient with pre-existing hypoparathyroidism, and (4) those who underwent parathyroid gland auto-transplantation. Patients who underwent hemithyroidectomy were excluded as this operation is associated with a very low rate of post-operative hypocalcemia.

Serum calcium was estimated before commencing operation, and at 8 AM on first postoperative days until discharge. PTH measurement was done for all patients.

Patients who developed hypocalcemia symptoms at early post-operative period were reviewed monthly for one year. Serum calcium and PTH were measured on monthly visit after stopping calcium treatment for 24 hours.

Post-operative hypocalcemia was diagnosed when serum calcium level of less than 2 mmol /L (reference range 2.1-2.6 mmol/L) with or without obvious clinical features of hypocalcemia including paresthesia, muscle spasm or seizures.

Regarding PTH, values less than 15 pg/mL were considered low [(10)].

Post-operative hypocalcemia is defined as a low calcium level persist for more than 6 months after thyroid surgery.

Indication for thyroid surgery was benign thyroid disease including simple goiter and thyrotoxic goiter, patients with various types of malignant thyroid tumors and patients with thyroiditis [(11)].

A standard approach was employed with a classical collar incision the parathyroid gland was identified macroscopically, and a meticulous dissection from the thyroid gland was performed, every effort was made to identify and preserve all parathyroid glands.

For all patients with mild post-operative hypocalcemia, oral calcium supplementation 500 mg of oral calcium monocitrate (2 tablets three times daily) along with vitamin D analogue (Calcitriol 0.25 mg) twice daily independent of their clinical symptoms.

In severe symptomatic cases (patients with severe neuromuscular manifestations), intravenous calcium gluconate 10% three to six times daily according to clinical response.

Patients with postoperative hypocalcemia were discharged when their serum calcium levels higher than 2.0 mmol/L. In these patients, levels of serum calcium and PTH were measured again within 2 weeks postoperatively after cessations of calcium and calcitriol substitution therapy for 24 hrs.

A final measurement of serum calcium and PTH level was performed 6 months after thyroidectomy. If serum calcium level returned to normal within 6 months, hypocalcemia was classified as transient; in all other cases, it was classified as permanent.

The following assays were employed in each laboratory investigation: chemiluminescence assay were used for detecting PTH (deferential values: 10-65 pg/mL); total serum calcium was estimated by autoanalyzer method (normal range 2.1- 2.6 mmol/L).

Statistical analysis

Data processing and statistical analysis were done by using mini-tab V.16 processor.

Results

One hundred ninety patients who met the inclusion criteria were included in this study. Of these, 95 patients (50%) were managed by total thyroidectomy (group 1) and 95 patients (50%) were treated by subtotal thyroidectomy (group 2). There were 144 (75.8%) females and 46 (24.2%) males. The mean age was 46 years (range 27 - 68 years).

Of the total number, serum calcium levels decreased from preoperative levels in 156 patients (82%), but still within normal range (2.1- 2.6 mmol/L)

The overall incidence of transient hypocalcemia was 42 patients (22%), 35 patients belong to group 1 and 7 patients to group 2, and that of permanent hypocalcemia (hypocalcemia persisted at the 6 months assessment) was 5

patients (2.6%), 4 patients belong to group 1 and 1 patient to group 2.
Most of the patients with hypocalcemia were asymptomatic, 37 (19.4%) and did not require calcium supplementation. Symptomatic hypocalcemia occurred in 10 (5.2%) patients.
Demographic and clinical characteristics of both groups are shown in Table 1.

Table 1. Incidence of postoperative hypocalcaemia in relation to different demographic and clinical variables (N = 190)

Parameter		Total No. (%)	Group 1	Group 2
Gender	Male	46 (24.2%)	23	23
	Female	144 (75.8%)	72	72
Age at the time of surgery	20-29 years	28 (14.7%)	7	21
	30-39 years	85 (44.7%)	39	46
	40-49 years	57 (30.0%)	33	24
	50-59 years	16 (8.4%)	9	7
	60-70 years	4 (2.1%)	2	2
Indication of surgery	Benign disease	70 (36.8%)	10	60
	Malignant disease	34 (17.9%)	32	2
	Thyrotoxic disease	65 (34.2%)	50	15
	Thyroiditis	21 (11.0%)	3	18

Hypocalcaemia was detected in the first postoperative day in 17 patients (8.9%), 15 patients from group 1 and 2 from group 2, and delayed up to 3rd postoperative day in 12 patients (6.3%), 9 patients from group 1 and 3 from group 2. details are shown in table 2.

Table 2. Time of onset of hypocalcemia in 47 patients with post-operative hypocalcemia

Time	No. of patients with hypocalcemia		
	Total	Group 1	Group 2
1st post-operative day	17	15	2
2nd post-operative day	8	6	2
3rd post-operative day	4	3	1
4th post-operative day	8	6	2
5th post-operative day	6	4	2
6th post-operative day	4	4	0

On analyzing various clinical situations independently, both transient and permanent hypocalcemia were significantly associated with hyperthyroidism (Grave's disease and toxic nodular goiter (TNG)).
Thyroidectomy for thyroid gland malignancy was associated with high incidences of both transient and permanent hypocalcemia.
Among the 21 patients with classic form of thyroiditis (Hashimoto thyroiditis and

lymphocytic thyroiditis), 3 patients (1.5%) developed low calcium levels post-operatively, while 4 patients (2.1%) from those with simple goiter developed post-operative hypocalcemia.
Of all risk factors, total thyroidectomy was most likely to result in postoperative hypocalcemia than subtotal one.
Gender and patients age at the time of surgery also emerged as independent predictors of postoperative hypocalcemia.
Patients with 20-30 years group and those of 50-60 years age had significantly lower likelihoods of developing hypocalcemia postoperatively as shown in table 3.
On average, patients undergoing thyroidectomy had a hospital length of stay of 1.9 days.

Table 3. Prevalence of hypocalcemia in different clinical and pathological situations

Pathological condition	No. of pts with hypocalcemia /total	Mean S. Ca mmol/L	Sex f/m	Age groups (years)				
				20-30	30-40	40-50	50-60	60-70
Simple MNG	4/70	1.86	4/0	1	1	1		1
Toxic goiter	19/65	1.81	14/5	1	9	9	1	
Malignancy	21/34	1.79	14/7	1	11	7	1	1
Thyroiditis	3/21	1.79	2/1		1	1	1	

Discussion

Postoperative hypocalcemia is a multifactorial, problematic source of morbidity for patients with thyroid surgery; resulting prolonged hospital stays with increase treatment costs [12].
Recently, many authors have advocated measurement of the PTH level several hours after operation in order to predict the development of hypocalcemia [13,14]. The principle behind this, is that the half-life of PTH is 2 to 5 minutes [15]; thus, the level of PTH in the immediate postoperative period, provides a very precise indication of parathyroid function. This practice has not been widely adopted [16]. However, the fast PTH assay is not available in the majority of the medical centers in our country.
In this study, serum calcium level was also analyzed as an indicator for hypoparathyroidism, furthermore, we analyze PTH level preoperatively and then one monthly just to ascertain that post-operative hypocalcemia is due to low level of PTH.
It was found that the overall incidence of hypocalcemia after thyroidectomy was 24.7% (22% transient and 2.6% permanent).
Few authors had noted a 50% of transient and 4% permanent hypocalcemia following thyroidectomy [17,18].
In this work, the incidence of permanent hypocalcemia appears low compared to some other comparable studies, this may be explained by the surgical technique we adopted (capsular dissection).
The current literature indicates that the incidence of hypocalcemia may be affected by a number of risk factors. The most important factor is the extent of surgery. More extensive thyroidectomy procedures, for instance, result in a greater incidence of hypocalcemia, though the exact mechanism behind the association is unclear [19].
Study analysis demonstrates that, hypocalcemia occurred significantly more often after total thyroidectomy than after subtotal thyroidectomy. Incidental parathyroidectomy is believed by many authors to explain the increased incidence of hypocalcemia with more extensive surgery [20].

Post-operative hypocalcemia should clearly be considered in discussions about the appropriate extent of thyroid surgery. Particularly with total thyroidectomy procedure, surgeons may take corrective measures to reduce the incidence of hypocalcemia and improve long-term outcomes. For instance, if incidental removal or devascularization of the parathyroid glands is noted, parathyroid auto-transplantation should be done to reduce the occurrence of permanent hypocalcemia among those patients [21].

When performing a total thyroidectomy with central compartment dissection, the inferior parathyroid glands are at risk of vascular damage or even inadvertent removal during clearance of pre-tracheal and para-tracheal lymph-nodes in the median part of the neck [22]. American Thyroid Association had revised the recommendation for central neck dissection and recommended prophylactic minimum dissection in selected patients only [23].

Blood supply of parathyroid glands was studied thoroughly by William Halsted as early as 1907 [24]. The technique of capsular dissection of thyroid gland ensures intact parathyroid glands with its blood supply. Sosa et al. noted that lateral ligation of inferior thyroid arteries (ITA) as a strong predictor of hypocalcaemia [25].

Some authors suggest that a surgeon's skill and experience affect the incidence of post-thyroidectomy hypocalcemia. However, one study found that patients operated on by surgical trainees had complications that were comparable to patients operated on by well-trained consultant surgeons [26].

In accordance with our findings, Cheah et al. [27] found that a greater proportion of the women in their study were affected by thyroid disease, but that there was no association between patient sex and hypoparathyroidism. The association between postoperative hypocalcemia and female gender also found in the literature may be due to women being more prone to calcium and vitamin D deficiency than men [28].

The incidence of hypocalcemia is relatively more common after thyroidectomy for thyrotoxicosis. This finding was observed by Michie and colleagues as early as 1965 [29].

Hypocalcemia was observed in some earlier studies associated with Grave's disease as noticed in some previous studies [28,30].

McHenry [31] found a strong association between post-thyroidectomy hypocalcemia and Graves' disease and explain this relationship by that the location and preservation of the parathyroid glands in patients with Graves' disease is more difficult. This is also in consistent with our finding in the present study.

Hyperthyroidism may lead to demineralization of bone. Transient hypocalcaemia may be related to osteodystrophy seen in thyrotoxicosis. Furthermore, surgical manipulation may cause thrombosis of parathyroid vessels due to autoimmune process of Grave's disease [32].

The results of present analysis demonstrate that, the incidence of hypocalcemia following thyroid surgery is more commonly associated with total thyroidectomy especially for malignant conditions Zedenus et al. [32] support our finding, the possible explanation is that the surgery in malignant disease is more extensive and more difficult due to adhesion, fibrosis of the thyroid tissues comparing with benign disease.

Finding of this study regarding the higher incidence of hypocalcemia among thyroid cancer patients is consistent with previous studies [33], who believe that malignancy is usually treated with a more aggressive approach to thyroid surgery, thereby leading to incidental damage or removal of the parathyroid gland and hypocalcemia.

The current study concluded that a more aggressive approach to thyroid surgery, leading to incidental damage or removal of the parathyroid gland with subsequent hypocalcemia. On the other hand, refined

surgical approach may decrease the incidence of hypocalcemia following thyroid surgery.

Preservation of parathyroid glands and its blood supply is essential to avoid this complication. If incidental removal or devascularization of the parathyroid glands is noted, parathyroid auto-transplantation should be done.

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Conflict of interest

The author has no conflict of interest to declare.

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Evaluation of the Anti-Inflammatory Effect of Telmisartan As an Adjuvant Therapy to NSAID in The Management of Knee Osteoarthritis; A Clinical Prospective Study

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Abstract

Background Osteoarthritis (OA) is a degenerative joint disease caused by gradual loss of cartilage. Telmisartan is an angiotensin II receptor antagonist, and act as a partial agonist on the nuclear peroxisome proliferator-activated receptor-gamma (PPAR-γ), that has been reported to exert anti-inflammatory effects.

Objective To evaluate the potential anti-inflammatory effect of telmisartan in patients with knee OA.

Methods Forty-two patients with painful knee OA were allocated into 2 groups, group (1): patients treated with naproxen tablets (500 mg/12 hr), telmisartan tablets (40 mg/day) and omeprazole (20 mg/day) for 3 months, while group (2): patients treated with naproxen tablets (500 mg/12 hr) and omeprazole (20 mg/day) for 3 months. The serum levels of IL-1β, high-sensitivity C-reactive protein (hs-CRP), TNF-α and erythrocyte sedimentation rate (ESR) were measured before and after 3 months of treatment.

Results Telmisartan when used in combination with naproxen resulted in significant decrease in serum levels of IL-1β and hs-CRP, higher than that produced by naproxen when used alone. The mean TNF-α level and ESR was decreased non-significantly in both study groups.

Conclusion Administration of telmisartan as an adjuvant therapy to naproxen in knee OA patients produced a significant decrease in the serum levels of IL-1β and hs-CRP, though no clear effect on TNF-α and ESR was noticed after 3 months treatment. Accordingly, many promising preventive strategies emerged from the available results since telmisartan relatively reduce the inflammatory burden in OA patients. The dose and duration of telmisartan treatment in this study did not indicate a risk of hypotension.

Keywords Telmisartan, naproxen, osteoarthritis, cytokines

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List of abbreviations: CK = Creatine kinase, COX-2 = Cyclooxygenase-2, DMOADs = Disease-modifying osteoarthritis drugs, ELISA = Enzyme-linked immunosorbent assay, ESR = Erythrocyte sedimentation rate, hs-CRP = High-sensitivity C-reactive protein, IL-17 = Interleukin-17, KL = Kellgren-Lawrence, IL-1β= Interleukin-1 beta, IL-6 = Interleukin-6, iNOS = Inducible nitric oxide synthase, MMP-1 = Matrix metaloproteinase-1, MMPs = Matrix metalloproteinases, mPGES-1 = Microsomal prostaglandin E synthase-1, NSAIDs = Non-steroidal anti-inflammatory drugs, OA = Osteoarthritis, PGE2 = Prostaglandin E2, PPAR-γ = Peroxisome proliferator-activated receptor-gamma, TENS = Transcutaneous electrical nerve stimulation, TNF-α = Tumor necrosis factor-alpha

Introduction

Osteoarthritis (OA) is a degradative joint disease occurs due to gradual loss of cartilage and resulting in bony spurs and cysts formation at joint edges [(1)]. It is a pathological result of several disorders, which cause functional impairment of synovial joints [(2)]. Primary OA is related to aging, while secondary OA tends to show up earlier in life, often due to a specific cause such as an injury, diabetes, or obesity [(3)]. The major risk factor for OA are family history [(4)], obesity, older age, female gender, previous knee injury and the presence of hand OA/Heberden's nodes [(5)]. Clinical features of OA include pain, stiffness [(6)],

crepitus, restricted joint motion, joint deformity, joint tenderness, effusion and muscle atrophy [7]. The diagnosis of OA is usually based on clinical and radiological features [8]. A typical plain X-ray of OA may elucidate one or more of the following features; (1) Joint space narrowing, (2) Subchondral sclerosis, (3) Peripheral osteophytes, (4) Subchondral cysts [9]. Normal adult articular cartilage is made up of extracellular matrix (water, collagen, proteoglycans and calcium salt) and chondrocytes [10]. Inability of chondrocytes to provide homeostasis between degradation of extracellular matrix ingredients and synthesis lead to OA [11]. A severe subchondral bone modifying lead to sclerosis of the tissue noticeable radiographically. These changes are often coexisted with subchondral cysts formation due to resorption [12]. Synovitis is believed to be induced at first by the cartilage matrix proteolytic degradation products [13]. Synoviocytes can produce inflammatory cytokines; proinflammatory cytokines such as interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), these cytokines are considered to interpose the degeneration related to OA [14]. Non-pharmacological management of OA includes exercise [15], weight reduction [16], balneotherapy [17], knee braces [18], self-management [19], transcutaneous electrical nerve stimulation (TENS) [20], acupuncture [21] and nutraceuticals [22]. Pharmacological treatment of OA includes acetaminophen [23], oral non-steroidal anti-inflammatory drugs (NSAIDs) [24], topical NSAIDs [23], intraarticular (IA) injections of corticosteroids [25], IA injection of hyaluronic acid [23], opioid analgesics [26], and disease-modifying osteoarthritis drugs (DMOADs) [27]. Surgical procedures are generally accepted in patients who are not obtaining adequate pain relief with a combination of pharmacological and non-pharmacological treatments [28]. The discovery that several factors such as cytokines or prostaglandins can raise the levels of matrix metalloproteinases (MMPs) by chondrocytes led to an inflammatory concept. OA is a complicated disease with inflammatory mediators liberated by the cartilage, bone and synovium [29,30]. These inflammatory mediators represent potential targets for therapeutic interventions designed to reduce both symptoms and structural joint damage in OA [31]. Cyclooxygenase-2 (COX-2) is a major Prostaglandin E2 (PGE2) synthetic enzyme and implicated in the pathogenesis of inflammation and pain in OA [32]. Cyclooxygenase-2 is upregulated in inflamed joint, its accountable for increased level of PGE2 in OA joint. PGE2 is implicated in inflammation, apoptosis, angiogenesis, and potentially morphological alteration in arthritis [33]. Over expression of COX-2 is mostly stimulated by pro-inflammatory mediators such as IL-1β, TNF-α, and IL-6 (interleukin-6) stimulation [34]. Proinflammatory cytokines are thought to have an essential role in OA pathophysiology, in particular IL-1β and TNF-α [35]. TNF- α operate the inflammatory cascade, while IL-1β is significant for cartilage destruction [36].

Telmisartan a highly selective AT1 receptor (Angiotensin II receptor, type 1) antagonist, it is a partial agonist on peroxisome proliferator-activated receptor-gamma (PPAR-γ), which improved to have anti-oxidative and anti-inflammatory actions. PPAR-γ partial agonist activity improves metabolic and inflammatory cascade [37]. Peroxisome proliferator-activated receptors (PPARs) are present in three types: PPAR-α, PPAR-β/δ, and PPAR-γ [38], they produce anti-inflammatory actions by preventing pro-inflammatory cytokines induction, adhesion molecules and extracellular matrix proteins production, or by enhancing the induction of anti-inflammatory particles. In addition, PPARs control the proliferation, differentiation and survival of immune cells as macrophages, B cells and T cells [39]. Several lines proposed that the PPARγ activation in OA may be therapeutically interested, PPARγ is functionally active in chondrocytes, and its activation modify the expression of several genes demonstrated to

be important in OA pathogenesis. PPARγ activation inhibits production of nitric oxide synthase, matrix metaloproteinase-13 (MMP-13), COX-2, and microsomal prostaglandin E synthase-1 (mPGES-1) in chondrocytes by inhibiting IL-1 induction [40,41]. In addition, activation of PPARγ inhibits proteoglycan degeneration which caused by IL-1 induction [42]. PPARγ activation in synovial fibroblasts inhibits IL-1, TNF-α and matrix metaloproteinase-1 (MMP-1) expression [43]. The effect of PPARγ activators has been demonstrated in many animal models of OA as guinea-pig [44]. Treatment of human OA chondrocytes with PPARγ agonist decreased nitric oxide, PGE2, inducible nitric oxide synthase (iNOS) and COX-2 expression [45]. The induction of NO production by interleukin-17 (IL-17) and TNF-α was also inhibited upon PPARγ activation [45]. Decreased expression of PPARγ in OA cartilage may resulted in increased production of inflammatory and catabolic genes, enhancing cartilage degradation. Diminished PPARγ expression in OA cartilage supporting PPARγ role in OA, and increase the possibility that upregulation of PPARγ may be beneficial in OA treatment [46]. Clockaerts et al concluded that PPAR-α activation results in anti-inflammatory effects in human OA cartilage [47]. Telmisartan decreased formaldehyde-induced chronic inflammation in rats in a dose-dependent pattern [48]. This study was designed to evaluate the potential anti-inflammatory effect of telmisartan in patients with knee OA.

Methods

This is a prospective randomized controlled open-label interventional study to evaluate the anti-inflammatory effect of telmisartan in the treatment of patients with painful knee OA. The study was conducted during the period from September 2015 to May 2016 and carried out on 42 randomly selected Iraqi patients newly diagnosed with knee OA; at the outpatient clinic at Baghdad Teaching Hospital. All patients have symptomatic and radiologic evidence of OA in one or both knee joints. Ethical approval was obtained from Ethics Committee by Pharmacy College/ University of AL-Mustansyriah, and Baghdad Teaching Hospital. All subjects gave written informed consent to participate in the study. Patients were diagnosed based on clinical and radiological features depending on Kellgren-Lawrence (KL) classification system [49]. Certain exclusion criteria were followed to avoid interference with the study design, include: patients with hypertension, ischemic heart diseases, asthma or diabetes mellitus; patients who are on treatment with drugs, which interfere with the tested drugs, patients with peptic ulcer, patients with end-stage radiological events of joint destruction, patients with positive history of allergic reactions to any one of the known tested drugs, and pregnant or lactating patients. The selected patients were allocated into two main groups as following: group (1): includes 22 patients with knee OA treated with naproxen tablets (500 mg/12 hr), telmisartan (Micardis® tablets 40 mg/day) and omeprazole (20 mg/day) for three months, while group (2): includes 20 patients with knee OA treated with naproxen tablets (500 mg/12hr) and omeprazole (20 mg/day) for three months. Omeprazole 20mg used in this study as a primary prophylaxis against NSAID-associated ulcer disease or dyspeptic symptoms.

Ten milliliters of venous blood were drawn from fasting patients, at baseline and after three months of treatment to follow up the changes in IL-1β, high-sensitivity C-reactive protein (hs-CRP), TNF-α and erythrocyte sedimentation rate (ESR). Blood samples were allowed to clot, then separated by centrifuge at speed of 3000 rpm for 10 minutes and stored at (-40 °C) until the time of examination for IL-1β, TNF-α and hs-CRP, unless worked immediately for the evaluation of ESR. Serum level of IL-1β, TNF-α and hs-CRP were determined using commercial enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Biotechnology, China), while ESR was measured using Westergren method [50].

Statistical analysis

The statistical analysis system- Minitab 16.1 (2010) was used. Data were expressed as (mean ± standard error "SE"). Chi-square test was utilized to detect significant differences among demographic variables, while paired t-test was used to compare between pre- and post-treatment results in same group, independent t- test used to compare pre- or post- treatment between group 1 and group 2. P value (<0.05) considered significant difference, P value (<0.01) considered highly significant difference.

Results

The demographic and baseline disease characteristics were evenly distributed for both groups as summarized in table 1.

After adjustment of baseline mean of patients' demographic and disease characteristics. There was no significant statistical difference (P>0.05) between both groups in respect to age, gender, X-ray finding, BMI, waist circumference and joint deformity finding (table 1).

Table 1. Patients demographics and disease characteristics

Variable		Study Groups		*P*-value
		Group 1	Group 2	
Age (years) mean±SE		51.91±1.9	47.65±1.4	0.084 NS
BMI (kg/m²) mean±SE		35.62±1.2	33.74±1.5	0.340 NS
Waist circumference (cm) mean±SE		113.95±1.9	109.30±3.0	0.196 NS
Gender (n (%))	Female	16 (72.72)	17 (85.0)	0.333 NS
	Male	6 (27.27)	3 (15.0)	
X-ray finding (n (%))	Grade I	5 (22.7)	6 (30.0)	0.618 NS
	Grade II	6 (27.3)	7 (35.0)	
	Grade III	11 (50.0)	7 (35.0)	
Joint deformity finding (n (%))	Negative	15 (68.0)	14 (70.0)	0.899 NS
	Positive	7 (32.0)	6 (30.0)	

Data presented as mean±standard error (SE), number of patients (n) and percentage (%). NS: No significant differences (*P*>0.05), (*) significant differences (*P*<0.05), (**) highly significant differences (*P*<0.01). BMI: body mass index

The results showed that telmisartan, when used in combination with a non-steroidal anti-inflammatory drug (naproxen) resulted in significant decrease (P<0.05) in the serum levels of IL-1β (table 2, figure 1), and a highly significant decrease (P<0.01) in the serum levels of hs-CRP (table 3, figure 2), higher than that produced by naproxen when used alone. While the serum levels of TNF-α level and ESR was decreased non-significantly (P>0.05) in both study groups, as shown in (table 4, figure 3), and (table 5, figure 4) respectively.

Table 2. Effects of treatment with naproxen alone, and combination of naproxen+ telmisartan on serum level of interleukin-1 beta (IL-1β) in osteoarthritic patients

IL-1β (pg/ml)	Study Groups		*P*-value
	Group 1	Group 2	
Pre-treatment	68.2±12.1	135.4±37.9	0.105 NS
Post-treatment	37.3±4.5	84.4±16.0	0.011*
***P* – value**	0.016*	0.174 NS	----------

Data presented as mean±standard error (SE), NS: No significant differences (*P*>0.05), (*) significant differences (*P*<0.05), (**) highly significant differences (*P*<0.01)

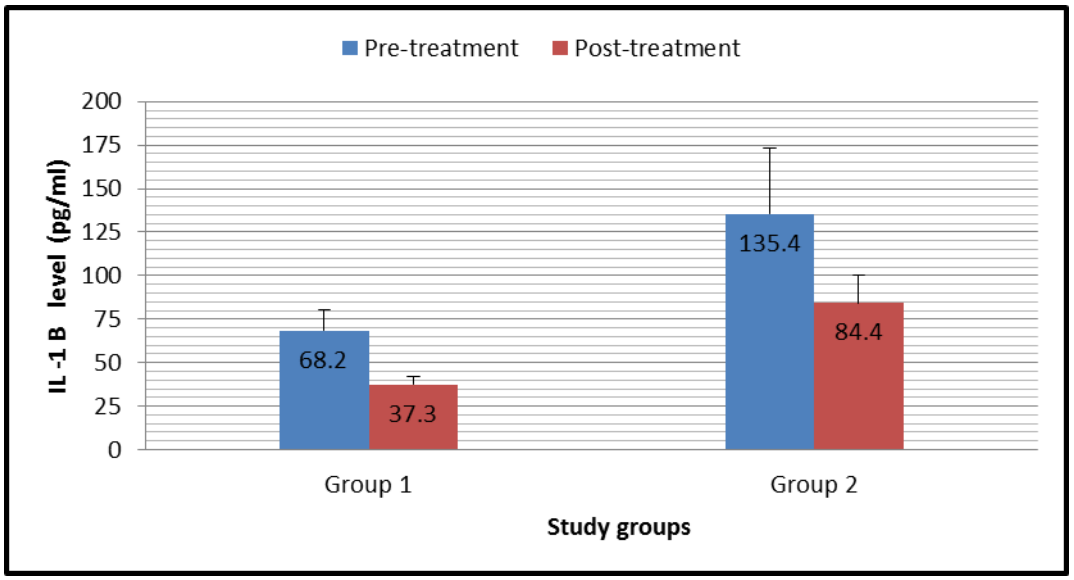

Figure 1. Effects of treatment with naproxen alone, and combination of naproxen+ telmisartan on serum level of interleukin-1 beta (IL-1β) in osteoarthritic patients

Table 3. Effects of treatment with naproxen alone, and combination of naproxen+ telmisartan on serum level of high sensitivity c-reactive protein (hs-CRP) in osteoarthritic patients

hs-CRP (mg/l)	Study Groups		*P*-value
	Group 1	Group 2	
Pre-treatment	8.74±1.2	7.91±0.9	0.589 NS
Post-treatment	6.12±0.8	6.24±0.8	0.916 NS
***P* – value**	0.009**	0.012*	----------

Data presented as mean±standard error (SE), NS: No significant differences (*P*>0.05), (*) significant differences (*P*<0.05), (**) highly significant differences (*P*<0.01)

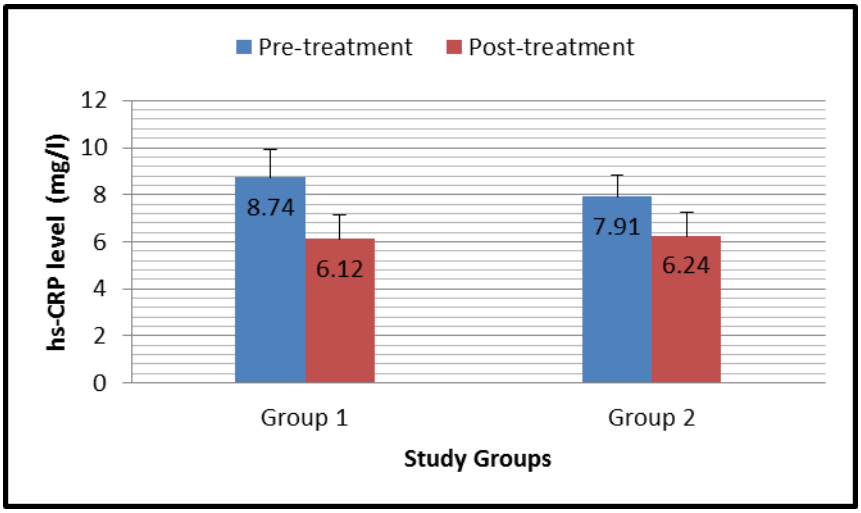

Figure 2. Effects of treatment with naproxen alone, and combination of naproxen+ telmisartan on serum level of high sensitivity c-reactive protein (hs-CRP) in osteoarthritic patients

Table 4. Effects of treatment with naproxen alone, and combination of naproxen+ telmisartan on serum level of tumor necrosis factor-alpha (TNF-α) in osteoarthritic patients

TNF-α (pg/ml)	Study Groups		*P*-value
	Group 1	Group 2	
Pre-treatment	43.0±11.4	46.81±9.1	0.793 ^{NS}
Post-treatment	38.3±10.0	37.9±11.0	0.975 ^{NS}
***P* – value**	0.520 ^{NS}	0.400 ^{NS}	----------

Data presented as mean±standard error (SE), NS: No significant differences ($P>0.05$), (*) significant differences ($P<0.05$), (**) highly significant differences ($P<0.01$)

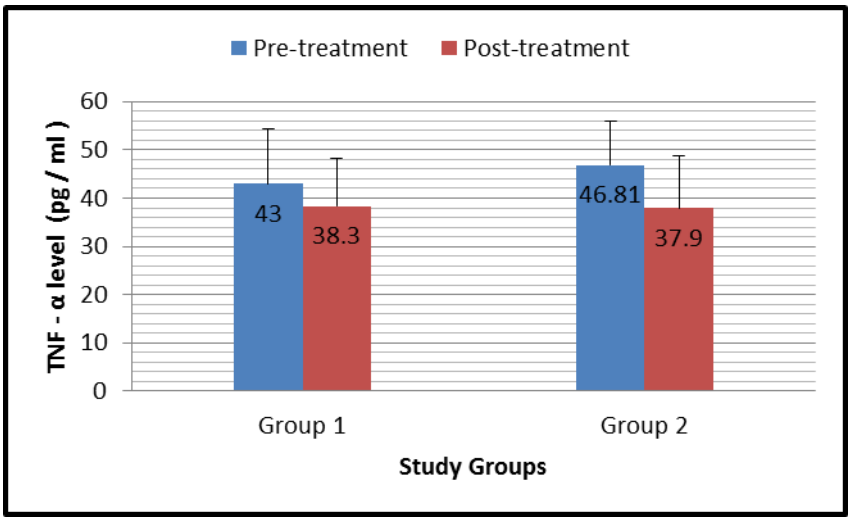

Figure 3: Effects of treatment with naproxen alone, and combination of naproxen+ telmisartan on serum level of tumor necrosis factor-alpha (TNF-α) in osteoarthritic patients

Table 5. Effects of treatment with naproxen alone, and combination of naproxen+ telmisartan on erythrocyte sedimentation rate (ESR) in osteoarthritic patients

ESR (mm/hr)	Study Groups		*P*-value
	Group 1	Group 2	
Pre-treatment	30.27±6.6	17.55±2.1	0.079 ^{NS}
Post-treatment	19.1±3.0	14.95±1.6	0.230 ^{NS}
***P* – value**	0.105 ^{NS}	0.072 ^{NS}	----------

Data presented as mean±standard error (SE), NS: No significant differences ($P>0.05$), (*) significant differences ($P<0.05$), (**) highly significant differences ($P<0.01$)

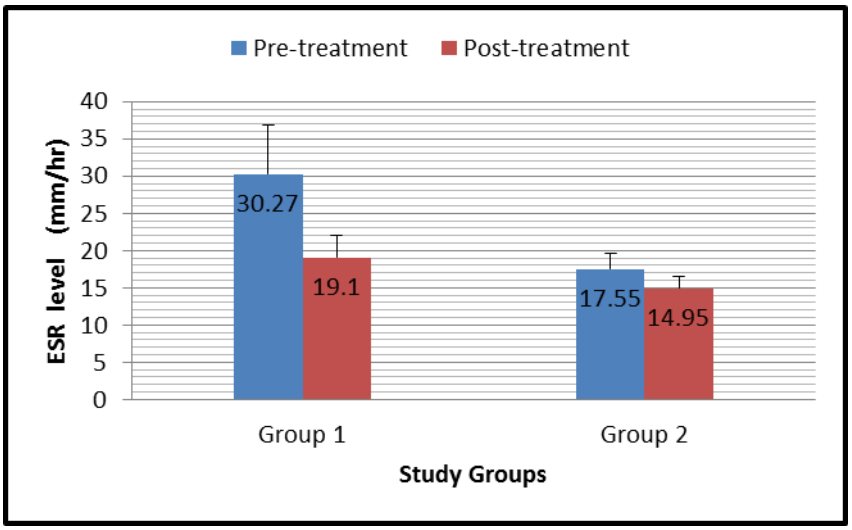

Figure 4: Effects of treatment with naproxen alone, and combination of naproxen+ telmisartan on erythrocyte sedimentation rate (ESR) in osteoarthritic patients

Discussion

Osteoarthritis is the leading cause of inability worldwide [51]. Although telmisartan was developed to treat hypertension by blocking angiotensin II (type 1) receptor, the finding that telmisartan can activate PPARγ has prospectively remarkable therapeutic modulations for treatment of other clinical diseases that might be responsive to PPARγ activators [52].

The ability of telmisartan to reduce the intensity of pain and inflammation that contributed to the pathophysiology of arthritis, with no serious adverse effects, has been reported in some animal model studies [53]. This may be considered a new therapeutic approach added to the current NSAIDs treatment for inflammation patients with knee OA. Up to the best knowledge, there is no clinical study reported for Iraqi population to explore the role of telmisartan in OA. Thus, the present study was undertaken to clinically evaluate whether or not telmisartan can reduce the intensity of inflammation in knee OA patients.

As mentioned earlier, the pro-inflammatory cytokines TNF-α and IL-1β, are among the critical mediators modulated in the pathophysiology of OA. These mediators regulate articular cartilage matrix degeneration, which makes them as major targets for treatment strategies. Although animal studies provide support for this approach, just a few studies have sought the effects of inhibiting these cytokines in OA [29].

In the present study, significant decrease of mean IL-1β level in group 1 patients was found after three months, meanwhile group 2 showed no significant decrease of this anti-inflammatory cytokine, which can be explained by the potential role of telmisartan in reduce concentrations of the pro-inflammatory cytokine IL-1β in group 1, with the fact that naproxen therapy failed to control IL-1β level in group 2. Animal study on rats with periodontitis showed that telmisartan reduces the inflammatory response. Treatment with telmisartan reduced levels of IL-1β, COX-2, MMP-2, MMP-9 and TNF-α [54]. Animal study on brains of neonatal Wistar rats was aimed to illustrate the role of angiotensin system in the modulation of glial functions and Alzheimer's disease pathology. Telmisartan highly reduced the production of TNF-α and IL1-β (by approximately 50% and 30%, respectively) which already induced by lipopolysaccharide (LPS) [55].

Animal study on ulcerative colitis in rats tested the anti-inflammatory effect of telmisartan. Telmisartan at 5 mg/kg exerts beneficial effects, these effects may be due to accelerated termination of acute inflammatory phase, due to decreasing TNF-α level [56]. As mentioned previously, telmisartan decreased formaldehyde-induced chronic inflammation in rats in a dose-dependent pattern [48]. The dose-response relationship of the anti-inflammatory activity of telmisartan was found to be relatively linear within the dose ranges utilized in the study, with best linearity between 0.6 and 3 mg/kg [48]. The present study found that TNF-α decreased in both study groups (1 and 2) after three months treatment, but not significant. Higher doses might be required to explore the effect clearly. Recent study search for the effect of telmisartan on the pro-inflammatory mediators secreted from adipocytes. Telmisartan was dissolved in a vehicle, the study showed that higher dose of telmisartan (1 µg/ml) on adipocytes culture decreased the levels of TNF-α, while lower dose of telmisartan (0.1 µg/ml) had non-significant effects on TNF-α levels [57].

Regarding hs-CRP in the present study, group 1 show a higher significant decrease in mean results of serum level of hs-CRP than group 2, suggesting the potential role of telmisartan. A study on Wistar rats was performed to research the effect of spironolactone, telmisartan, and their combination on isoproterenol-induced cardiac hypertrophy. The study concludes that chronic treatment with spironolactone, telmisartan, and their combination result in a significant decrease in the elevated levels of creatine kinase (CK) and CRP [58]. A recent study aimed to investigate the effects of telmisartan on inflammation and fibrosis after myocardial infarction in rats, the study showed that levels of CRP, TNFα, IL-6 and IL-1β were decreased markedly in telmisartan group [59].

The present study found that ESR decreased in both study groups (1 and 2) after three months treatment, but not significant. Longer duration of treatment and/or higher doses might be required to explore the effect clearly. Another study was aimed to estimate the potential effects of telmisartan in hypertensive HIV-positive patients with microalbuminuria. Males receiving stable combined antiretroviral therapy and 80 mg/day telmisartan for 6 months. A significant decrease in ESR, which may be correlated to the protective effect of telmisartan [60].

The present study has few limitations including small sample size with different disease severity, which required to verify the findings, short duration of patient follow up, as longer period may be required to explore a good result.

The current study concluded that: (1) Telmisartan when co-administered with naproxen, produced significant reduction in serum levels of IL-1β, highly significant reduction in serum levels of hs-CRP, compared to that of using naproxen alone. (2) Telmisartan when co-administered with naproxen produced non-significant reduction in TNF-α and ESR levels. The present study have few limitation including small sample size with disease severity of different stages, which required to verify the findings, short duration of patient follow up. In addition, inattention blood pressure measurement to explore the hypotensive effect of telmisartan in

normotensive patients. In the current study, many promising preventive strategies emerged from the available results since telmisartan relatively reduce the inflammatory burden in OA patients, suggesting that telmisartan plays effective role to be a good candidate as an adjuvant therapy. Further investigations for all drugs in angiotensin receptor blocker group for their pleiotropic effect must be undertaken so that new indications of these drugs can come to light.

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Authors Contribution:

Dr. Hmood and Dr. Mohammed and collected the data and analyzed it; Dr. Kamal arranged it and supervised the study; and Dr. Jasim interpreted and arranged drafting of this paper.

Conflict of interest

There is no conflict of interest that could be perceived.

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