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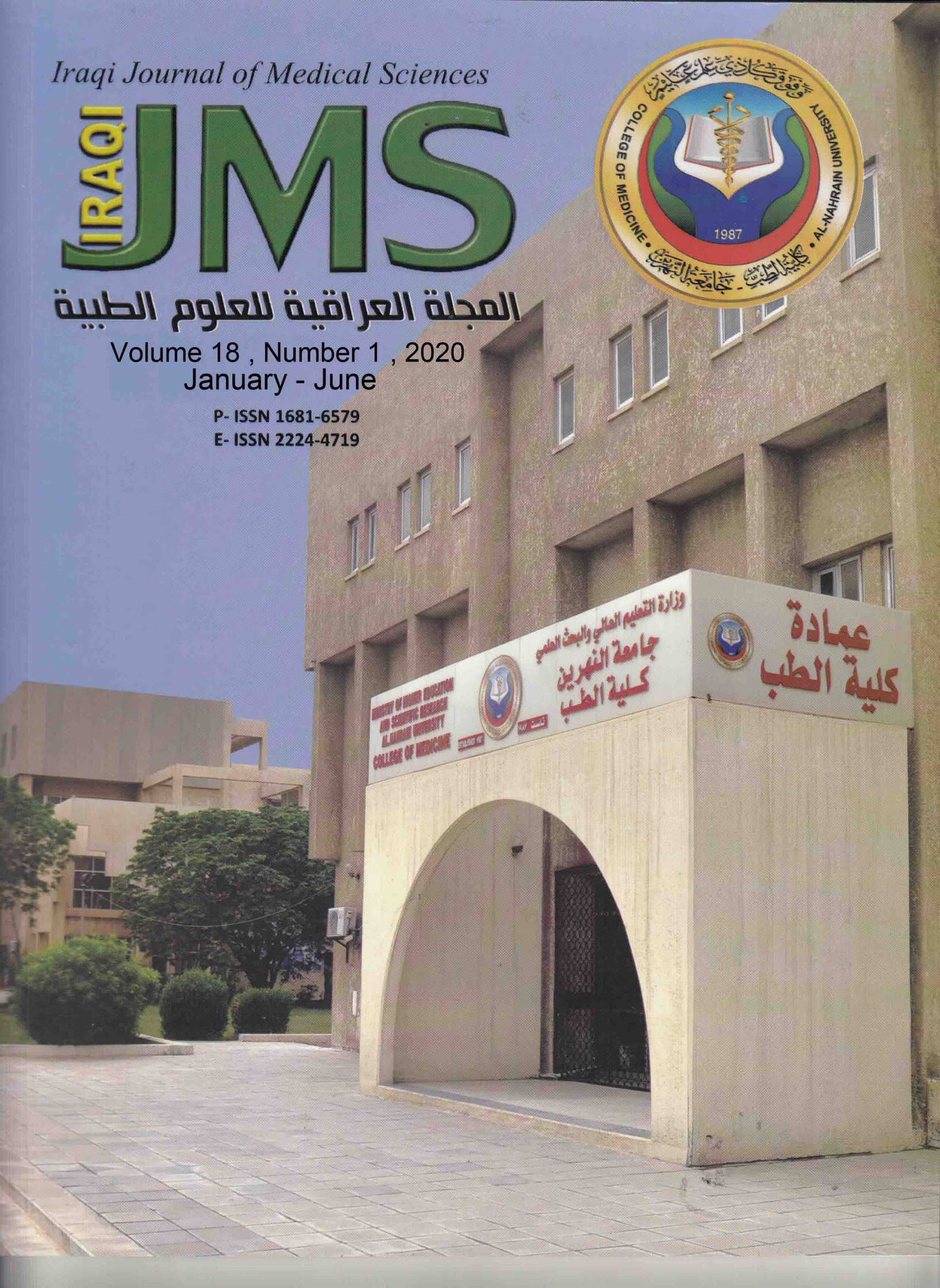

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A Review on Different Benefit for *Annona muricata* Fruits as Antioxidant

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Abstract

More than 200 chemical compounds have been identified and isolated from *Annona muricata* fruits and the most important such as alkaloids, phenols and acetogenins. Number of investigations have interested for this plant including antidiabetic, anticancer, anticonvulsant, anti-arthritic, anti-parasitic, antimalarial, analgesic hypotensive, anti-inflammatory and enhancing of immune system in addition to its content of high form antioxidants, which are vital to human health. Because these it the World Health Organization recommends the minimum consumption of fruit 120 kg/person/year.

Keywords *Annona muricata*, oxidation, health, cytotoxic activity, phytochemicals and traditional medicine

Citation Jassim HS. A review on different benefit for *Annona muricata* fruits as antioxidant. Iraqi JMS. 2020; 18(1): 1-3. doi: 10.22578/IJMS.18.1.1

Because the importance of the active materials of plants in agriculture and medicine has stimulated important scientific interest in the biological activities of these substances [1]. The appearance of *Annona muricata* (*A. muricata*) is an evergreen, the erect tree reaching from 5-8 m in height and open and the shape is a roundish canopy with large, glossy, dark green leaves. The fruits of the tree are large, heart-shaped and color green, the diameter between 15 and 20 cm [2]. *A. muricata* found in the warmest tropical region of Central and South America, Southeast Asia and Western Africa. It grows at high below 1200 m above sea level, and temperatures between 25 and 28 °C, with humidity between 60 and 80% [3].

The sixty or more kind of the genus Annona, family Annonaceae, the graviola, *A. muricata L.*, is the most tropical, the largest-fruited and it self well to preserving and processing. The leaves and bark tree contain cytotoxic acetogenins and also produces iso-quinoline and phenanthrene alkaloids. The Annonaceous acetogenins, lactones, alkaloids, tannins and coumarins are of a bioactive compound present in the Annona muricata leaves [4].

Ethno medicinal uses the fruit is as a natural medicine for arthritic pain, neuralgia, arthritis, diarrhea, dysentery, fever, malaria, parasites, rheumatism, skin rushes and worms, and it is also eaten stimulation to elevate a mother's milk after childbirth. In addition, the fruits are employed for the preparation of candy, ice creams, and syrups. In tropical Africa, the plant is used as an astringent, insecticide and pesticide agent and to treat coughs, pain and skin diseases. In India, used the fruit and flowers were employed as used against catarrh, while the root-bark and leaves as shown in figure (1) are benefit to have antiphlogistic and anthelmintic activities [2].

Traditional medicinal uses of *A. muricata* have been identified in tropical regions to treat

diverse ailments such as fever, pain, respiratory and skin illness, bacterial infections, hypertension, inflammation, diabetes, predominately breast and prostate cancer [5,6].

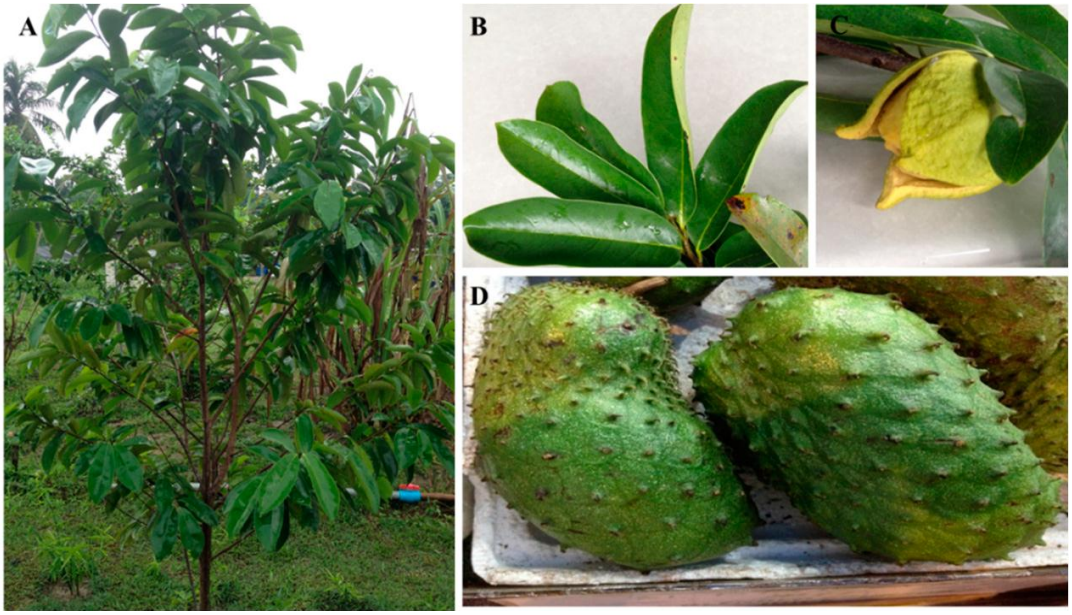

Figure 1. (A) *Annona muricata L.*; the appearance of the (B) leaves; (C) flowers and (D) fruits [2]

As shown in figure (2) the levels of non-enzymic antioxidants analyzed in fresh leaf of *A. muricata* are ascorbic acid and α-tocopherol. The ascorbic acid is distributed in both intracellular and extracellular fluid and is shown to function as scavenger for many free radical and the tocopherol is a protective agent that can act against the toxic effects of oxygen radicals within the membrane cell and can act as an excellent inhibitor of lipid peroxidation [7]. Many of bioactive compounds have been reported to be found in *A. muricata*. The major of phytochemicals has been identified by organic extract, but recently concentered have also been directed toward aqueous extracts. Several other compounds such as carbohydrates and essential oils [8,9].

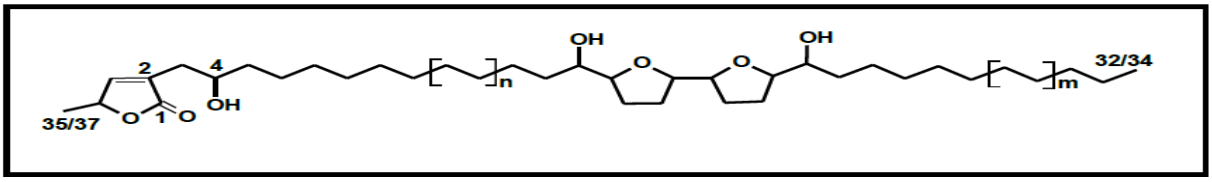

Figure 2. General structure of acetogenins [3]

There are many reported record the popular use the *A. muricata* in anticancer treatment. In addition of, ethnobotanically may be related to reports it assumed of its selective cytotoxic activity and this bioactivity is considered a selective as a some of the extracts studied in vitro were compered to be more toxic to cancer cell lines than to normal cells [8].

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The Possible Role of Epstein Barr Virus and Its Latent Proteins in Systemic Lupus Erythematosus Patients

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Abstract

Background Systemic lupus erythematosus is a chronic, systemic, idiopathic autoimmune disease. One of the suggested environmental factors that lead to development of systemic lupus erythematosus is infection with Epstein-Barr virus.

Objective First, detection and quantification of Epstein-Barr virus in peripheral blood of systemic lupus erythematosus patients compared to control. Second, estimation of mRNA level of latent and lytic genes and compare them with control groups.

Methods This a case-control study conducted on systemic lupus erythematosus patients during the period from (December 2018 to March 2019). A total of 40 patients were involved in this study. Blood samples were taken from Baghdad Teaching Hospital of Medical City. On the other hand, 40 blood samples were collected from apparently healthy subjects, as control samples from blood donor center in Al-Imamein Al-kadhimein Medical City.

Results Eleven of forty (27.5%) of systemic lupus erythematosus patients were positive with Epstein-Barr virus at mean viral load 815.72 copy/ml with (P value 0.59). And the rate of Epstein-Barr virus detection in blood of patients group was highest in severe cases rather than in less severe ones. Only 2/40 (5%) of control subjects were positive with Epstein-Barr virus at much lower mean viral load, 64.75 copy/ml (P Value 0.34). The expression of the latent genes in patients versus control groups, was 100% versus 77.5% for EBNA-2, 50% versus 15% for EBNA-3C, 82.5% versus 97.5% for late membrane protein -1, and 85% versus 75% for EA/D, respectively. EBNA-2 expression showed significant direct proportional correlation with viral load.

Conclusion Increased rate of Epstein-Barr virus DNA detection in systemic lupus erythematosus group compared to control group and higher rate of viral DNA detection within severe cases might indicate a possible defect in controlling viral infection and increased number of latent infected cell in systemic lupus erythematosus. Also increased positive EBNA-2 and EBNA-3C expression in systemic lupus erythematosus group rather than in control group indicates that these Epstein-Barr virus proteins might have ability to disrupt the normal immune system, and might trigger and/or promote the autoimmune status.

Keywords EBV, SLE, EBNA-2, EBNA-3C, LMP-1, EA/D

Citation Abdullah ZF, Abdulamir AS, Gorial FI. The possible role of Epstein Barr virus and its latent proteins in systemic lupus erythematosus patients. Iraqi JMS. 2020; 18(1): 4-11. doi: 10.22578/IJMS.18.1.2

List of abbreviations: EA/D = Early antigen/diffuse, EBV = Epstein-Barr virus, EBNA-2 = Epstein-Barr virus nuclear antigen 2, EBNA-3C = Epstein-Barr virus nuclear antigen 3C, LMP-1 = Late membrane protein -1, QRT-PCR = Quantitative Real Time polymerase chain reaction, SLE = Systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a chronic, systemic, idiopathic autoimmune disease [(1)]. SLE mainly

affects women in child bearing age. Female to male ratio is 9:1 to 15:1 [2]. The etiology of SLE is believed to be multifactorial including genetic and environmental factors that both take part in the development of this very complex disease.

Epstein-Barr virus (EBV) is a lymph trophic double stranded DNA virus with a172 kb genome. It is one of a human herpes virus belonging to the gamma herpes family [3]. After the primary lytic EBV infection, the virus existence continues in host through latent phase [4]. There are several lytic cycle antigens that participate in immune evasion. One of them EBV early antigen/diffuse (EA/D), which is vital for DNA polymerase to replicate the viral genome [5]. In the latent phase, EBV persists in memory B cells, in cryptic form [6]. To successfully escape from the immune response, EBV expresses many viral genes, one of them is EBV nuclear antigen 2 (EBNA2) [7]. It is one of the most essential transcription factors that transform infected B cells [8] to lymphoblastoid cell lines (LCLs). Also has capacity to control the expression of all other latent viral antigens [4]. EBV nuclear antigen 3C (EBNA3C) protein serves as a transcriptional co-regulator by cooperating with various cellular and viral factors [9]. It is responsible for B-cell immortalization. Late membrane protein -1 (LMP-1) is regarded as most important oncogenic EBV gene, that intermediates cell proliferation and inhibits apoptosis [10].

Recently, EBNA-2 was found to act as a transcriptional activator for almost half of SLE risk genes [11]. Increasing evidence in field that EBV might trigger or promote SLE disease as part of the multifactorial etiology of SLE rather than a mere consequence of the disease itself [4]. Hence, the current study was carried out on SLE patients for estimating the role of EBV and its expressed genes in SLE compared to healthy population.

Methods

Patients and controls

The current study is a case control study which was conducted on SLE patients in the period from (December 2018 to March 2019). A total of 40 SLE patients were involved in this study, blood samples were taken from Baghdad Teaching Hospital of Medical City.

Inclusion criteria

All patients were diagnosed with SLE according to 1982 American College of Rheumatology (ACR) criteria for the classification of SLE [12], and also depending on immunological tests, anti-dsDNA and anti-nuclear antibody. Moreover, only newly diagnosed SLE patients or those at off therapy stage were included in order to avoid the effect of immune suppressive drugs (Mycophenolate mofetil 50 mg, Azathioprine 50 mg) on the results of the current study.

Exclusion criteria

Patients with other chronic autoimmune diseases and immune suppressant conditions, such as cancer, were excluded

The control subjects were apparently healthy and age-, sex- matched volunteers; were taken from blood donor center in Al-Imamein Al-kadhimein Medical City. Written informed consents were obtained from subjects.

This study was approved by the Ethical Committee of the College of Medicine/Al-Nahrain University. The current study was conducted in the laboratories of the Microbiology Department at the College of Medicine, Al-Nahrain University.

Disease severity assessment

SLE disease activity index (SLEDAI) score was calculated according to the scale of SLEDAI-2K [12] in SLE patients to determine disease severity, and to explore the correlation of disease severity and rate of EBV detection, gene expression levels with disease severity.

Collection and storage of samples

Forty blood samples were collected from SLE patients who are either during off therapy stage or taking just prednisolone (20 mg). On the other hand, 40 blood samples were taken from apparently healthy subjects, as control samples. From all participant, (3 ml) of whole blood were collected in EDTA tubes, up to (1.5 ml) for RNA extraction and (1.5 ml) for DNA extraction. RNA extraction and conversion to complementary DNA (cDNA) was carried out on the same day of blood collection, and the residual blood was stored at -20C for later step of DNA extraction.

Viral DNA extraction

DNA extraction was carried out by using DNA extraction kit (Bosphore, Turkey, Cat, # ABXVD1) and the method of extraction was according to the manufacturer guidelines.

Viral RNA extraction

RNA extraction was conducted using (Bosphore, Turkey, Cat. #ABXVR1) and extracted RNA was then converted to cDNA by using GO Script reverse transcription system (promega, U.S.A, Cat. #A5000), After DNA, RNA extraction and conversion to cDNA, the yield and purity of the EBV DNA and cDNA were measured using Nano Drop following instruction of the manufacturer (ActGene NAS99).

Detection of viral DNA

EBV quantification kit V1 (Bosphore, Turkey Cat, # ABEBV3) used to detect and quantify viral DNA. Reaction mixture consists of (15 µl) of PCR Master Mix, (0.1 µl) of Internal Control was added/reaction, (10 µl) of DNA sample, four standards with copy number ($2x10^3$-$1x10^6$ copies/ml), Negative/Positive Control were added/ reaction to reach final volume (2 5 µl). Real-time PCR instrument used was Stratagen MX 3005P. The thermal protocol consisted of an initial denaturation at 95 °C for 14:30 min, followed by 50 cycles each with denaturation at 97 °C for 00.30 sec, annealing at 53 °C for 1:30 sec and elongation at 72 °C for 00:15 sec. Moreover, the standard curve is plotted using the data obtained from the defined standards, with the axes (Y-axis) for Ct- Threshold Cycle and (X-axis) Log Starting Quantity.

Estimation of viral gene expression

After RNA extraction and conversion to cDNA; the gene expression for EBV genes was estimated by using Syber green master mix ("SINTOL", Russian, Cat, No M-427). Reaction mixture consists of 10ul of master mix, and 1ul of each forward and reverse primers were added/reaction. (1.5 µl) of $MgCl_2$ along with (8.5 µl) of dd H_2O were added / reaction. (3 µl) of template were added / reaction to reach final volume (25 µl).

The sequences and amplicon size of primers listed in table (1).

Table 1. The primers used along with sequence and amplicon size

Gene		Primer sequence	Product size (bp)	Reference
EBNA2	F	-GTCTGGCACATGCAAGACA	154 bp	(11)
	R	TCTGCCACCTGCAACACTAA		
EBNA3c	F	GGCACATTGTCTTCCGTGTC	220 bp	(11)
	R	-TACAGACTACCGGCGAGCAT		
LMP1	F	CCAATAGAGTCCACCAGTT	78 bp	designed by sigma
	R	TCTTCCTAGCCTTCTTCCTA		
EA/D	F	TAAGGTGACACTCAATCC	83 bp	designed by sigma
	R	TCAGAGGCTTGTAGTCTA		
GADPH	F	GCACCGTCAAGGCTGAGAAC	138 pb	(11)
	R	TGGTGAAGACGCCAGTGGA		

Thermal profile for EBNA-2 includes initial denaturation 95 °C for 5min, followed by 40 cycles of denaturation 95 °C for 30 sec, annealing &extension 62 °C for 30 sec, followed by melting curve analysis. And thermal profile for EBNA-3C &GADPH, as reference gene, includes initial denaturation 95 °C for 5 min, followed by 40 cycles of denaturation 95 °C for 30 sec, annealing 58 °C for 30 sec, extension 72 °C for 30 sec, followed by melting curve analysis. The thermal profile for LMP-1 and EA/D includes initial denaturation 95 °C for 5 min, followed by 40 cycles of denaturation 95 °C for 30 sec, annealing and extension 60 for 30 sec, followed by melting curve analysis. The cycle threshold ΔΔCT method for mRNA was used to measure the relative level of genes expression and make comparison between SLE and control groups at gene expression level for the target genes.

Statistical analysis

The data of the current study was processed and analyzed using SPSS version 16.0.02. Quantitative data were first assessed in terms of normality tests and accordingly parametric (student t-test) or non-parametric tests (Mann-Whitney) were used. For nominal qualitative data, chi-square and Fisher Exact tests were used to measure the significance of association among different qualitative variables of the study. Correlation coefficient, or r, and linear regression analysis were also included. P values <0.05 were considered significant.

Results

This case control study included 40 blood samples of SLE patients with mean age 32 years (Std. error 1.51), and with mean duration of disease 3.5 years (Std. error 0.48), the mean age for controls 25 years (Std. error 2.0).

The positive detection and mean viral load of EBV in SLE versus control group

The findings of the current study showed that the rate of positive EBV, detected by real-time qPCR, was much higher in SLE patients than in control group (P < 0.01), as shown in table (2). In addition, interestingly the mean blood viral load of EBV was found to be 384.11 folds higher in SLE patients than in control subjects.

Table 2. Rate of EBV detection in SLE versus control group

			Case-control		Total
			Case	Control	
Detection of EBV	Negative	Count	29	38	67
		% within Detection of EBV	43.3%	56.7%	100%
		% within Case-control	72.5%	95.0%	83.8%
		% of Total	36.2%	47.5%	83.8%
	Positive	Count	11	2	13
		% within Detection of EBV	84.6%	15.4%	100%
		% within Case-control	27.5%	5.0%	16.2%
		% of Total	13.8%	2.5%	16.2%
Chi-square: P = 0.006 (highly significant), Odds ratio = 7.2, P = 0.014					

The expression of EBNA-2 in SLE compared to control groups

The results of the current study on the positive/negative gene expression of viral EBNA-2 gene, using relative real-time PCR, showed that all of SLE patients were with positive EBNA-2 gene expression (100%) while only (77.5%) of control subjects showed positive EBNA-2 gene expression (P < 0.01).The odds of SLE patients to have a detected

positive expression of EBNA-2 was extremely high, 24.22 times more than control subjects to have a positive EBNA-2 expression, as shown in table (3) .

Table 3. The positive/negative expression of EBNA-2 gene in SLE versus control groups

			Case-control		Total
			Case	Control	
Expression of EBNA2	Negative	Count	0	9	9
		% within Detection of EBNA2	0%	100%	100%
		% within Case-control	0%	22.5%	11.2%
		% of Total	0%	11.2%	11.2%
	Positive	Count	40	31	71
		% within Detection of EBNA2	56.3%	43.7%	100.0%
		% within Case-control	100.0%	77.5%	88.8%
		% of Total	50.0%	38.8%	88.8%
Fisher Exact: P = 0.002 (highly significant) Odds ratio = 24.22, P = 0.029					

The expression of EBNA-3C in SLE compared to control groups

Half (50%) of SLE patients were with positive expression of EBNA-3C versus only 15% in control group. Hence, the expression of EBNA-3C in SLE patients was much higher than in control group (P < 0.01), as shown in table (4).

Table 4. The expression of EBNA3C gene in SLE group versus control group

			Case-control		Total
			Case	Control	
Expression of EBNA3C	Negative	Count	20	34	54
		% within Detection of EBNA3C	37.0%	63.0%	100%
		% within Case-control	50.0%	85.0%	67.5%
		% of Total	25.0%	42.5%	67.5%
	Positive	Count	20	6	26
		% within Detection of EBNA3C	76.9%	23.1%	100%
		% within Case-control	50.0%	15.0%	32.5%
		% of Total	25.0%	7.5%	32.5%
Chi-square: P = 0.001 (highly significant) Odds ratio = 5.66, P = 0.001					

The expression of LMP-1 and Early antigen/D (EA/D) in SLE compared to control groups

On contrary to the expression of EBNA-2 and -3C, the present study revealed that the positive expression of LMP-1 gene was 82.5% in SLE patients compared to 97.5% in control subjects (P > 0.05). In regard to EA/D, although the positive expression of EA/D was a bit higher in SLE patients (85%) than in control (75%) group, the difference was non- significant (P > 0.05).

The quantitative assessment of the relative EBV genes expression in SLE patients

By calculating the relative gene expression of EBNA2, EBNA3, LMP-1, and EA/D, it was shown that EBNA2 was the highest gene expressed in

SLE patients in relative to control group, 22.45 folds, then EBNA3C, 5.68 folds, then EA/D, only 3.35 folds (P < 0.05). On the other hand, LMP-1 gene expression was lower in SLE than in control group, 0.16 folds.

The correlation between EBV load and the relative expression of EBV studied genes

The findings of this study showed that the relative expression of EBV in fold change, was positively correlated with that of EBNA-2 (correlation coefficient or r = 0.82), LMP-1 (r = 0.9), and EA/D (r = 0.86) (P < 0.01). The relative expression of EBNA2 was found to be positively correlated with that of EBNA3C (r = 0.6), LMP-1 (r = 0.85), and EA/D (r = 0.94) (P < 0.01).

Regression analysis between EBV viral load and the relative expression of EBV genes

Linear regression analysis was tested between the viral load of EBV, in folds, as independent variable and the relative expression of EBV genes, EBNA2, EBNA3C, LMP-1, and EA/D. Except for EBNA-3C, all other genes reached significance as dependent variables on EBV load (P<0.01). EBNA-2, LMP-1, and EA/D expression and EBV load fold regression analysis results show that the expression of these three genes was highly dependent on EBV load.

The Expression of EBNA2 gene as an indicator for the presence of EBV infection

Based on the results of the current study, the expression of EBNA2 gene was detected positively in all of the SLE patients while only a portion of SLE patients were with detected EBV via absolute qPCR. Therefore, EBNA-2 expression seems to be more sensitive for the presence of underlying EBV infection in SLE patients. Accordingly, the receiver operator characteristic (ROC) curve was used to assess the feasibility of using the relative expression of EBNA-2 gene as a reliable indicator for the presence of EBV infection. The accuracy, or the usefulness of using this test, was found to significantly high, 0.708 (P < 0.05) and the ROC curve showed that it is accurate to measure EBNA-2 relative expression in folds at cut-off value of 11 folds for the detection of an underlying active EBV infection without measuring directly EBV load, and the use of this cut-off value guarantees an acceptable sensitivity and specificity for the detection of underlying EBV infection, up to 72% and 66%, respectively.

Discussion

Many studies showed similar findings to these of the present study that there was a relation between EBV infection and development of SLE [12-15]. Increased viral load was related with disease activity and it was independent of intake of immunosuppressive medication [4,13]. The cause of elevated viral load in SLE patients might be due to impairment in controlling of infected cells, that leads to increased frequency of latent infection of memory B-cell and also increase in number of the replicating virus [16], and also, impairment of cytotoxic CD8+ T- cell function [14]. These immune defects might enable virus to proceed in persistent latent state and causes viral disease and may further propagate disease status [4]. Another study found that increased virally infected cells in severe cases of SLE rather than mild to moderate ones suggesting that EBV could highly be related to the disease flare in cause-effect manner [16].

The findings of the present study reveal that all SLE patients had positive EBNA-2 expression versus 77% in control group. These findings agree with a recent study showing that EBV can cause autoimmune disease by EBNA-2 transcription factor that help changing how infected B cells to act, and how body employment to those cells [12]. And this study also agrees with another study which showed that almost half of SLE risk alleles are occupied by the EBNA2 protein, triggering their transcription, indicating that an important mechanism by which EBV can perturb immunity [17].

Moreover, a correlation study for the relative expression of viral genes of EBV indicated that increased EBNA-2 expression is positively correlated with other latent genes, namely (EBNA-3C, LMP-1, EA/D) because EBNA-2 has

ability to control the expression of all other latent genes, and also the correlation between increased expression of EBNA-2 and that of EBNA-3C was due to the fact that EBNA-3C works as a co–activator for EBNA-2 [8]. This is exactly what was shown in the present study confirming the pivotal role of EBNA2 in SLE disease.

In the current study, the regression analysis for EBNA-2 expression showed clearly that it is dependent on the viral load and could use the expression of EBNA-2 as an indicator for the viral activity. This indicates several key points; first, EBNA2 and to a lesser extent EBNA3C are consistently and abundantly produced in the latent EBV infection in SLE patients while this phenomenon is not seen in healthy population. Second, the higher viral load observed in SLE patients is uniquely coupled with consistent high activity of latent cycle enabling EBV to possibly exert certain promoting or exaggerating effect on SLE condition. Actually, this shed light on a key point that the more deregulated immune system in severe cases of SLE is expected to have reactivation rather than an active latent cycle; however, this is not the case in SLE where both latent antigens and reactivation antigens are activated with stark favor to latent proteins. This shift in EBV cycle activity seen in SLE requires more investigation.

The findings of the current study on EBNA-3C expression reveal that half (50%) of SLE patients were with positive expression of EBNA-3C, which was much higher than in control group (15%). This result agrees and approach with another study that measured EBNA-3C expression from infected B-lymphocyte that induce lymphoma [18]. This study, besides our study, might explain the role of EBNA3C in contributing in disease pathogenesis through transformation and immortalization of lymphocytes. Hence, the association of the expression of these genes with the disease may be due to severe immune impairment during the flare of disease and that may enhance viral activity or may be the virus itself one of the factors for the flare up and progression of the disease, or both scenarios could be true.

The findings of EA/D expression in the current study showed that although the positive expression of EA/D was a bit higher in SLE patients (85%) than in control (75%) group, but the result was not significant. Anyhow, the high level of expression of EA/D means that there is some sort of EBV reactivation in SLE patients resulting in creation of new virions and ensure spread infection to other epithelial cell and B-cell. These findings may approach to other study findings; they found higher IgA antibody against EA/D in SLE patients compared to normal; this points to viral reactivation and endeavor of immune system to control of reactivation [13].

Overall, according to the findings of the current study, EBV seems to play an active role in SLE pathogenesis and it is not just a consequence of the immune impairment.

Lastly, the positive expression of EBNA-2 in all of systemic lupus erythematosus patients studied, even in cases with negative detection of virus, refers to that it is feasible to use detection of EBNA-2 expression as an indicator for viral infection.

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Author contribution

All authors contributed to this manuscript. Dr. Abdulamir: designed, interpreted and arranged this manuscript, Abdullah conducted the research, and Dr. Isho supervised the clinical aspect of this research.

Conflict of interest

There is no conflict of interest.

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Radiological Imaging Diagnosis of Adult Renal Cystic Diseases and Management

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Abstract

Background Renal cysts are a heterogonous group of cystic renal diseases that present in approximately 40% of the patients undergoing imaging.

Objective To describe the role of the radiological imaging methods for the diagnosis of adult renal cystic diseases and their management.

Methods A descriptive study carried out for 218 patients of renal cystic diseases in adults in two Private Hospitals in Aden Province during the period from February 2006 to December 2014, diagnosed by ultrasonography, contrasted urography and computerized tomography determined the age, gender, frequency, and complications and type of treatment.

Results Autosomal dominant polycystic kidney disease (ADPKD) presented in 96 patients (44%), simple renal cysts 90 (41%), medullary sponge kidney 25 (11%), localized cystic renal disease (LCRD) six (3%) and adult multilocular cystic nephroma one (1%). Ultrasound correctly interpreted 179 patients (82%), nonvisualized 25 (11%), and inconclusive 13 (7%) contrasted urography correctly interpreted 176 (76%), nonvisualized 13 (6%) and inconclusive 19 (9%) and computerized tomography (CT) scan 199 patients (91%), six (3%) and 13 (6%) respectively. Complications occurred in 65 patients (68%) of ADPKD, 23 (25%) of simple renal cysts, 15 (60%) of medullary sponge kidney (MSK) and three patients (50%) of LCRD. Conservative treatment performed in 171 (78%) and surgical intervention in 47 patients (22%).

Conclusion The commonest renal cystic disease is adult dominant polycystic kidney. Ultrasound is inconclusive for renal cystic masses. CT scan is the effective imaging study for localized and multifocal renal cystic diseases, complications and associated pathology. The frequent complications occur in adult dominant polycystic kidney disease and medullary sponge kidney.

Keywords Adults, renal cystic diseases, imaging, management

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List of abbreviations: ADPKD = Autosomal dominant polycystic kidney disease, CT = Computerized tomography, LCRD = Localized cystic renal disease, US = Ultrasonography, UTI = Urinary tract infection

Introduction

Renal cystic disease of the kidney in adults comprises a wide spectrum of hereditary, acquired, developmental and acquired conditions; they are a common cause of high morbidity and mortality worldwide [(1)]. Inherited cystic diseases in adults are linked to various genes involved in the formation and functioning of the primary cilia of the renal tubular epithelium. Dysfunction of primary tubular cilia leads to increased tubular epithelial proliferation and excessive fluid secretion. Nonhereditary renal cystic diseases are secondary to obstructive, stromal-epithelial mal-inductive and neoplastic mechanisms [(2-4)]. They may complicate by hemorrhage, infection

and ischemia, which produce a difficulty to be differentiated from renal cell carcinoma [5,6]. Clinically present with flank, back and abdominal pain, hematuria, renal swellings and hypertension [7]. Adult dominant polycystic kidney is the most common inherited cause of end-stage renal failure with more than 45% of patients requiring dialysis or transplantation by the age of 60 years. Implementation of genetic, cellular, and molecular techniques made accurate diagnosis, and carrier detection [8]. Actually, the use of various imaging diagnostic studies plays an important role in the diagnosis and management, detects their complications, disease progression and the response for treatment [9]. The use of Bosniak classification by computerized tomography (CT) scan findings differentiated by their attenuation, contrast enhancement, presence of calcification and septation differentiates benign from malignant cystic lesions, guides patient management and determine nonsurgical from surgical lesions (10). The management of renal cysts is best done with a multidisciplinary team that includes a urologist, nephrologist, geneticist and an internist with the assistance of a nurse practitioner or physician assistant facilities their management [11].

The main objective is to describe the importance of radiological diagnosis of adult cystic renal diseases and their complications.

Methods

A descriptive retrospective study carried out for a total of 218 adult patients with congenital and developmental cystic renal diseases including 117 men (54%) and 101 women (46.3%), during the period from February 2006 to December 2014 in two Private Hospitals in Aden Province named "Al Naquib and Al Mansorah". Age groups range from 18 to 70 years (mean age 52 years). A detailed history and thorough physical examination and abdominal ultrasonography, contrasted urography and CT scan. Clinically presented with abdominal, flank and back pain in 105 patients (48%), hematuria 33 (15%), recurrent urinary tract infection (UTI) 53 (24%) and abdominal palpable masses 27 (12%) respectively, determining the frequency of each adult renal cystic diseases, excluding the children and acquired renal cystic disease and those treated by endoscopic procedures, recognize the associated renal anomalies, complications and type of treatment whether medical or surgical.

Data retrieved from medical outpatient records and Hospitals registries. Ethical clearance was obtained and patients identifiers were not included and non-exposure of organs. Imaging Figures only to be used only in the scientific field. Data was processing using SPSS 21 version. Independent samples t-test was performed to determine the significance among variables.

Results

Congenital and developmental renal cystic diseases in adults presented in 117 men (54%) and 101 women (46%). Age group were as follows (18-30 years) eight patients (4%), (31-40 years) 33 (15%), (41-50 years) 48 (22%), (51-60 years) 63 patients (29%) and (61-70 years) 66 (30%). Frequency of renal cystic diseases included adult dominant polycystic kidney disease (ADPKD) 96 patients (44%), simple renal cyst 90 (41%), medullary sponge kidney (MSK) 25 (11.5%), localized cystic renal disease (LCRD) six (3%) and one patient 1% with adult multilocular cystic nephroma (AMCN) (Table 1).

Table 1. Adult renal cystic diseases regarding to age, gender and frequency in two Private Hospitals in Aden Province

Age group (years)	Males No. (%)	Females No. (%)	Total No. (%)	Frequency	No. (%)
18-30	5 (62)	3 (37)	8 (4)	ADPKD	96 (44)
31-40	15 (46)	18 (55)	33 (15)	SRC	90 (41)
41-50	27 (56)	21 (44)	48 (22)	MSK	25 (11)
51-60	33 (52)	30 (48)	63 (29)	LCRD	6 (3)
60-70	37 (56)	29 (44)	66 (30)	AMCN	1 (1)
Total	117 (54)	101 (46)	218 (100)	Total	218 (100)

ADPKD = Adult dominant polycystic kidney disease, SRC = Simple renal cyst, MSK = Medullary sponge kidney, AMCN = Adult multilocular cystic nephroma

Complications in ADPKD including hypertension 27 (41%), recurrent UTI 21 (32%), stones seven (11%), renal failure seven (11%) and renal cell carcinoma three (5%) with a total of 65 patients (68%). Simple renal cysts hypertension presented in seven patients (30%), UTI nine (39%), stones four (17%), malignant tumors two (9%), and renal failure one (4%) with a total of 23 patients (24%). Medullary sponge kidney hypertension found in two patients (13%), UTI seven (47%), stones five (33%), and renal failure one (7%) for a total of 15 patients (60%). Localized cystic renal disease, hypertension presented in one patient (33%), UTI one (33%), tumors one (33%) with a total of three (50%) of patients (Table 2).

Table 2. Patients with adult renal cystic diseases regarding to complications in two Private Hospitals

Cystic renal diseases	Hypertn-sion No. (%)	Recurrent UTI No. (%)	Stones No. (%)	Tumors No. (%)	Renal failure No. (%)	Total No. (%)
ADPKD	27 (41)	21 (32)	7 (11)	3 (5)	7 (11)	65 (68)
SRC	7 (30)	9 (39)	4 (17)	2 (9)	1 (4)	23 (25)
MSK	2 (13)	7 (47)	5 (33)	0 (0)	1 (7)	15 (60)
LCRD	1 (33)	1 (33)	0 (0)	1 (33)	0 (0)	3 (50)

ADPKD = Adult dominant polycystic kidney disease, SRC = Simple renal cyst, MSK = Medullary sponge kidney, AMCN = Adult multilocular cystic nephroma

Ultrasound carried in all patients, correctly interpreted renal cystic diseases 179 patients (82%), inconclusive 25 (11%) and non-visualized 13 (6%). Contrasted urography carried out in 209 patients, correctly interpreted 176 (82%), nonvisualized, 13 (6%), and inconclusive 29 (14%). CT scans, correctly interpreted in 199 (91%), nonvisualized six (3%) and inconclusive in 13 (6%) (Table 3). CT scan was the most effective imaging method for the diagnosis of renal cystic diseases in adults. Conservative treatment carried out in 171 patients (78%) and surgically intervened 47 (22%).

Table 3. Adult congenital cystic renal diseases according to results of imaging in two Private Hospitals in Aden Province

Imaging study	Correctly interpreted No. (%)	Nonvisualized No. (%)	Inconclusive No. (%)	Total No. (%)
Ultrasound	179 (82)	25 (11)	13 (7)	218 (100)
Contrasted urography	176 (76)	13 (6)	19 (9)	209 (100)
Contrasted CT scan	199 (91)	6 (3)	13 (6)	218 (100)

Discussion

The most common adult congenital and developmental renal cystic diseases included ADPK, followed by simple renal cysts (SRCs), medullary sponge kidney (MSK) and localized cystic renal disease (LCRD) respectively and adult multicystic nephroma was the rarest one consisting with reported [12-14].

Complications were more common in ADPKD and MSK. ADPKD was the most common cause of hypertension and end-stage renal failure. MSK was complicated by urinary stones and recurrent UTI Cystic dilatation of collecting tubules producing urinary stasis, which contribute to UTI and stones [15-17].

This study presented unilateral ADPKD associated with renal agenesis seen in three patients; one of them was an adult woman with right renal agenesis associated with incomplete duplications of left kidney with upper pole normal function, and lower pole hydronephrosis (Figure 1).

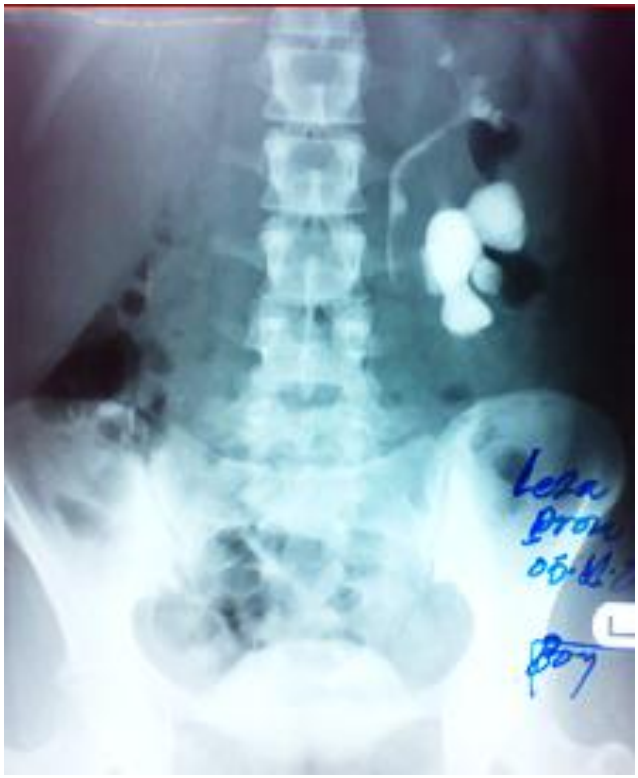

Figure 1. IVU shows right renal agenesis and double left pelvicalyceal system and lower pole hydronephrosis in ADOKD

LCRD presented in six patients. It is a rare benign non-progressive, non-surgical unilateral multiple renal cyst characterized by conglomerate mass of multiple simple renal cysts replacing a variable portion or an entire kidney separated by normal or atrophic renal tissue. Relatively few cases described in literature [18-23].

Multilocular cystic nephroma in adult diagnosed in one patient. Multlocular cystic nephroma is a rare finding and few cases reported in literature. In CT scan typically appears as a well-circumscribed encapsulated multicystic hypodense cyst of variable size which is separated by hyperechogenic septae (Figure 2). It is benign nonhereditary unilateral hypoechoic renal cyst lined by an epithelium and fibrous hyperechogenic septae contain mature tubules [24-26].

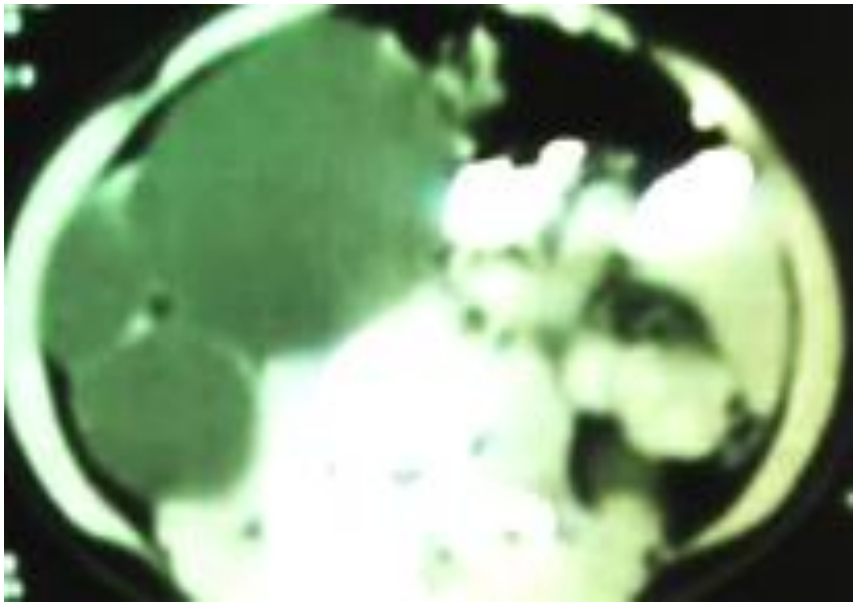

Figure 2. CT scan reveals hypoechoic renal cysts of different sizes separate by hyperechogenic septae diagnostic of adult multilocuar cystic nephroma

Ultrasonography (US) is the first study for assessment of cysts location and size of both kidneys but is inconclusive for cystic renal masses evaluation and ectopic kidneys. It is an excellent study for determination of cysts in other organs and follow-up. Pei et al. in 2015 [17] considered US as the gold standard for the diagnosis of ADPKD. Simple renal cyst is seen as a round or oval hypoechoic structure surrounds by a thin and smooth wall, while multiple bilateral renal cysts at the age of 20-30 years with large kidneys and extrarenal cysts most commonly seen in the liver confirms ADPKD.

Intravenous urography is an effective imaging method for diagnosis of medullary sponge kidney and ADPKD, which seen with thin prolonged calyces described as a spider-leg deformity; it determines the obstruction level and renal excretion [11,17,27].

CT scan is very sensitive imaging study for the diagnosis and characterizing of simple renal cysts and complex cystic mass with solid components or thick septae with enhancement suspected of renal cell carcinoma, detection of complications and associated abnormalities consistent with reported by Weibi et al. in 2017 [28].

Non-contrasted CT urography and US images detects non-radiopaque stones and used in renal failure [29].

MSK was diagnosed by contrasted urography; it shows a paint brush like appearance produced

by medullary collecting ducts. Koraishi et al. in 2014 [30] considered CT urography as the best diagnostic study for MSK.

Sporadic angiomolipoma is an extremely rare clinical entity; this study presented one patient with bilateral multiple simple cysts [11,31].

Medical treatment was the most frequent type of treatment and surgical intervention reserved for complicated patients and for renal cystic tumors [32]. The association of renal cysts with malignant renal cystic tumors is infrequent finding [33]. This study diagnosed four patients with malignant cystic lesions in adult renal cystic diseases, one of them seen in the left kidney (Figure 3) and other one in the right kidney, presented with thick calcification (Figure 4) and another one with cystic hypoechoic structure in horseshoe kidneys (Figure 5) and the later seen bilaterally in an adult dominant polycystic kidney disease (Figure 6).

This study concluded that:

1. Ultrasound is inconclusive for cystic renal masses.
2. CT scan is the most effective study for localized and multifocal cystic renal diseases, complications, and associated pathology.

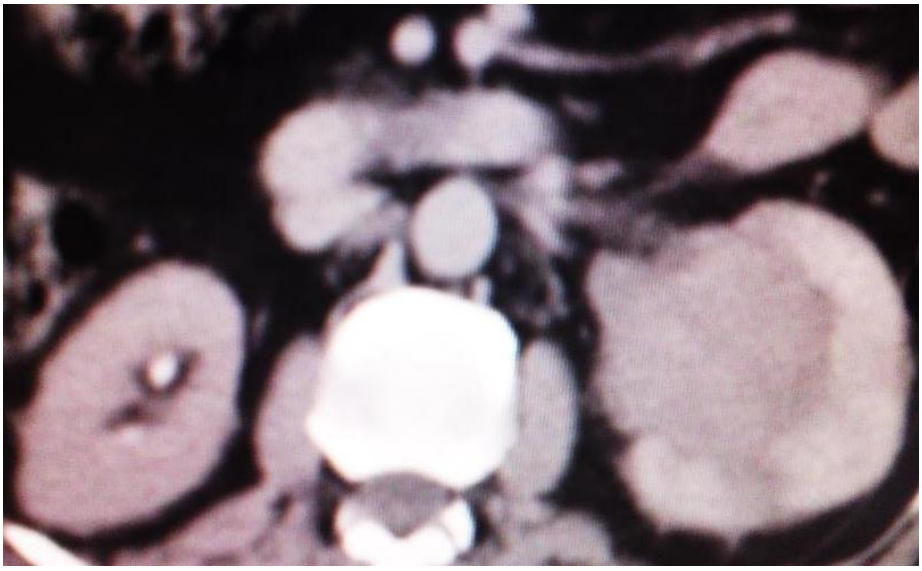

Figure 3. CT scan demonstrates malignant cystic mass in the left kidney

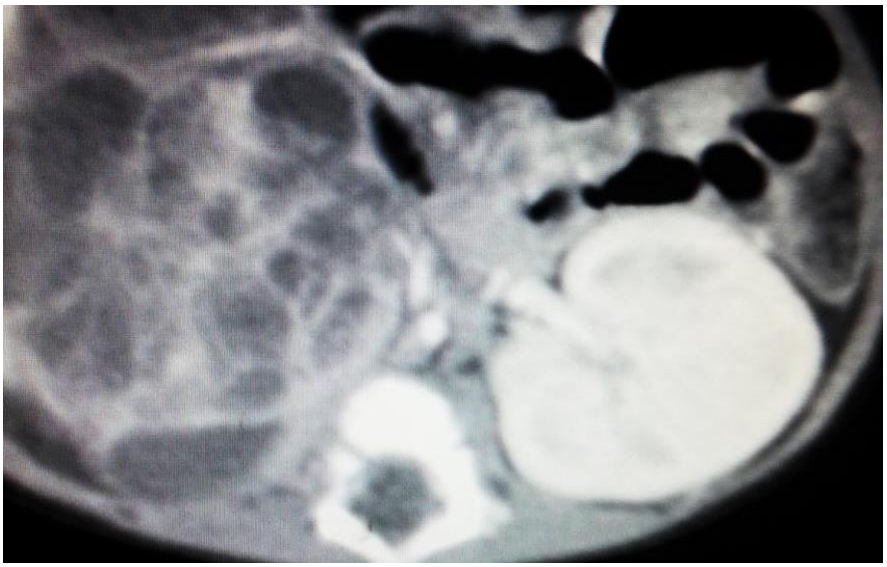

Figure 4. CT scan reveals right kidney enlargement and hypoechoic cystic tumor with hyperdense calcification

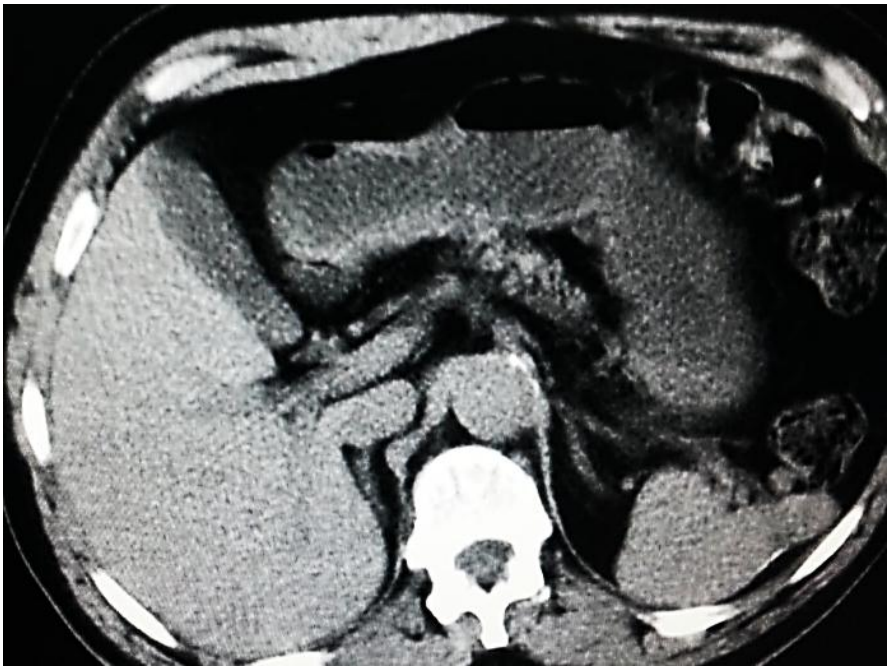

Figure 5. CT shows a hypoechoic cystic lesion in horseshoe kidney

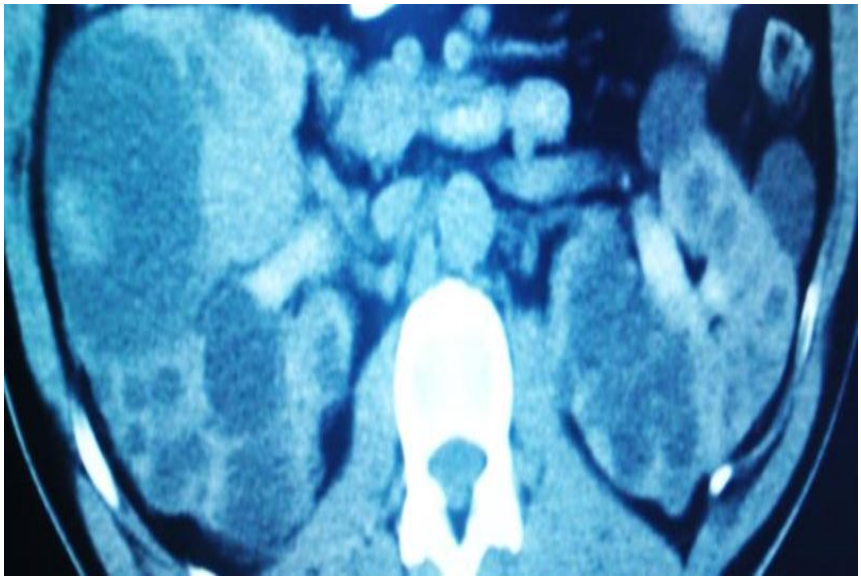

Figure 6. Contrasted CT scan reveals bilateral malignant cystic lesions in ADPKD

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Author contribution
Dr. Akares did the case selection and prepared the manuscript; Dr. Bahomil reviewed the radiological tests and prepared the manuscript.

Conflict of interest
The authors declare no conflicts of interest regarding the publication of this paper.

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Surfactant Protein Type-A in Diagnosis of Drowning Cases

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Abstract

Background Drowning is a main universal community health problem. In medico-legal practice, the autopsy diagnosis of drowning presents one of the major problems especially when there is delay in recovering the victim from water.

Objective To gather the autopsy findings with the serology test "Surfactant-associated protein A" (SP-A) procedures to reach more accurate diagnosis of drowning and to clarify the significance of serology test (SP-A) procedures.

Methods This study was performed at Medico-legal Directorate (MLD) in Baghdad for (12) months within the period from 1/1/2018 to 31/12/2018. Full proper autopsy including external and internal examination of the body for all cases was performed, after obtaining complete medico-legal history, in addition to serology test (SP-A) procedures to determine the cause of the death as due to drowning.

Results The study included (60) cases, (52) males and (8) females with their ages ranged between (15-44 years old) for male, while ages ranged between (1-44 years old) for females. Drowning was the cause of death in all cases. The most important result of this study is that the serum SP-A concentration showed increment alongside with period that had been passed since the event of drowning. The highest value was (1042.167 ng /L) after 48 hours from the event.

Conclusion The concentration of SP-A increases with increasing duration of immersion in water and an important marker in the diagnosis of drowning together with the autopsy findings.

Keywords Medico-legal study, SP-A, drowning

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List of abbreviations: ALI = Acute lung injury, ARDS = Acute respiratory distress syndrome, MLD = Medico-legal Directorate, SP-A = Surfactant-associated protein A

Introduction

Drowning is a main universal community health problem. It is the process of experiencing respiratory impairment from submersion / immersion in liquid as stated by world health organization [1,2]. In a medico-legal view, drowning is a type of asphyxia due to aspiration of fluid either water, milk, oil ...etc instead of air and immersion of whole body or nose and / or mouth under the level of fluid [3-6]. In drowning there is relation between liquid and air junction at the entrance of the airway prevents breathing air [7]. Drowning could be considered as a mixture of mechanical presence of water within the respiratory system (mechanical asphyxia) with liquid and electrolyte changes depending on the medium (either sea or fresh water) in which immersion has occurred [8]. Drowning cases are usually accidental in nature, they could be suicidal and rarely homicidal [6]. Drowning is a common method of suicide in India and (35%) of cases in Austria are positive

for alcohol [7]. It is the second leading cause of accidental death in children at 14 years old [9]. In medico-legal aspect, the problems with drowning start with the scenes involving long sections of sea, rivers or lakeshore, mainly in cases underwater disaster identification [10,11] and failure to find a body quickly [12].

Autopsy signs include immersion and drowning signs. Immersion signs are maceration (corrugation) of the skin, which is the first sign that starts within minutes in warm water, while in cold water it would be visible after a variable time, the minimum is (4 or 5) hours depending upon temperature other study suggest a longer duration from (12 to 48) hours. Maceration is obvious in hands and soles, the skin becomes wrinkled, pale and wet so-called "washerwoman's skin". Maceration could be seen also on the extensor surface of knees and elbows [9] and it was appeared in summer days within (30-60) minutes, in mild atmosphere in (3-4) hours and is delayed in cold season [13].

Cutis anserine (goose-flesh) is a common sign in immersed bodies [9].

It is usual for most corpses to float or hang in water with buttocks uppermost, while the head and limbs are hanging down. The hypostasis in cadaver pulled out cold water is pink color [2,9].

Mud, coal-slurry, oil, silt or sand present on the body, in addition to other artefacts such as seaweed, waterweed, algae. Mud may be adherent to the whole-body surface and clothing [9].

The corpses are colder in the depths of rivers, seas, canals, and ponds than the outer atmosphere. Contraction of scrotum may happen before or after death [8].

Algae growth on the skin is helpful to determine the position of cadaver. They may be found in trachea and stomach as a sign of immersion, but not of active inhalation of fluid [13-16].

In medico-legal practice, the autopsy diagnosis of drowning is one of the major problems specifically when there is delay in recovering the victim [17,18]. Drowning signs include froth in the air passages as a positive sign in fresh bodies. Froth is edematous fluid from the lungs and consists of a proteinaceous exudate and surfactant mixed with the water of the drowning medium [3,9]. It is white in color and may be pink or red-tinged, due to slight mixing up with blood from intrapulmonary bleeding. [9,13].

Generally, the weights of a lung in drowning is about 600-700 g, whilst the non-drowned is about 370-540 g [19].

Froth also observed in epilepsy, electrical shock, drug intoxication and cardiogenic pulmonary edema [8,19]. The lungs of a drowning victims commonly look like those seen in deaths associated with severe pulmonary edema, as in cases of arteriosclerotic heart disease [17,20].

The most important internal organ to observed and the most information about the cause of death in drowning are lungs. They are distended brick red in color, with signs of emphysema [9,13,21]. The edematous fluid in the bronchi locks the passive collapse that normally occurs at death, holding the lungs in the inspiratory position [9,15]. This is a positive sign of drowning at autopsy [15].

The increase in weight of lungs and is due to asphyxia and aspiration of water [20].

The heart and great veins are dilated and engorged with fluid blood, especially the right side, but this is non-specific [13,21,22]. Pleural fluid accumulation is associated with drowning, the volume of which controversially being said to reflect the post-mortem interval [8]. Subpleural hemorrhages (Paultauf's spots) may reflect hemolysis within intra-alveolar spaces and have been described in (50-60%) of cases of drownings [8,22].

In stomach, there is Wydlers sign due to swallowing of water or Mallory-Weiss syndrome (esophageal mucosal tear) [23].

Miscellaneous signs in drowning include bloody or watery fluid in the intracranial sinuses, engorgement of solid organs, reduced weight of the spleen, Tardieu spot on organ and muscular hemorrhages in the neck and back

and all are additional physical signs of drowning [8].

Cadaveric spasm is a positive sign and may be see in one or both hands. There may be grass, herbs, or gravel in the fist of the victim [8,9,15,20].

Surfactant-associated protein A (SP-A) is a lipoprotein & an innate immune system collectin. It is water-soluble. It is part of the innate immune system [21]. Pulmonary surfactant is covering the surface of the alveoli and essential for normal lung function as it maintains alveolar stability and prevents alveolar collapse by reducing surface tension at the air-liquid interface [22,23]. SP-A are very important constituents of pulmonary surfactant [24,25]. It is a hydrophilic and large collagen-like glycoprotein produced by the alveolar type (II) cells and bronchiolar epithelial Clara cells [26]. Function of SP-A in the alveolus is to facilitate the surface tension-lowering properties of surfactant phospholipids, to regulate surfactant phospholipid synthesis, secretion, recycling by alveolar type (II) pneumocytes and alveolar macrophages and to resist the inhibitory effects of plasma proteins released during lung injury [27]. SP-A is dependent on the presence calcium because it plays a strong role in the structure and function of pulmonary surfactant after secretion into the alveolar space [28].

Surfactant loss or changes of the proteinaceous fluid into the air spaces lead severe pathological significances and SP-A altered in a variety of pulmonary diseases such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). [29]. Clinical ALI and the ARDS represent a common response of the lung to a variety of insults, including trauma, infection, aspiration of water or gastric contents, inhalation of toxic gases, and pneumonia [30]. SP-A concentration changes in bronchoalveolar lavage fluid (BAL) and in serum have been investigated in ARDS/ALI found useful since it is correlated significantly with the level of SP-A [31]. In Japan, SP-A immunohistochemically studied as a marker of pulmonary function and possible usefulness for postmortem investigation of death involving asphyxiation and respiratory distress [32]. Increase membranous or linear SP-A staining on the intra-alveolar surface indicated as an increase of surfactant secretion due to various fatal stresses for example acute myocardial infarction and carbon monoxide (CO) intoxication [33,34]. SP-A has been also observed in a different immunohistochemical distribution pattern such as aggregated granular staining distributed both intracellular and extracellular in the intra-alveolar space, but the exact mechanism of massive aggregates production remains to be determined [35]. This aggregated form of SP-A useful tool to distinguish mechanical asphyxia from other hypoxic cases and the effect of drugs and poisons on respiratory function [36]. Massive aggregates of granular SP-A staining were found in (70.4%) of mechanical asphyxia cases e.g. hanging, strangulation, smothering and choking. A high score of intra-alveolar aggregates of SP-A was more frequently observed also in freshwater (66.6%) than saltwater (6.25%) drowning victims. (34) Not all cadavers recovered from water or found near water are drowned [37].

Drowning remains one of the most difficult diagnoses in forensic pathology because macroscopic and microscopic autopsy findings are unspecific [38]. In medico-legal aspect, histology and immunohistochemistry are significant tools for study of pulmonary tissue. An ideal diagnostic marker for drowning still needs to be developed, other conditions that lead to increase SP-A are asphyxiation, drowning and respiratory distress syndrome [39].

The aim of this study is to clarify the significance of serology test of SP -A to strengthen the diagnostic of fatal drowning cases.

Methods

Site and duration

Medico-legal Directorate (MLD) of Baghdad for one-year duration from 1\1\2018 till 31\12\2018.

Subjects
Sixty victims of drowning.

Inclusion criteria
Cases with circumstantial evidence favoring drowning death.

Exclusion criteria
Decomposition.

Methods
External examination, which include examination of clothes and external signs, followed by internal examination and taking blood samples for serology test of SP-A (Eliza test), and this includes:

1. Ten ml of blood were taken from the heart.
2. Samples were left for 10-20 minutes to be clotted.
3. Centrifugation at 300-400 RPM for 5-10 minutes.
4. Serum samples were taken in test tube and stored in deep freezing at -20 °C
5. Samples were sent to the serology lab for SP-A investigation.

SP-A investigation
ELISA Kit done by dilution standard solutions: In this kit provides one standard original concentration. Users may independently dilute in small tubes following the chart below:

- 480 ng/L Standard No.5 120 µl Original Standard + 120 µl Standard diluents
- 240 ng/L Standard No.4 120 µl Standard No.5 + 120 µl Standard diluents
- 120 ng/L Standard No.3 120 µl Standard No.4 + 120 µl Standard diluent
- 60 ng/L Standard No.2 120 µl Standard No.3 + 120 µl Standard diluent
- 30 ng/L Standard No.1 120 µl Standard No.2 + 120 µl Standard diluent
- Standard solution No.5 No.4 No.3 No.2 No.1

The number of stripes needed is determined by that of samples to be tested added by the standards. It is recommended that each standard solution and each blank well be arranged with multiple wells as much as possible.

Sample injection

1. Blank well: Do not added sample, anti SP-A antibody labeled with biotin and streptavidin-HRP; added chromogen reagent A & B and stop solution, each other step operation is the same.
2. Standard solution well: Added 50 µl standard and streptomycin-HRP 50 µl (biotin antibodies had united in advance in the standard, so no biotin antibodies are added).
3. Sample well: Added 40 µl sample and then 10 µl SP-A antibodies, 50 µl streptavidin-HRP. Then covered it with seal plate membrane. Shake gently to mix. Incubate at 37 ℃ for 60 minutes.
4. Distilled water for later use.
5. Washing: carefully removed the seal plate membrane, drained liquid and shacked off the remainder. Fill each well with washing solution, let stand for 30 seconds, then drained. Repeat this procedure five times then blot the plate.
6. Color development: First added 50 µl chromogen reagent A to each well, and then added 50 µl chromogen reagent B to each well. Shaked gently to mix. Incubate for 10 minutes at 37 ℃ away from light for color development.
7. Stop: Added 50 µl Stop Solution to each well to stop the reaction (color changes from blue to yellow immediately at that moment).
8. Assay: Taken blank well as zero, measure the absorbance (OD) of each well under 450 nm wavelength, which should be conducted within 10 minutes after had added stop solution.

According to standards concentrations and corresponding OD values, calculated the linear regression equation of the standard curve. Then according to the OD value of samples,

calculated the concentration of the corresponding sample.

Results

Throughout the total period of collecting samples for study, which extended from 1st January to 31st of December of 2018, death cases due to drowning was recorded in only (134 out of 6591) cases referred to the MLD in Baghdad and the ages of victims drowning were among (4-45 years) and history were taken from family about diseases and appeared most of cases not complain from chronic diseases of lungs while control cases were road traffic accident. Drowning was the 10th cause of death being responsible for only (2%) of cases referred to MLD during the period of study. This study was showed that the SP-A concentration increment with Increasing duration of immersion in water as the concentration at the beginning of the first 30 minutes was (10.655 ng/l) and at the end of 30 minutes (36.95 ng/l) while the concentration of SP- A was within 48 hours stay in water at the beginning (303.938 ng/l) and the end of it, (1402.167 ng/l) and all drowning cases were not decomposed because the decomposing was affected on serum SP-A result and SP-A was test for drowning diagnosis not definitive test as in Table (1) and Figure (1).

Table 1. Concertation of SP-A in serum with times in drowning cases

	Time					
	30 m	**60 m**	**360 m**	**12h**	**24h**	**48 h**
SP-A (ng/l)	10.655	53.406	101.737	160.708	179.966	303.938
	11.459	53.707	103.335	165.381	180.424	319.041
	13.088	62.175	105.414	165.463	186.932	333.573
	22.139	63.905	112.225	172.842	224.153	587.953
	31.575	69.231	117.936	175.398	237.214	673.967
	35.199	70.04	118.955	177.42	246.638	858.834
	36.395	70.544	121.064		249.434	1402.167
		70.571	122.112			
		70.688	125.651			
		74.692	126.922			
		76.115	127.082			
		77.114	130.15			
		77.844	130.635			
		82.351	135.483			
		85.246	144.092			
		88.97	146.544			
			152.21			

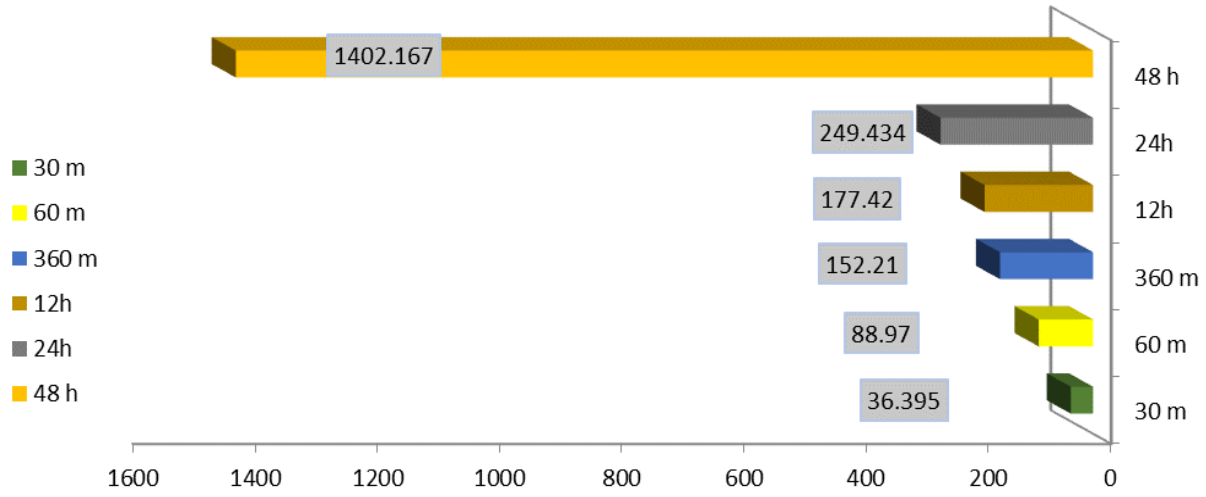

Figure 1. Highest value Concertation of SP-A (ng/l) in serum with times in drowning cases

Discussion

The SP-A concentration increment with longer period of time increasing duration of immersion in water before recovering the body from water, we observe a concentration at the beginning of 30 minutes was (10.655 ng/l) concentration while at the end of 30 minutes (36.395 ng/l) and also the concentration at the beginning of 48 hours (303.938 ng/l), at end was (1402.167 ng/l), the reason is inhaling water during drowning leads to the rupture the wall of alveoli, thus leading to the exit of SP-A into the blood stream while staying in water with longer period of time lead to increase damage to the wall of the alveoli and concentration of SP-A in the blood was increment. Some victims were having lung diseases that exerted an effect on alveolar wall leading to increment the concentration of SP-A in the serum. This study agreed with study in Italy the SP-A as a marker of asphyxiation and drowning. The postmortem diagnosis of drowning continues to be one of the most difficult in forensic pathology because of unspecific autopsy findings This study shows that the concentration of SP-A increment with prolongation of immersion in it is a good strengthening the diagnosis of drowning cases as it is a good marker of alveolar injury [39].

This study disagreed with study in Italy; the massive and dense intra-alveolar SP-A aggregates can only support the final diagnosis of drowning as well as duration and severity of respiratory distress. Intense membranous or linear SP-A pattern as well as low granular scores can only support the detection of pulmonary edema fluid on the intra-alveolar surface [40].

As a conclusion, this study showed an increment of the SP-A concentration with stay victims for long time inside water and serum SP-A test for diagnosis of drowning cases but not fully specific but is a good marker of alveolar injury.

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Author contribution

Dr. Hussein collected cases and wrote the research, Dr. Hashim and Dr. Abdulla did the statistic to research.

Conflict of interest

None to be declared.

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The Validity of Different F Wave Parameters in The Diagnosis of Diabetic Axonal Polyneuropathy

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Abstract

Background The underlying pathology of the vast majority of diabetic polyneuropathies is axonal degeneration. F wave study is one of the most sensitive indices of the severity of neuropathy.

Objective To test the validity of different F wave parameters including F minimum latency, F wave index and F Jitter in the diagnosis of diabetic axonal peripheral neuropathy.

Methods Eighty type 2 diabetics aged 52.57±5.62 years with disease duration of 1 to 18 years and 90 aged-matched healthy volunteers serve as the control group. Both groups were submitted to medical history, clinical neurological examination, and electrophysiological tests of both upper and lower limbs.

Results Tibial and ulnar F wave latencies were significantly prolonged in diabetic patients ($p < 0.001$). Tibial F index for male patients shows significantly lower value as compared to the control group. Ulnar F wave latency was 76.7% sensitive and 89.3% specific in female patients while tibial F wave latency was 80% sensitive and 81.3% specific in male patients.

Conclusion F wave is a precise parameter in detecting diabetic axonal peripheral neuropathy. Minimal F-wave latency is more sensitive than both F index and F Jitter in the diagnosis of axonal neuropathy in diabetic patients.

Keywords DM, Axonal neuropathy, Ulnar, Tibial, F-wave latency, F-jitter, F-index

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List of abbreviations: NCS = Nerve conduction study, ROC = Receiver operating characteristic

Introduction

Diabetic neuropathies are frequent chronic complications of diabetes mellitus [(1)]. Chronic distal symmetric polyneuropathy is the most common type and accounts for 75% of cases of diabetic neuropathies [(1,2)].

The diagnosis of diabetic polyneuropathy depends on the appropriate clinical history and clinical neurological examination [(3)]. According to the Toronto consensus criteria, probable neuropathy is defined as the presence of at least two of the following: neuropathic symptoms, reduced distal sensation, or diminished or absent ankle reflexes. Abnormal nerve conduction study (NCS) or a proven study of small-fiber function [(4)] would confirm the final diagnosis.

For an accurate diagnosis of diabetic sensorimotor neuropathy, studies always recommend the presence of a combination of neuropathic symptoms and signs plus certain and specific abnormalities in NCS criteria [(5)]. On many occasions, the diagnostic value of NCS is

questioned, i.e., patients presented with signs of atypical neuropathy may need an electrodiagnostic examination to diagnose his condition. On the reverse, patients with typical diabetic polyneuropathy may not need NCS to settle the diagnosis [6].

Seventy years have been passed since Magladery and McDougal [7] describes the importance of F-wave in the assessment of peripheral neuropathies, in particular, those of axonal type [8]. It serves as a sensitive measure for axonal polyneuropathy and radiculopathy and is used in the diagnosis of diabetic polyneuropathy, Guillain–Barrè syndrome, and amyotrophic lateral sclerosis. F waves also help in the early detection of abnormality in motor fibers [9].

Peripheral neuropathies can be documented by F-wave abnormalities even before any change in the compound muscle and sensory nerve action potentials and might be the only abnormality disclosing a neuropathy, precisely the diabetic type [10]. F wave slowing to a lesser degree may be seen in other axonal and mixed polyneuropathies [11] though, F wave latency slowing seems to be of value in nerve pathologies especially in patients with diabetes mellitus [12].

Various F wave parameters are used for the diagnostic evaluation of peripheral nerve disorder; among them are the minimal F wave latency, F wave chronodispersion, F wave persistence, and the F wave conduction velocity [11,13-16].

The objective of current study is to test the validity of different F wave parameters including F minimum latency, F wave index and F Jitter in the diagnosis of diabetic axonal peripheral neuropathy.

Methods

This is a case-control study carried out at the Neurophysiology Department of Al- Imamein Al-Kadhimein Medical City in Baghdad, for the period from Dec. 2017 to Jul. 2018.

The study was approved by the Iraqi Council of Medical Specialization (Decision number: 860, Date 12.02.2018). Written informed consent was obtained from all individual participants included in the study.

Subjects

Eighty type 2 diabetic patients (40 females and 40 males) with clinical signs and symptoms of peripheral neuropathy were recruited for the study. Their ages range from 40 to 60 years (mean±SD = 52.57±5.62 years) with a disease duration of 1-18 years. Another 90 aged-matched healthy volunteers (43 females and 47 males) with a mean age of 51.93±6.66 years served as the control group.

Exclusion criteria

Those patients who had a history of carpal tunnel syndrome, Guillian-Barre syndrome, ulnar and tibial neuropathy, myopathy, hypothyroidism, neuromuscular disorders, fractures of upper or lower limbs, and patients with pacemaker were excluded from the study.

Methods

History and clinical examination

The patients were referred by senior Neurologist and/or Endocrinologist after taking brief medical history from each patient including age, onset, and duration of symptoms, past medical history, and signs and symptoms of peripheral neuropathy based on the Toronto Clinical Score [17] (which included the presence of clinical features such as unpleasant, unusual or abnormal sensation such as burning pain, electric shock-like sensations, tingling, pins and needles formication, prickly feeling and cramp-like sensation in the lower and upper limb).

Also, a clinical neurological examination was done for each patient, including motor, sensory and cranial nerve examination. Deep tendon reflexes were graded based on the amplitude of the response [18] and muscle strength (power) grading was measured according to the extended MRC scale [19].

Electrophysiological assessments

Key point (Medtronic functional Diagnosis A\S - DK-2740 Skovlunde, Denmark) EMG machine

was used throughout the study. The room temperature was monitored between (25-28 °C) during the test procedures and skin temperature between (32-34 °C) was ensured using a skin thermometer.

According to the methods adopted by Preston and Shapiro [20], The following electrophysiological tests were performed:

1. Bilateral sensory nerve conduction (SNC) of the median, ulnar, and sural nerves.
2. Bilateral motor nerve conduction (MNC) of the median, ulnar, common peroneal and tibial nerves recorded from abductor digiti minimi, abductor pollicis brevis, extensor digitorum brevis, and abductor hallucis brevis, respectively.
3. Bilateral F wave elicited by distal stimulation of the ulnar and tibial nerves at the wrist or ankle and recording from abductor digiti minimi and abductor hallucis brevis at relaxed state, respectively.

A total of 10 stimuli were considered appropriate to explore the full potential of F waves. To be clearly identifiable, F waves should be at least 20 µV in peak-to-peak amplitude to differentiate them from background noise. The conventional stimulus intensity is 25 percent above maximal for eliciting a direct response. This provides a consistent physiologic environment for eliciting F waves.

The following F wave parameters were studied:

a) F wave minimum latency which represents conduction of the largest and fastest motor fibers and measured from the start of the stimuli to the onset of the response.
b) F persistence which is a measure of the number of F waves obtained for the number of stimulations.
c) F choronodispersion which denotes the degree of scatter among consecutive F waves and is determined by the difference between the minimal and maximal F wave latencies. It indicates the range of motor conduction velocities between the smallest and largest myelinated motor axon in the nerve.
d) F index: calculated by the following equation: F wave index = [F persistence × Arm length) / (F latency × F chronodispersion] [21].
e) F jitter: stands for the latencies of consequent F waves. If a specifically recorded trace showed and absent F wave, it will be omitted and the next trace was analyzed instead. F jitter = (|f2 – f1| + |f3 – f2|+ |f4 – f3)…. / (n – 1) [22].

An obstetric tape measure was used for limb length. In the upper limbs, the surface measurement from the stimulus point to the C7 spinous process with the limb extended and abducted 90 degrees pronated via the axilla and midclavicular point gives a close estimate of the nerve length. For the lower limb, the nerve course is measured from the stimulus site to the T12 spinous process by way of the knee and greater trochanter of the femur.

The electrophysiologic settings for adequate display of F waves were an amplifier gain of 200 or 500 µV per division and a sweep of 5 or 10 msec per division.

Statistical analysis

Microsoft excel 2016 and SPSS (statistical package for social sciences) version 23 were used as a software to do the statistics. Continuous data were presented as mean±standard deviation, and comparison between means of study groups was done by using unpaired student t-test. A p-value of less than 0.05 was considered significant. Cutoff values of the prolonged F minimum latency, F jitter, and decreasing F index and accordingly the sensitivity and specificity were evaluated by using the receiver operating characteristic (ROC) curve.

Results

Table 1 shows the demographic and neurophysiologic data of the diabetic patients. The impact of gender differences in limb length reflected as significant difference between females and males considering the upper and lower limbs ($p = 0.007$; $p < 0.001$). No significant difference was noticed between the age of the patients 52.57±5.62 years and control subjects 51.93±6.66 ($p = 0.692$).

No significant difference was noticed in the mean values of ulnar and tibial F latency, F index and F jitter between the right and left side in both genders of the control and patient groups (Table 2).

Table 1. Demographic and neurophysiologic data of diabetic patients

Age (years)		52.57±5.62
Sex	Females	40
	Males	40
HbA1c %		8.99±2.31 (6.5-13.5)
Disease duration (years)		1-18
Limb length (cm)		
Females	Upper limb (female)	72.94±4.7
	Lower limb (female)	86.35±3.87
Males	Upper limb (male)	78.03±4.35
	Lower limb (male)	92.1±3.29
Compound muscle action potential amplitude (mV)	Median	4.2±1.3
	Ulnar	6.04±1.2
	Peroneal	2.42±0.6
	Tibial	3.8±1.22
Sensory nerve action potential amplitude (µV)	Median	14.31±1.7
	Ulnar	12.21±1.7
	Sural	5.62±3.1
Motor conduction velocity (m/sec)	Median	53.73±3.7
	Ulnar	51.12±2.3
	Peroneal	42.6±2.7
	Tibial	45.2±2.2
Sensory conduction velocity (m/sec)	Sural	42.35±2.4
	Median	53.61±3.8
	Ulnar	52.3±2.7

CMAP = compound muscle action potential; SNAP = sensory nerve action potential; MCV = motor conduction velocity; SCV = sensory conduction velocity.

Accordingly, these data were pooled together and tabulated as one group for females and males for future comparisons. Table 3 illustrates longer tibial and ulnar F latency ($p < 0.001$) in female patients as compared to the control group. Also, the tibial F index was significantly lower ($p = 0.038$) in female patients when compared to female controls.

For males, significantly longer tibial and ulnar F latency in the patient group when compared to the control group ($p < 0.001$). likewise, the tibial and ulnar F index was significantly reduced ($p < 0.001$; $p = 0.001$, respectively) in male patients as compared to male controls.

Sensitivity and specificity of F wave parameters

A ROC analysis curve was constructed for F minimum latency, F index, and F jitter for the tibial and ulnar nerves in both genders. Regarding female patients, tibial F latency was 73.3% sensitive and 82.1% specific which is the highest estimated value among other parameters. For the ulnar nerve, the F latency was 76.7% specific and 89.3% specific which was the highest estimated value.

For male patients, tibial F latency has the highest sensitivity (80%) and specificity (84.4%) among the three F wave parameters. Also, ulnar F latency demonstrates 80% sensitivity and 81.3% specificity which is the highest among other parameters (Table 4, Figures 1 and 2).

Table 2. Ulnar and tibial F latency, F index and F jitter in the diabetic patients and controls (unpaired t-test)

Nerve/parameter		Female controls		P-value	Female patients		P-value
		Right side N=43	Left side N=43		Right side N=40	Left side N=40	
Tibial	F latency	47.43±4.02	48.41±3.92	0.520	53.82±5.51	53.74±5.19	0.968
	F index	62.14±23.26	67.14±23.35	0.575	55.33±38.52	43.33±20.59	0.296
	F jitter	0.28±0.2	0.18±0.14	0.128	0.27±0.21	0.38±0.53	0.468
Ulnar	F latency	24.89±1.41	24.98±1.13	0.849	27.13±1.98	26.98±1.9	0.830
	F index	102.86±32.45	132.14±63.39	0.136	95.33±43.89	100.67±39.18	0.728
	F jitter	0.19±0.15	0.2±0.14	0.771	0.19±0.09	0.17±0.13	0.569
Nerve/parameter		Males controls		P-value	Male patients		P-value
		Right side N=47	Left side N=47		Right side N=40	Left side N=40	
Tibial	F latency	50.36±4.31	50.52±4.6	0.919	60.15±9.21	59.08±8.33	0.740
	F index	61.25±19.96	69.38±36.05	0.436	35.33±25.03	39.33±27.64	0.681
	F jitter	0.32±0.26	0.33±0.15	0.888	0.28±0.19	0.3±0.18	0.837
Ulnar	F latency	26.63±2.16	26.96±1.96	0.653	31.03±3.96	30.73±4.3	0.847
	F index	118.75±79.32	131.25±71.45	0.643	80.67±44.15	66.67±29.92	0.318
	F jitter	0.27±0.14	0.21±0.14	0.238	0.21±0.27	0.5±0.94	0.267

N = number of subjects

Table 3. Ulnar and tibial F latency, F index and F jitter of the patients and controls (unpaired t-test)

Nerve/parameter		Females		P-value	Males		P-value
		Control N=86	Patients N=80		Control N=94	Patients N=80	
Tibial	F latency	47.92±3.93	53.78±5.26	<0.001	50.44±4.39	59.62±8.65	<0.001
	F index	64.64±23.01	49.33±30.95	0.038	65.31±28.96	37.33±25.99	<0.001
	F jitter	0.23±0.18	0.32±0.4	0.245	0.32±0.21	0.29±0.18	0.491
Ulnar	F latency	27.06±1.9	24.93±1.25	<0.001	26.79±2.04	30.88±4.06	<0.001
	F index	98±40.97	117.5±51.61	0.115	125±74.53	73.67±37.74	0.001
	F jitter	0.18±0.11	0.2±0.15	0.643	0.24±0.14	0.36±0.69	0.355

N = represent the number of limbs examined (right and left)

Table 4. The area under the curve, sensitivity, specificity, and cutoff value for tibial and ulnar F wave parameters in patients of both genders

Nerve/parameter		AUC	Sensitivity	Specificity	Cutoff value
Females					
Tibial	F latency	0.828	73.3%	82.1%	52.05
	F index	0.693	46.4%	73.3%	65.0
	F jitter	0.562	50.0%	50.0%	0.24
Ulnar	F latency	0.827	76.7%	89.3%	26.35
	F index	0.610	46.4%	66.7%	0.105
	F jitter	0.510	50.0%	56.7%	0.195
Males					
Tibial	F latency	0.850	80.0%	84.4%	54.55
	F index	0.808	68.8%	73.3%	45.0
	F jitter	0.538	50.0%	56.7%	0.315
Ulnar	F latency	0.832	80.0%	81.3%	28.65
	F index	0.768	65.6%	70.0%	85.0
	F jitter	0.549	59.4%	63.3%	0.205

AUC = area under the curve

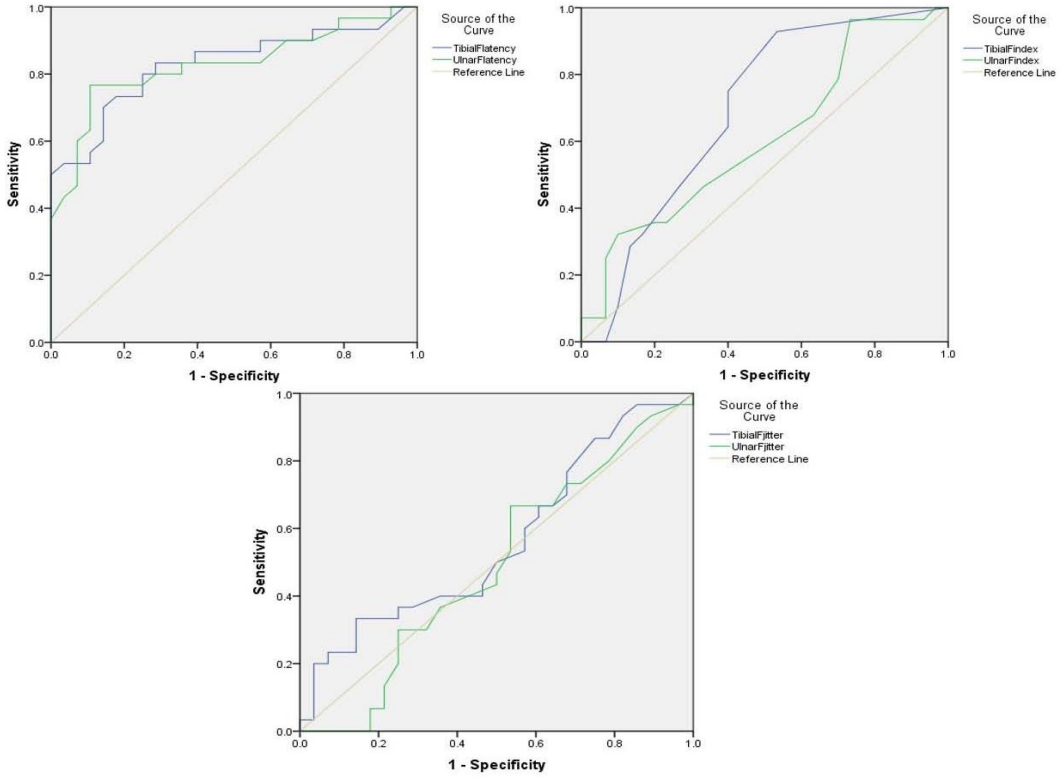

Figure 1. Receiver operating characteristics curve for tibial and ulnar. (upper left) F latency (upper right) F index in male patients (bottom) F jitter in female patients

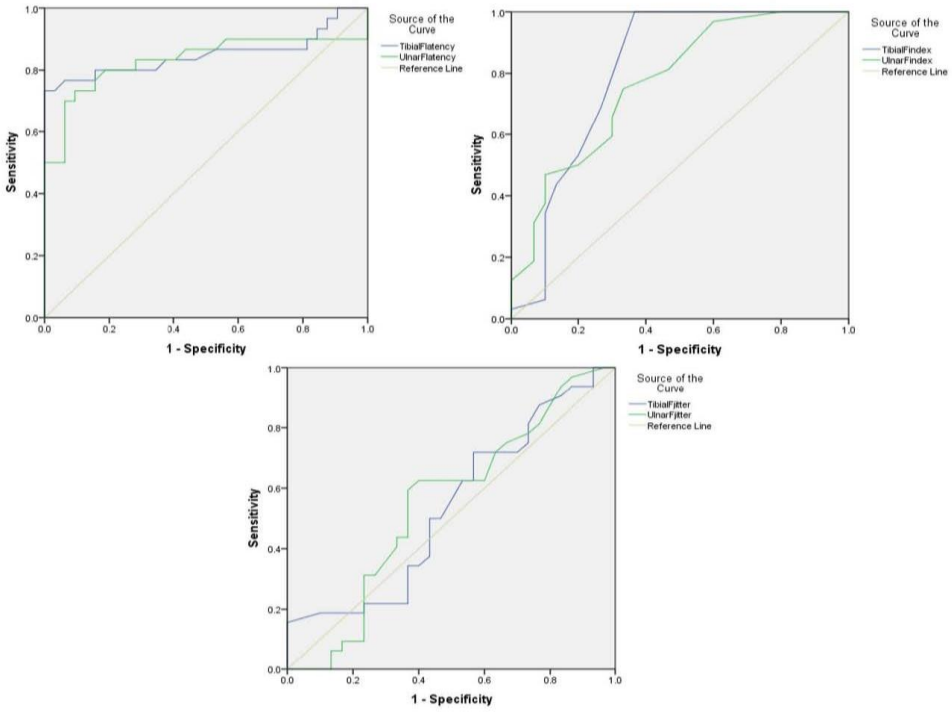

Figure 2. Receiver operating characteristics curve for tibial and ulnar. (upper left) F latency (upper right) F index in male patients (bottom) F jitter in male patients

Discussion

In peripheral neuropathies, F-wave minimal latency is usually prolonged and it could be abnormal in cases where the motor conduction studies are normal [23]. Moreover, it is also crucial in axonal neuropathies when compared to conventional motor conduction studies [24]. Furthermore, it was the most stable and consistent parameter for serial NCS in the same subjects [23] and is the reliable measurement in patients with diabetic neuropathies [16].

Previously, during routine electrophysiological studies of patients with diabetes mellitus, F wave has been studied to assess the proximal parts of the motor nerves with almost conflicting outcomes. As some researchers did not notice any differences between the proximal and distal nerve segments, others have revealed slightly but significantly more distinct slowing in the distal nerve segments [11].

Because the minimal F wave latency is a direct estimate of the conduction along the entire length of the nerve, it can correlate with height or limb length. Therefore, it is more reliable and can amplify nerve conduction in detecting peripheral neuropathies than using the conventional compound muscle action potential method, which is limited to a small segment of a peripheral nerve.

The recorded F-wave minimum latencies of ulnar and tibial nerves were per that reported by other researchers [25,26]. No significant side to side difference was demonstrated in the F-wave minimum latency of upper and lower extremities. This was also reported by others [27,28] but contrary to the results of other researchers [29] who reported intrasubject variability in F-minimum latency of upper and lower limb nerves. The latter discrepancy could be due to the difference in the subjects' age studied.

The present study has verified the direct relationship between minimum F wave latency and height for all nerves as documented by others. The taller the subject, the longer the nerve, and hence, the latency of conduction also prolongs. F wave latency has been documented to prolonged with height by 0.2 ms/cm in the upper and 0.4 ms/cm in the

lower limbs [30,31]. The present data confirm in apart the well-known correlation of F wave latencies to the limb length and height.

In the present study, a significant effect of limb length on all tested F wave parameters was found; since they all depend on the distance from which the impulse traveled antidromically from the site of stimulation and up to the spinal cord and then descend again. Distance effect on different F wave measurements is demonstrated as follows:

1. F wave minimum latency calculated as the time needed for the impulse to travel up reaching to proximal spinal cord segment and then descend again.
2. F wave jitter corresponds to latencies of consequent F waves [22].
3. F index calculation includes the limb length as one of its factors (F wave index [F persistence × arm length) / (F latency × F chronodispersion] [21].

A significant difference between males and females concerning their limb length (leg and arm length), they were dealt with as two separate groups. Gender had a significant effect on F minimum latency [32]. The effect of gender on nerve conduction parameters can be explained based on gender-wise differences in anatomical and physiological factors [33]. This gender difference in NC parameters could be due to the difference in height as the action potential through the nerve has to travel greater distance [34].

Prolonged F-wave latency was demonstrated in diabetic patients of the current study as compared to the control subjects. This finding was also noticed by other researchers [35]. Moreover, Pan et al. denote that F- waves of the tibial nerves are the most sensitive measure to detect subclinical or overt diabetics [10].

Two factors could contribute to the prolonged F-wave minimal latency; first is the diminished excitability of the anterior horn cells and second is the selective loss of the fastest conducting axons [14].

The present study demonstrates a reduced F index in diabetic patients with peripheral neuropathy as compared to the control subjects. This finding was also reported by Sathya et al [21].

About F- jitter, the results of the current study were contradictory insignificant. In female patients, it shows increment in the tibial nerve and decrement in the ulnar nerve and almost the reverse in male patients. This finding was in contradiction to that of Uludağ et al. [22] which denotes significant increment in F jitter in patients with polyneuropathy. This discrepancy could be attributed to the smaller sample size in their study and type of polyneuropathy (as they did not recognize the peripheral neuropathy whether axonal or demyelinating).

In the present study, F minimum latency shows the highest sensitivity and specificity as compared to the F index and F jitter in the patient group. These findings were in disagreement with the results of Sathya et al. [21] considering F index and Uludağ et al. [22] considering F jitter. This discrepancy could be ascribed to the difference in height and limb length, smaller sample size and type of neuropathy.

The F minimal latency was the parameter used most frequently and of which sensitivity is highest in demyelinating polyneuropathy [9]. This study confirms that F-wave minimal latencies can detect early changes in predominantly axonal neuropathy. Adding F-wave latencies to the motor and sensory conductions improved the sensitivity of the detection of electrophysiologic abnormalities from 3% to 36% in asymptomatic patients of diabetes [10].

This study concluded that F wave is a sensitive parameter in detecting diabetic axonal peripheral neuropathy, minimal F-wave latency is more sensitive than both F index and F Jitter in the diagnosis of diabetic axonal neuropathy, and measurement of F wave latency highly correlated with the length of the studied nerve. The authors recommend a study with larger sample size, study twenty F wave responses rather than 10 as in this study to increase the chance of detecting any possible changes in F wave and any alteration of motoneuronal excitability, and study the F repeater to assess the available motor neurons with each firing.

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Author contribution
All the authors have directly participated in the preparation of this manuscript and have approved the final version submitted. Dr. Yaqoub contributed to the collection of cases and drafted the manuscript. Dr. Kaddori and Dr. Hamdan conceived the study and participated in its design and interpretation. Dr. Kaddori and Dr. Hamdan supported manuscript drafting. All the authors have read and approved the final manuscript.

Conflict of interest
The authors declare no conflict of interest.

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Association of CD46 Cellular Receptor Gene SNP in Measles Vaccine Response

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Abstract

Background Measles is a highly contagious viral disease. It remains an important cause of death among young children globally, despite the availability of a safe and effective vaccine. Measles transmitted via droplets from the nose, mouth or throat of infected persons.

Objective To evaluate the immune response to measles infection among immunized school aged children, and to detect the cluster of differentiation 46 single nucleotide polymorphism (CD46 SNP) in association with the immune response.

Methods The current study is a cross sectional study including 158 hospitalized patients were previously vaccinated with two doses of measles-mumps-rubella vaccine. The ages of patients were 5-10 years school aged children. All samples were collected from blood collection unit in Al-Imamein Al-Kadhimein Medical city during the period from December 2018 to April 2019. The detection was based on the presence of IgG Antibody to measles. The positive results were considered according to enzyme linked immunosorbant assay and conventional polymerase chain reaction to detect CD46 SNP.

Results Among those 158 subjects' male children count for 62% and female children were 38%. Forty-one (41) immunized children (with mean age 7.98±1.92 years) were low immune response to measles vaccine (MV). On the other hand, only two cases (with mean age 5.50±0.71 years) were negative to measles virus vaccine.

Conclusion Ensuring two doses of MV give protective immune response to MV may declines with aging. CD46 cellular receptor gene SNP (rs7144) may play role in reduction in immune response to MV.

Keywords Measles, MMR, CD46, SNP, Iraq

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List of abbreviations: CD46 = Cluster of differentiation 46, ELISA = Enzyme linked immunosorbant assay, MMR = Measles, mumps rubella vaccine; MV = Measles vaccine, PCR = Polymerase chain reaction, SNP = Single nucleotide polymorphism

Introduction

Measles is a highly infectious disease that results from infection with the measles virus. Measles was one of the top causes of childhood morbidity and mortality and responsible for over 2 million childhood deaths each year before the introduction of measles vaccines and the increase in global measles vaccine coverage resulting from the Expanded Program on Immunization (EPI) that started in the 1980s [(1)]. Measles incidence and mortality have declined substantially in the last 20 years due to the improvement in socioeconomic status, better nutrition, and the increasing use of live attenuated measles vaccines administered through routine childhood immunization programs and mass vaccination campaigns [(2)].

The World Health Organization (WHO) reports annually the number of reported measles cases, the estimated number of deaths, as well as on national measles vaccination coverage. Reported measles cases decreased worldwide from 850,000 to 250,000 between 2000 and 2015 [3]. During the same time period, estimated measles deaths, derived from a model based on reported cases, vaccine coverage, and age-specific fatality ratios [4], decreased by almost 80% [2]. Global measles vaccine coverage with the first dose of measles vaccine (MV) increased from 72 to 85% from 2000 to 2010, but since then has plateaued at about 85% [3]. In 2012, the World Health Assembly endorsed the Global Vaccine Action Plan with the objective of eliminating measles [5]. The encouraging reductions in measles incidence and mortality have led to five out of six WHO regions adopting the Global Vaccine Action Plan with targets to eliminate measles by 2020 [5]. Studies had shown that measles vaccine immune response declines due to genetic variants in CD46 cellular receptor that is responsible for the introduction and enhancement of immune response to MV ([6, 7]. This study aimed to evaluate the immune response to measles infection among immunized school aged children, and to detect the CD46 SNP in association with the immune response.

Methods

The current study is a cross sectional study including 158 hospitalized patients were previously vaccinated with measles, mumps and rubella (MMR) vaccine.

This study under the agreement of the ethical approval of the Institutional Board Review (IRB) in the College of Medicine, Al-Nahrain University under the No. 165 dated 17/12/2018.

The ages of patient 5-10 years school aged children. From all selected patients, 5 ml of whole blood were collected. Two ml of blood were collected in EDTA tubes for DNA extraction and conventional PCR and store at (-20 °C). DNA extraction was done according to DNA extraction protocol (Geneaid Kit, Taiwan)

The residual 3ml of blood were separated into serum for enzyme linked immunosorbant assay (ELISA) detects IgG Ab level to measles.

Conventional polymerase chain reaction (PCR) was used to detect the presence of SNP in CD46 cellular receptor gene that expected to affect the IgG antibody level in patient who is previously vaccinated with MMR vaccine. Primers were designed in online version of primer design software by Integrated DNA Technologies website and synthesized using Oligo Synthesis Service in Accuoligo® Bioneer, Korea. as showed in table (1) and prepared for PCR reaction according to manufacture instructions. All primers that used in this experiment are demonstrate in table (1).

Table 1. The primers used in this study

Gene		Primer sequence (5'----------3')	Product size (bp)	Reference
CD46 rs2724384	F	CAAGTCCATTTCCTCCACTG	228	(Clifford 2010) [7]
	R	GGTTTACCAATGAGCTCCATA		
CD46 rs7144	F	AAGTGAACACTGTAGTCTTGTT	272	(Clifford 2010) [7]
	R	TCTGCCTTTTAGGAGATGAG		

Results

This study included 158 hospitalized school age children were previously vaccinated with measles virus vaccine (MV). Forty-one children were low immune response to MV with mean age 7.98±1.92 years. Two cases were negative to measles virus vaccine with mean age 5.50±0.71 years. Eighty-nine cases were

moderate immune response to MV with mean age 6.81± 1.57 years. Twenty six cases were high immune response to MV with mean age 6.81±1.10 years.There was a significant statistical difference in the means of ages among the study groups as shown in table (2).

Table 2. The mean and standard deviation of vaccinated school age children

Age	Vaccine response				
	Negative (n=2)	Low (n=41)	Moderate (n=89)	High (n=26)	Total (158)
Mean	5.50	7.98	7.98	6.81	7.75
Standard Deviation	0.71	1.92	1.57	1.10	1.66
Median	5.50	8.00	8.00	7.00	8.00
Percentile 25	5.00	6.00	7.00	6.00	6.00
Percentile 75	6.00	10.00	10.00	8.00	9.00
Minimum	5.00	5.00	5.00	5.00	5.00
Maximum	6.00	10.00	10.00	9.00	10.00
P value	0.002				

The study also classified patients according to their age groups, the results shows low immune response 12.9% in age group 7 years. While in age 10 years was 36.3%. Moreover, the moderate immune response was 41.9% in age range from (5-6) years. While in age groups 7 and 10 years was around 64%. High immune response was measured in (5-6) years age group and it was 27.9%. While no one had high immune response, in age group 10 years (0.0%). There were statistically significant differences (P=0.032) between age groups as shown in table (3).

Table 3. Patients' categories according to age groups

Vaccine Immune Response	Age group (year)				
	5-6 years	7 years	8 years	9 years	10 years
Negative	2	0	0	0	0
%	4.7	0.0	0.0	0.0	0.0
Low	11	4	6	7	13
%	25.6	12.9	22.2	33.3	36.1
Moderate	18	20	16	12	23
%	41.9	64.5	59.3	57.1	63.9
High	12	7	5	2	0
%	27.9	22.6	18.5	9.5	0.0
Total	43	31	27	21	36
P value	0.032*				

p value < 0.05

On the other hand, the study shows variation in immune response to MV vaccine according to number of vaccine doses; 60.8% of cases were immunized by three doses of MV vaccine with moderate immune response. In addition to that, 47.9% cases were immunized by 2

doses of MV vaccine with moderate immune response. There were statistically significant differences (P=0.008) between age groups as shown in table (4).

Table 4. Vaccine categories according to vaccine doses

Vaccine Response Category	Number of vaccines Doses			Total
	1.00	2.00	3.00	
Negative	1	1	0	2
%	12.5	2.1	0.0	1.3
Low	2	19	20	41
%	25.0	39.6	19.6	25.9
Moderate	4	23	62	89
%	50.0	47.9	60.8	56.3
High	1	5	20	26
%	12.5	10.4	19.6	16.5
Total	8	48	102	158
%	100.0	100.0	100.0	100.0
P value	0.008*			

P value < 0.01 highly significant

The study also showed the frequency of heterozygous mutant (CT) 25% was slightly more in age group 10 years, a marked rise in homozygous mutant genotype among the same groups (22.2%). Moreover, there were significant elevated in homozygous wiled genotype (TT) 80.6% in age group 7 years. Likewise, the frequency of minor allele (CT) 34.7% in age group 10 years. There were elevated in minor allele (TT) among age group 5-6 years. There were statistically significant differences among age groups as shown in table (5).

Table 5. Vaccine categories according to vaccine doses

Age Group	Genotype			Total
	Homozygous mutant	Heterozygous	Homozygous wild	
5-6 years	1	7	33	41
%	2.4	17.1	80.5	100
7 years	4	2	25	31
%	12.9	6.5	80.6	100
8 years	0	6	21	27
%	0.0	22.2	77.8	100
9 years	3	3	15	21
%	14.3	14.3	71.4	100
10 years	8	9	19	36
%	22.2	25.0	52.8	100
Total	16	27	113	156
%	10.3	17.3	72.4	100
P value	0.001*			

P value < 0.01 highly significant

In addition to that, this study showed vaccine response level according to the polymorphism in CD46 gene there were significant elevated in heterozygous genotype (CT) 36.4% in subjects with low immune response to Measles vaccine. While there were elevated in homozygous wild genotype (TT) 85.5%. Moreover, there were no significant differences in heterozygous and homozygous mutant genotypes except in high immune response as shown in table 6.

Table 6. The association of vaccine response level according to the polymorphism in CD46 gene

Age Group	Genotype			Total
	Homozygous mutant	Heterozygous	Homozygous wild	
Low	26	40	44	110
%	23.6	36.4	40.0	100
Moderate	6	14	118	138
%	4.3	10.1	85.5	100
High	0	0	64	64
%	0.0	0.0	100.0	100
Total	32	54	226	312
%	10.3	17.3	72.4	100
P value	<0.001*			

P value < 0.01 highly significant

Based on allele specific technique (Figure 1), this polymorphism had only one genotypes in vaccinated school age children which were CC. The results showed the frequency of homozygous mutant genotype of CD46 receptor gene CC polymorphism was found in patient with lower level of IgG antibody response.

Figure 2 shows the polymorphism had only two genotypes in patient that immunized with measles vaccine which where TT and CT. It showed the frequency of genotype and allele of CD46 receptors gene polymorphism in immunized children. Heterozygous genotype CT was higher in patient with intermediate immune response to measles mumps vaccine.

Discussion

There are several reasons for an outbreak of measles among children, like variability in MV response between children. Poor immunogenicity and vaccine failure in young children contribute to the ongoing burden of measles. This study aimed to identify whether genetic variation in the MV receptor CD46 gene contributed toward measles-specific antibody responses in an Iraqi cohort of children following their contact with measles vaccine. This study also aimed to investigate whether CD46 polymorphisms were associated with functional effects on the CD46 receptor in order to determine a possible mechanism through which the genetic variant acts on antibody responses. Three CD46 gene polymorphisms were associated with measles IgG levels (rs7144, rs11118580, and rs2724384), supporting the hypothesis that CD46 gene polymorphism plays an important role in how a child responds to measles vaccine [6]. There is significant association between rs7144 CT and rs7144 CC and lowering the IgG Ab level particularly in children with aged between 9-10 years, there were significant decrease in IgG Ab due to the heterozygous, homozygous mutant allele CC, CT respectively in compared to TT homozygous wiled type allele that have been association with elevated IgG Ab level, this is agreed with Clifford et al. (2012) [7].

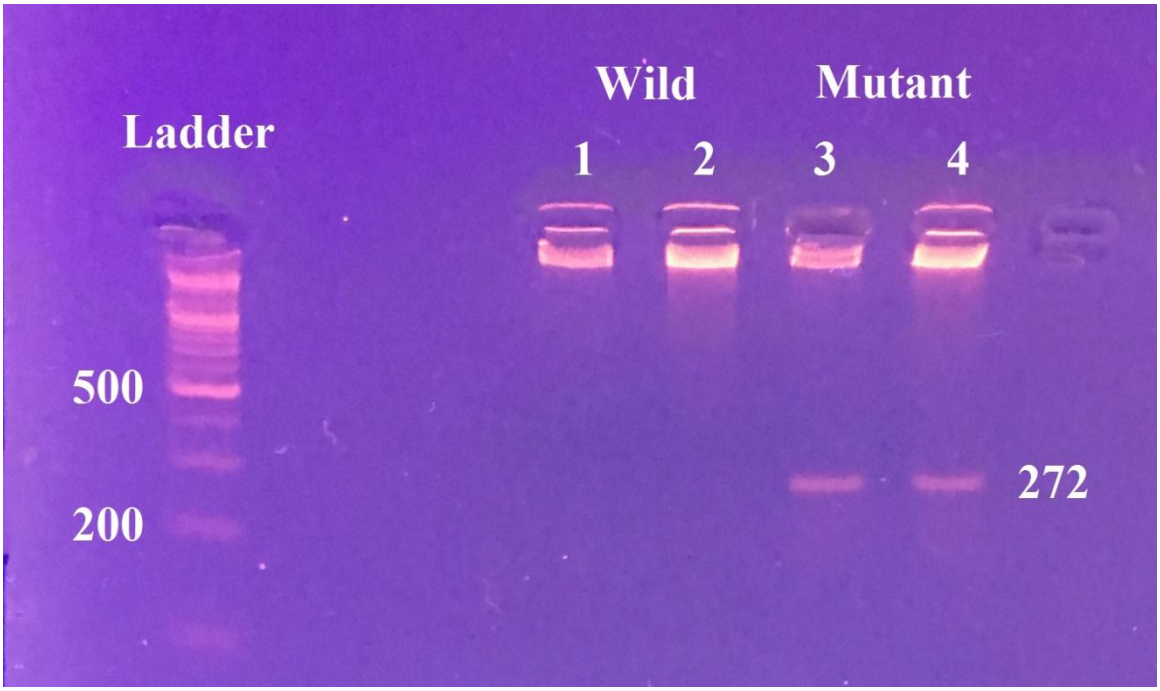

Figure 1. Gel electrophoresis for CD46 gene PCR products visualized U.V light after staining with ethidium bromide. Ladder:100-2000 bp; lanes 3,4; homozygous genotype CC, lanes 1,2; homozygous wild genotype TT

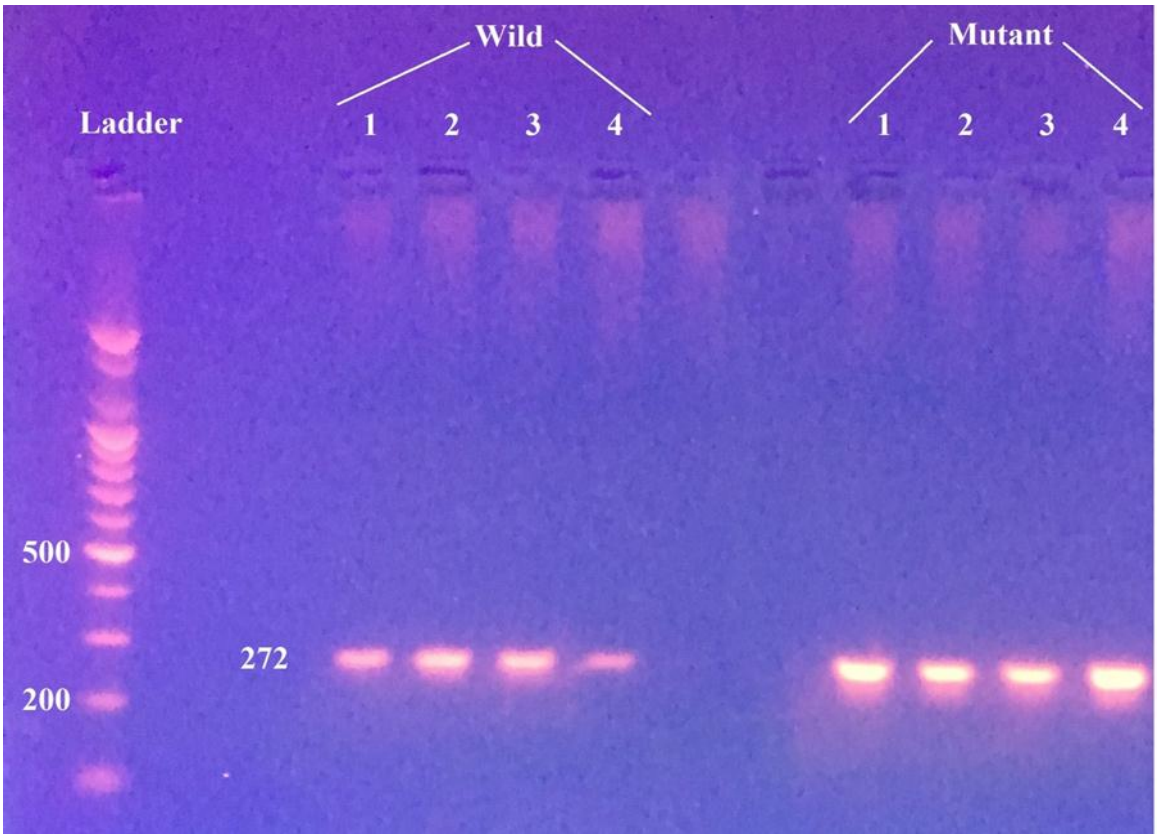

Figure 2. Gel electrophoresis for CD46 gene PCR products visualized U.V light after staining with ethidium bromide. Ladder: 100-2000 bp; the right lanes 1,2,3 and 4: homozygous mutant genotype CC, and the left lanes1,2,3and 4: heterozygous mutant genotype CT

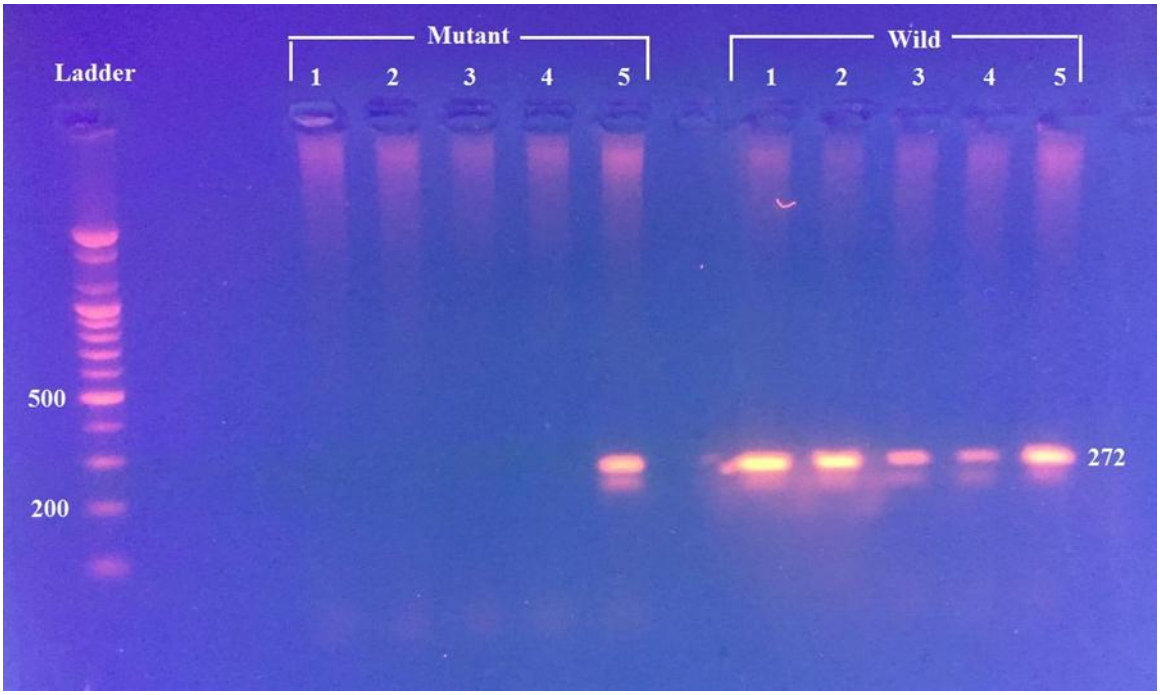

Figure 3. Gel electrophoresis for CD46 gene products visualized U.V light after staining with ethidium bromide. Ladder: 100-2000 bp;1,2,3 and 4 homozygous wild genotype TT. Lane 5; heterozygous CT

This study also showed the elevated proportion in number of subjects who were taken three doses of MMR vaccine, they showed moderate immune response (60.8%), in addition to those subjects with two doses of MMR vaccine they showed low immune response (47.9%), this could be due to the possibility of existence of heterozygous and homozygous genotype (CT, CC) (10.1%, 4.3%) respectively in subjects with moderate immune response to MV response, on the other hand, the frequency of minor allele (CT, CC) were showed in subject with low immune response (36.4%, 36.6%) respectively these results agreed with Dhiman et al. (2007) [6].

The study also showed that children unknown history of vaccination was about 12.5% with negative immune response to MMR vaccine, 25%, 50% subjects with low and moderate immune response respectively to MMR vaccine who had an unknown vaccination history or sub-optimal measles vaccine coverage. The majority of those with an unknown vaccination history were among the cases reported from those living outside the capital city of the province [8]. A study showed the immune response to MV was measured as low response 12.9% in age group 7 years. While in age 10 years was 36.3%. Moreover, the moderate immune response was 41.9% in age range from 5-6 years, while in age groups 7 and 10 years was around 64%. High immune response was measured in (5-6) years age group and it was 27.9%. While no one had high immune response, in age group 10 years (0.0%). This could be due to that immune response may decline with age [9,10].

In conclusions, this study showed that CD46 SNP may affect immune response to MV, in addition to the possible reduction in immune response to this vaccine with age.

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Author contribution

Al-Gburi: did the sampling and lab works; Dr. Kadhim supervised the study; Dr. Ghazi prepared the manuscript and the statistical analysis.

Conflict of interest:

Authors declare no conflict of interest.

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The Potential Role of Human Herpes Virus-6 in Idiopathic Facial Nerve Paralysis

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Abstract

Background Bell's palsy (BP) is an acute, generally unilateral, paralysis or weakness of facial musculature consistent with peripheral facial nerve dysfunction; different factors suspected to contribute to the development of BP including herpesviruses.

Objective To detect human herpes virus 6 (HHV-6) in the saliva samples of patients in the early presentation of BP.

Methods A case-control study included saliva samples taken from 50 patients with BP and 50 apparently healthy controls without any neurological deficit, viral DNA was extracted from the saliva and then subjected for quantitative real time polymerase chain reaction (PCR) for detection of HHV-6 viral load (VL) in saliva.

Results HHV-6 DNA was positive in 22 (44%) out of the 50 patients, and in 12 (24%) of the controls (p=0.028), with a significantly higher mean viral load in patients than control (p=0.002), in addition, 70% of HHV-6 positive patients had severe grades with a significantly higher VL (p=0.007) and (0.015), respectively.

Conclusion HHV-6 could play an important role in the development of BP and HHV-6 might have a neuro-pathological effect in severe cases of BP.

Keywords HHV-6, Bell's palsy, Saliva, real time PCR

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List of abbreviations: • BP = Bell's palsy, HHV-6 = Human Herpes Virus 6, VL = Viral Load

Introduction

Bell's palsy (BP) is the most common condition involving a rapid and unilateral onset of peripheral paresis/paralysis of the seventh cranial nerve. It affects 11.5–53.3 per 100,000 individuals a year across different populations. Bell's palsy is a health issue causing concern and has an extremely negative effect on both patients and their families. Therefore, diagnosis and prompt cause determination are key for early treatment [(1)].

Despite its severe effects, the exact etiology of BP remains unclear. The Guideline Development Group (GDG) [(2)] has identified the diagnosis of BP as one of exclusion, requiring careful clinical elimination of other potential etiologies of facial paralysis/paresis, such as trauma, neoplasms, postsurgical facial paralysis/paresis, or infection by agents including herpes viruses and Lyme disease [(1,3)]. Some studies stressed on the role of Human herpes virus 6 (HHV-6) in the development of Bell's palsy [(4,5)]. HHV-6 (family Herpesviridae, subfamily Beta herpes virinae, genus Roseolo virus, species Human herpes virus 6) is

neurotropic virus that reactivate frequently in immune compromised persons [6,7].

Several studies had been conducted on the possible role of HHV-6 in the etiology of Bell's palsy, it is suggested that the high level of HHV-6 DNA is only a consequence of the patient's immune status, this may be the main predisposing factor for the occurrence of the palsy although the contribution of HHV-6 reactivation/replication cannot be ruled out [4,8,9].

This study aimed to detect the frequency of HHV-6 in the saliva of patients with BP and its association with the grade of the neurological deficit.

Methods

A case-control study was conducted from October 2019 to February 2020. Fifty patients with idiopathic unilateral facial nerve paralysis (Bell's Palsy) were collected from Al-Imamein Al-Kadhimein Medical City and Baghdad Teaching Hospital, and 50 apparently healthy age- and sex-matched volunteers as controls. A written informed consent was taken from each subject enrolled in this study. The study was approved by the Institutional Review Board (IRB) of the College of Medicine-Al-Nahrain University, approval number (269-15-10-2019). The research was conducted in the Microbiology Department at the College of Medicine-Al-Nahrain University.

From all patients and controls about 1-2 ml saliva (without induction) were collected in plane tubes. Saliva samples preserved in deep freeze (-20 °C), and then used for viral DNA extraction. Viral nucleic acid was extracted using (Geneaid/Taiwan) kit, and real time polymerase chain reaction (PCR) amplification kit (Sacace, Italy) was used for quantitative detection of HHV-6 in the saliva samples. Fifteen µl of master mix were added to all PCR tubes and 10 µl of (DNA sample, negative control, positive control or standards) were added to master mix. Real time PCR instrument used in this work was STRATAGENE MX 3005P (Agilent Technologies, USA). For real-time PCR the following amplification protocol was used: 1 cycle at 95 °C for 15 min followed by 5 cycles consisting of 5 s at 95 °C, 20 s at 60 °C, and 15 s at 72 °C, and then 40 cycles consisting of 5 s at 95 °C, 30 s at 60 °C, and 15 s at 72 °C.

Statistical analysis

The Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA), Version 20 used for statistical analysis. Categorical data formulated as count and percentage. Odd ratio and Chi-square test used to describe the association of these data. Alternatively, Fisher exact test was used if there is 25% of cells less than expected count. Numerical data were described as mean and standard deviation. Independent sample t-test used for comparison between two groups. The lower level of accepted statistically significant difference is equal or less than 0.05.

Results

In this study HHV-6 viral load (VL) was measured in the saliva samples of Bell's Palsy patients and normal controls. HHV-6 DNA was positive in 22 (44%) out of the 50 patients, and in 12 (24%) of the controls (p=0.028), and the mean viral load was also significantly higher in patients than in the controls (p=0.002) (Tables 1 and 2).

Table (3) demonstrated no significant association between HHV-6 positivity with patients' age, sex, side of the weakness or duration of BP, however there was a significant association between HHV-6 and the severity of BP, in which 70% of HHV-6 positive cases were in the severe grades (p=0.007), also the mean HHV-6 VL is significantly higher in the severe grades (p=0.015).

Table 1. Comparison of the number of HHV-6 DNA positive cases between patients and controls

		Patients	Controls
HHV-6	Positive	22	12
	%	44.0	24.0
	Negative	28	38
	%	56.0	76.0
Total		50	50
%		100.0%	100.0%
p value		0.028	
Odds Ratio (Confidence interval)		2.488 (1.06-5.85)	

Table 2. Comparison of the mean Viral Load (VL) of HHV-6 between patients and controls

HHV-6	Patients	Control
Mean VL (Copies/ml)	4026.00	117.04
Standard Deviation	9912.57	252.71
Median	0.00	0.00
Percentile 25	0.00	0.00
Percentile 75	3000.00	0.00
P value	0.002	

Table 3. The associations between HHV-6 DNA positivity and viral load (VL) with patients' clinical and demographic data

		HHV6		P value	Mean VL Copies/ml	Standard Deviation	P value
		Positive	Negative				
Age groups	≤25 years	9 (45.0%)	11 (55%)	0.907	6640.00	14686.42	0.125
	>25 years	13 (43.3%)	17 (56.7%)		2283.33	4105.51	
Sex	Female	8 (66.7%)	4 (33.3%)	0.073	6608.33	13971.04	0.305
	Male	14 (36.8%)	24 (63.2%)		3210.53	8321.77	
Side	Left	9 (39.1%)	14 (60.9%)	0.577	2265.22	4514.58	0.250
	Right	13 (48.1%)	14 (51.9%)		5525.93	12761.50	
Severity	Moderate	10 (30.3%)	23 (69.7%)	0.007	630.30	1072.52	0.015
	Severe	12 (70.6%)	5 (29.4%)		10617.65	15121.85	
Duration days	1	4 (40%)	6 (60%)	0.522	1000.00	1333.33	0.253
	2	3 (30%)	7 (70%)		1330.00	2921.21	
	3	15 (50%)	15 (50%)		5933.33	12385.35	

Discussion

HHV-6 is a common neurotropic virus which has been associated with conditions such as febrile convulsions, encephalitis, and multiple sclerosis and is another candidate in Bell's palsy. HHV-6 has been directly or indirectly associated with several neurological diseases [8,10,11], in cases of primary infection in immune-competent young children, reactivation in

healthy adults [12], or in immunosuppressed patients [13].

The present study found that both the number of HHV-6 positivity and its mean VL in saliva were significantly higher in patients than in the controls, which is supported by the study of Turriziani et al. in 2014 [4] which showed that the value of HHV-6 DNA copies was significantly higher than that detected in healthy subjects. In addition, patients with the highest levels of HHV-6 DNA showed no change in facial palsy HB grade (The House-Brackmann FacialNerve Grading System) or even an increase of at least one HB grade at the first visit. This suggests that the high level of HHV-6 DNA is only a consequence of the patient's immune status: this may be the main predisposing factor for the occurrence of the palsy although the contribution of HHV-6 reactivation/replication cannot be ruled out. These finding also agrees with results of the current study in which 70% of HHV-6 positive cases were in the severe grades (p=0.007), also the mean HHV-6 VL is significantly higher in the severe grades (p=0.015).

One study searched for HHV-6 in the tear fluid of patients with BP, finding a significantly higher detection rate of HHV-6 than that in healthy controls [8]. In addition, recently a study of HHV-6 in saliva of children and adolescents with BP found even a higher frequency of HHV-6 in patients than the current result [14]. However, it is still uncertain whether HHV-6 is involved in the development of the disease or that the underlying disease mechanism might predispose patients to HHV-6 reactivation. Perhaps the major limitations of this study are that the species of HHV-6 detected in samples were not characterized, and absence of follow up VL levels in these patients.

Turriziani et al. in 2014 [4] suggested that co-infection with this HHV-6 is possible, and could be more implicated in the pathogenesis of BP. The combined role of viruses also could be explained by the autoimmune theory in which viral replication may provoke an autoimmune reaction against peripheral nerve myelin components, leading to the demyelination of cranial nerves, especially the facial nerve [15]. This is supported by studies on multiple sclerosis which could be caused by autoimmune reaction to multiple herpesviruses interactions especially antibodies against HHV-6 that cross react with myeline basic protein [16].

Recently Ptaszynska-Sarosiek et al. in 2019 [9] have revealed a common presence of the herpesviruses (including HHV-6 and EBV) in trigeminal and facial nerve ganglia post-mortem among a random group of Polish population and demonstrated simultaneous infection of the ganglia with different herpesviruses.

In conclusions, the significantly high frequency and viral load of HHV-6 in Bell's palsy make it possible player in the pathogenesis of this disease. The high HHV-6-frequency and viral load in severe HB grades of BP strengthens its role in this neurological condition.

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Author contribution

Rhaif did the sampling and run the real time PCR. Dr. Al-Obaidi put the research plan and writing the manuscript. Dr. Al-Khazrajee did the selection of patients and grading of BP.

Conflict of interest

Authors declare no conflict of interest.

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Evaluation of Autophagy Flux LC3 Gene Expression and Serum IFN-γ in Pulmonary Tuberculosis Patients

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Abstract

Background *Mycobacterium tuberculosis* (Mtb) is the major causal pathogen of human tuberculosis (TB). Autophagy is a highly conserved cytosolic pathway influencing the immune responses and the elimination of intracellular pathogens including Mtb.

Objective To evaluate the effect of Mtb on autophagy flux with autophagy related genes of LC3I and LC3II, beside the evaluation of serum interferon-gamma (IFN-γ) level in patients with pulmonary TB.

Methods A total of 50 blood samples were collected from patients with pulmonary TB, besides 30 as healthy control. The real-time polymerase chain reaction (PCR) method was determined the measuring of mRNA expression of autophagy-related genes of LC3I and LC3II. Serum IFN-γ protein concentration was measured using sandwich Enzyme Linked ImmunoSorbant assay.

Results The present study showed an increasing in LC3-I level in newly-diagnosed pulmonary TB patients rather than in the control group despite statistically non-significant P>0.05, while LC3-II showed decreasing in all pulmonary TB groups but statistically non-significant P>0.05. Autophagy flux ratio of LC3-I and LC3-II genes showed a statistically significant decrease in pulmonary TB groups especially in newly- diagnosed (p value=0.02) rather than control groups. Moreover, the study of the serum level of IFN-γ showed an increase in the level of IFN-γ with p=0.0001 in pulmonary TB patients in comparison with the control group. In addition, the correlation between autophagy-related genes and IFN-γ have been shown a positive significant correlation (p value =0.013) in the multidrug-resistant (MDR) TB group.

Conclusion Autophagy flux ratio showed a statistically significant decrease in pulmonary TB groups particularly in newly-diagnosed TB patients rather than control groups, this indicates the different modulation factors that may affect the process of autophagy. The only positive correlation within biomarkers of the present study has been shown that LC3-II is a dependent factor on IFN-γ in MDR group.

Keywords Pulmonary tuberculosis, autophagy flux, LC3-I, LC3-II, diagnosis, IFN-γ

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List of abbreviations: DCs = Dendritic cells, Eis = Enhanced intracellular survival gene, ESAT6 = Early secreted Ag of 6 kDa, Mtb= Mycobacterium tuberculosis, TB = Tuberculosis, Th1 = T helper 1, Th2 = T helper 2, miRNAs= MicroRNAs

Introduction

Tuberculosis (TB) is an infectious bacterial disease. It is considered to be one of the world's deadliest illnesses [(1)]. The prevalence of TB rod bacilli with the droplet of respiratory secretions is related to coughing or sneezing of the infected individual [(2)]. It is an

infection that becomes active, which is most frequently occurred in almost 90% of reported lung cases. Only about 10 percent of people infected with *Mycobacterium tuberculosis* (Mtb) developed to active TB disease within their lifetime [3]. Although, improved and transformative TB diagnostics are still needed to overcome the severe restrictions on older, non-standard and inaccurate techniques currently used in most circumstances [4].

Autophagy is a newly identified innate and adaptive immunity defense upon intracellular pathogens [5]. Autophagy can capture and eradicate intracellular microbes, including Mtb [6]. There were much has been reported about the functions of autophagy besides the mechanisms by which the autophagic pathway stimulates the host innate effector response against Mtb infection [7]. The phrase "autophagic flux" is utilized to describe the dynamic process of autophagy. In particular, autophagic flux refers to the whole process of autophagy, including autophagosome formation, maturation, fusion with lysosomes, subsequent breakdown and the release of macromolecules back into the cytosol [8]. Autophagy aids with antigen processing of intracellular and extracellular element for major histocompatibility complex (MHC) class I and class II presentation for recognition by CD8+ and CD4+ T cells, respectively [9]. Predominantly, T helper 1 (Th1) cytokines stimulate autophagy in order to kill intracellular Mtb [10]. Th1 cytokines (IL-2, TNF-α, and IFN-γ) are recognized as autophagy inducers while T helper 2 (Th2) cytokines (IL-4, IL-5, IL-6, IL-10, and IL-13) and anti-inflammatory cytokines are observed as autophagy repressors [11]. Mtb has evolved numerous approaches to counteract autophagy. For instance, Mtb Eis (enhanced intracellular survival) gene represses macrophage autophagy and cell death by a reactive oxygen species (ROS)-dependent pathway. In addition, (ESAT6), a significant ESX-1-mediated secretory protein, performs a great role in the suppression of late-stage autophagy in human dendritic cells (DCs) [12]. The current study aimed to: First, to evaluate the mRNA expression levels of autophagy-related genes of (LC3-1) and (LC3-II) in TB patients through autophagy flux. Second, to evaluate the serum level of IFN-γ in pulmonary TB patient groups. And third, to determine the correlation between autophagy- related genes and IFN-γ.

Methods

This case control study included fifty pulmonary TB patients were collected from the National Reference Laboratory (NRL) of Tuberculosis Respiratory, Baghdad during the period from January 2019 to September 2019. This study was approved by the Institutional Review Board (IRB) of the College of Medicine-Al-Nahrain University, approval code (208).

Study design

Patients were divided into three groups: multidrug-resistant (MDR) patients group consists of 20 samples, newly diagnosed (ND) group which consists of 20 samples and old case (OC) group with 10 samples. Besides 30 samples of apparently healthy persons as control groups were collected from the Blood Donation Center, Baghdad Medical City.

Inclusion criteria

Patients with pulmonary TB, age group include eighteen years old and above.

Exclusion criteria

Patients having an autoimmune disease (Crohn's disease, systemic lupus erythromatosus, multiple sclerosis), central nervous system disease, human immune deficiency dis, patients on radio and chemotherapy, and atypical mycobacteria.

Total RNA purification of LC3-I and LC3-II from whole blood

Total RNAs were isolated from sample according to the protocol of TRIzol™ reagent (Thermo Scientific, USA).

Measuring of mRNA expression level of autophagy-related genes LC3-I and LC3-II by Relative RT Real time polymerase chain reaction (RT-PCR)

The PCR primers were listed in table (1). GoTaq® 1-Step RT-qPCR System combines GoScript™ Reverse Transcriptase and GoTaq® qPCR Master Mix (Promega, USA), which enables detection of RNA expression levels using a single-step real-time amplification reaction. Quantitative PCR used for cDNA quantification, this method allows the assessment of the amount of a given sequence present in a sample and quantitatively determine levels of gene expression. Hence, in this study, this method was used to measure levels of gene expression of mRNA for LC3-I and LC3-II target genes. By the use of RT-PCR system software program, the comparative quantification experiment type was selected from the programming window. Then RT-PCR machine was programmed according to the conditions illustrated in table (2). The threshold was set manually at the lowest point of the parallel rise of the logarithmic amplification curve to obtain a cycle threshold (Ct) value for each reaction tube.

Table 1. Primers and its sequences

Primer Name	Primer ID	Sequence
LC3-I	F*	5`-GCTACAAGGGTGAGAAGCAGCT-3`
	R**	5`-CTGGTTCACCAGCAGGAAGAAG-3`
LC3-II	F	5`-AATCCCGGTGATAATAGAAC-3`
	R	5`-TTTCATCCCGAACGTCTCC-3`
GAPDH	F	5`-AGA AGG CTG GGG CTC ATT TG-3`
	R	5`-AGG GGC CAT CCA CAG TCT TC-3`

*: forward primer, R**: reverse primer, LC3I [13], LC3II [14], GAPDH [15]

Table 2. Conditions for Real-time PCR run

Real time PCR program			
Steps	**Temperature**	**Time**	**Cycle**
Real time enzyme activation	37 °C	15:00	1
Initial denaturation	95 °C	10:00	
Denaturation	95 °C	00.20	40
Annealing	60 °C	00.20	
Extension	72 °C	00.20	

Gene expression calculation

The relative expression of a targeted gene was calculated using the formula 2- ΔΔCt. The folding = $2^{-\Delta\Delta CT}$, $\Delta\Delta CT = \Delta CT_{Treated} - \Delta CT_{Control}$, $\Delta CT = CT_{gene} - CT_{House\ Keeping\ gene}$.

Serum IFN-γ protein measurement using sandwich Enzyme Linked ImmunoSorbant assay (ELISA)

The current experiment based on utilizing the Sandwich-ELISA principle (elabscience, china). The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human IFN-γ. The optical density (OD value) of each well was determined at once with a

micro-plate reader set to 450 nm. Finally, for results calculation, four-parameter logistic curve has been plotted on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis figure (1).

Statistical analysis

The data of the present study were processed using SPSS version 16.0.0, Microsoft Excel 2010, and Graphpad Prism version 7.04. Differences were considered statistically significant at $p < 0.05$. Accordingly, the proper statistical tests were used, t-test and ANOVA test were used for parametric data to measure the significance of difference in means taking into account whether variables of analysis sharing different or equal variance. Correlation coefficient tests or, r, among variables were used to assess the nature of correlation in terms of positive, negative or indifference.

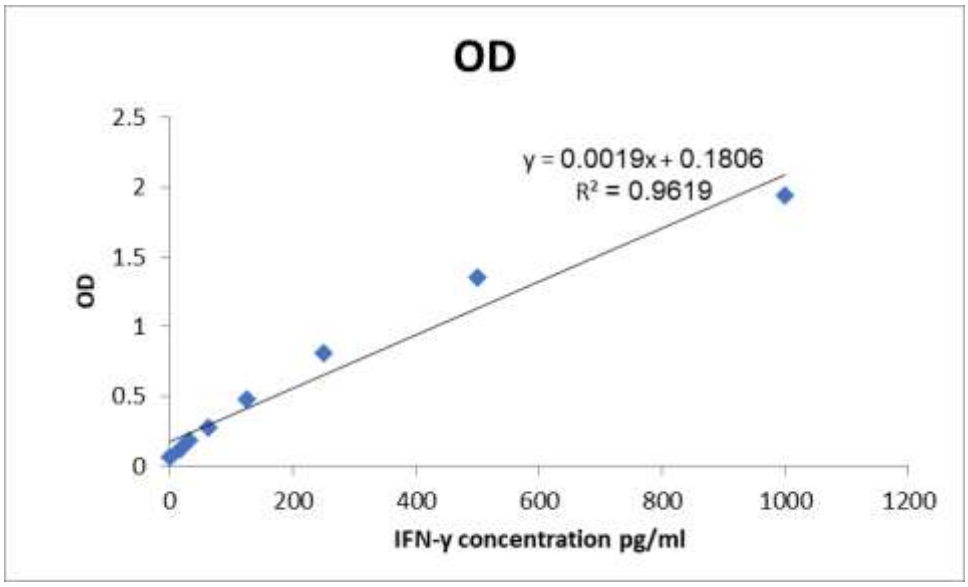

Figure 1. Standard curve of interferon-γ

Results

Regarding the expression level of autophagy-related genes, the results showed that the mRNA levels of LC3-I were increased in pulmonary TB group than control group, but statically not significant (P>0.05). There is no statistically significant variation in LC3II folds between PTB patients and the control group (P>0.05) (Table 3).

Furthermore, autophagy flux ratio has been calculated in order to access the entire process of autophagy in pulmonary TB groups. The results have been shown significant decreasing in the ratio of autophagy flux (p value =0.027) between pulmonary TB groups and control groups as shown in figure (2). The highest significant decreasing has been shown in newly diagnosed group with (p value=0.02), and MDR group with (p value =0.05) rather than control group. While in old cases group was non-significant (p value=0.6). The mean ± SD of autophagy flux in newly diagnosed, MDR, old-cases and control groups were (0.90±0.96, 1.26±1.38, 2.56±3.62, 3.70±6.48) respectively.

Besides the results of the current study showed that IFN-γ serum concentration was with highly significant differences (p=0.0001) between PTB groups and control group. IFN-γ serum level were significantly increased (p<0.05) in newly diagnosed ND and old cases OC group than the control, while MDR group reach the borderline (p value =0.06). Furthermore, among patients'

groups, there were no significant differences (P>0.05) in the levels of IFN-γ between MDR and ND, MDR and OC and ND with OC. As shown in table (4), the serum levels mean ±SD of MDR, ND, OC and controls were 91.61±82.04, 1.066±65.57, 90.72±64.93, 54.93± 26.61, respectively.

Further correlations have been made between LC3II gene expression and IFN-γ. the outcome shows that LC3-II is a dependent factor on IFN-γ in the MDR TB group with a positive significant correlation (r = 0.54, P=0.013) (Table 5 and Figure 3).

Table 3. LC3I and LC3II gene expression in TB patients and control groups

	Control vs TB	N	Mean	Std. Deviation	P value
LC3I folds	Control	30	2.12	3.27	0.292^{NS}
	TB group	50	24.61	115.87	
	Control	30	2.12	3.27	0.671^{NS}
	MDR-TB	20	1.76	2.33	
	Control	30	2.12	3.27	0.264^{NS}
	Newly Diagnosed TB	20	48.11	180.14	
	Control	30	2.12	3.27	0.183^{NS}
	Old TB cases	10	23.31	46.86	
LC3II folds	Control	30	2.25	2.85	0.144^{NS}
	TB group	50	1.39	1.60	
	Control	30	2.25	2.85	0.061^{NS}
	MDR-TB	20	1.13	1.14	
	Control	30	2.25	2.85	0.382^{NS}
	Newly Diagnosed TB	20	1.60	1.99	
	Control	30	2.25	2.85	0.442^{NS}
	Old TB cases	10	1.51	1.58	

NS=none statistical significance (p>0.05)

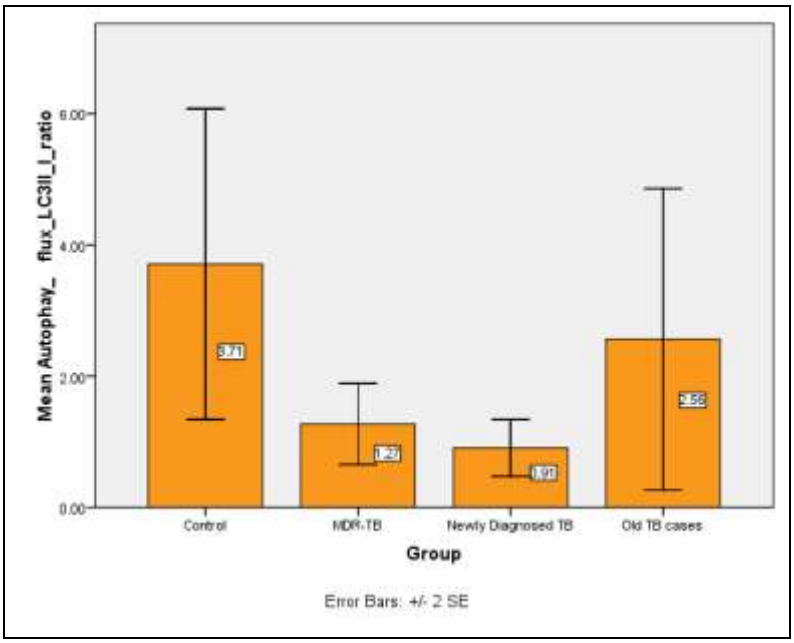

Figure 2. Autophagy flux ratio between pulmonary TB and control groups

Table 4. Serum level of IFN-γ in PTB patients and controls

Groups	N	Mean	Std. Deviation	P value
Control TB group	30 50	54.93 97.45	26.61 71.48	0.0001**
Control MDR-TB	30 20	54.93 91.61	26.61 82.04	0.062
Control Newly Diagnosed	30 20	54.93 1.0666E	26.61 65.57	0.003*
Control Old TB cases	30 10	54.93 90.72	26.61 64.93	0.017*

*=statistical significance (p≤0.05) **=High statistical significance (p≤0.001)

Table 5. Correlations between LC3-I, LC3II genes expression and IFN-γ in MDR group

		LC3II folds	IFN-γ concentration
LC3I folds	Pearson corr. coefficient Sig. (2-tailed)	0.342 0.140	-0.034 0.887
LC3II folds	Pearson corr. coefficient Sig. (2-tailed)		0.544* 0.013

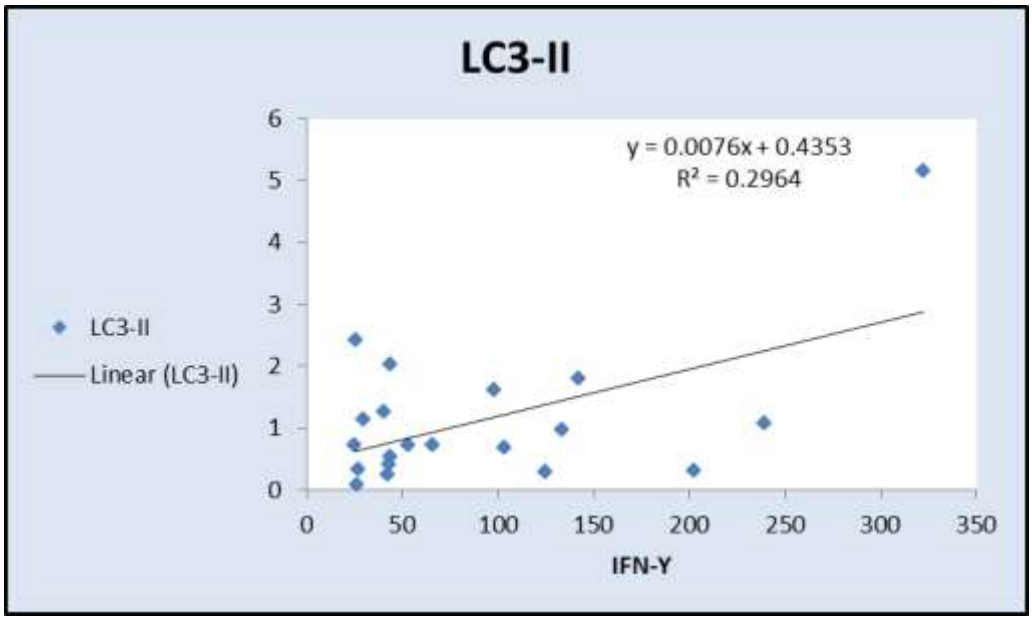

Figure 3. LC3-II is a dependent factor on IFN-γ in MDR TB group at correlation coefficient r = 0.544, P=0.013, and at regression index R2= 0.294 with linear curve equation LC3-II (folds)= 0.0076xLC3-I(folds)+0.4353

Discussion

Autophagy, a lysosome-dependent degradation process, considered as a crucial innate host defense mechanism in macrophages toward intracellular bacteria, particularly Mtb [16]. Autophagy flux refers to the entire process of autophagy, the present study showed block in autophagy flux with significant (P value <0.05), which may refer to several factors including autophagy modulators such as miRNAs, cytokines, vitamin D3. It has been shown that mycobacterial virulence factors like: PhoP, system 1 (ESX-1) secretion-associated protein B (EspB) and early secretory antigenic target 6 (ESAT-6) are highly linked to immunogenicity and virulence of this organism which could inhibit the process of autophagy. Genetic mutations in humans may cause changes in autophagic flux, also it has been implicated in neurodegenerative diseases and cardiovascular diseases besides cancers [17].

Lambelet et al. in 2018 examined the processed LC3B-II by western blot, together with electron microscopy for autophagosome formation, which have the mainstays for autophagy detection. It has been found that LC3 expression levels can vary markedly between different cell types and in response to different stresses, and there is further concern that over-expression of tagged versions of LC3 to facilitate imaging and detection of autophagy interferes with the process itself [18]. Another study worked on autophagy flux in Mtb-infected macrophages and they observed that there is no interesting change in the autophagy flux in macrophages, which disagreed with this study [19].

The findings of serum level of cytokines IFN-γ in pulmonary TB of the present study revealed that the serum level of IFN-γ was significantly higher (p=0.0001) between PTB groups and control group, this provides new insights into (IFN-γ) as a powerful pro-inflammatory cytokine that plays a major role as biomarker candidates in the pathogenesis and the immune response of pulmonary TB. The findings of the current study in agreement with study by Hussain in 2010 who observed that the levels of interferon-gamma in the blood of tuberculosis patients were significantly higher (P value <0.001) in compared to the control group [20]. The present study disagreed with Al-Jubouri et al. (2018), they observed that there were no significant raised in the serum levels of

IFN-γ in patients with pulmonary TB [21]. Regulation of cytokine production and autophagy activation seem to be mutually regulated by each other; cytokines regulate autophagy, but the opposite is also true. Furthermore, the present study showed an agreement with Bento et al. (2015) who's studied the correlation between autophagy and inflammation during Mtb infection and observed that IFN-γ was shown to be an important cytokine in the regulation of mycobacteria clearance by autophagy by Increases production of reactive nitrogen species in macrophages and induces autophagy [22].

As described before, autophagy plays an important role in the host immune response against Mtb and therefore the development of promising autophagy-based therapies including IFN-γ to combat TB represents an appealing strategy through enhancing autophagy also increases the efficacy of the only TB-prophylactic method available, the BCG vaccine.

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Author contribution

Dr. Wahwah: did the sampling and lab works; Dr. Abdulrahman and Dr. Mankhi supervised the study and participated in its design and interpretation.

Conflict of interest

Authors declare no conflict of interest.

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Amorphophallus konjac and Polycystic Ovary Syndrome

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Abstract

Background Polycystic ovary syndrome (PCOS) is that the commonest reproductive endocrine disorder among women of reproductive age, affecting high of population worldwide. It's the most typical multisystem endocrinopathy having diverse etiopathogenesis in women, causing menstrual irregularities, hirsutism and anovulatory infertility.

Objective To determine the effect of Glucomannan, Amorphophallus konjac supplementation on body weight, body mass index (BMI), Insulin level, insulin resistance indexes, fasting glucose level, luteinizing hormone (LH) level, testosterone, sex hormone binding globulin (SHBG) and lipid profile in over weight and obese women patients with PCOS.

Methods Thirty obese and overweight women with PCOS, mean BMI = 32 kg/m^2 with mean age = 29 yr were enrolled in the study. They were received Glucomannan in a dose of 2300 mg daily) for three months. The parameters were measured before and after three months of treatment are BMI, fasting plasma glucose, serum insulin levels, Insulin resistance indexes; homeostasis model assessment of β-cell function (HOMA-B), homeostasis model assessment of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI), lipid profile; serum cholesterol, triglycerides, very low density lipoprotein (VLDL-C), low density lipoprotein (LDL-C) and high density lipoprotein (HDL-C), LH, testosterone and SHBG.

Results Glucomannan shows highly significant decrease in weight, glucose, insulin level, HOMAB, HOMAIR, QUICKI, testosterone, LH, serum cholesterol, triglycerides, VLDL-C and LDL-C and highly significant increase in HDL-C and SHBG concentrations, no effect on BMI.

Conclusion Glucomannan was effective in management of patients with PCOS through their ability to decrease the glucose, insulin levels, insulin resistance indexes, lipid profile, testosterone and LH and increase the HDL-C and SHBG.

Keywords PCOS, Glucomannan, obese, overweight, SHBG

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List of abbreviations: GLUC = Glucomannan, HOMA = Homeostasis model assessment of insulin resistance, LH = luteinizing hormone, PCOS = Polycystic ovary syndrome, SHBG = Sex hormone binding globulin

Introduction

Under normal conditions, the hormone insulin rises briefly after eating. It stimulates the liver and muscles to take up sugar from the blood and convert it to energy. That then causes blood sugar to fall, and then insulin to fall. With normal insulin sensitivity, both sugar and insulin are normal on a fasting blood test. With insulin resistance, blood sugar may be normal, but insulin is high. Why? Because the pancreas has to make more and more insulin to try to get its message through. Too much insulin generates inflammation and causes weight gain. It can

also lead to type 2 diabetes and heart disease. Too much insulin is also an underlying physiological driver of polycystic ovary syndrome (PCOS) [(1)].

PCOS is a heterogeneous disorder that affects at least 7% of adult women, the National Institutes of Health Office of Disease Prevention, reported that PCOS affects approximately five million women of childbearing ageing the U.S. it is one of the leading causes one of the leading causes of female infertility [(2)].

Research submits that 6-11% of females; 18-44 years of age are affected by PCOS, reproduction it the most common endocrine abnormality amongst women of reproductive age in the world. Women looking for help from health care professionals to resolve issues of obesity, acne, amenorrhea, excessive hair growth, and infertility often receive a diagnosis of PCOS. Women with PCOS have higher rates of endometrial cancer, cardiovascular disease, dyslipidemia, and type-2 diabetes mellitus [(3)].

It is a relationship between the presence of polycystic ovaries and signs of hirsutism, menstrual disturbances (e.g. amenorrhea, oligomenorrhea) and obesity [(4)].

Insulin resistance is a key feature of both obese and lean PCOS. It occurs in 70-95% of people with obese PCOS and 30-75% of people with lean PCOS, high insulin can impair ovulation and cause the ovaries to make excess testosterone [(5)].

Glucomannan (GLUC), which is a soluble, fermentable, and highly viscous dietary fiber. It is derived from the root of konjac plant, which is native to Asia. GLUC consists of a polysaccharide chain of beta-di glucose and beta di mannose with attached acetyl groups in a molar ratio of 1:1.6 with beta 1-4 linkages. Then human salivary and pancreas amylase enzyme cannot split (beta 1, 4 linkages), GLUC passes relatively unchanged into the colon, where it is highly fermented by colonic bacteria [(6)].

GLUC has a high molecular weight and can absorb up to fifteen times its weight in water, it is considered one of the most viscous dietary fibers known. In the stomach, the GLUC turns into gelatin and induces a sense of satiety. Thus, GLUC inhibits the absorption of cholesterol and fats and reduce sugar absorption; it prevents the blood glucose peak by reducing the release of insulin from the pancreas and thus prevents hypoglycemia. It has been shown that GLUC long-term supplementation reduces fasting glucose and LDL cholesterol plasmatic levels in diabetic patients [(7)].

The aim of this study is to explain the effectiveness of GLUC in the management of Iraqi women patients with PCOS.

Methods

Patients

This prospective study was conducted during the period from July 2018 to August 2019. The Ethics Committee of the Al-Nahrain Institutional Review Board Medical College approved this study. Women included in this study were among those who attended Clinic of Gynecology and Obstetrics in Al-Imamein Al-Kadhimein Medical Teaching City; Baghdad, Iraq, Patients were selected randomly according to the day of presentation to the clinic. All participants were given informed consent before they were included in this study.

The patients were; thirty women diagnosed with PCOS in their reproductive age (18-40 years). All the patients had at least 2 out of 3 of Rotterdom criteria.

Each patient was involved to detailed clinical history, physical examination and typical appearance of polycystic ovaries by ultrasound according to the criteria of Rotterdam consensus meeting 2003.

Safety Assessment

Safety assessment included medical history, physical examination, hormonal level, and serum chemistry at all visits and the monitoring of drug-related adverse events by recordation in patient diaries.

Laboratory investigations

Weight, length, body mass index (BMI). [BMI = weight (kg)/ height $(m)^2$].

Hormonal assays include sex hormone binding globulin (SHBG), testosterone, and luteinizing hormone (LH) by (TOSOH Corporation, Japan).

Fasting plasma glucose, serum insulin levels, insulin resistance indexes:

- HOMA-B = homeostasis model assessment of β-cell function; [HOMA-B= (20 x insulin in mIU/mL)/(glucose in mmol/L- 3.5]
- HOMA-IR = homeostasis model assessment of insulin resistance; [HOMA-IR= (glucose in mmol/L x insulin in mIU/mL)/22.5]
- QUICKI = quantitative insulin sensitivity check index; [QUICKI = 1/(log [FIRI in mU/l] + log [FPG in mg/dl]).

Lipid profile: serum cholesterol, triglycerides, very low-density lipoprotein (VLDL-C), low density lipoprotein (LDL-C) and high-density lipoprotein (HDL-C) concentrations by (Selectra pro, Elitech, France).

Study Design and Treatments

The enrolled patients were asked to take 2300 mg of Gluc (NOW company, USA) orally for three months. Clinical examination, biochemical assay, and hormonal assay were performed at baseline and after three months of therapy. All patients provided their written informed consent and completed the entire trial.

Statistical analysis

The data were analyzed using Statistical Package for Social Sciences (SPSS) program version 25 by the National Opinion Research Center (NORC), at the University of Chicago. Results were reported as mean±SD. The total variations were analyzed by performing the statistical design t-test. Probability levels of less than 0.05 were considered significant.

Results

GLUC treatment was well-tolerated by all patients. None of the subjects suspended the therapy due to side effects, although some experienced transient flatulence during the first month of treatment. The mean weight and BMI decreased significantly during the treatment time, from (88.90±8.41) kg to (81.21±7.68) kg, (34.43±5.16) kg/m^2 to (31.45±4.71) kg/m^2 respectively (Table 1).

The results (Mean±SD) of glucose (mg/dL), insulin (uIU/ml), HOMAB, HOMAIR and QUICKI comparison of the GLUC group between before and after treatment show highly significant decrease ($p<0.001$) in the values (104.72±7.23 vs. 84.48±5.96), (10.31±2.19 vs. 6.64±0.70), (119.48±32.52 vs. 90.99±24.22), (2.67±0.61 vs. 1.39±0.18), (1.48±0.22 vs. 1.34±0.05) respectively (Table 1).

In this study, results (mean±SD) before vs. after 3 months of treatment with GLUC of total cholesterol, triglycerides, LDL-C, VLDL-C, LH and testosterone show highly significant decrease ($p<0.001$) in the values as (173.71±10.54 vs. 165.17±7.52), (131.48±6.32 vs. 98.31±9.58), (98.55±10.02 vs. 80.97±6.76), (31.38±3.49 vs. 27.00±2.39), (23.09±3.52 vs. 16.32±2.71), (65.56±19.68 vs. 31.59±8.87) respectively (Table 1) (Figures 1-4). Where the results (Mean±SD) before versus after 3 months of treatment with GLUC of HDL-C and SHBG showed highly significant increase ($p<0.001$) in the values as (43.78±3.67 vs. 57.21±3.69), (42.97±5.79 vs. 57.04±6.83) respectively (Table 1) (Figures 5).

Table 1. Comparison of weight, height, body mass index, glucose, insulin, insulin resistance indexes, lipid profile, LH, SHBG and Testosterone, in patients' group between before and after three months treatment of glucomannan

Parameter	Glucomannan		P value
	Before	After	
Wt (kg)	88.90±8.41	81.21±7.68	0.005
BMI (kg/m^2)	34.43±5.16	31.45±4.71	0.012
Glucose (mg/dl)	104.72±7.23	84.48±5.96	<0.001
Insulin (uIU/ml)	10.31±2.19	6.64±0.70	<0.001
HOMAB	90.99±24.22	119.48±32.52	0.010
HOMAIR	2.67±0.61	1.39±0.18	<0.001
QUICKI	1.48±0.22	1.34±0.05	<0.001
TG (mg/dL)	131.48±6.32	98.31±9.58	<0.001
Cholesterol (mg/dL)	173.71±10.54	165.17±7.52	0.001
VLDL-C (mg/dL)	31.38±3.49	27.00±2.39	<0.001
LDL-C (mg/dL)	98.55±10.02	80.97±6.76	<0.001
HDL-C (mg/dL)	43.78±3.67	57.21±3.69	<0.001
LH (mIU/ml)	23.09±3.52	16.32±2.71	<0.001
Testosterone (mIU/ml)	65.56±19.68	31.59±8.87	<0.001
SHBG (mIU/ml)	42.97±5.79	57.04±6.83	<0.001

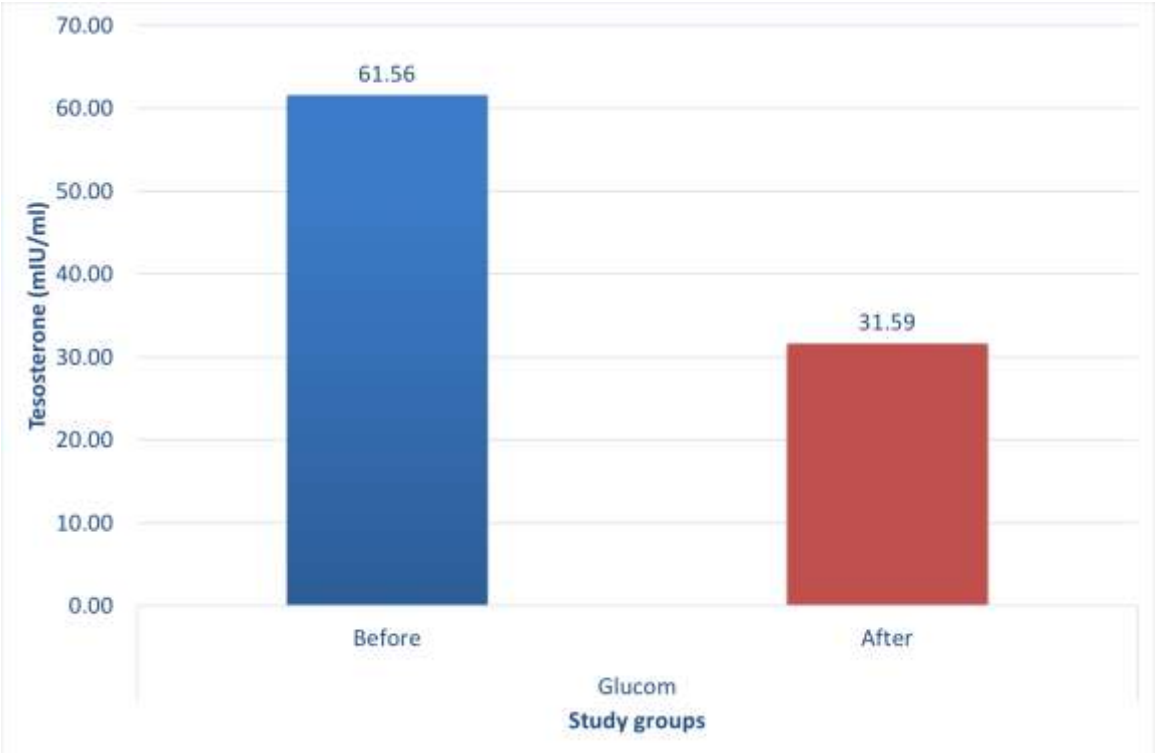

Figure 1. Comparison of mean Testosterone (mIU/ml) between study group before and after treatment

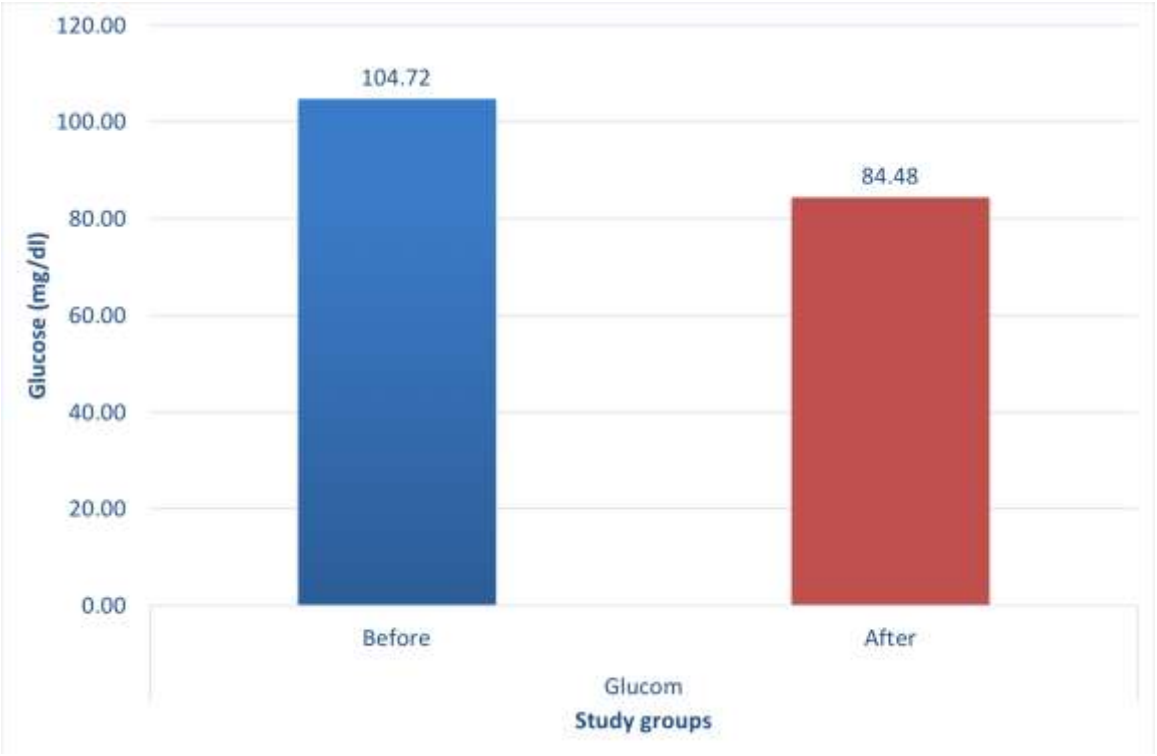

Figure 2. Comparison of mean glucose (mg/dl) between study group before and after treatment

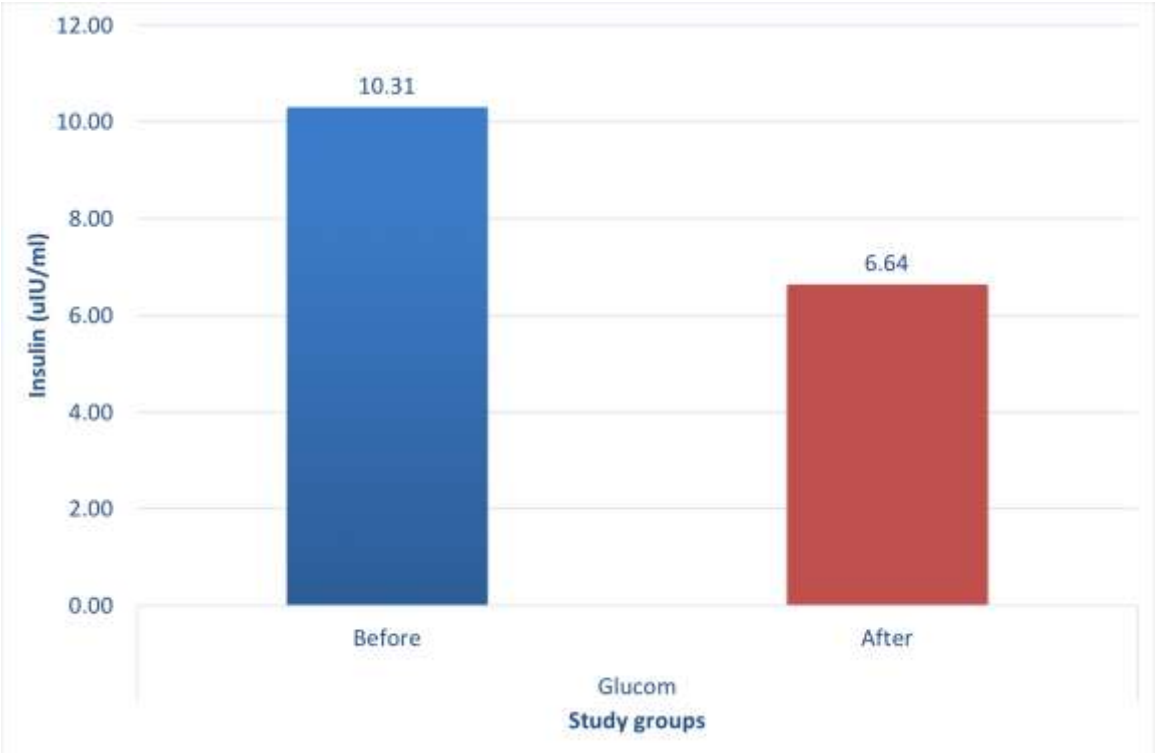

Figure 3. Comparison of mean insulin (mIU/ml) between study group before and after treatment

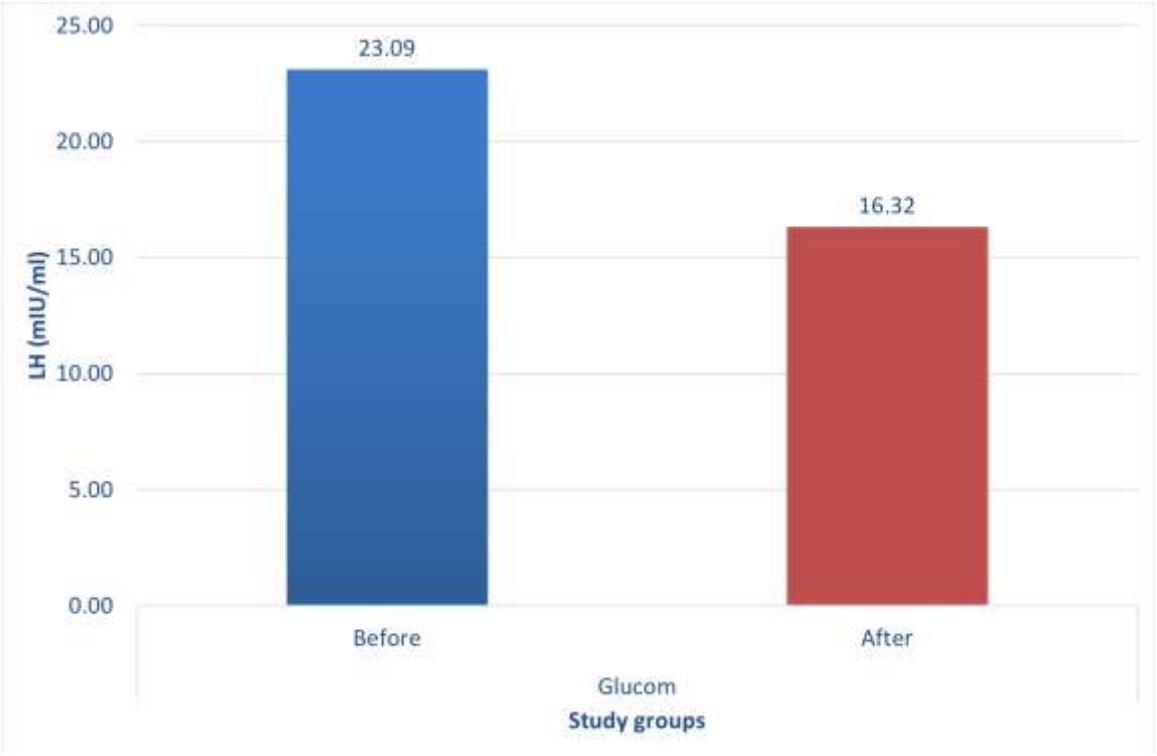

Figure 4. Comparison of mean luteinizing hormone (mIU/ml) between study group before and after treatment

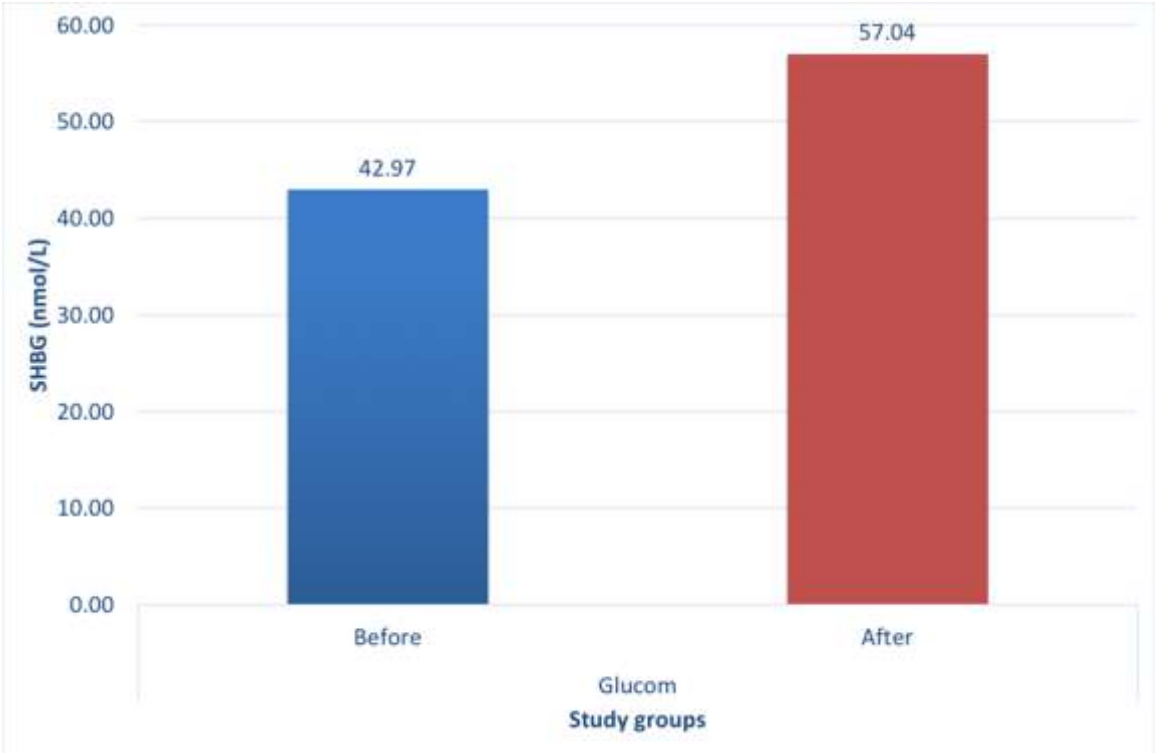

Figure 5. Comparison of mean sex hormone binding globulin (nmol/L) between study group before and after treatment

Discussion

PCOS is multifaceted disorder have neuroendocrine and ovarian dysfunction, high incidence of metabolic disorder, such as obesity and glucose intolerance [4,8].

The results (Mean±SD) of glucose (mg/dL), insulin (uIU/ml), HOMAB, HOMAIR and QUICKI comparison of the GLUC group between before and after treatment show highly significant decrease ($p<0.001$). The high insulin level stimulates the thecal and stromal cells of ovarian to secrete androgen [9]. The insulin resistance with compensatory hyperinsulinemia, leading to hyper androgenism. The action of insulin on the production of androgens in the ovary is thought to be through insulin like growth factor-1 (IGF-1) receptors on theca and stromal cells [10,11].

In recent years in Iraq, PCOS has become a very common disease due to the complicated lifestyle of social and environmental conditions and the lack of societal culture towards proper nutrition, so stress, bad eating habit, sweets and gaseous drinks has a role in increasing cases of PCOS between the women.

The mean weight and BMI decreased significantly during the treatment time due to GLUC is a water-soluble fiber, it's encouraged weight loss in several ways; it is very low in calories [12]. It takes space in stomach and promotes a feeling of fullness (satiety) then reducing food intake and delays stomach emptying time, due to increased satiety [13].

One of the studies showed a correlation between altered gut bacteria and body weight, it mentioned that GLUC enhances the good bacteria in your intestine, which due to short chain fatty acids like butyrate that protect against fat gain in some animal studies [14].

GLUC differs from other soluble fibers, as it's especially viscous, making it effective for weight loss. Weight reduction was significantly greater among those who supplemented with GLUC [15].

Many other studies agree with these results. GLUC caused modest weight loss in overweight and obese individuals when regularly ingested before a meal [16,17].

In the gastrointestinal tract GLUC conforms to gelatin and induces a satiety. It inhibits the absorption of cholesterol, fats and decrease sugar absorption; it prevents the blood glucose increasing by reducing the release of insulin from the pancreas, this wonderful in part to better control of glucose and reduce insulin resistance [6,18,19].

Many studies have steadily shown that GLUC taken with a meal significantly reduces the glucose and insulin response by as much as one-half., adding GLUC to your diet could potentially lower your risk of developing heart disease and type 2 diabetes [18].

GLUC reduces plasma glucose level and make alteration of plasma insulin when taken in obese and healthy subjects. Insulin resistance indexes were decreased in female subjects taking GLUC but increase in the placebo-taking females [20].

GLUC appears to beneficially affect total cholesterol, LDL-C, triglycerides, body weight, and FBG, but not blood pressure [21].

Insulin sensitivity or β-cell function, according to HOMA-IR, QUICKI and HOMA-B indexes show change after treatment; however, significantly ($p < 0.001$) in GLUC group. This agrees with, an increased response to insulin and reduction in glycemia in diabetic patients consuming GLUC [22].

In this study, the patients in group that receiving GLUC show decreased in total cholesterol, LDL-C, triglycerides and increased in HDL-C. These results agree with study reported that cardiovascular benefits of GLUC are due to its effect on lipids. Studies have shown that GLUC is not only statistically significant, but probable is also clinically significant [23].

These effects on total cholesterol, LDL-C, and HDL-C are comparable to those observed several meta-analysis studies that suggests effect of soluble fibers demonstrate reductions in triglycerides with soluble fibers. The cause of GLUC ability to especially lower triglycerides compared with other soluble fibers is not known, probable due to higher viscosity and its ability to alter the metabolic pathways of hepatic cholesterol and lipoprotein metabolism [24], increased fecal neutral sterol and bile acid

contents thus improving blood lipid levels [25]. Compared to other gel-forming fibers, GLUC has three-to five folds stronger lipid-lowering effect [26]. Two previous studies showed Glucomannan and others soluble fiber believed to decrease the risk of cardiovascular disease risk by modifying plasma lipids [27].

Patients getting GLUC had statistically significantly lower total cholesterol, LDL-C cholesterol, triglycerides, body weight, and fasting glucose level, it is having greatest cardiovascular benefits due to its effect on lipids profile [23].

This research is a novel due to first time studying the GLUC effects on women with PCOS, we see a significant statistical difference in the hormones levels testosterone, LH and SHBG in the GLUC group before and after treatment ($p \leq 0.001$); the effects of the GLUC on the hormones were probably due to GLUC have a hypoglycemic effect to lower blood glucose level and decrease the insulin resistance lead to decrease in ovarian androgen level, free testosterone, increase SHBG, decrease hypothalamic GnRH pulses, and this lead to low in the LH level [28].

Administration of an insulin sensitizing agent has beneficial role in lowering serum testosterone level by exerting its action over serum insulin and increasing insulin sensitivity of tissues in PCOS [18].

The benefit of GLUC in PCOS treatment as a fiber supplement should be used to get a significant weight reduction. In addition, reduces fasting glucose increment, improve insulin sensitivity and decrease an elevation of serum lipids [29].

GLUC was effective in management of patients with PCOS throw their ability to decrease the glucose, insulin levels, insulin resistance, lipid profile, testosterone and LH and increase the HDL and SHBG.

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Author contribution

Hussein: Acquisition of data with participation. All authors participated in the design of the study, analysis, interpretation of data and writing of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Screening of Silent Myocardial Ischemia in Type 2 Diabetic Patients with Additional Atherogenic Risk Factors; The Applicability of Exercise Stress Test

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Abstract

Background Silent myocardial ischemia (SMI) is a major clinical entity. SMI is highly prevalent and occurs more commonly in patients with diabetes. It is a predictor of mortality.

Objective To assess SMIs in type 2 diabetic patients with atherogenic risk factors using exercise stress test (EST) and to evaluate its applicability as a screening test.

Methods The study was conducted on sixty-three type 2 diabetic symptomless patients (37 male, 26 female patients), and without resting electrocardiographic (ECG) signs of ischemia. Patients were divided into 2 groups; Group I: patients with one or none of the atherogenic risk factors and Group II: patients with 2 or more of the atherogenic risk factors or peripheral vascular disease (PVD) alone. EST was carried out for all patients. Ischemia was diagnosed by ECG. Other tests were carried out as echocardiography, ultrasound Doppler of carotid arteries.

Results Only 57/63 (90.5%) patients had a diagnostic EST; 11 (19.3%) tested to be positive and 46 (80.7%) tested to be negative. ECG indicated ischemic features in 10 patients with positive EST. Ultrasound Doppler showed PVD in 3 out of 41 patients. Using multivariate analysis, there was a significant association of positive EST with PVD, hyperlipidemia, smoking, and hypertension.

Conclusion As the number of the atherogenic risk factors in diabetic patients increase, the incidence of SMI increases. Therefore, EST is applicable and practicable for screening of SMI in type 2 diabetic patients with 2 or more atherogenic risk factors.

Keywords Silent Myocardial ischemia, Exercise Stress test, Diabetes mellitus.

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List of abbreviations: CAD = Coronary artery disease, ECG = Electrocardiography, EST = Exercise stress test, IHD = Ischemic heart disease, OHA = Oral hypoglycemic agents, PVD = Peripheral vascular disease, SMI = Silent myocardial infarction

Introduction

Ischemic heart disease (IHD) is the most common, serious, chronic, life-threatening illness in developed countries. Diabetes mellitus (DM) accelerates coronary and peripheral atherosclerosis and is frequently associated with dyslipidemia and increases the risk of angina, myocardial infarction, and sudden coronary death. Asymptomatic or silent myocardial ischemia (SMI) is more frequent in diabetic than in nondiabetic patients [(1)].

Diabetes is a common disease with prevalence rates that are predicted to grow significantly over the next several decades [(2)]. Its complications affect many organ systems, especially through vascular changes

(atherosclerotic changes) and are responsible for the majority of morbidity and mortality associated with the disease.

SMI screening is particularly necessary for those patients who show additional risk factors other than diabetes [3]. The exercise stress test (EST) is the low-cost, non-invasive screening test used most widely for SMI [4].

This study aimed to assess SMIs in type 2 diabetic patients with atherogenic risk factors using EST and to evaluate its applicability as a screening test.

Methods

A cross sectional single center study was conducted on 63 type 2 diabetic patients attending the Diabetic Clinic in Al-Imamein Al-Kadhimein Medical City from November 2004 to May 2005. All patients had a previous diagnosis of type 2 diabetes mellitus. The diagnosis was based on WHO criteria and under supervision of an internal medicine specialist.

Diabetic patients with any of the following criteria were excluded: typical angina or chest pain; signs of myocardial ischemia during resting echocardiography (ECG); severe and poor prognosis systemic diseases (e.g., liver failure or chronic renal failure); age >70 years; claudication observed at <400 m; and left bundle branch block on resting ECG.

The study was conducted according to the principles of declaration of Helsinki. Patients were divided into 2 groups; Group I: patients with one or none of the atherogenic risk factors and Group II: patients with 2 or more of the atherogenic risk factors or peripheral vascular disease (PVD) alone.

All patients were recruited and underwent the EST to detect silent myocardial ischemia in relation to the presence or absence of additional atherogenic risk factors for coronary artery disease (CAD). The patients were informed about the benefits and the risks of the test with full explanation of the test procedure by which they have signed a written consent. If the patient was on β-blocker or a calcium-channel blocker, the EST was postponed in order to stop these drugs gradually and substituted by an alternative one (e.g., captopril). EST was done with abstinence of these drugs for at least 48 hours. Prior to the EST, the following measures were done: resting ECG; measurement of blood pressure (BP), pulse rate, HbA1c, Lipid profile (total cholesterol, triglyceride, high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL)), fundoscopy, ultrasound doppler of carotid arteries except some patients. Echocardiography was done for all patients as soon as possible (1/4 – 1 hour) after the EST.

Treadmill exercise test (CASE 16 exercise testing system, Marquette Medical System, Inc.1998, Milwaukee, Wisconsin, USA) according to Bruce protocol was carried out for all patients participating in this study. Each stage lasts for 3 minutes. At the end of each stage and recovery period, which lasts for 3-5 minutes, a 12-lead ECG record was printed and BP measurement was recorded. The EST was halted when one of the following endpoints was reached:

1) Target heart rate, 85% of the anticipated heart rate (220 beats/minus age in yrs);
2) Extreme fatigue;
3) A reduction of the systolic blood pressure;
4) Hypertensive response (plus systolic blood pressure >230 mmHg and/or blood pressure diastolic >120 mmHg);
5) Arrhythmias which are life threatening;
6) Severe depression of ST segment >3 mm; Acute elevation of ST segment.
7) Desire of the patient.

Echocardiography (Diagnostic Ultrasound Scanner, Combison 530D, kretz-Technik, Inc.1997, Zipf, Austria) was carried out for all patients after the EST. It was done to detect the ischemic changes like hypokinesia, dyskinesia, poor left ventricular function, and impaired diastolic relaxation.

Fundoscopy (Topcon, Optical CO., LTD; Tokyo, Japan) was carried out for patients by two ophthalmologists to detect diabetic retinopathy. It was preceded by visual acuity examination and pupillary dilatation.

Ultrasound Doppler (Sonoline Elegra, Siemens Medical Systems, Inc., Ultrasound Group; Issaquah, WA, USA) of the carotid arteries was carried out for 41 out of 57 patients (71.9%) to detect peripheral vascular disease. The latter was considered, if the lumen stenosis was ≥40%.

Biochemical tests: The HbA1c (Glycated hemoglobin) was measured in the central laboratory of Al-Imamein Al-Kadhimein Medical City, and lipid profile including serum total cholesterol, serum triglycerides, serum HDL, serum LDL and VLDL was measured in the laboratories of Al-Nahrain College of Medicine.

Statistical analysis

The statistical analysis was performed with SPSS for Windows software v.10. Results are expressed as means ± SD or n (%), and P values < 0.05 were considered to indicate significance. Comparisons of data were performed using Student's t test and Fisher's exact test for continuous and categorical data, respectively. Odd ratios (OR) of relative risk were calculated and the results were given as OR and 95% confidence interval (CI). OR expressed the increased risk of SMI per unit increase of the atherogenic risk factor itself. Multivariate analysis by multiple logistic regression[105] was computed for the determination of independent risk factors for CAD.

Results

Sixty-three type 2 diabetic patients (group I & II) were recruited. They comprised 37 male and 26 female patients aged 35 to 69 year with duration of DM ranging from 2 months to 24 years having characteristic features shown in table 1.

Table 1. Characteristics of the patients in terms of demographic data and metabolism

Parameters	Results
Number	63
Male/female	37/26
Age (years)	52.9 ± 8.9
Age of onset of DM (years)	46.3 ± 8.7
Duration of DM (years)	6.2 ± 6.6
HbA1c (%)	8.8 ± 1.8
Cholesterol (mmol/L)	5.4 ± 1.4
LDL cholesterol (mmol/L)	3.4 ± 1.4
HDL cholesterol (mmol/L)	1.1 ± 0.4
Triglyceride (mmol/L)	1.8 ± 0.6
Hyperlipidemia [n (%)]	29 (46.0)
Family history of CAD [n (%)]	20 (31.7)
Hypertension [n (%)]	31 (49.2)
Smoking [n (%)]	19 (30.1)
PVD [n (%)]	3 (4.8)
Retinopathy [n (%)]	9 (14.3%)
Treatment	
Diet [n (%)]	4 (6.3)
OHA (Daonil) [n (%)]	47 (74.6)
Insulin / insulin + OHA [n (%)]	12 (19.1)
Antihypertensive agent [n (%)]	24 (38.1)
Aspirin [n (%)]	5 (7.9)

The results of EST were non-diagnostic (non-conclusive) in 6 patients (as shown in patients' flow chart) (Figure 1): 2 because of fatigue, 2 because of hypertensive response, 1 because of dizziness, and 1 because of poor compliance. These conditions resulted in early termination of EST before reaching the target heart rate. Those patients were excluded from the study. The results of EST were diagnostic in the remaining 57 patients (22 from group I, and 35 from group II). These patients were divided into those having a positive EST (n=11, 19.3%) and those having a negative EST (n=46, 80.7%). The positive EST results were more in group II than in group I. Of group II (n=35, 61.4%), 10 patients (28.6%) had positive EST and 25 patients (71.4%) had negative EST. While in group I (n=22, 38.6%), 1 patient (4.5%) had positive EST and 21 patients (95.5%) had negative EST (Figure 1, Table 2).

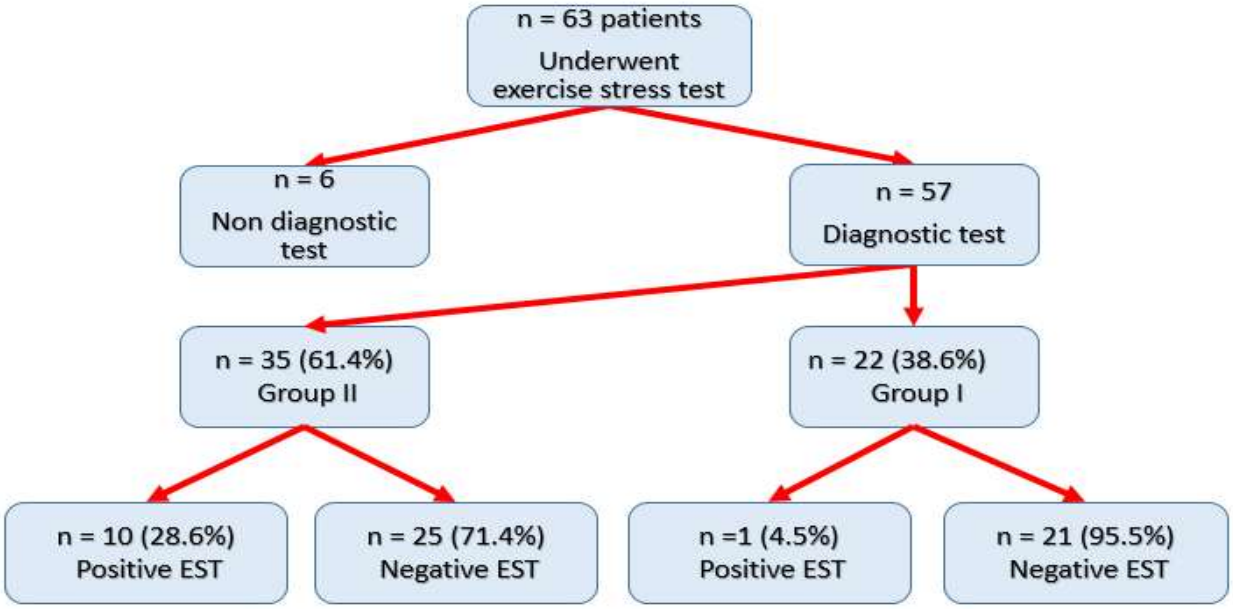

Figure 1. Patients' flow chart

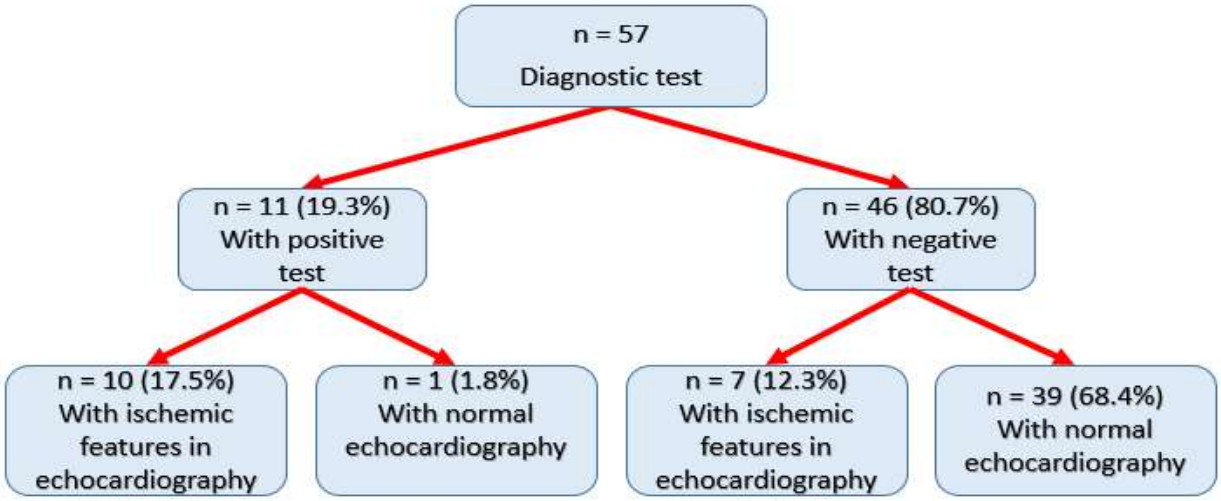

Figure 2. Echocardiographic results in 57 type 2 diabetic patients regarding their positive and negative results in exercise stress test

Table 2. Positive and negative patient clinical and metabolic data at exercise stress test

Clinical and metabolic data	Positive N=11	Negative N=46	P value
Male/female	6/5	27/19	1.000
Age (years)	53.8 ± 8.9	53.1 ± 9.0	0.817
Age of onset of DM (years)	47.6 ± 8.6	46.0 ± 9.2	0.602
Duration of DM (years)	5.7 ± 5.2	6.7 ± 6.1	0.618
HbA1c (%)	8.7 ± 2.3	8.9 ± 1.8	0.755
Cholesterol (mmol/L)	5.6 ± 1.3	5.4 ± 1.4	0.668
LDL cholesterol (mmol/L)	3.7 ± 1.4	3.4 ± 1.5	0.549
HDL cholesterol (mmol/L)	1.1 ± 0.6	1.2 ± 0.3	0.427
Triglyceride (mmol/L)	1.9 ± 0.6	1.8 ± 0.6	0.621
Hyperlipidemia [n (%)]	9 (81.8)	17 (37)	0.016*
Family history [n (%)]	5 (45.5)	15 (32.6)	0.491
Hypertension [n (%)]	9 (81.8)	20 (43.5)	0.041*
Smokers [n (%)]	5 (45.5)	12 (26.1)	0.275
PVD [n (%)]	2 (18.2)	1 (2.2)	0.092
Retinopathy [n (%)]	1 (9.1)	8 (17.4)	0.673
Treatments			
Diet	1 (9.1)	3 (6.5)	1.000
OHA (Daonil)	8 (72.7)	33 (71.8)	1.000
Insulin/insulin + OHA	2 (18.2)	10 (21.7)	1.000
Antihypertensive agent	6 (54.5)	16 (34.8)	0.305
Aspirin	1 (9.1)	4 (8.7)	1.000

Unpaired ttest for comparison of means, Fisher exact test for comparison of frequencies, * significant

The positive EST results were also more in patients with three atherogenic risk factors. Of the total number of patients with positive EST (n=11, 19.3%), 8 patients (14%) had three risk factors, 2 patients (3.5%) had two risk factors, and only 1 patient (1.7%) had one risk factor. The positive EST was diagnosed by a horizontal ST segment depression of ≥1 mm (0.1 mV) calculated at 0.08 s after the J point between QRS and ST segment None of the patients with positive EST had chest pain or down sloping or slow upsloping ST segment depression. Of the 11 patients with positive EST, 10 patients (17.5%) (from group II) had one or more of the ischemic features in echocardiography (hypokinesia, dyskinesia, poor left ventricular function) and in the remaining patient (1.8%) (from group I) the echocardiography was normal. In the 46 patients with negative EST, 7 patients (12.3%) (5 patients from group II & 2 patient from group I) showed abnormal echocardiography with ischemic features, while the remaining 39 patients (68.4%) (20 patients from group II & 19 from group I) the echocardiography was normal (Fig. 2). All patients had good LV functions with ejection fraction > 60 %. Three patients had infrequent ectopics. None of the patients had other abnormality in echocardiography like valvular heart disease or arrhythmias. Echocardiography was interpreted by two independent specialized persons in echocardiography. Ultrasound Doppler was carried out for 41 patients (65.1%). Of the 11 patients with positive EST, 2 patients (18.2%) (from group II) had carotids stenosis > 40 % of the lumen, and 6 patients (54.5%) (from group II) had normal Doppler study. The test was not

done for the remaining 3 patients with positive EST. While 1 patient (2.2%) (from group II) with negative EST had carotids stenosis > 40 % of the lumen, and 32 patients (78.1%) (20 patients from group II & 12 patients from group I) had normal carotids. Diabetic retinopathy was observed by fundoscopy in 9 patients (14.3%): Only one patient (9.1%) (from group II) with diabetic retinopathy had positive EST. The remaining 8 patients (17.4%) (6 patients from group II and 2 patients from group I) had negative EST. The fundoscopy was done by two ophthalmologists. In 26 patients (41.3%) who had abnormal lipid profile test (hyperlipidemic patients), 9 patients (81.8%) (all of them from group II) had positive EST. While the other 17 hyperlipidemic patients (37%) (14 patients from group II & 3 patients from group I) had negative EST. HbA1c was elevated above normal levels (i.e., >6.5%) in 50 patients (79.4). 9 (81.1) out of 11 patients with positive EST had elevated HbA1c (9.2±2.4 %). While 41 (89.1) out of 46 patients with negative EST had elevated HbA1c (8.9±1.9%) (Figure 2, Table 2).

A multiple logistic regression analysis (multivariate analysis) was performed with silent myocardial ischemia as a dependent variable and the following atherogenic risk factors as predictive variables: PVD, family history of myocardial ischemia, smoking, hypertension, and hyperlipidemia. Analysis showed that PVD (OR=10.9, 95% CI=1.9-48.4; P=0.010), hyperlipidemia (OR=9.3, 95% CI=1.8-37.1; P=0.012), smoking (OR=2.4, 95% CI=1.6-9.4; P=0.020), and hypertension (OR=5.6, 95% CI=1.1-28.8; P=0.048) were significant predictors of silent myocardial ischemia in type II diabetes. The role of family history of myocardial ischemia was non-significant (OR=1.9, 95% CI=0.5-7.3; 0.740) (Table 3).

Table 3. Multivariate analysis was performed with silent myocardial ischemia as a dependent variable and the following atherogenic risk factors as predictive variables

Clinical and metabolic data	OR	95% CI	P
Family history	1.9	0.5-7.3	0.74
Hyperlipidemia	9.3	1.8-37.1	0.012 *
Hypertension	5.6	1.1-28.8	0.048 *
Smoking	2.4	1.6-9.4	0.02 *
PVD	10.9	1.9-48.4	0.01 *

* significant

Risk of SMI in diabetic female patients slightly exceeded that of male patients, but with no significant difference. Their results were (1.9, 95% CI=0.5-6.9; P=0.521) and (OR=0.8, 95% CI=0.2-2.9; P=0.628) respectively. No relation was found between retinopathy and SMI in both groups of patients (OR=0.2, 95% CI=0.02-1.8; P=0.321) (Table 4).

Table 4. Risk of SMI according to gender and retinopathy according to Fisher's exact test

Clinical data	OR	95% CI	P
Males	0.8	0.2-2.9	0.628
Females	1.9	0.5-6.9	0.521
Retinopathy	0.2	0.02-1.8	0.321

Discussion

Detection of subclinical CAD in diabetic patients is becoming more focused as diabetes becomes more common and additional information and tools to reduce the risk of death from CAD are becoming more available [5]. Epidemiological studies clearly show the independent major impact to diabetes CAD, but also show cumulative risk rise of other atherogenic factors [6]. A higher mortality risk significantly associates with ST segment depression during EST, regardless of the presence or absence of angina [7]. Mortality among patients with SMI is higher in diabetics than in non-diabetic subjects. Furthermore, the age-adjusted annual cardiovascular mortality rate is higher in type 2 diabetic patients with 2 or 3 cardiovascular risk factors than those with less than 2 risk factors, being 1.2-5.4% and 0.3-0.6% [8], respectively. Taken together, our data strongly indicates that SMI should be screened for those patients with type 2 DM that have additional CAD risk factors.

This study has evaluated two groups of type 2 diabetic patients without any clinical or electrocardiographic evidence of CAD to detect SMI among these patients. Diabetic patients comprising group 2 (with 2 or more atherogenic risk factors) had showed more positive EST than those in group I (with 1 or no risk factor), 17.5 % and 1.8 %, respectively. This is in agreement with other studies [9] and support the idea that only patients with 2 or more atherogenic risk factors should be screened with treadmill for SMI, otherwise it would be costly, exhausted for time and effort, and impracticable [10]. The traditional (basic) atherogenic risk factors were considered. These are peripheral vascular disease, family history of myocardial ischemia, smoking, hypertension, and hyperlipidemia. There are other risk factors revealed to be associated with SMI or CAD in some studies, were not considered, except for retinopathy and hyperglycemia (HbA1c), for example: microalbuminuria [11], autonomic neuropathy [12], and apolipoprotein (a) [13]. This study had showed an interesting finding, that is: as the number of the basic risk factors increases, the results of positive EST (which most likely suggests the occurrence of SMI) increases in type 2 diabetic patients.

In this study, the percentage of SMI in type 2 diabetic patients was 19.3%, SMI prevalence data reported in diabetic patients are quite variable from 9% to 48% [14]. This variability may be due to the selection parameters and diagnostic approaches for the different patients, e.g. age, type and duration of DM.

Based on the results of multivariate analysis, PVD, hyperlipidemia, smoking, and hypertension appear to be risk factors that play a role in identification of these patients. No significant role of family history of myocardial ischemia was found. This study revealed no significant difference in SMI between men and women, and this finding is consistent with the assumption that diabetic women lose their inherited protection against CAD [15]. Furthermore, no differences were found in the detection of SMI in relation to age of the patient, duration of DM, age of onset of DM, hyperglycemia (HbA1c), and retinopathy.

The non-significant role of age could be attributed to the absence of restriction criteria for selection of age of the patients, where young and elderly patients were included in this study.

Diabetic patients selected in our study had various duration of DM (2 months- 26 years). For this reason, probably, the duration of DM showed no significant correlation with SMI as a risk factor.

Uncontrolled hyperglycemia (which can be best assessed by periodic measurement of HbA1c [16] was reported as risk factor for CAD and poor outcome in some studies. Retinopathy is not a risk factor for SMI, as in other previous studies [17]. Retinopathy was of very low frequency in patients with positive EST, as well as patients with negative EST. This might explain the insignificance of retinopathy as a risk factor for SMI by the multivariate analysis of the data. Moreover, it explains also as is a microvascular not macro vascular complication of DM [18].

This study concluded that diabetic patients with atherogenic risk factors have a higher risk for SMI than those without risk factors and as the number of the atherogenic risk factors increases, the risk for SMI increases. EST can be considered as a practicable screening test for SMI in type 2 diabetic patients with 2 or more atherogenic risk factors and echocardiography can play a role in supporting the results of EST, when coronary angiography is not available. Therefore, EST is recommended in asymptomatic type II diabetic patients with 2 or more atherogenic risk factors, unless contraindicated.

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Author contribution

Dr. Al-Ethary: substantial contributions to acquisition and interpretation of data, writing the manuscript and revising it critically for important intellectual content, performing the calculations and statistical analysis. Dr. Alrikabi: selection of patients and interpretation of EST results.

Conflict of interest

Authors declare no conflict of interest.

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