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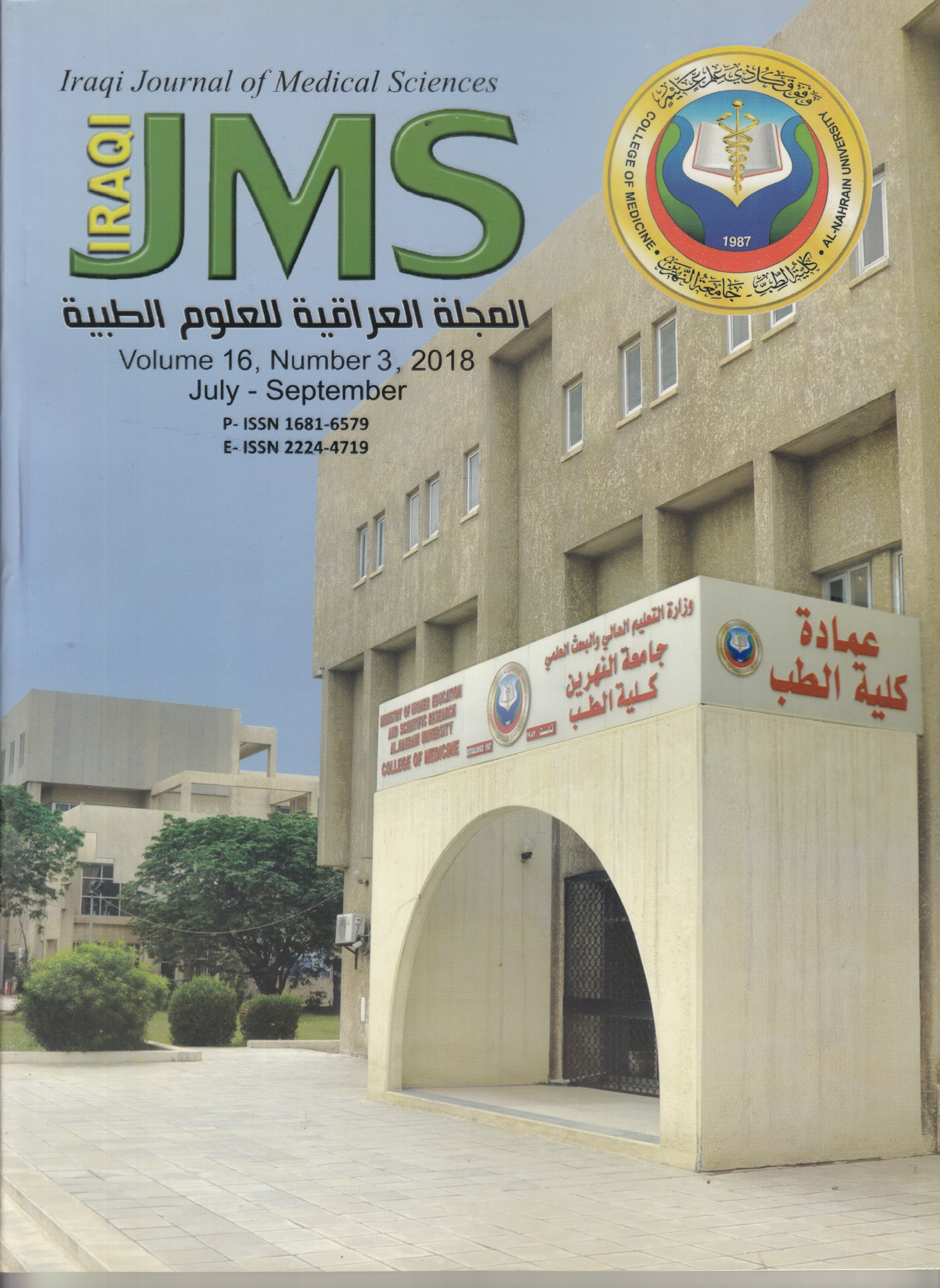

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The Interstitium (The Pre-lymphatic Region), Is It a Newfound 'Organ'

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Abstract

Many of the human anatomists wouldn't expect to discover a new body part, however, researchers claim that they found a network of fluid-filled spaces in connective tissues all over the body that hadn't been seen before and it acts like an "open, fluid-filled highway" that is supported by a lattice of thick collagen "bundles".
The researchers stated that tissue fixation during the procedure of histological tissue processing causes collapse of this network of fluid-filled spaces, and these spaces were not seen during light microscopic examination till the new imaging technique were developed to allow examination of living tissues on a microscopic level.

Keywords Interstitium, anatomy, neworgan, cancer

Citation Mubarak HJ. The Interstitium (the pre-lymphatic region), is it a newfound 'organ'. Iraqi JMS. 2018; 16(3): 230-231. doi: 10.22578/IJMS.16.3.1

List of abbreviation: None

Introduction

Researchers in 2018 reported the description of a network of fluid-filled spaces in connective tissues all over the body as an organ called (the interstitium). This description utilized the examination of the living tissue by the power of confocal laser endo-microscopy. This technique combines an endoscope with a laser, and sensors that showed the spaces where fluid accumulates, those spaces are pre-lymphatic and appeared to drain into lymph nodes [(1)].

This finding may have many implications in fields of medicine, including cancer research. It may explain why cancer can spread to the lymph nodes, as this fluid - filled spaces are a source of the fluid (called lymph) drained into the lymphatic system [(2)]. When cancer cells metastasized through the blood stream or the lymph system, the interstitium spaces may act as conduits, and thus direct sampling of the interstitial fluid may be a proper diagnostic tool for cancer researches [(1)].

The human body contains about 60 percent water, two-thirds of this water is intra-cellular, and one-third is extra-cellular (known as "interstitial" fluid). The interstitial tissue and interstitial fluid are well known in medical biology. The interstitial" fluid is estimated to form about 20% of body volume, which is equivalent to about 10 liters in a young adult [(3)].

The new insights suggested that the (interstitial fluid) is a previously unrecognized feature of human anatomy and raising the idea of calling the interstitium an "organ". The newly described interstitial spaces were suggested to be important possibly in many aspects as, generating the supportive collagen, housing stem cells, or playing a role in conducting electrical signals during cellular movements and stretch. The interstitium likely acts as a

kind of shock absorber for the rest of our interior [1].

The traditional histopathological biopsy sampling involved dehydration of tissue samples, and the interstitium is collapsed by dehydration and appeared as a dense layer when examined after traditional histopathological tissue preparation [4]. When the tissue samples were quickly frozen, it allowed the fluid-filled spaces could be seen under a microscope [1].

Is interstitium as an organ a pseudoscientific belief, or a fact?

Calling interstitium as "organ" is one of the controversies among scientists, the other is about the idea that this discovery could explain many of modern medicine's mysteries.

Many have the feeling of a bit of déjà vu for this discovery. Recently, researchers reported also the discovery of "new" organ: the mesentery! [5].

In order to illuminate answers for these debates, the definition of an organ needs to be settled [6]. Does human body have 79 organs, or 80 organs, or 1,000 organs. The number depends on the definition of what an organ actually is. No two anatomists will agree on a list of organs in the body.

Googling "what is an organ"? Results suggested that (an organ is composed of two or more tissues, is self-contained and performs a specific function). While, tissue is defined as (the structure made from specific and similar type of cells that are grouped or organized together and assigned to perform the specific function).

In consideration to debates of anatomist to the definition of the term (organ), we must report that all the body parts are important regardless of whether being called as organ or not. We still need it to function.

In conclusion; the distinction for the interstitium as an (organ) needs further investigations in order to clarify the possible changes that may be associated with / or altered in this organ during disease, and to establish whether this organ have a role in driving diseases.

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Corneal Changes in A Sample of Pseudoexfoliation Iraqi Patients

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Abstract

Background Pseudoexfoliation syndrome is a common eye disorder that can affect different parts of the eye causing significant morbidity.

Objective To compare corneal endothelial changes between patients with pseudoexfoliation syndrome and normal age matched patients.

Methods Specular microscopy was used to measure central corneal thickness, average size of endothelial cells and coefficient of variation in cell area, endothelial cell density, as well as hexagonality of 238 eyes from 238 patients (120 eyes from patients with pseudo exfoliation syndrome and 118 eyes from normal age-matched patients).

Results Patients with pseudoexfoliation syndrome had lower central corneal thickness (p value 0.049) compared to the control (491.6±28.86 vs 502.5±35.77 µm), lower endothelial cell density (p value 0.02) compared to control (2458.9±430.8 vs 2585.3±378.8 cell/mm^2), lower hexagonality (P value 0.006) compared to control (48.25±18.76 vs 55.06±12,44), also a significantly higher coefficient of variation (p value 0.046) compared to control (36.15±7.381 vs 33.4±6.22).

Conclusion Pseudoexfoliation syndrome is associated with a significant change in the corneal endothelium, including reduction of endothelial cell density, increased variation of cells shape (pleomorphism) and increased variation of cells area (polymegathism). There was a significant change of central corneal thickness.

Keywords Corneal endothelium, specular microscopy, pseudoexfoliation syndrome, central corneal thickness

Citation Rasheed AM, Kadum DJ. Corneal changes in a sample of pseudoexfoliation Iraqi patients. Iraqi JMS. 2018; 16(3): 232-238. doi: 10.22578/IJMS.16.3.2

List of abbreviations: CCT = Central corneal thickness, CD = Endothelial cell density, CV = Coefficient of variation, HEX =Hexagonality, PEX = Pseudoexfoliation syndrome

Introduction

The corneal endothelium is the inner layer of the cornea that is in contact with the aqueous humor in the anterior chamber of the eye. It consists of a single layer of cuboidal cells that are linked to each other by desmosomes and hemidesmosomes [(1)]. When cells are viewed from the posterior surface, there is an overall hexagonal pattern. The number of endothelial cells fall steadily with age and there is increased variation of cell size. The adult mean cell density is about 2500 cell /square mm, and this number falls gradually with age and it is estimated that between the ages of 20 and 80 years the reduction in cell density averages 0.52% per year [(2)].

The maintenance of a transparent cornea depends upon the endothelium producing a state of relative stromal dehydration [(3)]. The proteoglycan matrix that surrounds each collagen fiber of the stroma produce an imbibition pressure (60 mm hg), which tends to draw water into the cornea. Tight junctions between epithelial cells of the cornea form a

barrier to reduce the flow of water from the tear film into the stroma, but the absence of tight junctions between endothelial cells permits the free flow of aqueous into the stroma. If this water is allowed to accumulate it produces stromal swelling and clouding. The pump-leak concept of corneal hydration proposes that there is a dynamic equilibrium between the tendency of the stroma to swell and the active transport of ions by the endothelial pump to oppose the inward movement of water [4].

The normal function of the endothelial pump of the cornea may be affected by diseases that involve the endothelium like pseudoexfoliation and endothelial dystrophy.

Pseudoexfoliation syndrome (PEX) is an idiopathic age-related systemic disease manifesting itself primarily in the eyes [5]. It is diagnosed clinically by the deposition of abnormal fibrillar material on various intraocular structures including the corneal endothelium, anterior lens surface, iris, trabecular meshwork, zonules, and ciliary body [6]. This may lead to various ocular complications such as chronic open-angle glaucoma, lens subluxation, poor mydriasis and zonular dehiscence, capsular rupture or vitreous loss [7,8]. Corneal endothelial changes have also been reported [9,10], and this can affect the corneal hydration, thickness, and transparency. In patients with PEX, quantitative and qualitative morphological changes of the corneal endothelium have been demonstrated in specular and electron microscopic studies [9,11-13]. These changes represent an abnormal or unstable endothelium, predisposing to an endotheliopathy that may be more susceptible to the effects of intraocular surgery [9,14].

The aim of this study is to study the corneal endothelial changes in patients with pseudoexfoliation syndrome and to compare them with normal aged match subjects.

Methods

Two hundred thirty-eight eyes were examined, 120 eyes of patients with PEX and another 118 eyes of normal aged match subjects. Informed verbal consent was obtained from all patients. Exclusion criteria included patients with PEX glaucoma, previous ocular surgery or trauma, eye inflammation, corneal dystrophy, and metabolic disease like diabetes. Each eye was examined by slit-lamp for the anterior segment, intraocular pressure by Goldmann tonometry, and optic nerve head examination by non-contact volk magnifying lens +90. Specular microscopy then was done by non-contact Topcon sp-3000 p. For each eye 100±20 endothelial cells were counted in each image for analysis, and the following measurements were determined: Central corneal thickness, cell density, coefficient of variation, and hexagonality. Data were divided into four groups according to patients age as follows: Group A for those between 50-59 years; group B for those between 60-69 years; Group C for those between 70-79 years; and Group D for those more than 80 years. T test was used to assess the differences in mean for continuous variables, while chi square test was used to assess the differences in mean for continuous variables, while chi square test was used to assess the differences in distribution of categorical variables. Statistical analysis was performed using SPSS software windows version 21 and Student's independent t-test to reveal any significant association.

Results

There were 238 cases included in this study. Mean age of patients and control did not differ (for patients = 66.28±7.45 years and for control = 66.25±7.47 years), male to female ratio in the patient group was 1.66:1 while in the control group it was 1.45:1, so there is male predominance in both control & patient group (Tables 1 & 2).

By comparing central corneal thickness (CCT) between control corneas, patient's cornea, there is a significant decrease of mean CCT for patient group, mainly for age groups A, B, C except D where there is no significant difference in CCT (Table 3).

Table 1. Baseline characteristics of the study group

Age groups	Gender	Control		Patient	
		No.	%	No.	%
Group A	Male	12	17.1	12	16.0
	Female	11	22.9	11	24.4
Group B	Male	30	42.9	33	44.0
	Female	21	43.8	17	37.8
Group C	Male	23	32.9	26	34.7
	Female	15	31.3	15	33.3
Group D	Male	5	7.14	4	5.3
	Female	1	2.08	2	4.4
Total	Male	70	100	75	100
	Female	48	100	45	100

Table 2. Descriptive of age group (control and patient)

	Control		Patient		t-test	P-value	Sig.
Age groups	Mean	SD	Mean	SD			
Group A	55.8	2.92	56.2	2.72	0.405	0.689	NS
Group B	64.3	2.37	63.8	2.45	1.174	0.246	NS
Group C	72.6	2.48	72.4	2.36	0.462	0.646	NS
Group D	82.3	1.86	83.3	1.86	0.889	0.415	NS

*P>0.05 Non-significant

Table 3. Descriptive of central corneal thickness

	Control		Patient		t-test	P-value	Sig.
Age groups	Mean	SD	Mean	SD			
Group A	512.3	31.3	494.8	38.09	2.475	0.022	S
Group B	497.2	34.5	491.3	21.23	2.477	0.029	S
Group C	507.9	35.86	492.3	31.07	2.464	0.018	S
Group D	477.2	49.75	476.3	26.65	1.982	0.058	NS

*P<0.05 Significant, **P>0.05 Non-significant

Endothelial cell density (CD) significantly decreases for patient group in comparison with the control group, also it is only for group A, B, C while in group D there are no significant changes in endothelial cell count in comparison with the control group (Table 4).

With Regard to the percentage of hexagonal cells (hexagonality), there is a significant reduction in the mean of the patient group for age group A, B, C in comparison with the control group while group D shows nonsignificant reduction in hexagonality in comparison to control group (Table 5).

Table 4. Descriptive of central corneal thickness

Age groups	Control		Patient		t-test	P-value	Sig.
	Mean	SD	Mean	SD			
Group A	2701.1	184.3	2445.9	469.5	2.2624	0.015	S
Group B	2608.5	351.4	2565.1	470.2	2.047	0.049	S
Group C	2538.8	474.8	2369.4	358.9	2.323	0.047	S
Group D	2238.2	276.8	2236	99.7	1.892	0.092	NS

*P<0.05 Significant, **P>0.05 Non-significant

Table 5. Descriptive of hexagonality

Age groups	Control		Patient		Z-test	P-value	Sig.
	Mean	SD	Mean	SD			
Group A	59.8	7.71	47.4	22.48	2.409	0.025	S
Group B	56.47	9.83	49.54	17.1	2.453	0.018	S
Group C	53.57	13.74	49.0	19.27	2.021	0.049	S
Group D	34.0	17.84	33.16	2.639	1.72	0.145	NS

*P<0.05 Significant, **P>0.05 Non-significant

As for Coefficient of variation (CV), there is increased variation in individual cell areas for all age groups for the patient group in comparison to the control group as shown in the table 6:

Table 6. Descriptive of coefficient of variation control and patient

Age groups	Control		Patient		Z-test	P-value	Sig.
	Mean	SD	Mean	SD			
Group A	32.4	4.74	35.03	7.28	2.043	0.049	S
Group B	33.9	6.698	36.09	5.994	3.012	0.035	S
Group C	32.95	6.407	36.36	9.02	2.39	0.041	S
Group D	34.6	6.804	39.5	6.31	2.422	0.045	S

*P<0.05 Significant

In comparison with non PEX control corneas, PEX corneas (patient group) have lower central corneal thickness (CCT) (491.6 vs 502.5 µm), lower endothelial cell density and hexagonality (2458.9 vs 2585.3 cell/mm^2, 48.25 vs 55.06% respectively), a significant increase in endothelial cell size, standard deviation of cell size and coefficient of variation (CV) (36.15 vs 33.4) as shown in the table 7.

Corneal endothelial changes regarding gender distribution in both patient & control group. All data reveal a higher mean for male except for CV which is higher for female in both patient & control group (Table 8).

Table 7. Overall differences in corneal parameters between pseudoexfoliation patients and control

Parameter	Control		Patient		t-test	P-value	Sig.
	Mean	SD	Mean	SD			
Central corneal thickness	502.5	35.77	491.6	28.86	2.012	0.049	S
Endothelial cell density	2585.3	378.8	2458.9	430.8	2.365	0.02	S
Hexagonality	55.06	12.44	48.25	18.76	2.772	0.006	S
Coefficient of variation	33.4	6.22	36.15	7.381	2.136	0.046	S

*P<0.05 Significant

Table 8. Corneal parameters difference between pseudoexfoliation patients and control with regards to gender

Parameter	Gender	Control		Patient		t-test	P-value	Sig
		Mean	SD	Mean	SD			
Central corneal thickness	Male	509.2	37.25	501.04	36.2	2.322	0.047	S
	Female	492.8	31.38	500.2	34.25	2.143	0.034	S
Endothelial cell density	Male	2595.7	381.9	2484.9	439.9	2.696	0.008	S
	Female	2569.9	377.7	2433.1	417.3	2.773	0.004	S
Hexagonality	Male	56.08	13.23	50.37	18.62	2.033	0.047	S
	Female	53.58	11.16	47.02	18.53	2.354	0.046	S
Coefficient of variation	Male	32.07	5.951	33.51	5.84	1.89	0.089	NS
	Female	35.22	6.21	36.3	9.96	1.99	0.065	NS

*P<0.05 Significant, **P>0.05 Non-significant

Discussion

The pseudoexfoliation syndrome affects all structures of ocular anterior segment. By electron microscopy, large clumps of typical pseudoexfoliation material can be found adhering to the corneal endothelium, and masses of pseudoexfoliation material are incorporated into the posterior Descemet membrane [12]. These may lead to early corneal endothelial decompensation. In this study, we found that the thinnest corneas occur in the eyes of patients with PEX (P<0.049) and this was similar to the result found by Inoue et al [15] and Yazgan et al. [16] in their study of corneal biomechanical comparison of PEX, pseudoexfoliative glaucoma and healthy subjects. They found that the mean CCT were 546.3± 28, 525.5±35 and 509±36 µ, in healthy subjects, PEX and PEXG, respectively. The differences on CCT were also significant among the three groups ($p < 0.001$). However, Hepsen et al. [17] and Arnarsson et al. [18] reported that patients with PEX have a higher CCT than the control group. (P=0. 56, P= 0. 23, respectively) but these results are statistically non-significant.

Interesting conclusions regarding the influence of PEX on the corneal stroma were included in the study published by Zheng et al. [19]; the authors, using confocal microscopy, identified deposits of pseudoexfoliating materials in the cornea itself. They also showed that the number of keratocytes in the corneal stroma of the eyes of patients with PEX (per unit of area) was smaller than in the group of people without PEX. They concluded that the presence

of the pseudoexfoliating material induces apoptosis of corneal stroma keratocytes and in the end leads to the impoverishment of its extracellular structure. This may result in the thinning of the cornea and its greater susceptibility to elevated intraocular pressure.

The normal density of corneal endothelial cells in adults is approximately 2500 cells/mm^2 and it is reduced by about 0.6% a year so normally endothelial cell density decreases with age. In this study, it was found that there is a significant reduction of endothelial cell density in eyes with PEX in comparison with normal aged match eyes (p = 0.02). This finding was similar to reported studies by Miyake et al. [9]; Zheng et al. [19]; Yüksel et al. [20]; Tomaszewski et al. [21]; Wang et al. [22]; Quiroga et al. [23]; Kovaliunas et al. [24].

Research presented above clearly shows that PEX significantly influences cell density of corneal endothelium of people with this disease. The cause of the lower endothelial cell density of patients with PEX is the pseudoexfoliation material, appearing at the earliest stages of PEX, which settles on the endothelium penetrating it in the direction of the Descemet's membrane and breaking the connections between individual six-sided cells, which results in local accelerated apoptosis of these cells. Other factors recognized by researchers, excluding the accumulation of PEX material causing the reduction of the number of cells within the layer of the corneal endothelium, include hypoxia of the anterior chamber, changes in the fibroblasts of the endothelium, and elevated concentration of TGF-α1 [6].

The average size and coefficient of variation (CV) of corneal endothelial cells were found to be significantly increased in eyes with PEX (P<0.046). The increase in coefficient of variation (CV) indicates the presence of polymegathism in which endothelial cells enlarge to fill the gaps between adjacent cells. This study also showed that the percentage of hexagonal cells were significantly reduced (P<0.006), indicating the presence of pleomorphism. These results were similar to those obtained by Miyakeet al. [9]; Yüksel et al. [20]; and Wali et al. [25].

However, Inoue et al. [15] and Wang et al. [22] found that the difference of coefficient of variation of cell size and percentage of hexagonal cells between the PEX eyes and the control eyes was not statistically significant.

Another aspect of the study result is the fact that this syndrome is strongly age-related. In this study, the age factor was evident. Despite the bulk of our study depend on Group B but we found that Group A is more affected by pseudoexfoliation syndrome than others, in addition to that, for patients older than eighty there are no significant changes apart from polymegathism in comparison with the control group.

These observations suggest that the corneal endothelial changes represent a consistent finding in eyes affected with pseudoexfoliation.

This study concluded that there is a decrease in corneal endothelial cells in patients with pseudoexfoliation syndrome, in addition to pleomorphism, polymegathism and corneal thinning.

The authors recommended that ophthalmic surgeons should pay special attention while doing surgery in eyes with pseudoexfoliation, and this may include doing preoperative endothelial cell study by specular microscopy to detect eyes at risk of endothelial decompensation.

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Authors contribution

Dr Rasheed: collection of study cases. Dr Kadum: collection of study cases and writing of the article.

Conflict of interest

Authors declare no relation with any institute or personal that have any influence on the results of this study.

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Detection of New Delhi Metallo-Beta-Lactamase-1 (*bla*$_{NDM-1}$) in Carbapenem-Resistant *Pseudomonas aeruginosa* Isolated from Clinical Samples in Wasit Hospitals

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Abstract

Background *Pseudomonas aeruginosa* (*P. aeruginosa*) infections are clinical problem, it is a difficult to treat because of high resistant to many antibiotics (Multi-drug resistant) and a high risk of emergence of resistance during therapy. Carbapenems are therapeutic choice against infections caused by Gram-negative bacilli including strains of *P. aeruginosa*. New Delhi metallo-β-lactamase-1 (*bla*$_{NDM-1}$) gene, an acquired class B carbapenemase. Dissemination predominantly involves transfer of the *bla*$_{NDM-1}$ gene among promiscuous plasmids and clonal outbreaks. Bacteria with NDM-1 are typically resistant to nearly all antibiotics.

Objective To detect *bla*$_{NDM-1}$ in the isolates of *P. aeruginosa*, which were recovered from various clinical samples from hospitalized patients in Wasit hospitals.

Methods This cross-sectional study involved 200 clinical samples were collected from three major hospitals in Wasit province. Samples were inoculated in Mackonkey and blood agar for primary isolation and then biochemical tests were used to confirm diagnosis of *P. aeruginosa*. The susceptibility test for 14 types of antibacterial drugs were tested by using disk diffusion method. Chromosomal and plasmid DNA were extracted by using special methods.

Results Out of 36 carbapenems resistant *P. aeruginosa* (CRPA) isolates, there were 18 isolates (50%) positive for *bla*$_{NDM-1}$ gene.

Conclusion Rate of occurrence of *bla*$_{NDM-1}$producers is highest among carbapenem-resistant *P. aeruginosa* isolated from clinical samples in Wasit hospitals. Therefore, its recognizable proof in clinical bacterial diseases will be suspected in any carbapenem resistance *P. aeruginosa*.

Keywords *P. aeruginosa*; carbapenems; metallo-β-lactamase; *bla*$_{NDM-1}$

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List of abbreviations: BHI = Brain-heart infusion, bla-NDM1 = New Delhi metallo-β-lactamase-1, CRPA = Carbapenem- resistant *Pseudomonas aeruginosa*, ESBLs = Extended-spectrum beta lactamases, MDRs = multi-drug resistance

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an aerobic Gram-negative rod-shaped. It is widely distributed in nature and can adapt to many environments, it can be isolated from nearly any conceivable source within hospitals [(1)]. It is an important cause of both community and hospital-acquired infections. Infections with these bacteria have been associated with high mortality and morbidity when compared with other bacterial pathogens [(2)]. *P. aeruginosa* infections are clinical problem, it is a difficult to treat because of high resistant to many

antibiotics (Multi-drug resistant) and a high risk of emergence of resistance during therapy [3].

Beta-lactam as antibacterial agent are broadly used to treat diseases caused by Gram-negative Pathogens However, the adequacy of these medications is lessened impressively because of the presence of extended-spectrum beta lactamases (ESBLs) and the consequent emergence of multi-drug resistant (MDRs) strains [3].

Carbapenems are a group of β-lactam antibiotics with a broad spectrum of antibacterial activity. Their structure makes them highly resistant for most β-lactamases [4]. They include meropenem and imipenem, which are among the few therapeutic options still available for treating infections caused by *P. aeruginosa* [5]. Carbapenems are considered to be antimicrobial agents of choice and are frequently used for the treatment of hard-to-manage *P. aeruginosa* infections. However, carbapenem resistance in *P. aeruginosa* has been reported to increase steadily over the years across the world, but the relative contribution of different carbapenems resistance mechanisms is not well established [6,7]. bla_{NDM-1} is an enzyme that cleaves the amide bond of β-lactam ring and provides resistance against major classes β-lactam antibiotics [8]. New Delhi Metallo-β-lactamase-1 gene (bla_{NDM-1}) codes for NDM-1 [2]. An association with other resistance mechanisms makes majority of *P. aeruginosa* with blaNDM-1 gene extensively resistant to antibiotics.

The goal of this research was to detect the presence of NDM-1 producers in clinical *P. aeruginosa* isolates producer between clinical P. aeruginosa isolates in Wasit hospitals.

Methods

Clinical isolates

Over six months from November 2016 to April 2017, different samples including (burn swab, ear swab, urine, sputum and wound swab) from two hundred patients admitted to (Al-Zahraa Teaching Hospital, Al-Karama Teaching Hospital and Al-Kut Hospital for Gynecology, Obstetrics and Pediatrics) in Wasit province were enrolled in this study. In the case of swab samples, two swabs were taken from each patient, while sputum and urine were divided directly into two parts, the first one was prepared for wet smear preparation (Gram stain), and the other was used for culturing on different culture media for further isolation and characterization of the causative agents. The isolated bacteria were identified by standard laboratory methods and API20E system (BioMerieux), *P. aeruginosa* isolates in Brain-Heart Infusion (BHI) broth containing 15%, and the tubes were stored in deep freezing at -20 °C [9].

Antimicrobial susceptibility testing

Resistance patterns of the *P. aeruginosa* isolates to different antibiotics was determined using disk diffusion test (Kirby-Bauer) on Muller Hinton agar media [10], the antibiotic discs used in this study was Levofloxacin (5 µg), Meropenem (10 µg), Imipenem(10 µg), Aztreonam (30 µg), Ceftazidime (30 µg), Amikacin (30 µg), Gentamicin (30 µg), Ciprofloxacin (10 µg), Piperacillin (10 µg), Colistin sulphate (25 µg). The standard isolates from central public health laboratory *E. Coli* ATCC25922 used as a negative control. When the incubation was completed temperature and time, the resulting zones of inhibition were measured and compared with the break points standard value of Clinical Laboratory Standards Institute CLSI (2016) [10]. The minimum inhibitory was determined by Vitik2-System (VITEK MS, bioMérieux, Nürtingen, Germany), and standard agar dilution method [11] according to the CLSI (2016) [10].

Phenotypic detection of metallo-β-lactamases (MBL)

All imipenem and meropenem-resistant isolates were examined for MBL production using the IMP-EDTA double disk synergy test as described by [12], furthermore, Modified Hodge test (MHT) was used for detection of carbapenemases production *P. aeruginosa*

isolates according to CLSI guidelines using 10 µg meropenem susceptibility disk, which was placed in the center of the test area. P. aeruginosa was streaked in a straight line from the edge of the disk to the edge of the plate. The plate was incubated overnight at 37 °C in ambient air for 16-24 hours. After 24 hours, MHT positive test showed a clover leaf-like [13].

DNA Extraction and polymerase chain reaction (PCR) amplification

In this study, both plasmid DNA and chromosomal DNA were extracted, plasmid DNA was extracted according to (14), while chromosomal DNA was extracted by Genomic DNA Mini Kit (Genaid) according to company instructions. All carbapenem-resistant isolates were screened by standard PCR conventional using specific primers for *bla*$_{NDM-1}$ gene as shown in table (1). PCR reaction tubes were transferred into thermal cycler (Agilent, USA) that was programmed as following: initial denaturation for 5 mints at 95 °C, (the conditions for each cycle were: 30 sec. at 94 Ċ, 30 sec. at 60 °C and 30 sec. at 72 °C), and final extension at 72 °C for 5 mints. Amplified products were electrophoresed on 1.5% agarose for 90 mints at 5 V/cm.

Table 1. Sequences of primer that used in the detection bla$_{NDM-1}$ gene

Gene		Nucleotide sequences (5'→3')	Products size bp	References
bla$_{NDM-1}$	F	GGG CAG TCG CTT CCA ACG GT	475	(15)
	R	GTA GTG CTC AGT GTC GGC AT		

Statistical Analysis

Statistical analysis was performed with Graph Pad Prism version 6 software, percentages were used for the comparison between samples of the study. Data analysis was done using Chi-square for the comparison of categorical data.

Results

A total of two hundred samples were enrolled in this study which include, burn swabs (n=105, 52.50%), ear swabs (n=19, 9.50%), wound swab (n=36, 18.00%), sputum from patients with lower respiratory tract infection (n=7, 3.50%) The patient's ages ranged from one to older than 61 years (Table 2).

Table 2. Distribution of *Pseudomonas aeruginosa* isolates according to age groups

Age groups	*P. aeruginosa*	Others bacteria	Negative	Total
1-10 yr	11	10	2	23
11-20 yr	10	9	2	21
21-30 yr	20	12	4	36
31-40 yr	27	13	2	42
41-50 yr	11	14	3	28
51-60 yr	9	7	4	20
≥61 yr	15	13	2	30
Total	**103**	**78**	**19**	**200**

One hundred and eighty-one bacterial species were isolated from these samples with the percentage of *P. aeruginosa* (n=103, 51.50%) followed by *E. coli* (n=28, 14.00%) and the lowest percentage were *K. pneumoniae* (n=2, 1.00%). There is non-significant association between Pseudomonas infections and age groups as shown in table (3).

Table 3. Distribution of *Pseudomonas aeruginosa* and other bacteria according to the growth

Type of Bacteria	Samples	%
Negative	19	9.50%
Klebsiella pneumoniae	2	1.00%
Staphylococcus epidermidis	4	2.00%
Acinetobacter baumanni	7	3.50%
Pseudomonas putida	7	3.50%
Streptococcus pyogenes	12	6.00%
Staphylococcus aureus	18	9.00%
Escherichia coli	28	14.00%
Pseudomonas aeruginosa	103	51.50%
Total	200	100%

Antimicrobial susceptibility test

The results of antibiotic susceptibility test for isolated *P. aeruginosa* indicated different antibiotic profiles as shown in table (4). In total, 55.5% (n=103) resistance to the third-generation ceftazidime 57.28% of the isolates exhibited resistance to the fourth generation cefepime. While the resistance to monobactams, aztreonam was 51.46%. The highest resistance percentage was found against gentamicin (91.26%). According to the results of the fluoroquinolones susceptibility testing, 83.50% and 60.19% of the isolates were resistant to ciprofloxacin and levofloxacin, respectively (Table 4).

Table 4. Susceptibility patterns of *Pseudomonas aeruginosa* to different antibiotics

Antibiotic	Sensitive (S)		Intermediate (IR)		Resistant (R)	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
Imipenem	67	65.05	0	0	36	34.95
Meropenem	67	65.05	0	0	36	34.95
Ciprofloxacin	12	11.65	5	4.85	86	83.50
Levofloxacin	38	36.89	3	2.91	62	60.19
Amikacin	9	8.74	6	5.83	88	85.44
Gentamicin	5	4.85	4	3.88	94	91.26
Cefepime	42	40.77	6	5.83	55	53.40
Ceftazidime	39	37.86	5	4.85	59	57.28
Aztreonam	40	38.83	10	9.71	53	51.46
Piperacillin	17	16.50	16	15.53	70	67.96
Piperacillin/Tazobactam	50	48.54	8	7.77	45	43.69
Colistin	102	98.03	0	0	1	0.97
Ticarcillin/Clavulanic acid	47	45.63	5	4.85	51	49.51
Ticarcillin	40	38.83	7	6.80	56	54.37

Phenotypic detection of MBLs

From 36 *P. aeruginosa* carbapenem-resistant isolates, MHT revealed 16 (44.44%) were positive showing their ability to produce carbapenemases, moreover, double disc synergy indicates that in 32 (88.89%) isolates, MBLs were produced. Those isolates, which were found MBL positive by Double disc synergy test and were also found to be MBL positive MHT.

PCR screening for NDM-1 encoding gene

PCR using specific primers for NDM-1 was performed on all the IMP-resistant isolates for generation of specific amplification band with certain molecular weight that were 475 bp fragment which represented blaNDM-1 gene. The results showed MBL gene *bla*$_{NDM-1}$ (475 bp) was detected in 18 (50.00%) of the carbapenem-resistant isolates on plasmid DNA, while MBL gene *bla*$_{NDM-1}$ not found on chromosomal DNA (Figure 1).

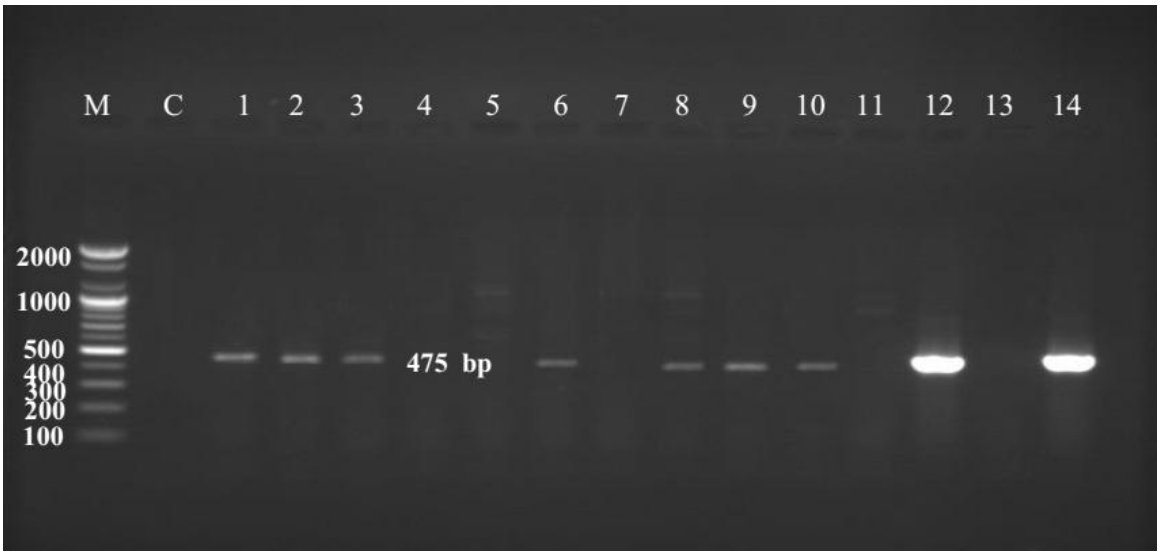

Figure 1. Gel electrophoresis of amplified plasmid DNA for detection of MBL bla-NDM1 gene (475bp) using PCR with specific primers; 1,5% Agarose for 90 minutes at 70 V\cm. Lane M: Marker DNA ladder Size (100bp), Lane C: Negative control and Lanes (1-14) positive for *bla*$_{NDM-1}$ (475 bp) except (4,5,7,11,13)

Discussion

The current emergence of *P. aeruginosa* carbapenem-resistant represents a major threat to the clinical approach because it exhibits intrinsically decreased susceptibility to a range of antimicrobials and possesses a great ability to develop resistance to multiple classes of agents [16]. Among two hundred samples were enrolled in this study, the mean age of the patients were 36.61 years. Results of current study revealed that, there is non-significant association between Pseudomonas infections and age. It is noteworthy to mention that result was disagreed with a study conducted by Magliano et al. [17] who was reported the high rate of *P. aeruginosa* infection among age group (≥ 60 years). In the present study, *P. aeruginosa* has been the predominant bacterial isolated among study group followed by *E. coli* (14%) and the lowest percentage were *K. pneumoniae* (1%). These findings are compatible with study conducted in Egypt by Gad et al. 2007 [18], the present study is incompatible with a study in Baghdad by Al-Huraishi [19] who found that *Acinetobacter baumannii* (31%) is even more common than *P. aeruginosa* (12%), and study conducted in Baghdad by Al-Kadhmi in 2016 [20], who reported that *S. aureus* (30%) was the most common agents, then *P. aeruginosa* (14.6%) this difference in results can be attributed to sample difference and kind of test

used in isolation and diagnosis of different bacterial species.

Carbapenems are a class of β-lactam antibiotics with good antimicrobial activity against *P. aeruginosa* but the arises and spread of acquired carbapenem-resistance in this species have challenged the success of therapeutic and control efforts [21]. Result in current study showed there was no different found in activity of imipenem and meropenem to *P. aeruginosa* (both of them have the same percentage 34.95% resistant, respectively), which disagree with Gupta et al. 2006 who found that the imipenem had a better activity than meropenem [22]. Furthermore, current finding indicated that higher resistance against imipenem and meropenem have compared with study in Najaf by Al-Shara in 2013 [23], who reported that the resistance rate was 7.4% and 14.8%, respectively. The percentage of fluoroquinolone-resistant isolates was 83.50% and 60.19% of isolates resistant to ciprofloxacin and Levofloxacin, respectively identified in this study is considerably higher than that reported in study conducted in Najaf Hospitals, in which resistance were 73.4 % for ciprofloxacin and 55.5% for Levofloxacin [23] also it is in harmony with previous study in Najaf [24]. Fluoroquinolone resistance among *P. aeruginosa* isolates looks to be increasing in the Wasit hospitals, perhaps because of high increasing fluoroquinolone use, and the lack of adherence to approved infection control practices by hospitals. The *P. aeruginosa* isolates were most resistant to amikacin (85.44%) and gentamicin (91.26%), the resistance rate was higher when compared with other study reported by Al-Shara [23] in Najaf, who revealed that only 64.8% of the P. *aeruginosa* isolates resistant to this antibiotic. However, the findings of *P. aeruginosa* antibiogram in the present study disagree with a study done in United States of America [25]. Results in the current research, showed that 51.46% of the *P. aeruginosa* isolates were resistant to aztreonam, Present findings are higher than previous study done by Abdullah and Mehdi, who showed low rate of aztreonam resistance among *P. aeruginosa* clinical isolates [26]. Colistin resistance is not dependent upon bacterial metabolic activity and acquired resistance is rare [27]. In present study, the resistance of the isolates to Colistin was 2.78%, this result disagreed with the study in Turkey, who mentioned that all multidrug-resistant strains were 100% susceptible to Colistin [28]. The present investigation showed that Colistin was only antibiotic that may remain highly active against carbapenem resistance *P. aeruginosa* (CRPA) isolates, these results accepted with Goli et al. study in Iran [28]. This might be explained by the high cost of Colistin and limited use out of the hospitals. The high rate of resistance observed in *P. aeruginosa* isolates in this study, may be explanted by incorrectly prescribed antibiotics, extensive of antibiotics in animal food which in turn transfers to humans by meat and egg consumption, and availability of few new antibiotics.

The production of MBLs is the most common mechanism for carbapenem resistance in *Enterobacteriaceae* and *P. aeruginosa* isolates [29,30]. The resistant isolates were tested by MHT revealed 16 (44.44%) were positive isolates, in addition double disc synergy test showed that 32 (88.89%) isolates out of 36 *P. aeruginosa* (CRPA) were positive. Present study revealed that these two tests may be useful in screening for MBL, but these tests cannot be routinely performed in all national laboratories. The current results showed that 50% percentage of *P. aeruginosa* (CRPA) isolates have $bla_{\text{NDM-1}}$ gene in plasmid DNA, the percentage of $bla_{\text{NDM-1}}$ gene in the current study was higher than previous study in Najaf who showed only 2 (5.6%) isolates harbored $bla_{\text{NDM-1}}$ gene [23]. In Slovakia, study was reported $bla_{\text{NDM-1}}$ gene in 6 isolates; 20%, this result disagreed with current study [30]. The spread of $bla_{\text{NDM-1}}$ gene on a large scale of serious things because it leads to the absence of any effective antibiotic against MDR bacteria [31]. The current result of $bla_{\text{NDM-1}}$ gene represent highest ratio recorded in Iraq, 50% of *P. aeruginosa* (CRPA) isolates, this percentage higher than the result recorded by Al-Shara, 2013 in Najaf who was showed low findings that 2 (5.6%) isolates harbored $bla_{\text{NDM-1}}$ gene [23], that's because it was easily transferred and

rapidly disseminated to other *Enterobacteriaceae* as it is plasmid borne. Moreover, it contained a variety of other resistance determinants, including a gene encoding another broad-spectrum β-lactamases and genes inactivating ciprofloxacin, erythromycin, chloramphenicol and rifampicin. In addition, the genetic element encoded an efflux pump that capable of producing additional antimicrobial resistance and growth promoters that insured the transcription of the genes contained in the genetic element [31].

This study concluded that the rate of occurrence of bla_{NDM-1} producers is highest among carbapenem-resistant *P. aeruginosa* isolated from clinical samples in Wasit hospitals. Therefore, its recognizable proof in clinical bacterial diseases will be suspected in any carbapenem resistance *P. aeruginosa*.

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Authors contribution

Hussein: conducted the sampling, isolation, and diagnosis, the molecular work and writing the manuscript. Dr. Kadhim and Dr. Hassan supervised the work, edit and finalize the writing of the study.

Conflict of interest

Authors declare no conflict of interest.

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Sodium Valproate Effects on Lipid Profile and Glucose Level in Normal and Diabetic Rabbits

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Abstract

Background Sodium valproate has turn out to be the most commonly prescribed antiepileptic drug (AED) worldwide. It is also prescribed to diabetic mellitus (DM) patient for treatment of neuropathic pain. Changes in lipid levels and lipid metabolism usually accompanied with diabetic disease leading to increases in circulating levels of free fatty acids (FFA), triglycerides and dense low-density lipoprotein together with reduced levels of high-density lipoprotein cholesterol levels.

Objective To investigate the effect of sodium valproate on lipid profile and glucose level and highlight these effects with and without diabetic disease.

Methods Thirty-two healthy, local domestic rabbits of both sexes were used in the present study. Animals were allocated into two main groups, group A and B. According to induction of diabetes both groups received the same food and put in the same environmental condition. blood glucose level and lipid profile has been done for all groups.

Results There was significant difference in the blood glucose level in the normoglycemic rabbits treated with sodium valproate (200 mg/kg) compared to the untreated (negative control group). The effect of valproate was also observed by the significant difference of blood glucose level in alloxan-induced diabetic rabbits treated with sodium valproate (200 mg) compared with the positive control group. Also, we have statistically observed the significant increase in total cholesterol (TC), low density lipoprotein – cholesterol (LDL-C), and triglyceride (TG) levels in normoglycemic rabbits treated with sodium valproate (200 mg/kg) compare to the negative control group, however, there is no significant difference regarding the high-density lipoprotein – cholesterol (HDL-C) level. As for the alloxan-induced diabetic rabbits, there was a statistically significant difference observed indicated an increase in level of TC, LDL-C, and TG and a decrease in HDL-C.

Conclusion The effect of sodium valproate on glucose level and lipid profile in diabetic rabbits could reflect a possible hypoglycemic and dyslipidemic effects which could be dangerous if the patient is on anti-diabetic drugs with or without cardiovascular accident, although it may be dose-dependent and still falls in the hypothesis field but further experimental studies on human are needed to explore this theory.

Keywords Sodium valproate, anticonvulsants, glucose, lipid

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List of abbreviations: FFA = Free fatty acids, HDL = High density lipoprotein, LDL = Low density lipoprotein, NSAIDs = Non-steroidal anti-inflammatory drugs, TG = Triglyceride, VLDL = Very low density lipoprotien

Introduction

Epilepsy and seizures affect approximately 70 million people worldwide (~ 1-2% of the population), research in this area is active. Sodium valproate (VPA) is the most widely prescribe antiepileptic drug (AED). It has wide pharmacological effects with a variety of mechanisms, from increasing gamma-aminobutyric acid (GABA)-ergic transmission to reduce release and/or effects of excitatory amino acids, blockade of voltage-

gated sodium channels and modulation of dopaminergic and serotoninergic transmission [1], it is almost completely metabolized in the liver, mainly by glucuronidation. It then undergoes further metabolism with oxidation, which is complex and involves several cytochrome P450 enzyme systems. It has multiple metabolites which may contribute to both its efficacy and toxicity [2,3].

VPA has earlier been used in antiepileptic and antitumor treatments. Currently, it has also been applied in the treatment of nerve degeneration, cardiovascular disease, autoimmune diseases and diabetes mellitus [4].

Because epilepsy and bipolar disease require lifelong treatment, the continuous and long-term use of VPA becomes a major concern to patients due to the potential adverse effects. The majority of adverse effects during VPA treatment are mild to moderate in severity, appear early in therapy, and do not require dosage adjustment. The most common adverse effects of VPA are gastrointestinal disturbances, weight gain, and neurological (e.g. somnolence, fatigue, tremor, dizziness). Rare severe adverse effects like hepatotoxicity and hematologic changes during VPA treatment have also been reported [5,6].

Diabetic neuropathy defines as the existence of symptoms and/or signs of peripheral nerve dysfunction in a patient with diabetes, after the omission of other causes [7]. 20-90% of diabetic patients over time will develop diabetic neuropathy [8]. The etiological factors credited to diabetic neuropathy can be grouped into those having a definite role (e.g. poor glycemic control, duration of disease) and those with a probable added influence (e.g. hypertension, age, smoking, hyperinsulinemia, dyslipidemia) [9].

The main drug in the management of Diabetic neuropathy include non-steroidal anti-inflammatory drugs (NSAIDs), anti-depressants and anti-epileptic drugs but there are problems with side-effects and contraindications for all these drugs which could be appear upon long-term treatment [10]. Other drugs include clonazepam, gabapentine, lamotrigine, baclofen, i.v. lidocaine or mexiletine, aldose reductase inhibitors, gamma linolinic acid, nucleosides and nerve growth factor [11].

Sodium valproate, which has proved to be effective in trigeminal neuralgia and migraine prophylaxis, has also shown significant role in the subjective improvement of painful diabetic neuropathy, with a unique advantage of low toxicity and favorable side effect profile [12].

In patient with DM, a change in lipid levels and subsequent disorders of lipid metabolism and stress have been observed [13]. Such as increase in free fatty acids (FFA), triglycerides and dense low-density lipoprotein together with low levels of high-density lipoprotein cholesterol levels [14]. FFA from exogenous or produced in the cell are essential to maintain proper nutrient induced insulin secretion. Acutely FFA generate an increase in glucose induced insulin secretion, while chronic exposure to high lipids results in cell collapse, impaired secretory response to glucose, and finally, initiation of cell apoptosis [15-17].

This study aimed to explore the effect of sodium valproate on lipid profile and glucose level and highlight these effects with and without diabetic disease.

Methods

Drugs

Drug that used in the experiment is sodium valproate 200 mg/kg/day.

Experimental animals

Thirty-two healthy, local domestic rabbits of both sexes weighing 0.5-2.5 kg were used in the present study. They were supplied from Center of Technical Institution, Al-Nahrain University, housed one per cage, which is provided with a wire mesh floor. They were reserved in a well-controlled hygienic environment. Rabbits were taken a standard food and water was given ad libitum.

Animals design

In order to evaluate the effects of sodium valproate on normal and on diabetic rabbits,

Animals were allocated into two main groups, group A and B. According to induction of diabetes, both groups received the same food and put on the same environmental condition. Each group has been given same dose of sodium valproate as follows group A (sodium valproate administered without diabetic induction), the group B (sodium valproate administered with diabetic induction).

Induction of diabetes

The rabbits were injected with alloxan monohydrate dissolved in sterile saline (0.9% NaCl) as single dose of 150 mg/kg I.P. Fasting blood glucose levels were determined before administration of alloxan. After 6 hours of alloxan administration, 5% glucose solution was given orally in feeding bottle for a day to overcome the early hypoglycemic phase as a result of acute massive pancreatic release of insulin. Hyperglycemia was established by elevated glucose level, determined at 3rd day post-induction. Rabbits that became hyperglycemic (fasting blood glucose level around 200-250 mg/dl) and stable were included in the study [18].

The rabbits were shifted to placement restraints (wooden holder) and the drugs administered orally, using a catheter. The blood samples (3-5 ml each time) were collected from the heart of the rabbits at 5 and after 20 days intervals during the test, the sample collection in group A started after 2 weeks from presence in the cage, while the first sample collected in group B was after the induction of the rabbits with alloxan.

After 12 hours fasting, the blood of the rabbits was collected in plane tube. Then blood samples were centrifuged at 3000 rpm for 10 min. After centrifugation and isolation of cellular fraction; the obtained plasma fraction was stored frozen at degree -4 ˚C until analysis performed [19].

Measurement of serum glucose level (FSG)

Glucose level was evaluated with a readymade kit for this purpose, which is based on enzymatic oxidation of glucose to form glucuronic acid and hydrogen peroxide by the action of glucose oxidase enzyme, and the reaction of the later with phenol and formation of quinonimine (colored complex) was followed spectrophotometrically at 505 nm. Results were measured as mg glucose/l, based on comparison with a standard glucose solution treated with same method [20].

Serum total cholesterol (TC) measurement

Serum TC was anticipated according to, where a readymade kit is used for this purpose, based on the oxidation of cholesterol, which resulted in the formation of H_2O_2, and when the latter is reacted with phenol, a red colored quinonimine was formed and the intensity of color was measured at 505 nm and compared with standard cholesterol solution [21].

Serum triglyceride (TG) measurement

Serum TG levels were determined according to the method of Fossati and Prencipe and a readymade kit was utilized for this purpose, based on enzymatic oxidation of the glycerol-3-phosphate, which is generated from the hydrolysis of triglyceride moiety. The oxidation process resulted in the formation of H_2O_2 which is measured spectrophotometrically as indicated before [22].

Determination of serum high and low-density lipoprotein cholesterol (HDL-C and LDL-C) [23]

Serum HDL-C levels were estimated according to the method of Burstein; through which LDL-C and VLDL-C was determined calorimetrically by measurement of light absorbance at 505 nm, using a readymade kit for this purpose.

Statistical analysis

The results were expressed as mean±SD. Student t-test for paired and unpaired samples and ANOVA test was used to evaluate the degree of significance, P-value less than 0.05 considered significant

Results

Highlight the results obtained from treated normal rabbits with of sodium valproate 600 mg/kg/day for 20 day The result has been shown that no significant differences in normal

negative control group from day 5 to day 20 of the experiment, on the other hand the results after administer of sodium valproate was found that glucose level has been decrease to 77.4±1.3 mg/dl with highly significant differences in compare to untreated negative group (Figure 1), with a highly significant differences observed as a results of increase in the lipid profile include the cholesterol (Fig Figure 2), triglyceride (Fig Figure 3), and LDL (Figure 4), in compare to untreated negative as shown in table 1.

Table 1. Comparison of blood glucose, cholesterol, TG, LDL and HDL levels negative and (600 mg/kg/day) valproate treated group between day 5 to day 20 of the experiment

Day	-ve untreated glucose l(mg/dl)	-ve treated glucose (mg/dl)	-ve untreated cholest. (mg/dl)	-ve treated cholest. (mg/dl)	-ve untreated TG (mg/dl)	-ve treated TG (mg/dl)	-ve untreated LDL (mg/dl)	-ve treated LDL (mg/dl)	-ve untreated HDL l(mg/dl)	-ve treated HDL (mg/dl)
5.0	87.0 ±1.4	89.0 ±1.02	67.6 ±1.1	66.0 ±1.5	85.0 ±0.9	86.0 ±1.21	46.0 ±1.3	46.1 ±0.9	33.60 ±1.4	34.0 ±1.22
20.0	88.0 ±1.1	77.40 ±1.3 **#	68.0 ±1.3	74.0 ±1.56 **##	86.0 ±1.0	160.0 ±0.98 **##	47.0 ±1.2	67.3 ±1.23 **##	32.60 ±1.1	33.0 ±1.4
% C	1%	-13%	1%	12%	1%	86%	2%	46%	-3%	-2%

Blood glucose, cholesterol, TG, LDL and HDL levels in negative controlled group (rabbits did not expose to any induction)

* significant differences in compare between day 5 and day 20 of the experiment in the same group (p> 0.05)

** highly significant differences in compare between day 5 and day 20 of the experiment in the same group (p> 0.01).

significant differences in compare between valproate un-treated group on day 20 of the experiment (p> 0.05)

highly significant differences in compare between valproate un-treated group on day 20 of the experiment (p> 0.01)

(% C) represent the percentage of changes between day 5 to day 20 of the negative treated group.

The other group (alloxan-induced diabetic group) has been started with administration of 600 mg/kg/day valproate after the induction of diabetic by alloxan and the results has shown that valproate decrease in the increment of glucose level between the positive treated and untreated group (Figure 5) while the cholesterol (Figure 6), triglyceride (Figure 7) and LDL (has shown a significant difference increased in compare between positive treated and positive untreated group (Table 2).

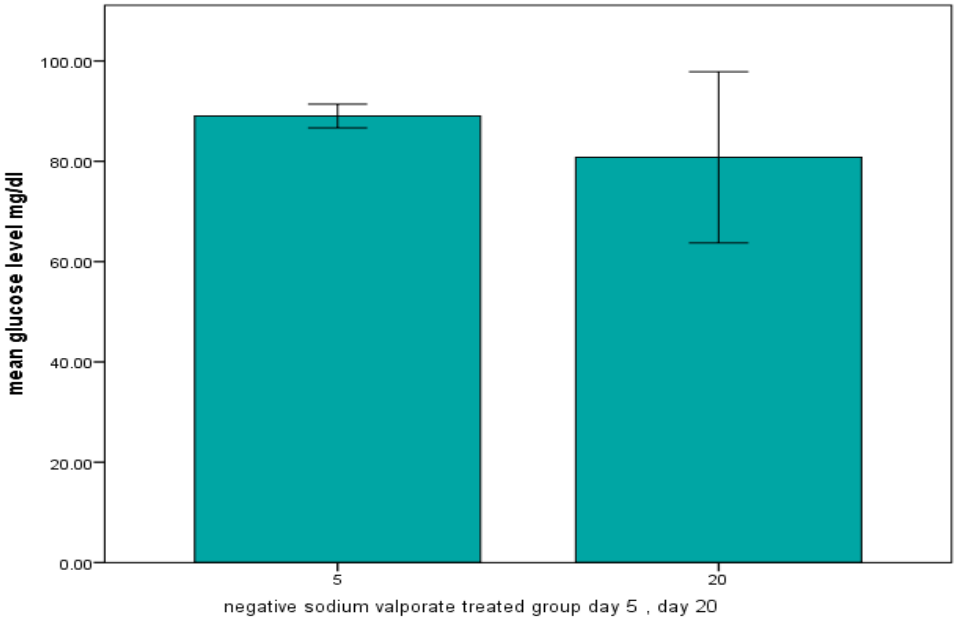

Figure 1. The difference in glucose levels of non-diabetic group (treated with valproate without induction of diabetic) between day 5 and 20 of the experiment

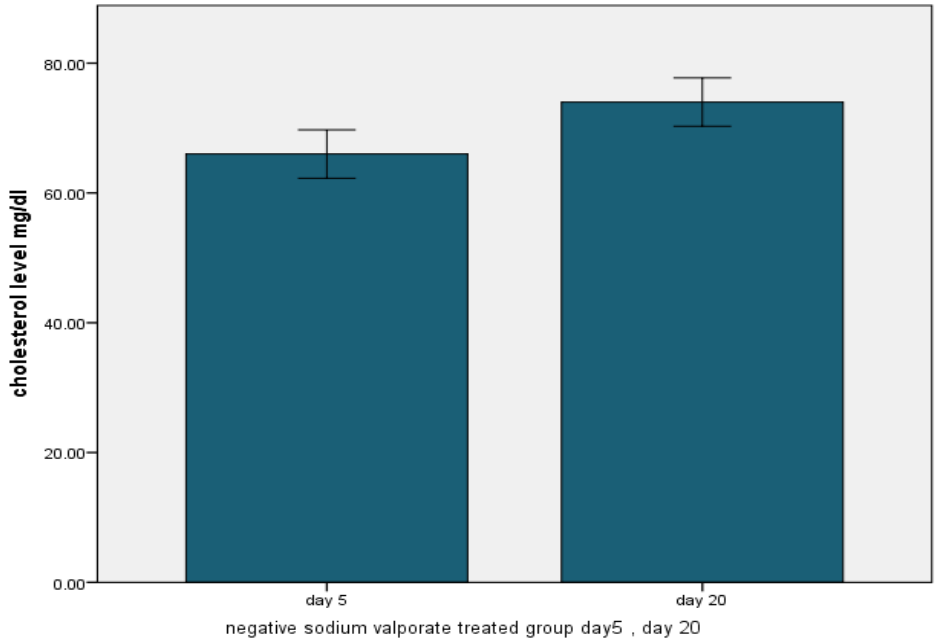

Figure 2. The difference in cholesterol levels in treated group between day 5 and 20 of the non-diabetic (treated group)

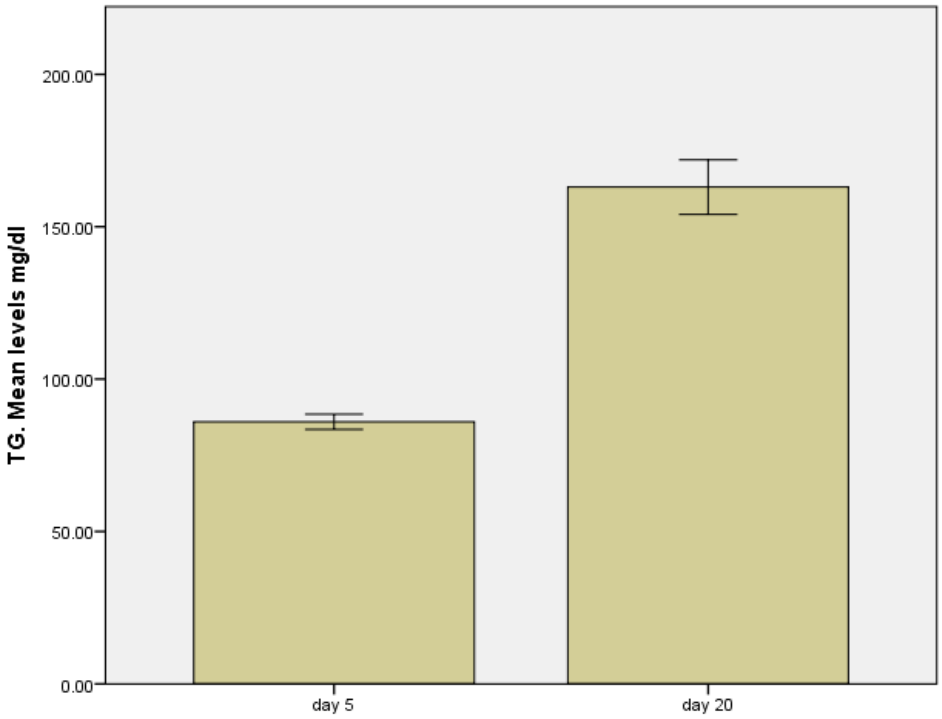

Figure 3. The difference in triglyceride levels in treated group between day 5 and 20 of the non-diabetic (treated group)

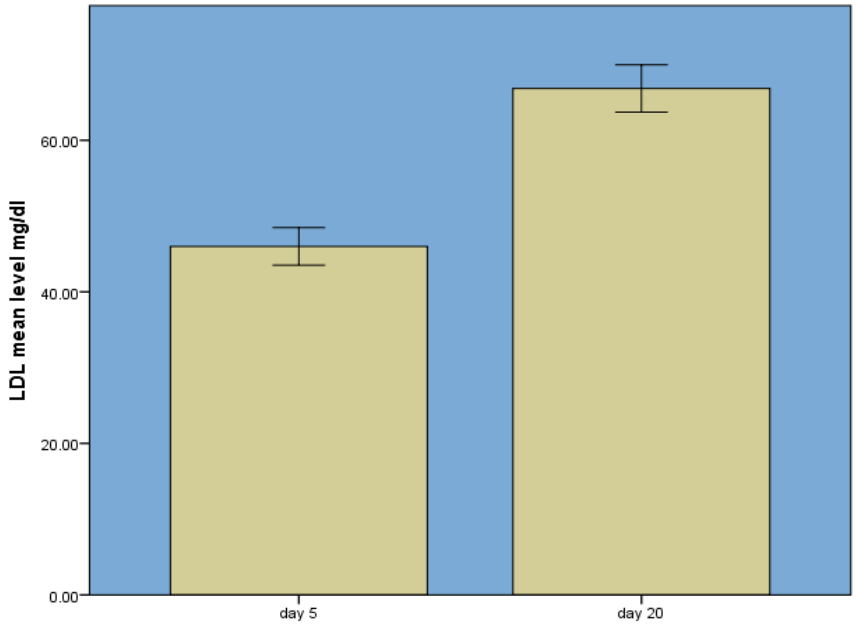

Figure 4. The difference in LDL levels in negative treated group from day 5 to 20 of the experiment

Table 2. Comparison of blood glucose, cholesterol, TG, LDL and HDL levels in positive controlled group (alloxan–induced diabetes) untreated with sodium valproate and alloxan–induced diabetes rabbits treated with 600 mg/kg/day sodium valproate from day 5 to day 20 of the experiment

Day	+ve diabetic glucose l(mg/dl)	+ve valproate treated diabetic glucose (mg/dl)	+ve diabetic cholest. (mg/dl)	+ve valproate treated diabetic cholest. (mg/dl)	+ve diabetic TG (mg/dl)	+ve valproate treated diabetic TG (mg/dl)	+ve diabetic LDL (mg/dl)	+ve valproate treated diabetic LDL (mg/dl)	+ve diabetic HDL l(mg/dl)	+ve valproate treated diabetic HDL (mg/dl)
5.0	200.0 ±1.2	205.0 ±1.3	182.0 ±1.5	185.0 ±1.4	133.0 ±1.45	150.0 ±1.44	100.0 ±1.54	101.0 ±1.56	27.33 ±0.9	26.0 ±1.1
20.0	265.0± 1.1 **	259.0± 1.2 **#	200.0± 1.4 *	230.0± 1.33 **#	150.0± 1.33 *	260.0± 1.5 **##	120.0± 1.45 *	133.0± 1.05 *#	20.0±1. 3	18.0 ±0.76
% C	32%	26%	9%	24%	12%	73%	20%	31%	-o.25%	-30%

Blood glucose, cholesterol, TG, LDL and HDL levels in positive controlled group (rabbits exposed to induction diabetic)
* significant differences in compare between day 5 and day 20 of the experiment in the same group (p> 0.05)
** highly significant differences in compare between day 5 and day 20 of the experiment in the same group (p> 0.01).
# significant differences in compare between valproate untreated group on day 20 of the experiment (p> 0.05)
highly significant differences in compare between valproate untreated group on day 20 of the experiment (p> 0.01)
(% C) represent the percentage of changes between day 5 to day 20 of the negative treated group

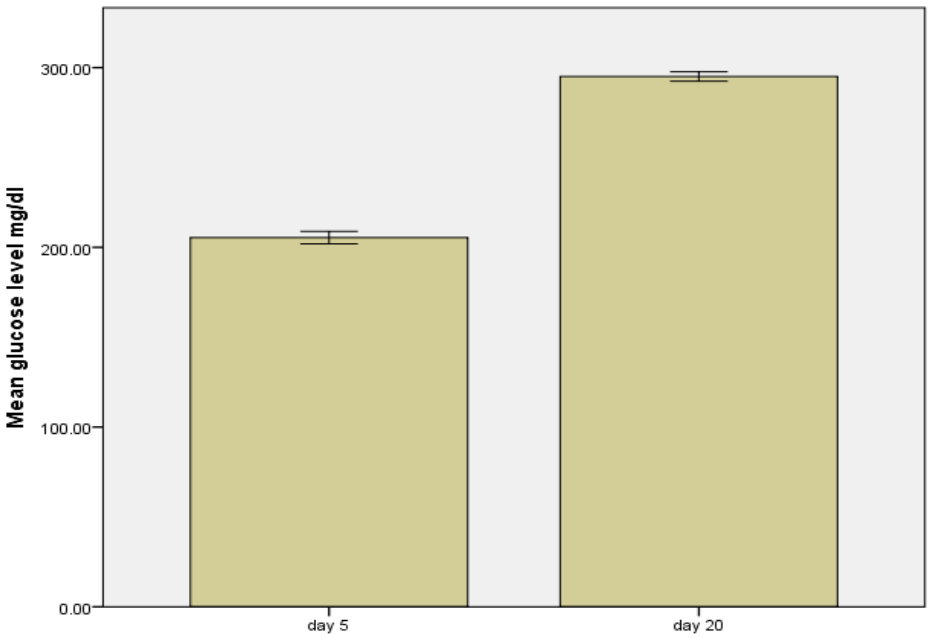

Figure 5. The difference in glucose levels on diabetic treated group between day 5 to day 20

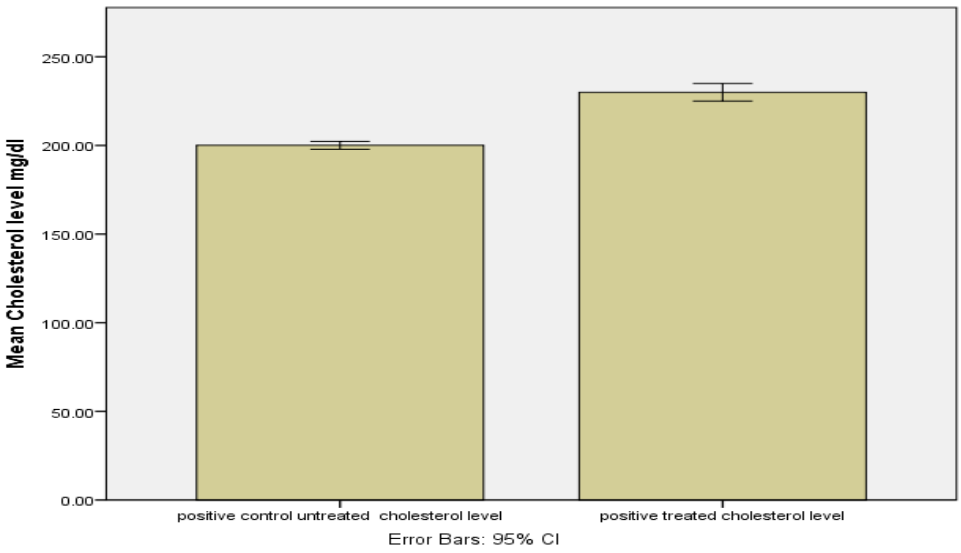

Figure 6. The difference in cholesterol levels between diabetic treated and untreated group on day 20 of the experiment

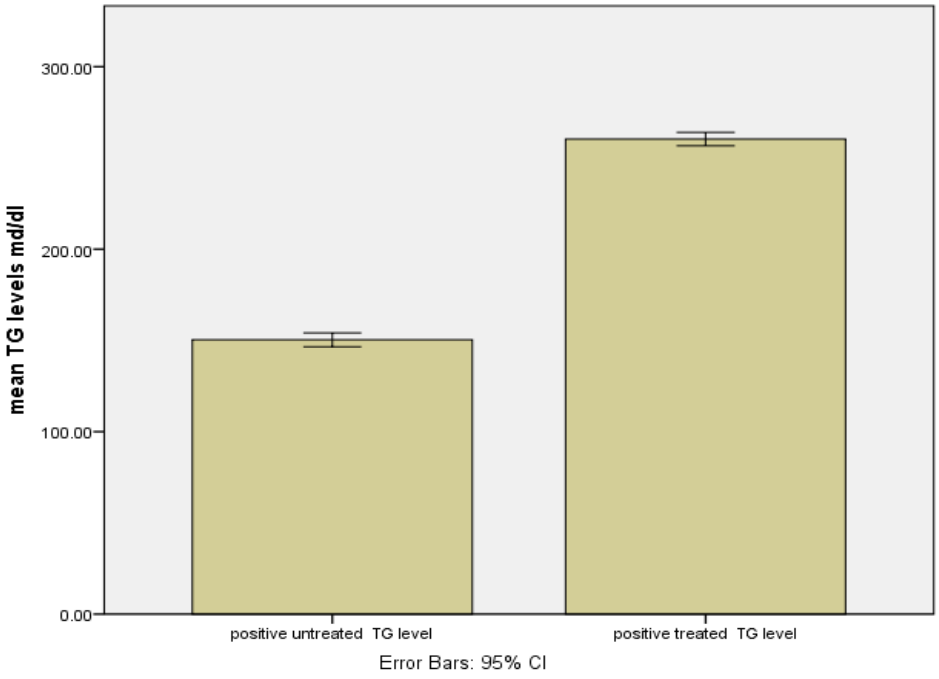

Figure 7. The difference in triglyceride levels between diabetic treated and un treated group on day 20 of the experiment

Discussion

The present study is designed to investigate the effect of the antiepileptic drug sodium valproate on lipid profile and glucose level for normal and diabetic rabbits to highlight the possible effects especially because it has been used for treatment of several disease that could be associated with diabetic.

Sodium valproate has been reported in various clinical studies to induce hypoglycemia in

patients as the study performed by Abraham et al. 1985. who stated that a three weeks oral treatment with valproate has been associated with reduction in blood glucose [22].

Other studies also considered the risk of hypoglycemia in infants exposed to valproate and found that they were at a significantly elevated risk of hypoglycemia [23,24].

Another study was performed by Akindele et al. in 2015 reported that the hypoglycemic effect of valproate is a dose-dependent as it didn't significantly reduce the blood glucose level of experimental rats (both diabetics and normoglycemic) except at the 600 mg/kg dose, the findings of this study suggested that valproate has a beneficial antidiabetic effect at a dose of 600mg, also stated that the effect of insulin is potentiated by valproate [25].

Another study suggested that valproate increase both the post-prandial insulin and pro-insulin levels, which explains the hypoglycemic phenomenon [26].

As for our present study it basically supports the relationship between valproate and hypoglycemia indicated by the previous studies as we can observe (between day 5 and day 20 of the experiment) the highly significant difference ($p > 0.01$) in the blood glucose level in the normoglycemic rabbits treated with sodium valproate (200 mg/kg) compared to the untreated (negative control group) (-13%).

The effect of valproate on glucose level of diabetic rabbits was estimated and a significant difference of blood glucose level in alloxan-induced diabetic rabbits treated with sodium valproate (200 mg) compared with the positive control group, the glucose level increased by 32% after 20 day of induction while it increases only by 26% by valproate treated group.

In respect of lipid profile, it is documented that chronically administered of valproate could be relate with the buildup of fats in tissues, which is associated with the cardiovascular complications that is a major cause of death in diabetes mellitus [27].

In 2010, Verrotti et al. studied the association between metabolic changes and valproate in children and adolescents treated with it. The results stated a high total serum cholesterol concentration (50%) of patients, high serum triglyceride concentrations (35%), and low high-density lipoprotein (HDL) (75%) [28].

Another study by Chang et al. in 2010 indicated that valproate treatment was associated with significant higher plasma triglyceride, as well as lower HDL levels [29].

Some studies contradict with these previous ones, as the one performed by Demircioğlu et al. in 2000, which studied the effect of carbamazepine and valproate on the serum lipids and results in that carbamazepine treatment alters the serum lipid profile of the children in such a way that it facilitates the development of atherosclerosis while valproate did not [30].

A study done in 2014 by Manimekalai et al. evaluated the effects of antiepileptic drugs on serum lipid and concluded that CYP enzyme inducer antiepileptic drugs like phenytoin and oxcarbazepine is strongly associated with increased levels of TC, LDL-C, and TG whereas valproate and levetiracetam showed no significant change [31].

The lack of association between many studies could be attributed to the difference in the dose of the sodium valproate given. In 2005, Green et al. conducted a study to discuss the dose-related effect of sodium valproate in migraine prophylaxis and resulted in a dose-dependent reduction in serum cholesterol within the first 3 months of therapy, there was a decrease from baseline in total cholesterol of 3% reduction with 500 mg/day, 4% reduction with 1000 mg/day; and 7% reduction with 1500 mg/day [32].

In the current study, it was statistically observed that the significant increase in TC, LDL-C, and TG levels in normoglycemic rabbits treated with sodium valproate (200 mg/kg) compared to the negative control group, however there was no significant difference regarding the HDL-C level.

As for the alloxan-induced diabetic rabbits there was a statistically significant difference observed indicated an increase in level of TC, LDL-C, and TG and a decrease in HDL-C. This dyslipidemic effects could be related with hyperinsulinemia and insulin resistance, hyperleptinemia and leptin resistance that

could be associated with valproate treatment [33].

This study concluded that the hypoglycemic effect valproate could serve as a motivation for using sodium valproate in diabetic patients. Especially valproate has been used for years in patients with diabetes to treat neuropathic pain. On the other hand, prescribed valproate for epileptic patient suffering from hypoglycemia could lead to dangerous consequences. As for its effect on lipid profile it maybe dose-dependent in diabetic patients but this still falls in the hypothesis field and further experimental studies are needed to deeply explore this theory.

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Conflict of interest

There is no conflict of interest that could be perceived.

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Safe Primary Repair of Colorectal Injuries Without Diverting Colostomy

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Abstract

Background Colostomy was used for treatment of colorectal injuries since the 2nd World War, and it is a lifesaving procedure. There is a trend towards primary closure of colorectal injuries without colostomy in hemodynamically stable patients.

Objective To evaluate the safety of primary closure of colorectal injuries without colostomy in hemodynamically stable patients.

Methods A cross sectional study was performed at Al-Imamein Al-Kadhimein Medical City for patients with colorectal injuries over the period from July 2011 to July 2017. Management was started with active resuscitation of the patient, explorative laparotomy (securing hemorrhage) and assessment of the colorectal injuries. When the patients were stable hemodynamically without associated injuries to other parts of the body; debridement of the wound edges of the colon and primary repair by suturing in two layers using 2/0 absorbable suture (polyglactin) on a round needle with or without colostomy. But if the patients were in a shock state with multiple associated injuries to other parts of the body, with severely devascularized lacerated colon; proximal colostomy was done as a part of damage control surgery with resection of the devascularized segment and suturing of the distal end of the colon.

Results A total of 231 patients sustained colorectal injuries; 143 (61.90%) males and 88 (38.09%) females. The age of the patients ranged from 6-76 years, mean age was 32.16±76 year. Colostomy was done for 134 (58.01%) patients. Primary repair without colostomy were done for 97 (41.99%) patients. Postoperative follow up of the patients treated with primary repair of colorectal injuries without colostomy were detected collection and leaking repaired segment of colon in 5 (5.15%) patients. Re-exploration of the abdomen and colostomy were done for them. There was no mortality in patients treated without colostomy. The mortality rate was 9 (6.71%) for patients treated by colostomy due to associated multiple traumas to other parts of the body.

Conclusion Primary repair of colorectal injuries without colostomy are safe in a hemodynamically stable patient without associated injuries to other parts of the body.

Keywords Colorectal injury, primary repair, colostomy.

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List of abbreviations: AAST = American Association for the Surgery of Trauma, ATLS = Advanced Trauma Life Support, CT scan = Computerized tomography scan, FAST = Focused assessment with sonography for trauma.

Introduction

Colon and rectal injuries are common due to both blunt and penetrating abdominal trauma. Primary repair was the main management of colon and rectal injuries during the First World War, and the mortality was 50%. Colostomy was used for the first time during the second World War, and then it becomes an important operative procedure to save life of patients with colorectal injuries [(1)]. The surgical management of colon was changed during the late 1980's in selected patients with injury to the antimesenteric side of the colon, to repair and exteriorization of

that segment of the colon with protection of the exteriorized colon and monitoring for three to five days to be returned back to the abdominal cavity after that time. This procedure was not used anymore because of the high failure rate of the repair and breakdown of the exteriorized segment, which may occur in about half of the patients who re-explored again and colostomy done for them [2]. Direct penetrating injury to the colon and rectum by sharp objects are the most common cause of the injury [3], which may be due to knife, and more commonly gunshot and shell injuries during war time [4]. Motor vehicle crash, falls from a height, and direct abdominal trauma may also cause colon and rectal injuries. Colon injury must even be suspected in seat belt injury [5]. Blast injury and explosive device may cause contusions or tears of the colon which may end with its rupture [6].

Management of patients with colorectal injuries start with resuscitation of the patient that follows the Advanced Trauma Life Support (ATLS) protocol [7], starting the resuscitation with securing the airway, breathing and circulation, treatment of shock, and a decision is made whether the patient may need immediate laparotomy. Detection of free fluid in the peritoneal cavity without solid organ injury by Computerized tomography (CT) scan may indicate colon and rectal injury in blunt abdominal trauma [8-10].

According to the injury scoring scale (AAST) "the American Association for the Surgery of Trauma" (Table 1); when there is severe laceration that involves more than half the circumference of the colon with devascularization, resection of the devascularized colon and colostomy may be indicated. Anastomosis may be indicated if there are no devascularization and minimal fecal contamination. When the patient was sustained multiple trauma with shock, damage control surgery with stapling of the ends of the colon and planned second exploration may be indicated [11-13]. After effective resuscitation and hemodynamic stability of the patient, re-exploration of the patient should be done, and if the ends of the colon are viable with good blood supply, and approximation of both ends of the colon is possible, anastomosis may be done without tension, and the anastomosis site may be protected by omentum.

Table 1. Colon injury scale

Grade	Type of injury	Description
I	Hematoma	Contusion or hematoma without devascularization
	Laceration	Partial thickness, no perforation
II	Laceration	Laceration <50% of circumference
III	Laceration	Laceration ≥50% of circumference without transection
IV	Laceration	Transection of the colon
V	Laceration	Transection of the colon with segmental tissue loss
	Vascular	Devascularized segment

Penetrating rectal injuries may be associated with injury to the urinary bladder and risk of fistula formation. Assessment of rectal injury was done according to AAST injury scale (Table 2). Colostomy is indicated if rectal injury is below the peritoneal reflection [14-20]. Intraperitoneal rectal injury can be treated by primary repair. In some cases of extraperitoneal rectal injuries, which are not associated with injury to the urinary bladder and no pelvic vascular injuries can be treated without colostomy [21].

Table 2. Rectum injury scale

Grade	Type of injury	Description
I	Hematoma	Contusion or hematoma without devascularization
	Laceration	Partial thickness laceration
II	Laceration	Laceration < 50% of circumference
III	Laceration	Laceration ≥ 50% of circumference
IV	Laceration	Full-thickness laceration with extension into perineum
V	Vascular	Devascularized segment

Not all abdominal wounds need explorative laparotomy especially if it is due to stab wounds. CT scan and laparoscopy can aid in decision making regarding indication for exploration [22,23]. Sometimes even gunshot may cause tangential injury to the abdomen without penetrating the peritoneal cavity [22]. Closed observation and follow up of the patient clinically can determine the need for explorative laparotomy. If there are no signs of peritonitis and the patient not in a shock states with normal CT scan, it may be safe to continue with conservative treatment with follow up [24,25]. If the patient's condition deteriorates or signs of intra-abdominal organ involvement by injury appear, then laparotomy is indicated [26-28]. Most of shell injury and gunshot to the abdomen especially in war time need explorative laparotomy because any missed injury to the intestine and/or internal bleeding may have catastrophic complications and may lead to death of the patient.

Laparoscopy can be used for assessment of patient with abdominal trauma to exclude any colorectal injuries that may need explorative laparotomy. Isolated small transvers colon injuries by stab wound can be managed by laparoscopy; penetrating injuries to the diaphragm can be diagnosed and repaired with the aid of laparoscopy [29]. Endoluminal device to bypass the extraperitoneal rectal injury can avoid the need for colostomy.

The aim of the study was to evaluate the safety of primary closure of colorectal injuries without colostomy in hemodynamically stable patient.

Methods

After ethical approval of the study by Institution Review Board in the College of Medicine, Al-Nahrain University, a cross sectional study was performed at Al-Imamein Al-Kadhimein Medical City for patients with colorectal injuries, over the period from July 2011 to July 2017. Inclusion criteria include patients with abdominal trauma operated upon and had colorectal injuries.

Exclusion criteria include:

1- Patients not operated upon for abdominal trauma.
2- Patients whom were underwent colorectal operations for diseases other than trauma.

Patients were attending the emergency department of the hospital with acute abdominal trauma whether it is due to war (shells, bullets, explosions and blast injuries); or abdominal trauma due to civilian life (motor vehicle crash, fall from a height, or stab wound injury to the abdomen).

Management of patients started with resuscitation following the ATLS protocol, starting with securing the airway, breathing and circulation, treatment of shock (intravenous fluid, blood transfusion, insertion of Folly's catheter into the urinary bladder and collection of urine output, antibiotics, and other resuscitative management), ultrasound examination of the abdomen (FAST) "Focused

Assessment with Sonography for Trauma" to detect the presence of free fluid in the abdomen, X-ray of the chest and X-ray of other parts of the body if indicated; and a decision is made whether the patient may need immediate laparotomy. Sometimes CT scan of the abdomen especially for patients with blunt trauma may be needed.

After hemodynamic stabilization of the patients, explorative laparotomy was done through a midline incision, securing hemorrhage, and dealing with other abdominal injuries (solid organ injuries like spleen, liver, kidney; or hollow organ injuries like the small bowel),exploration and assessment of colorectal injuries according to AAST injury scale was made in a systematic way with especial concern regarding the retroperitoneal colon in cases of penetrating injury to the back or flank and in a tangential wounds.

Primary repair of colorectal injuries was done by debridement of the wound edges and closure of the colorectal defect by suturing in two layers using 2/0 absorbable suture (polyglactin) (Vicryl suture) on a round needle; first continuous layer and second interrupted layer with invagination of the first layer).

Grade 1 injury were treated conservatively; Grade II patients were treated by debridement of the wound edges and primary repair with suturing in two layers using 2/0 vicryl suture on a round needle in two layers. For Grade III colorectal injuries; if the patients were stable hemodynamically and had isolated colon or rectal injury (without associated injuries to other abdominal organs nor associated injuries to other parts of the body like head injury or fractures), those patients were treated by primary repair without protecting colostomy; but if the patient was unstable hemodynamically (shock) or had associated injuries to other abdominal organs or the patient had multiple trauma to other parts of the body; then the primary repair was protected by proximal colostomy.

Patients with Grade IV, and Grade V injuries were presented with multiple associated trauma and injuries to other parts of the body, they were treated by resection of the devascularized segment with closure of the distal end of the colon and proximal end colostomy (Hartman's operation) as a part of damage control surgery.

The operative procedure done for the patients were either:

1. Debridement of the injured ends of the colorectal wounds and primary repair in two layers with or without proximal protective colostomy.
2. Resection of the devascularized segment with proximal end colostomy, and closure of the distal segment (Hartman's operation).
3. Right hemicolectomy with end ileostomy and mucous fistula of transvers colon.

Postoperative follow up of patients, by clinical abdominal examination, vital signs (fever, tachycardia), blood test white blood cell counts, and ultrasound examination of the abdomen looking for any signs of anastomotic leak or intra-abdominal collection.

All the patients were kept in the hospital postoperatively under closed observation till they had positive bowel motion, normal vital signs, and soft abdomen. Some patients with Grad III colorectal injuries who were treated by primary repair without protective colostomy were developed leaking anastomosis, and they were re-explored again and colostomy done for them.

Statistical analysis used:

- Categorical variables: frequency, and percentage %s.
- Continuous variables: Means ± standard deviation SD.

Results

Over the period from July 2011-July 2017, there were 687 laparotomies for abdominal trauma due to penetrating and blunt injuries; 231 patients of them were sustained colorectal injuries, the incidence of colorectal injuries was 33.62%. There were 143 (61.90%) males and 88 (38.09%) female patients. The age of the patients ranged from 6-76 years, mean age was 32.16 year. Table (3) shows the age and gender of patients. There were 97 (41.99%) patients

treated by primary repair of colorectal injuries without colostomy, while colostomy was done for 134 (58.01%) patients.

Table 3. The age and gender of patients with colorectal injuries

Age group	Males	Females	Total
1-9 years	3 (2.09%)	1 (1.13%)	4 (1.73%)
10-19 years	16 (11.18%)	8 (9.09%)	24 (10.38%)
20-29 years	67 (46.85%)	34 (38.63%)	101 (43.72%)
30-39 years	28 (19.58%)	23 (26.13%)	50 (21.64%)
40-49 years	15 (10.48%)	17 (19.31%)	33 (14.28%)
59-59 years	7 (4.89%)	3 (3.40%)	10 (4.32%)
60-69 years	5 (3.49%)	1 (1.13%)	6 (2.59%)
70-79 years	2 (1.39%)	1 (1.13%)	3 (1.29%)
Total	143 (61.90%)	88 (38.09%)	231

The causes of colorectal injuries were due to war in 171 (74.02%) patients (multiple shell injuries, explosions, high velocity bullet injuries and blast injury); while civilian injuries cause colorectal injuries in 60 (25.97%) patients (motor crash injuries, fall from a height, and stab wound injuries). Table (4) shows the causes of colorectal injuries. The site of colorectal injuries in patients with colostomy were; in the right colon 29 (12.55%), transverse colon 38 (16.45%), left colon 23 (9.95%), sigmoid colon 28 (12.12%), and rectum 16 (6.92%) patients.

The site of colorectal injuries in patients without colostomy were; in the right colon 22 (9.52%), transvers colon 26 (11.25%), left colon 9 (3.89%), sigmoid colon 21 (9.09%), and rectum 19 (8.22%) patients. Table (5) shows the site of colorectal injuries.

The grades of colorectal injuries according to AAST injury scale were as follows: in patients with colostomy; there were 32 (13.85%) patients sustained grade III injuries, 65 (28.13%) patients grade IV injuries, and 37 (16.01%) patients grade V injuries. While the patients without colostomy; there were 27 (11.86%) patients sustained grade I injuries, 39 (16.88%) patients grade II injuries, and 31 (13.41%) patients grade III injuries. Table (6) shows the Grades of colorectal injuries.

Table 4. The causes of colorectal injuries

Causes of abdominal trauma	Primary repair with colostomy	Primary repair without colostomy	Total
Motor vehicle crash		38 (16.45%)	38 (16.45%)
Fall from a height		9 (3.89%)	9 (3.89%)
Stab wound injury		13 (5.62%)	13 (5.62%)
War injury by Shells (explosions)	112 (48.48%)	31 (13.41%)	143 (61.90%)
high velocity bullets	19 (8.22%)	6 (2.59%)	25 (10.82%)
Blast injury	3 (1.29%)		3 (1.29%)
Total	134 (58.01%)	97 (41.99%)	231

Table 5. The sites of colorectal injuries

Site of injury	Primary repair with colostomy	Primary repair without colostomy	Total
Right colon	29 (12.55%)	22 (9.52%)	51 (22.07%)
Transvers colon	38 (16.45%)	26 (11.25%)	64 (27.70%)
Left colon	23 (9.95%)	9 (3.89%)	32 (13.85%)
Sigmoid colon	28 (12.12%)	21 (9.09%)	49 (21.21%)
Rectum	16 (6.92%)	19 (8.22%)	35 (15.15%)
Total	134 (58.01%)	97 (41.99%)	231

Table 6. The grades of colorectal injuries in both groups

Site of injury	Primary repair with colostomy	Primary repair without colostomy	Total
Grade I		27 (11.86%)	27 (11.68%)
Grade II		39 (16.88%)	39 (16.88%)
Grade III	32 (13.85%)	31 (13.41%)	63 (27.27%)
Grade IV	65 (28.13%)		65 (28.13)
Grade V	37 (16.01%)		37 (16.01)
Total	134 (58.01%)	97 (41.99%)	231

There were 5 (5.15%) patients treated by primary repair without colostomy was developed collection and anastomotic leak postoperatively, all of them were grade III injuries. Re-exploration of the abdomen and proximal protective colostomy were done for them. So, the failure rate was 5 (5.15%) patients. There was no mortality in patients treated by primary repair without colostomy, because those patients were stable hemodynamically and were highly selected group without trauma to other parts of the body.

The mortality rate was 9(6.71%) patients; all of them were died due to the associated severe trauma to other parts of the body and their complications (head and chest injuries with fractured long bones).

Discussion

During the decades of war in Iraq, thousands of patients underwent laparotomy for colorectal injuries; the vast majority of them were managed by repair with colostomy. Even inside cities of Iraq, there were many explosions, which cause severe penetrating abdominal injuries for people in all age groups. Although colostomy is life saving and simple procedure to divert fecal material away from the abdominal cavity, but it carries its complications, and needs second operation for closure. Recently there is a trend towards primary repair without diverting colostomy in especial circumstances.

In this study, there were 687 laparotomies for abdominal trauma due to penetrating and blunt injuries; 231 patients were sustained colorectal injuries, the incidence of colorectal injuries was 33%. The gender of the patients was 143 (61.90%) males and 88 (38.09%) female patients. The age of the patients ranged from 6-76 years, mean age was 32.16 years. Children, females, and elderly people were affected by explosions and shell injuries inside cities while young male patients were affected during fighting in combat in the war

Destructive injuries were due to war injury (explosions and high velocity bullet injuries), while non-destructive injuries were due to civilian injuries (motor crash injuries, fall from a height and stab wound injuries).

In review of literatures; treatment of the destructive colorectal injuries may require resection of the destructed segment [30]. In the civilian life 80-90% of colorectal trauma are non-destructive [30], while in war time 72% are destructive [6]. About 60-93% of the non-destructive colorectal injuries can be managed with primary repair, but the management of destructive injuries is still controversial [31]. Missed colorectal injuries may lead to fatal septic complications; therefor there should be high index of suspicion of colorectal injuries in patient with abdominal trauma [32].

There were 39 (16.88%) patients with grade II injuries were treated by debridement of the wound edges and primary repair with suturing in two layers using 2/0 vicryl suture on a round needle in two layers without colostomy. There were 63 (27.27%) patients had grade III colorectal injuries; about half of them 32 (13.85%) patient were treated by primary repair and protective proximal colostomy, because there were associated multiple traumas to other parts of the body (head injury, chest injury, fractured long bones), and the patients were unstable hemodynamically (shock state), and there was severe fecal contamination with prolonged period of time from the injury to the time of laparotomy.

While the other 31 (13.41%) patients with grade III colorectal injuries were treated with primary repair without colostomy because the patients were stable hemodynamically, and there were no associated injuries to other parts of the body with little fecal contamination and short period of time from the injury to the time of laparotomy.

Grades IV, V, are serious injuries, and were associated with multiple injuries to other intra-abdominal organ (lacerations of liver, spleen, and pancreas), as well as multiple trauma to other parts of the body (head and chest injuries with fractured long bones), those patients were treated by resection of the severely de-vascularized lacerated segment of the colon and closure of the distal segment with protective proximal end colostomy to save life as a rapid and safe procedure (part of damage control surgery).

Damage control surgery was used to save life of multiply injured patients (control bleeding and fecal contamination due to bowel injury), and postponed definitive reconstructive surgery in unstable patient to prevent rapid progress to death due to hypothermia, coagulopathy, and acidosis [33].

On the contrary to the above data, one study performed on 2009 showed that there were no differences whether colostomy done or not done for patients underwent damage control surgery with primary repair and anastomosis of colorectal injury [12].

There were 5 (5.15%) patients treated by primary repair without colostomy was developed collection and anastomotic leak postoperatively, all of them had grade III colorectal injuries. Re-exploration of the abdomen and protective proximal colostomy were done for them. So, the failure rate was 5.15%.

There were 9 (6.71%) patients died in patients treated with colostomy due to severe trauma to other parts of the body (head injury, chest injury, fractures, and severe trauma and lacerations to other abdominal organs with shock and sepsis). There was no mortality in patients treated without colostomy because they were highly selected group and stable hemodynamically without associated injuries to other parts of the body.

Ott advised that if the patient is unsuitable for primary repair and anastomosis, it is better to do fecal diversion with colostomy or ileostomy [13]. Broad-spectrum antibiotics which should cover aerobic and anaerobic bacteria are mandatory once a colorectal injury was diagnosed and the antibiotics should be continued for at least one day after control of fecal contamination [34]. Retrospective studies showed that excessive intravenous fluids more than 10.5 liters in the first three postoperative days have been associated with a fivefold increased risk of leaking anastomosis [35].

The incidence of colorectal injuries in this study was 33.62%, while the incidence of isolated

colorectal trauma in some studies is less than 1% from all the trauma patients, 43.9% following blunt abdominal trauma, and 56.1% following penetrating abdominal trauma with 25.6% mortality rate [36]. During wartime the incidence of colorectal injuries was increases to 5-10%. An American study of over 3,400 injured soldiers during Iraqi invasion shows colorectal injuries in 5.1% of them [37,38]. Other American study on 2010 revealed that penetrating colorectal injuries occurred in 71%, blast injuries in 23%, and blunt injury in 5 percent [6]. The incidence of colorectal injuries is much lower in civilian life, and it is around 0.6-3% in North American studies, but the mortality rate depends on the mechanism of injury, and there was a high mortality rate around 10-25.6% following high-energy trauma, while the mortality rate was 0.1-0.5% following blunt abdominal trauma [39,40]. The mechanisms of colorectal injuries are significant in determining the severity of the injury; the incidence of blunt trauma in urban hospitals is low, and it is around 0.02% [41].

There are many different modalities and approaches to patients with multiple traumas involving colorectal injuries [42]. Extra peritoneal rectal injuries and severe damage to anal canal and perineum may need colostomy which is important for their healing.

One of the previous retrospective studies showed that there were 2.4% incidence of leaking anastomosis in civilian trauma injuries [43], but during wartime the incidence is much higher and it were 13-30% due to the differences in mechanism of colorectal injuries (more destructive injuries) and the associated other injuries [44,45]. Shock with septic complications increases the mortality rate [32].

Although fecal diversion and colostomy may be advised during wartime, one study on 2009 advised primary repair of all colorectal injuries [46].

Mesenteric hematoma or colonic wall hematoma (grade I) need observation unless the hematoma is expanding or there is devascularization of the wall of the colon [47]. Large mesenteric hematoma should be assessed properly to exclude serious vascular injury [48].

One large study from 14 trauma centers covering 517 patients revealed that leaking anastomosis and intraperitoneal sepsis as a complications of damage control surgery was increased with large volume intravenous fluid resuscitation and increased number of re-explorative laparotomies [49].

Although colostomy may be lifesaving in colorectal injuries, but colostomy reversal is not free from complications. One of the studies showed that complication rate 25-44%, mortality rate 0.65-4.3%, and higher mortality rate (4.7%) following Hartman's operation reversal [50]. The complications were including minor wound infections (21.8%), ileus (5.7%), anastomotic leak (13.0%) without enterocutaneous fistula, small bowel obstruction (11.5%), anastomotic leak with enterocutaneous fistula formation (3.8%), and intra-abdominal abscess (1.1%). The complication rates increased when the patient had low serum albumin level or the patient used steroid treatment. Some patients may never have their stoma reversed [51].

This study concluded that primary repair of colorectal injuries without colostomy were safe in patients with grade III injuries (who were stable hemodynamically, without associated severe trauma to other parts of the body, with short period of time from the injury to the time of laparotomy, and with little fecal contamination.

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Conflict of interest
None.

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Nissl Stain Expression in the Frontal and Parietal Cortices of the Newborn Mice After Prenatal Exposure to Ketamine

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Abstract

Background Ketamine is an N-methyl-D-aspartate (NMDA) receptor blocking agent, which is used for induction and maintenance of anesthesia. It affects the cerebral cortex and has an impact on learning and memory functions; suggesting that any changes in NMDA receptors function will have an adverse outcome on learning and memory abilities.

Objective To assess the histological changes in the frontal and parietal cortices of mice offspring's after prenatal exposure to therapeutic doses of ketamine.

Methods Thirty pregnant mice were included in this study. They were divided into two groups named experimental and control groups (15 mice for each group). Those of experimental group were injected intraperitoneally with ketamine in a dose of 50 mg/kg/day on the 5th, 10th, 15th and 20th days of gestational age to showing effect of the ketamine after injection of it in all trimesters of pregnancy, while those of the control group were injected with distal water only with the same volume. The paraffin block sections of frontal and parietal cerebral cortices of newborn mice were stained by nissl stain.

Results In the control group, the mean number of Nissl stained cells in the frontal cortex showed a statistically significant increase compared to that of parietal cortex, while statistical non-significant decrease in the mean number of nissel stained cells of frontal cortex compared to that of parietal cortex.

Conclusion Iatrogenic apoptotic changes were seen in the cerebral cortex of the experimental mice after prenatal exposure to ketamine and it is more considerable in the frontal cortex than the parietal cortex.

Keywords Frontal cortex, parietal cortex, ketamine, nissl stain, development

Citation Najm MS, Mubarak HJ, Jarullah HA. Nissl stain expression in the frontal and parietal cortices of the newborn mice after prenatal exposure to ketamine. Iraqi JMS. 2018; 16(3): 268-278. doi: 10.22578/IJMS.16.3.6

List of abbreviations: CNS = Central nervous system, dep = Deep layer, NIH = National Institute of Health, NMDA = N-methyl-D-aspartate, SPSS = Statistical package of social sciences, sup = Superficial layer

Introduction

Ketamine is an N-methyl-D-aspartate (NMDA) receptor blocker [(1)], it is commonly used for induction and maintenance of anesthesia [(2)] chiefly in the developing world [(3)]. The NMDA receptors are present in high concentration in the cerebral cortex and play an essential role in learning and memory, and there is evidence suggesting that any changes in NMDA receptor function have an impact on learning and memory abilities [(4)].

The early annotation of ketamine neurotoxicity was of concern, and evidence that the more commonly used anesthetics can produce neuro-degeneration in neonatal animals [(5)].

The cerebral cortex is composed of gray matter and has been estimated to contain

approximately 10 billion neurons. The cerebral cortex, like gray matter elsewhere in the central nervous system, consists of a mixture of nerve cells, nerve fibers, neuroglia, and blood vessels [6].

The cerebral cortex can be divided into six layers, the cerebral cortex has the following five types of neurons: pyramidal cells, stellate cells, cells of Martinotti, horizontal cells of Cajal and fusiform cells [7].

The cortex contains supporting neuroglial cells (astrocytes), oligodendroglia and microglia [8].

The neuroglial cells function is the mechanical support, metabolic, and protections of neurons collectively form the neuroglia [9].

The trial studies in animals (rats, mice, guinea pigs, piglets, and non-human primates) have shown that exposure of the anesthetic agents during developmental periods can lead to neuronal apoptosis (programmed cell death) or neuronal degeneration [10-14].

The objectives of this study was to assess the histological changes in the frontal and parietal cortices of mice offspring's after prenatal exposure to therapeutic doses of ketamine

Methods

The animals used in this study were obtained from Laboratory Animal House at College of Medicine, Al-Nahrian University. A total of (30) pregnant females' adult (mus musculus) and aged about (8-12) weeks were used in this study. Weight of the animals was between (20-40 gm). After mating, the pregnancy was confirmed the following morning by finding vaginal plug and this was considered as day 0 of gestation [15].

All animals were treated according to National Institute of Health (NIH) Guidelines for the care and use of laboratory animals.

The 30 pregnant mice were divided into two groups; namely the experimental group and the control group (15 mice for each group). The pregnant mice of experimental group were injected intraperitoneally in a single shot with 50 mg/kg ketamine hydrochloride [16] during (5^{th} day, 10^{th} day, 15^{th} day, and 20^{th} day) of pregnancy. The control group was injected intraperitoneally with just distal water during the same gestational days and same volume.

Mice newborn brains were fixed in 10% formalin, the fixed tissues were then passed for routine paraffin wax embedding process including dehydration, clearing, infiltration, and embedding [17]. Sagittal sections of the paraffin blocks, at 5µm thickness, were prepared. Cresyl violet (Nissl) stain was used for examining the neural tissue, were captured by genex camera (5 mega pixels) which was associated built in the microscope.

Results

Result of nissl stain in frontal cortices of control group

The mean number of cells in the superficial layers (layers I, II and III) of the frontal cerebral cortex was 1598 cells, while the mean number of cells in the deep layers (layers IV, V and VI) of the frontal cerebral cortex was 1360 cells (Figures 1, 2 and 3).

Result of nissl stain in parietal cortices of control group

The mean number of cells in the superficial layers (layers I, II and III) of the parietal cerebral cortex is 1446 cells, while the mean number of cells in the deep layers (layers IV, V and VI) of the parietal cerebral cortex was 1243 cells (Figures 4, 5 and 6).

The mean number of cells in the frontal and parietal cerebral cortices

The mean number of total cells (in both superficial and deep layers) of frontal cerebral cortex was 1479, while that of the parietal cortex was 1334.50. These results showed statistically significant variability (p value = 0.004) (Figure 7).

Result of nissl stain in frontal cortices of experimental group

The average number of cells in frontal cortices in superficial layers (layers I, II and III) of experimental group is 817 cells, and the number of cells in deep layers (layers IV, V and

VI) of frontal cortex of experimental group is 701 cells (Figures 8, 9 and 10).

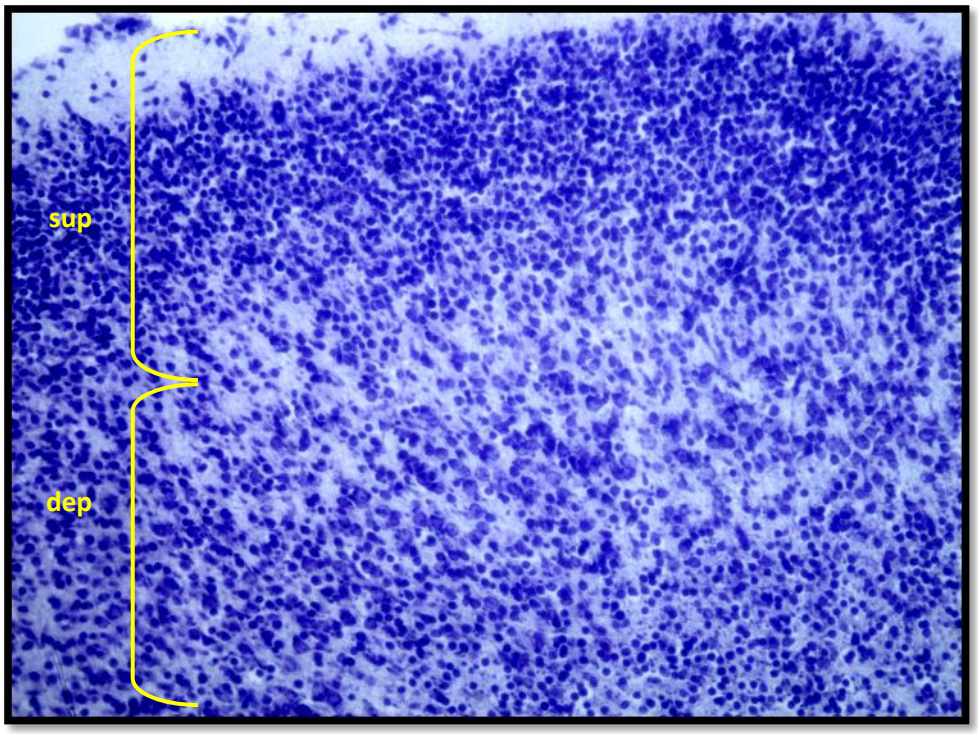

Figure 1. Frontal cortex of control group showing high number of cells on superficial and deep lamina. (sup) superficial layer of cerebral cortex, (dep) Deep layer cerebral cortex. Nissl stain. X200

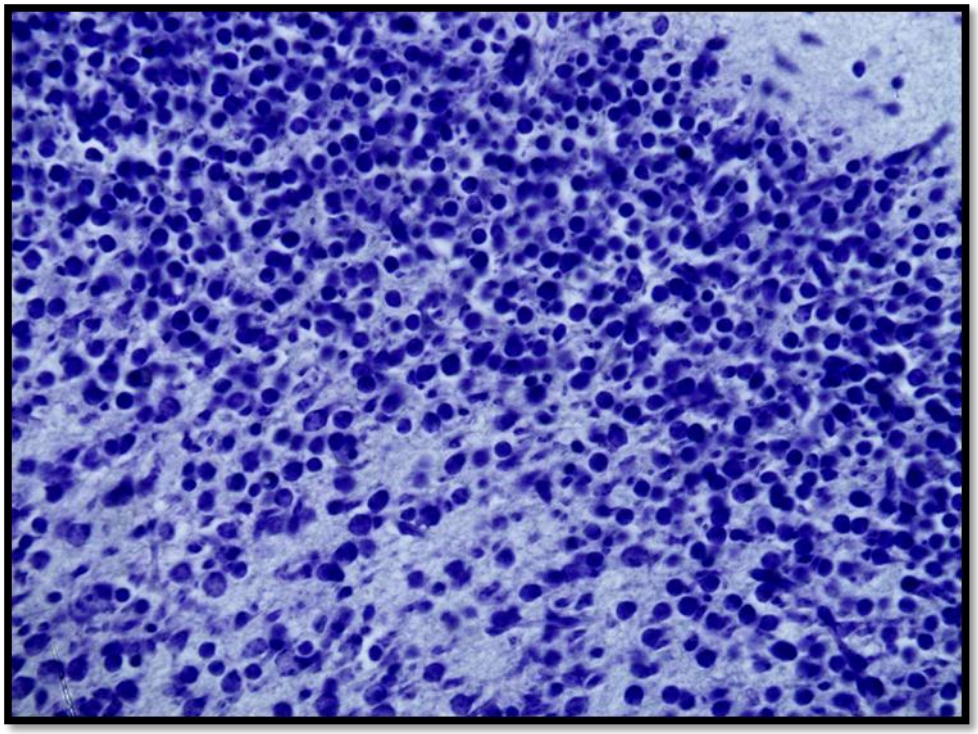

Figure (2): Nissl stain frontal cortex of control group. X400

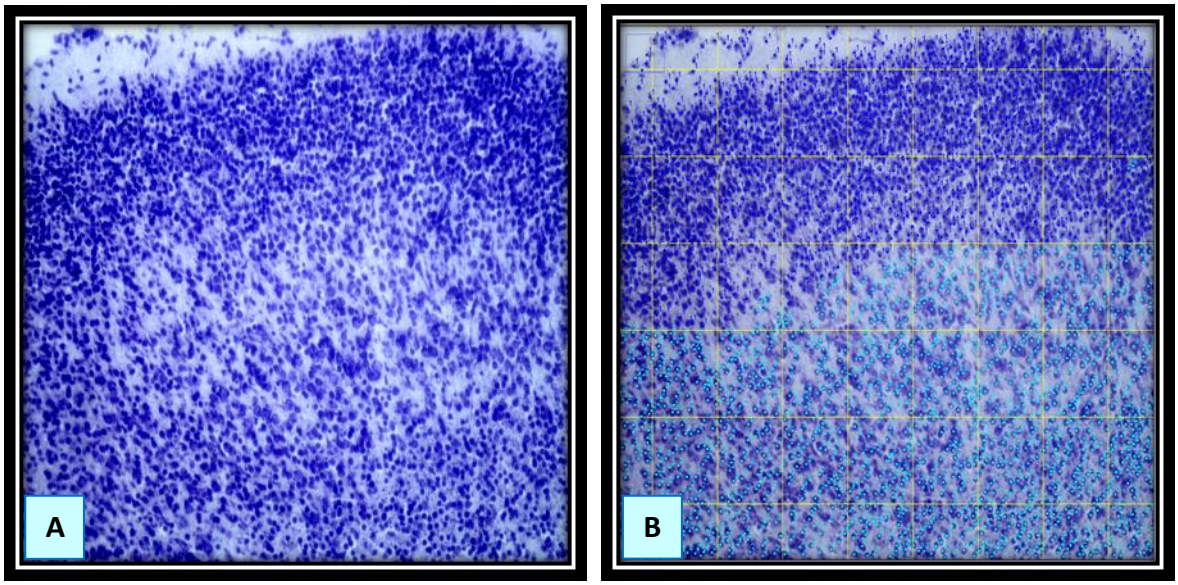

Figure 3. (A) Nissl stain frontal cortex of control group the picture showing shape of cells. X200. (B) The snap shoot as analyzed by image j program showing divided the picture into regular square and counting number of cells in deep and superficial layer

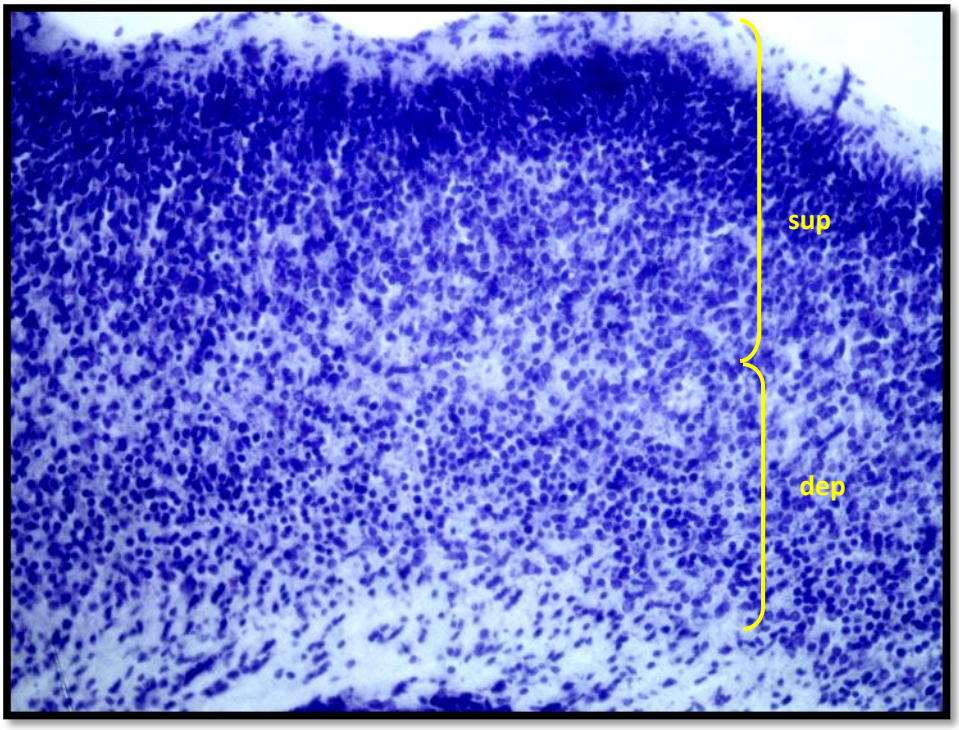

Figure 4. Parietal cortex of control group showing high number of cells on superficial and deep lamina. (sup) superficial layer, (dep) deep layer. Nissl stain. X200

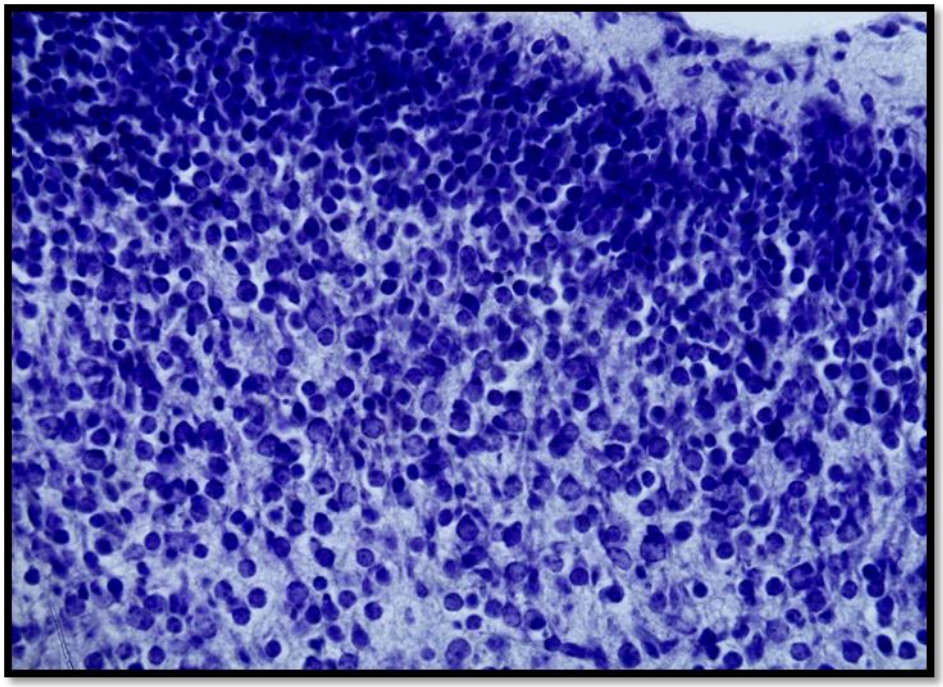

Figure (5): Nissl stain parietal cortex of control group. X400

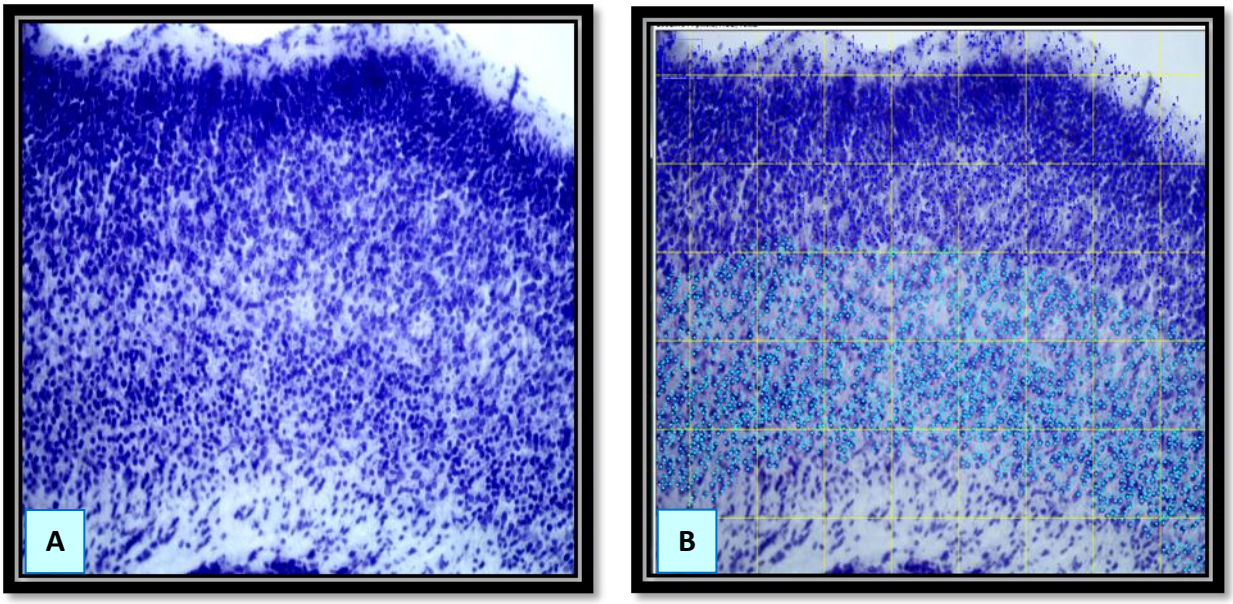

Figure 6. (A) Nissl stain parietal cortex of control group the picture showing shape of cells. X200. (B) The snap shoot as analyzed by image j program showing divided the picture into regular square and counting number of cells in deep and superficial layer

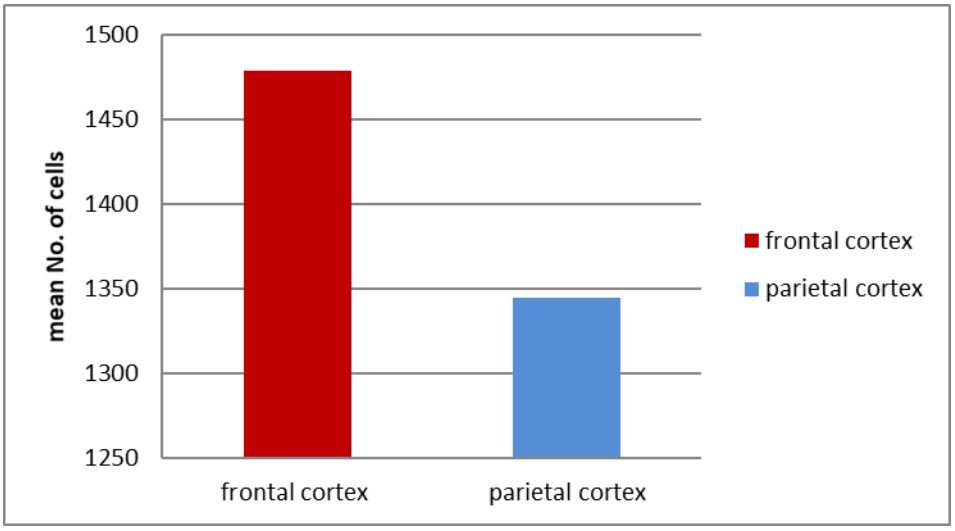

Figure 7. The values of number of cells obtained from frontal and parietal cortices in newborn mice in the control groups

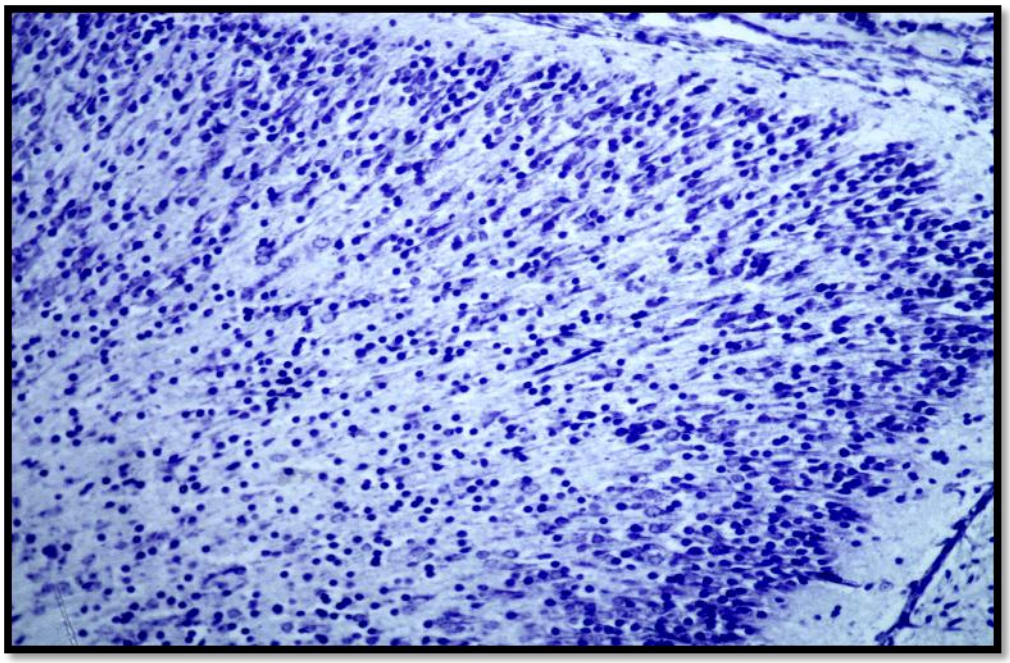

Figure 8. Frontal cortex of experimental group. Showing low number of cells on superficial and deep lamina when compare to control group. Nissl stain. X200

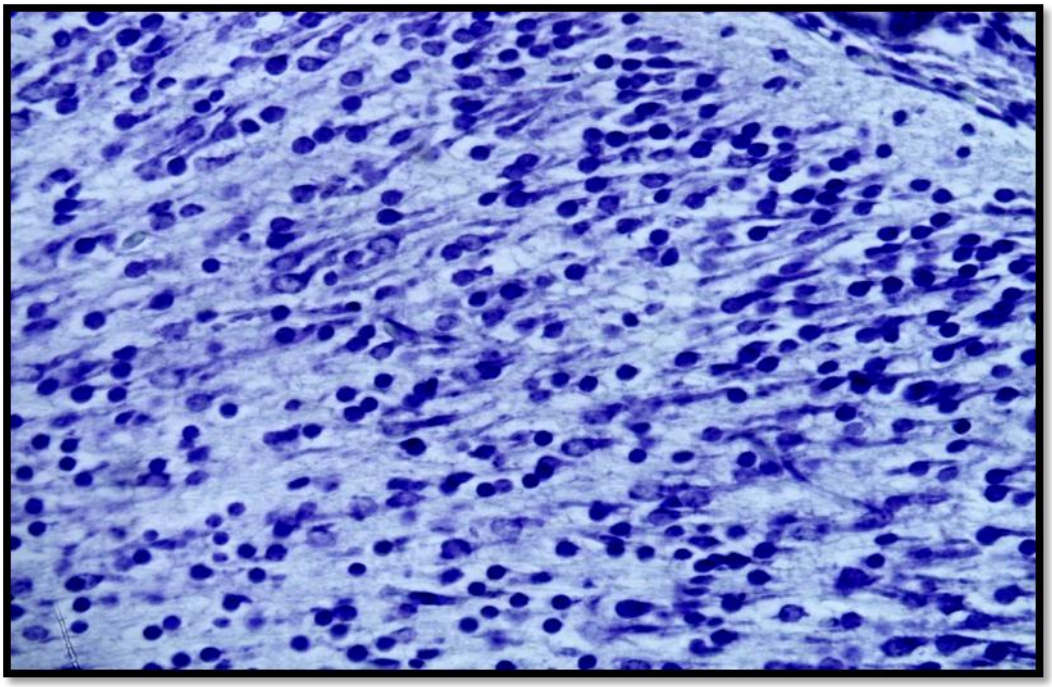

Figure 9. Frontal cortex of experimental group. Showing low number of cells on superficial and deep lamina when compare to control group. Nissl stain. X400

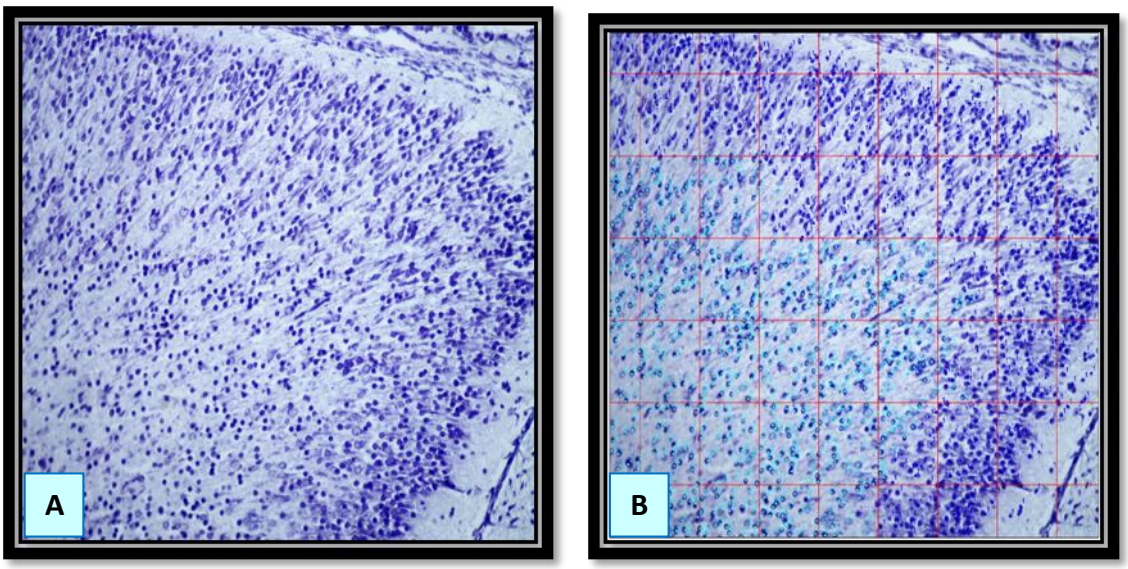

Figure 10. (A) Nissl stain parietal cortex of control group the picture showing shape of cells. X200. (B) The snap shoot as analyzed by image j program showing divided the picture into regular square and counting number of cells in deep and superficial layer

Result of nissl stain in parietal cortices of experimental group

The number of cells in parietal cortices in superficial layers (layers I, II and III) of experimental group is 853 cells, and the number of cells in deep layers (layers IV, V and VI) of parietal cortex of experimental group is 728 cells (Figures 11, 12, 13).

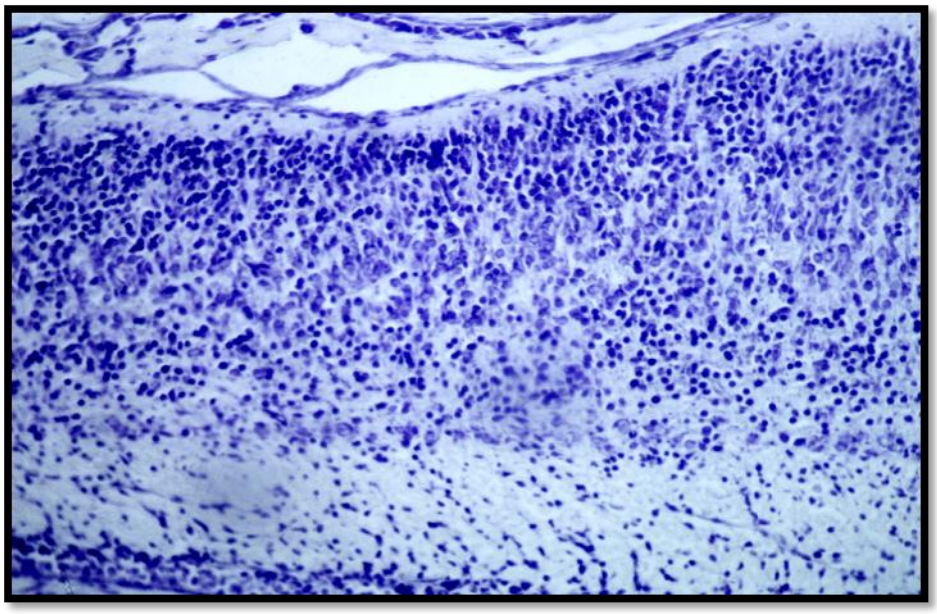

Figure 11. Parietal cortex of experimental group. Showing low number of cells on superficial and deep lamina when compare to control group. Nissl stain X200

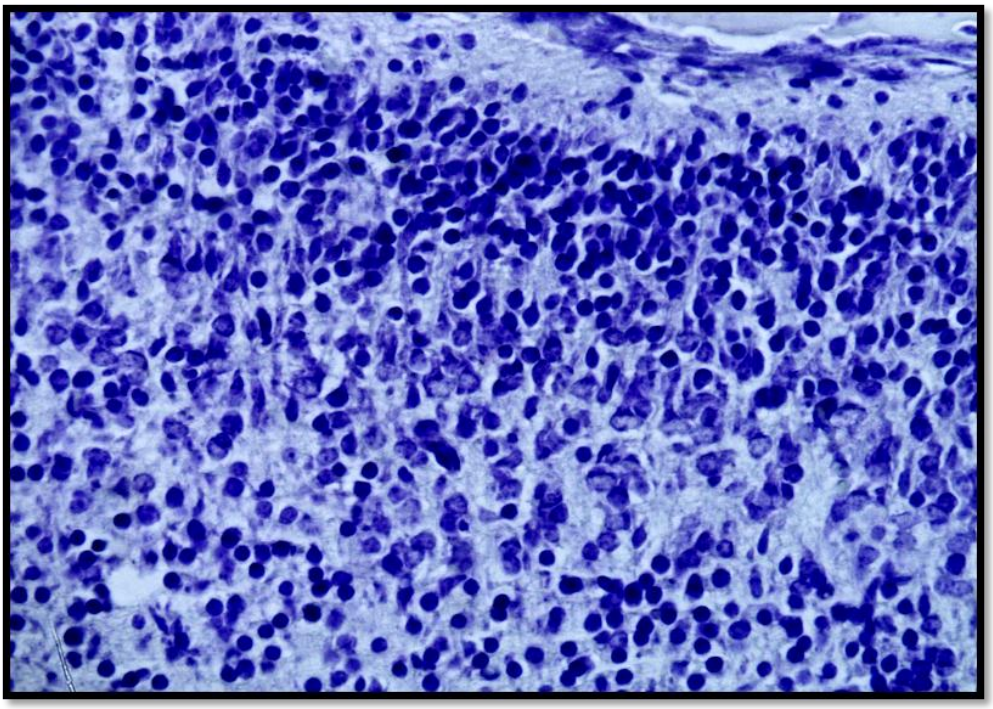

Figure 12. Parietal cortex of experimental group. Showing low number of cells on superficial and deep lamina when compare to control group. Nissl stain X400

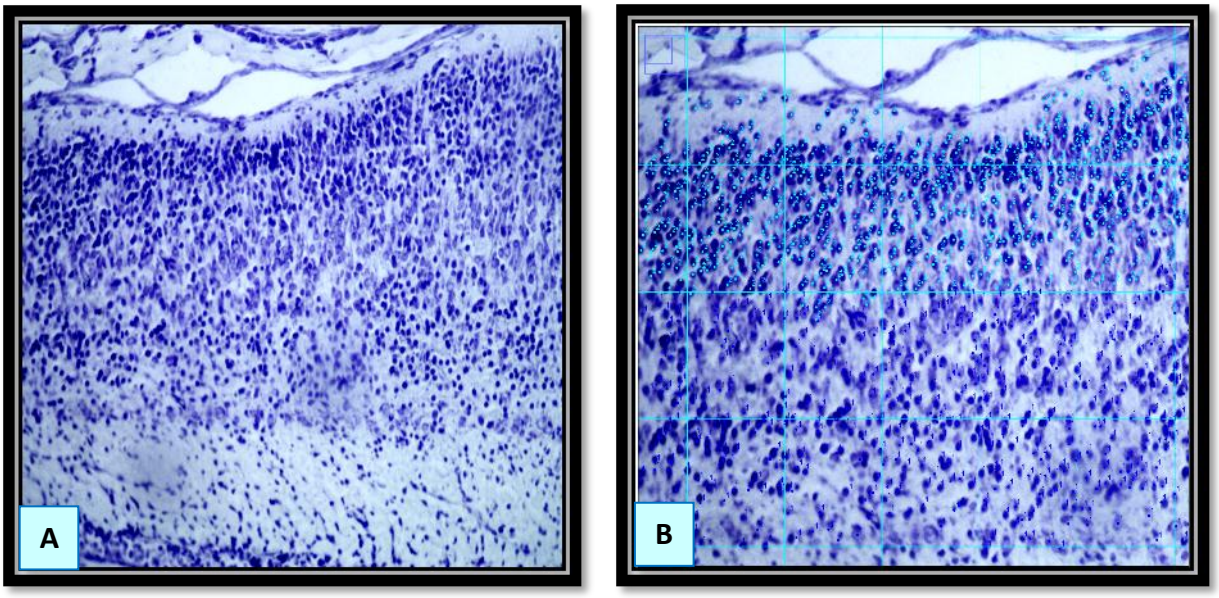

Figure 13. (A) Nissl stain parietal cortex of experimental group the picture showing shape of cells. X200. (B) The snap shoot as analyzed by image j program showing divided the picture into regular square and counting number of cells in deep and superficial layer

The comparison between frontal and parietal in experimental groups

The statistical analysis of the number of cells in frontal and parietal cortices in the experimental group showed non- significant variability, and the (p values = 0.195) for the frontal and parietal cortices in experimental group, as the mean of frontal cortex was 759.00, while the mean number of parietal cortex was 790.50 (Figure 14).

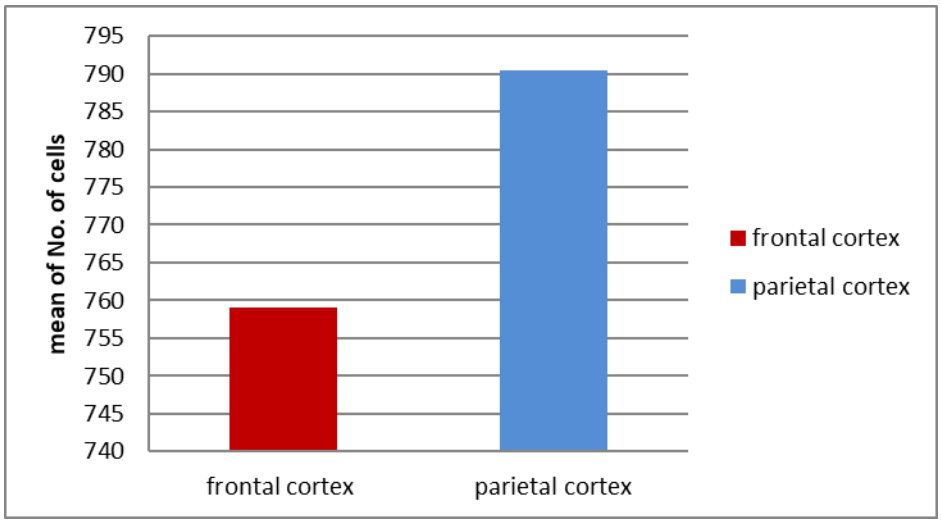

Figure 14. The values of number of cells obtained from frontal and parietal cortices in newborn mice in the experimental groups

Discussion

It was reported that quantitative neuroanatomical studies lack detailed cytological descriptions of neurons and glial cell types especially in the primate brain, the evaluation of the cellular density (both neurons and glial cells) done in the cerebral cortices of the animals involved in this study is experiment to elaborate the quantitative neuroanatomical aspect of histological changed resulting after prenatal ketamine exposure, this classical Nissl's staining technique had been used in this study because it labels all cells in the brain in distinct ways [18]. The procedure to compare the percentage of cell number in the deeper lamina (IV, V and VI) with that in the superficial lamina (I, II and III) had been submitted in accordance to the followings:

First, laminar structure of cerebral cortex is determined during early development in which, post mitotic cells migrate out to form cortical laminae in an inside–out manner in which the deeper cortical layers are formed before the more superficial ones [19].

Second, Ketamine leads to marked apoptotic changed in the period of peak synaptogenesis [20]. This fact had also been considered in the appraisal of comparing the percentage of cells in deep and superficial lamina in congruence to reports that neurons completing their migration showed synaptogenesis earlier in deeper layers than in more superficial ones [21].

These findings agreed with the report that cellular density in the brain coincides with neurological pathologies as well as with changes in brain associated with pharmacological treatment [22].

Nevertheless, are different techniques used previously to count the number of neurons in the cerebral cortex [23].

This study concluded that iatrogenic apoptotic changes were seen in the cerebral cortex of the experimental mice after prenatal exposure to ketamine and it is more considerable in the frontal cortex than the parietal cortex.

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Authors contribution

Dr. Najm: The MSc candidate performing the laboratory research work. Dr. Mubarak: The advisor of the research performing the interpretation of the results. Dr. Jarullah: Analysis data of the result.

Conflict of interest

The authors disclose no any financial and personal relationships with other people or organizations that inappropriately influence (bias) our work.

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Adenovirus Infection in a Sample of Iraqi Kidney Transplant Recipients: Molecular and Hematological Study

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Abstract

Background Human Adenovirus (ADV) is one of highly prevalent viruses worldwide, after primary infection, it remains latent and then might reactivate in immunocompromised patients. High ADV viremia seen in renal transplant recipients (RTR) with clinical presentations range from asymptomatic viremia to respiratory and gastrointestinal disease hemorrhagic cystitis, graft dysfunction and severe disseminated disease.

Objective The objectives of this study are to determine the rate of occurrence of ADV viremia by quantitative Real time PCR (QRT-PCR) in RTR and correlate them with urine cytology results, renal function tests and patients' hematological parameters.

Methods Seventy-one renal transplant recipients (RTR) were enrolled in this study. Whole blood samples (3 ml) divided into two parts, one part for complete blood picture and differential count and other part from which plasma separated and subjected to viral DNA extraction and then ADV Taqman QRT-PCR analysis for viral load measurement. Five ml urine specimens were collected for Pap-stained urine cytology.

Results Out of 71 RTR, 15 (21.12%) had positive ADV viremia by QRT-PCR, with a mean viral load 4.0 $x10^7$± 1.9 $x10^8$ copies/ml, and 80% (12 out of the 15) of positive viremia patients aged more than 40 years (p=0.011). All of RTRs 15/15 (100%) had symptomatic urinary tract infection (UTI) (p=0.039), and 5 out of 9 patients who had lymphopenia had positive viremia (p=0.007). Pap-stained urine cytology smears showed that 39/71 (55.71%) of the RTRs had positive decoy cells (DC), but there was no significant correlation between ADV viremia and the presence of DC (p=0.107).

Conclusion The present study showed the prevalence of ADV viremia in RTRs, with very high viral load, which is associated with lymphopenia and overt clinical features, this suggests that ADV might be an important cause of morbidity in RTRs.

Keywords Adenovirus, renal transplantation, real-time PCR, urine cytology

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List of abbreviations: ADV = Adenoviruses, CYC = Cyclosporine, DC = decoy cells, IS = Immunosuppressive drugs, MMF = Mycophenolate, QRT-PCR = Quantitative real time polymerase chain reaction, RTR = Renal transplant recipients, TAC = Tacrolimus, UTI = urinary tract infection

Introduction

Since first isolation from adenoid tissue over 60 years ago, human adenoviruses (ADV) belonging to Adenoviridae family, and the Mast adenovirus genus that have made continuous challenges in different clinical manifestations. Adenoviruses are divided into seven species, from A through G [1-3]. It has been reported more than 67 ADV types according to the gene bank for human adenovirus genotype classification [4]. Human ADVs known to cause a number of clinical manifestations, including keratoconjunctivitis, gastroenteritis, hemorrhagic cystitis, and

common cause of upper and lower respiratory tract infections, that produce in vitro cytolysis in these tissues [5].

Adenoviruses play an important role in patients with impaired immune responses, in whom viral diseases cause high morbidity and mortality. In the solid organ transplant recipients (SOT), the primary site of ADV disease is mainly related to the transplanted organ. Some of the clinical presentations occur in lung, liver, renal, and small bowel transplantations including pneumonia, hepatitis, nephritis, hemorrhagic cystitis, enteritis, and less commonly disseminated disease [6].

In renal transplant recipients (RTRs), the most common presentation is acute hemorrhagic cystitis and, to a lesser extent, pneumonia, with a 17% fatality rate [7]. Adenoviruses are included in the pathogens responsible for infections in the immediate post-transplantation period (PTP) as either primary infection or reactivation of a previous infection [8], in less than 2% of ADV cases, which presents as pneumonitis, nephritis, diarrhea, and hemorrhagic colitis or cystitis, the infection can become generalized and cause multiple organ failure [9,10].

Adenovirus infection was shown earlier after kidney transplantation, correlated with low absolute lymphocyte counts, and such patients develop more severe complications and progressive disease, in whom lymphocyte count can be used as a predictor of adenovirus disease and patient outcome [11]. In addition, ADV infection in kidney transplantation could be predicted when there is decoy cells (DC) in Pap-stained urine cytology but less commonly than polyomaviruses [12-14].

Progress in molecular detection methods especially quantitative real time PCR (QRT-PCR) has made the detection and monitoring of adenovirus diseases easily applicable in the clinical practice, in addition real time PCR is available for assessment of risk and accurate rapid diagnosis of invasive ADV infection [15].

In Iraq, several studies had been conducted on viral infections in RTRs, including human cytomegalovirus, BK and JC polyomaviruses, Epstein Barr virus, Human Herpes virus-6 and Parvovirus B19 [16-22]. However, to the best of our knowledge, this study is the first to investigate the prevalence of ADV viremia in Iraqi RTRs using QRT-PCR.

The objectives of the present study are detection of ADV viremia in kidney-transplanted patients by QRT-PCR, Screening of Pap-stained urine cytology smears for viral inclusions, correlate ADV viremia with patients' clinical presentation and renal function tests, and study the relation between the level of ADV viremia and patients' hematological parameters.

Methods

Study Population and Sampling

Cross sectional study conducted from November 2016 to April 2017, seventy-one RTRs were collected from the (Center of Kidney Diseases and Transplantation) in the Medical City of Baghdad. A consent letter obtained from all patients enrolled in the study. This study approved by the ethical committee of the College of Medicine-Al-Nahrain University. The study was conducted in the Microbiology Department at the College of Medicine-Al-Nahrain University.

Clinical parameters (immunosuppressive regimens, acute rejection episodes, transplant renal function, any signs and symptoms, and late complications) obtained from patient's medical records. Two main Standard immunosuppressive regimens were mainly followed in RTRs; either the cyclosporine (CYC), mycophenolate (MMF), and prednisolone, or the regimen that included tacrolimus (TAC) instead of CYC, in addition to MMF and prednisolone. And induction with monoclonal anti-CD25 antibodies (Basilixibam/Daclizumab)

From all 71 RTRs, three ml blood samples collected and divided in to two parts, one part for complete blood picture and differential lymphocytes count, and other part from which

plasma separated and subjected to viral DNA extraction and then ADV Taqman quantitative Real time PCR analysis for viral load measurement. Five ml urine specimens collected and preserved in 95% ethanol 1:1 for Pap-stained urine cytology smear.

Viral DNA Extraction

For viral DNA extraction from the blood samples; Geneius™ Viral Nucleic Acid Extraction Kit III (Geneaid, England) was used. One ml plasma sample used in viral DNA extraction, according to the manufacturer protocol.

Real Time PCR for Measuring ADV Viremia

For the quantitative detection of ADV; Adenovirus [R-gene®Ref.:69-010B France] is a Real-Time test, which is based on the principle of the so-called- "TaqMan" probe. Fifteen µl of Master Mix added into PCR tubes, and 10 µl of the (sample DNA, sensitivity controls, or standards) were added to the master mix. The final reaction volume was 25µl. All components were kept at +2 °C to +8 °C during the PCR preparation. Real time PCR instrument used in this work was7500 Real Time PCR System Applied biosystems (USA). The thermal protocol for Adenovirus R-gene® PCR kit is composed of a two hold steps, a one amplification cycle. The real-time data is collected at the third step of the amplification cycle. The size of the amplified fragment is 138 bp and is located in the Hexon gene coding for hexagonal capsomeres which form the sub-units of the adenovirus capsid protein. An Internal Standard (IC2) is included in the reaction mix controlling the possible inhibition of the PCR reaction. IC2 positive amplification is detected in the HEX fluorophore fluorescence channel. A range of 4 quantification standards is provided with the ADENOVIRUS Rgene®kit ranged from 5000 copies/µl to 5 copies/µl. These quantification standards used to generate a new standard curve in the software provided with the thermocycler. The quantification of Adenovirus genome in unknown samples is extrapolated from this standard curve. At the end of the thermal protocol, the 7500 Real Time PCR System Applied biosystems instrument software automatically calculates the baseline cycles and the threshold. The standard curve is plotted using the data obtained from the defined standards, with the (Y) axis is the Ct-Threshold Cycle, and the (X) axis is the viral DNA copy number. According to the manufacturer instructions, ADV DNA copies was calculated according to the following formula [23]:

$$\text{copy/ml} = \frac{\text{SC x EV}}{\text{IV}}$$

SC = Sample Concentration (copy/µL)
EV = Elution Volume (µl)
IV = Isolation Volume (ml)

Results

Among these 71 RTR; 49 (69.01%) were males, and 22 (30.98%) were females; their mean age was 37.06±12.73 years, ranging between 18 and 63 years, and the mean Post-Transplantation Period (PTP) was 10.44±5.54 months. The mean body weight of the RTRs was 75.54±8.5 Kg, while their mean serum creatinine value was 1.16±0.28, and the mean of their creatinine clearance was 92.08±20.58, and among all the RTRs; 9/71 (12.6%) had lymphopenia. All the RTRs received their allografts from living donors, and out of the 71 RTRs; 32 (45.07%) received their allograft kidney from living related donors, while the remaining 39 (54.93%) received their kidney allograft from living unrelated donors. Regarding the type of immunosuppression, 37/71 (52.11%) were on (CYC) and the reminder 34/71 (47.88%) were on (TAC) regimen.

Depending on the patient's files and questioner at the time of collection, the majority RTRs enrolled in this study had clinical presentations

and complications at the time of sampling, symptomatic urinary tract infections (UTI) were the highest among these presentations as shown in the table (1). Out of the 71 RTRs 40 (56.33%) were hypertensive and 10 (14.08%) were diabetic

Table 1. Clinical presentations of RTRs at the time of sampling

Clinical presentation	No. (%) out of 71 RTRs
UTI	58 (81.69%)
Respiratory disease	39 (54.92%)
Gastroenteritis	36 (50.70%)
Hematuria	23 (32.39%)
Eye infection	15 (21.12%)

Urine cytology smears were Papanicolaou-stained and microscopic examination showed that 39/71 (55.71%) of the RTRs had positive Decoy cells. Figure (1) shows various types of decoy cells among RTRs.

QRT-PCR for detection and quantitation of ADV (a hexon gene which is common to all adenoviral species) in plasma sample (viremia), was positive in 15 out of 71 (21.12%) RTRs. The mean of ADV Viremia was $4.0 \times 10^7 \pm 1.9 \times 10^8$ copies/ml. The standard curve of QRT-PCR included the four quantification standards ranged from 5000 copies/µl to 5 copies/µl. Statistical data in table (2) demonstrated that 80% (12 out of the 15) patients who had positive viremia aged more than 40 years which is statistically significant (p=0.011). There is no significant difference in gender, nevertheless, 9 out of 15 (60%) positive ADV viremia patients were males, while females were 6 out of 15 (40%) ADV positive cases (p=0.395).This study found no significant association between positive viremia and gender type, transplantation period, serum creatinine, creatinine clearance test, and the type of immunosuppression. Regarding the association between ADV viremia and clinical presentations all of RTRs 15/15 (100%) who had positive ADV viremia; had symptomatic UTI, which was statistically significant (p=0.039). Although there were no significant association between ADV viremia and other clinical presentations, however, about 30%, 25% and 23% of those patients who had hematuria, gastroenteritis, and respiratory infections had positive adenovirus viremia, respectively. Pap-stained urine cytology results showed no significant correlation between ADV viremia and the presence of decoy cells (DC), but 73.3% (11 out of 15 positive viremia cases) had positive DCs in urine. On the other hand, (55.6%) 5 out of 9 patients who had lymphopenia (lymphocytes count in the blood below 1.0X109) had positive ADV in plasma, which was statistically significant (p=0.007). In addition, there was a significant association between the value of viral load and lymphopenia in RTRs (p=0.023).

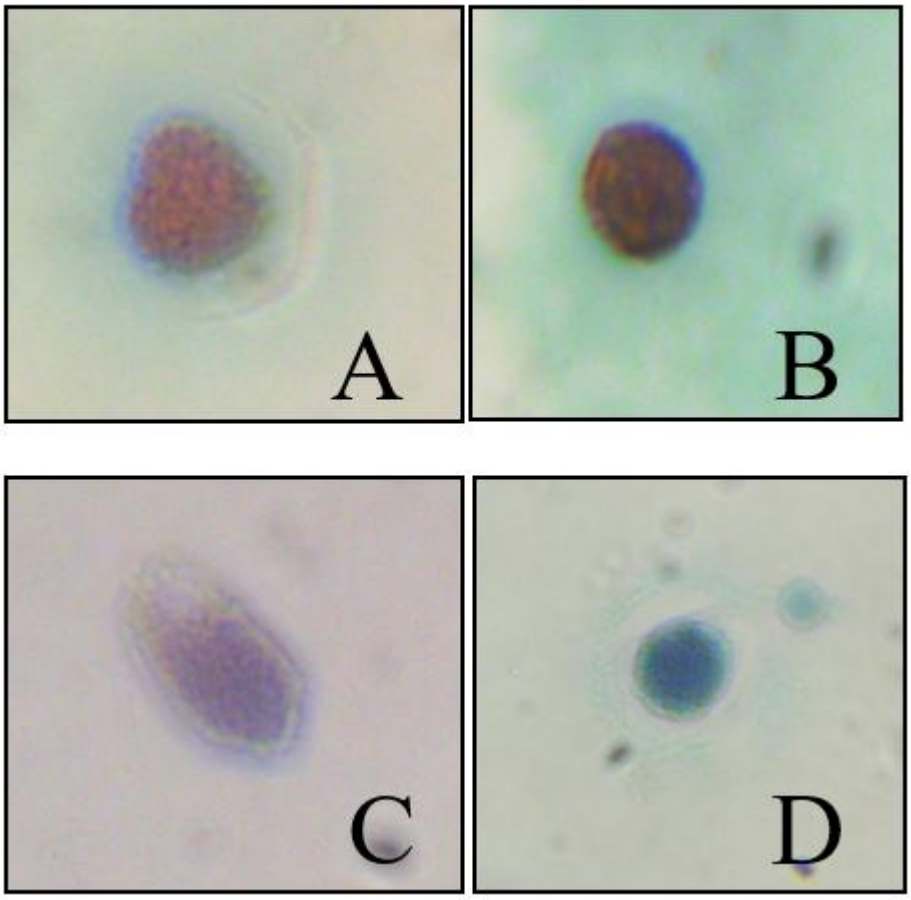

Figure 1. Pap-stained urine cytology smears demonstrate: (A and B) Common ground glass virus-infected (decoy cells (DC)). (C) Common comet-shaped DC. (D) Uncommon clumped variant DC. Magnification power for A (100X), B, C, D (40X)

Discussion

In this study, ADV was investigated in the plasma sample of RTRs using QRT-PCR, and the results showed that more than one-fifth of the RTRs (15 out of 71 (21.12%) had positive ADV viremia in plasma, this frequency is in the range of other studies, that spam between 5 to 22% [24,25]. These results were significantly correlated with RTRs who had low lymphocytes count (lymphopenia). ADV infection in RTRs may be a consequence of a primary infection, reactivation of latent infection or acquired through donor organs, and it is believed that majority of the cases are due to reactivation of latent infection [26]. The mean of ADV viremia was 4.0 $x10^7$ ± 1.9 $x10^8$ copies/ml, which is a very high viral load associated with wide range of clinical manifestations, in which 30%, 25% and 23% of those patients who had hematuria, gastroenteritis, and respiratory infections had positive ADV viremia, respectively. These results are in line with other previous studies on ADV infection in RTRs that showed high mean viral load in immunocompromised patients [27-29]. In immunocompromised patients, the immune escape and persistence of ADV mediated by different mechanisms. Specific viral proteins can block responses to anti-inflammatory and cytolytic cytokines, intrinsic cellular apoptosis, and innate and adaptive cellular immune responses [30]. Moreover, the viral protein E3 can down regulate major histocompatibility complex (MHC) class I molecules, there by affecting antigen presentation and reducing T-cell attack of the infected cells [31,32].

Table 2. Comparisons of the patients with positive ADV viremia and negative viremia including transplants' demographic, clinical and laboratory data

		Positive ADV	Negative ADV	Total	p value
Age	< 40 years	3 (8.6)	32 (91.4)	35	0.011
	≥ 40 years	12 (33.3)	24 (66.7)	36	
Gender	Male	9 (18.4)	40 (81.6)	49	0.395
	Female	6 (27.3)	16 (72.7)	22	
PTP	≤ 1 year	9 (19.1)	38 (80.9)	47	0.568
	> 1 year	6 (25.0)	18 (75.0)	24	
Relatedness	Related	7 (21.9)	25 (78.1)	32	0.980
	Unrelated	8 (21.6)	29 (78.4)	37	
IS Drugs	CYC	9 (24.3)	28 (75.7)	37	0.491
	TAC	6 (17.6)	28 (82.4)	34	
Rejection	No	15 (22.1)	53 (77.9)	68	0.360
	Yes	0 (0.0)	3 (100)	3	
Frequency of transplantation	Once	15 (22.1)	53 (77.9)	68	0.360
	Twice	0 (0.0)	3 (100)	3	
Respiratory Infection	Yes	9 (23.1)	30 (76.9)	39	0.657
	No	6 (18.8)	26 (81.3)	32	
Gastroenteritis	Yes	9 (25.0)	27 (75)	36	0.417
	No	6 (17.1)	29 (82.9)	35	
Eye Infection	Yes	1 (6.7)	14 (93.3)	15	0.122
	No	14 (25.0)	42 (75)	56	
UTI	Yes	15 (25.9)	43 (74.1)	58	0.039
	No	0 (0.0)	13 (100)	13	
Hematuria	Yes	7 (30.4)	16 (69.6)	23	0.184
	No	8 (16.7)	40 (83.3)	48	
Hypertension	Yes	9 (22.5)	31 (77.5)	40	0.747
	No	6 (19.4)	25 (80.6)	31	
Diabetes	Yes	3 (30.0)	7 (70.0)	10	0.458
	No	12 (19.7)	49 (80.3)	61	
Urine cytology	Yes	11 (28.2)	28 (71.8)	39	0.107
	No	4 (12.5)	28 (87.5)	32	
Lymphopenia	Yes	5 (55.6)	4 (44.4)	9	0.007
	No	10 (16.1)	52 (83.9)	62	
Creatinine Clearance	Abnormal	4 (20.0)	16 (80.0)	20	0.884
	Normal	11 (21.6)	40 (78.4)	51	
Serum Creatinine	Normal	9 (19.1)	38 (80.9)	47	0.568
	Abnormal	6 (25.0)	18 (75.0)	24	

PTP: Post-transplantation period, IS: Immunosuppressive, CYC: Cyclosporine, TAC: Tacrolimus, UTI: Urinary tract infection

In addition, studies found this high viral load is associated with many manifestations like pyelonephritis [33,34], hemorrhagic cystitis [35,36], respiratory infections [37-39], and GIT infections [40-42]. However, it should be noted that the high number of patients with upper and lower respiratory tract infections could be mainly because the time of collecting the patients in

this study, which was mainly during the winter season (November to April).

The present study found that 7/15 (46.6%) of RTRs who had positive ADV viremia had hematuria as clinical symptom during collecting the sample this is supported by Hofland et al. 2004, [43] who reviewed 37 cases of ADV hemorrhagic cystitis in kidney transplant recipients, and showed that in RTRs, the most common manifestation is hemorrhagic cystitis. Also, the current study had significant association between ADV viremia and UTI that could be supported by other studies, which found a correlation between adenovirus infection and cystitis and pyelonephritis, with white cell casts in urine [34,44]. Kolankiewicz et al. in 2010 [34] published a case report and a review of the literature in which the patients commonly presented with gross hematuria and dysuria (10/11), fever (9/11), and acute renal failure (9/11), and 27% of the patients had significant graft function impairment after adenoviral nephritis. Rady et al. in 2014 [44] described a case of necrotizing tubulointerstitial allograft nephritis due to adenovirus infection, a significant proportion of patients presented within 8 months of transplant with gross hematuria, dysuria, fever and acute renal failure.

Although there was no statistically significant association of ADV viremia with respiratory infection, nevertheless, 9 out of 15 ADV positive patients had respiratory disease, which was also showed in other studies Watanabe et al. in 2013, in which all patients enrolled in the study had acute respiratory tract infections [37]. Respiratory tract disease ranged from mild upper-tract involvement (URI), typically presenting nonspecific cold like symptoms, to severe pneumonia [39] which agrees with the patients' presentations in this study. In addition, this study found that 9 out of 15 ADV positive patients had gastrointestinal symptoms, mostly diarrhea, although there was no statistically significant association between ADV viremia and GIT disease, another study also showed that GIT infection occurred in RTRs, symptoms ranged from mild diarrhea to hemorrhagic colitis [32].

Results of the current study found a statistically significant association between ADV viremia and low lymphocytes count (lymphopenia) 5 out of 15 (33.3%) (p=0.007) which is supported by the study of Watcharananan et al. in 2011 who showed that early ADV infection appeared to have significantly lower lymphocytes count at several time points compared to those with late infection [11]. This finding underscores the influence of immune recovery during the course of infection ([45,46]. Heemskerk et al. in 2005 found exogenous lymphocyte therapy in the form of donor lymphocyte infusions has been successful in some cases [46]. Cohort of retrospective study of Kim et al. in 2015, found that a low absolute lymphocyte count within 3 months in allogeneic transplantation recipients was significantly associated with poor overall survival, progression-free survival, and mortality [47]. Ison in 2006 [5] demonstrated an association between lymphocyte recovery and recovery from adenoviral infections in RTRs.

On the other hand, there was no significant association between ADV infection and the level of serum creatinine and creatinine clearance (CrCl), a result might exclude the possibility of an associated renal impairment with adenoviremia, and this result is supported by other studies in RTRs that excluded the role of ADV in renal impairment or rejection [48]. Nanmoku et al. in 2016 [49] also showed there is no significant difference was seen before, during, or after disease onset of ADV infection. However, most of the studies reported sporadic cases of renal allograft impairment associated with ADV viremia [13,44,50].

Although the results of present study showed no significant association between ADV viremia and urine cytology findings, however, (73,3%) 11 out of these 15 ADV positive cases had positive decoy cells in Pap-stained urine cytology smears. A result can support the high association of ADV viremia with different urinary, gastrointestinal and respiratory complaints in these RTRs. Studies showed that although it is rare, but adenovirus is one of the viruses that are associated with urinary decoy cells shedding [5]. Viral cytopathic changes (decoy cells), which are typically associated with polyomavirus infection, have been

reported in the urine of RTRs with ADV infection [51,52]. Storsley et al. in 2011 found that four patients demonstrated decoy cells in their urine over the course of a few months, during which time the urine culture and PCR was positive for ADV while BKV virus was negative [53]. Surveillance studies of asymptomatic adult RTRs have shown an incidence of adenoviral viremia by PCR testing of 6.5% and viruria by 11% [5,54]. Asymptomatic viral shedding in the urine makes urinary cultures unreliable in the absence of signs and symptoms of disease activity. The presence of white cell casts with decoy cells on urinalysis may increase the suspicion for ADV infection [54].

In our transplantation center in Baghdad, there is no protocol biopsy for RTRs, and this makes the exact diagnosis of renal pathology extremely difficult. The presence of high adenoviral load in plasma, with high decoy cells shedding, and the significant association of viremia with lymphopenia (both qualitatively and quantitatively) (p=0.007, and 0.023) respectively, all these give strong association between this virus and the patients' clinical manifestations. However, other infections should be excluded.

In conclusion, ADV infection is an important cause of many diseases in RTRs, which is evident by high frequency of this virus with high viral load in these RTRs.

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Authors' contribution

Ahmed: Collection of specimens, DNA extraction, and real time-PCR, writing of the references. Dr. Hussein: Consultant Nephrologist help in providing all patients' data and in selection of patients. Dr. Al-Obaidi: Supervision and performance of viral DNA extraction and real time-PCR run, writing of the manuscript. Dr. Kadhim: Final editing of the manuscript. Dr. Ghazi: Statistical analysis.

Conflict of interest

Authors declare no conflict of interest.

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Pre and Post Extracorporeal Shock Wave Lithotripsy (ESWL) Urine Culture as A Guide for Antibiotics Management

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Abstract

Background The lifetime prevalence of kidney stone disease is estimated at 1-15%. The positive urine cultures can be obtained not only from Struvite stones, but also from calcium oxalate stones and also high levels of endotoxins are found both in infection stones (Struvite and carbonate apatite stones), and in non-infection stones. High concentrations of endotoxins (lipopolysaccharides) are thought to be released in the systemic circulation during stone treatment, inducing a systemic inflammatory response (SIRS) and this leads to urosepsis. Pre-Extracorporeal shock wave lithotripsy (ESWL) prophylactic antibiotics have an important role in reducing post-SWL infections, however, previous studies reported conflicting results. The issue of administering prophylactic antibiotics remains controversial in patients with sterile urine undergoing ESWL.

Objective To evaluate possible risk factors for post ESWL bacteriuria and consequently to identify patients with higher danger for urinary tract infection (UTI) or sepsis.

Methods Urine samples from 50 patients underwent ESWL, were collected by clean catch mid-stream urine collection method in sterile containers. Those patients were attending and admitting to Al-Imamein Al-Kadhimein Medical City during the period from October 2016 to January 2017. All patients had a urine culture performed before and after shock wave lithotripsy. Statistical analysis was performed with Epi-Info 7 and Excel programs. Statistical significance was evaluated using the Fisher's exact test with $p < 0.05$ considered statistically significant.

Results A total of 50 patients who underwent ESWL during; the 2- months study period was enrolled in the study. Thirty-three 33 (66%) out of 50 were men and 17 (34%) out of 50 were women. 10 (20%) of patients had hypertension and 5 (10%) had diabetes mellitus. Fifty urine samples were collected from patients enrolled in this were cultivated on blood and MacConkey agar Pre-and post-ESWL Regarding Pre-ESWL results revealed 14 (28%) were urine culture positive while 23 cases were post-SWL urine culture positive.

Conclusion Antibiotic prophylaxis is not justified without defined risk factors such as positive urine culture before ESWL, an external bladder catheter or nephrostomy tube and a history of infectious stones or recurrent urinary tract infection.

Keywords Extracorporeal shock wave lithotripsy (ESWL), urine culture, renal stone and antibiotics

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List of abbreviations: AUA = American Urological Association, EAU = European Association of Urology, ESWL = Extra Corporal Shockwave Lithotripsy

Introduction

The lifetime prevalence of kidney stone disease is estimated at 1% to 15%, varying according to age, gender, race, and geographic Location [(1)]. The most common component of urinary calculi is calcium, which is a major constituent of nearly 80% of stones. Calcium oxalate comprises about 60% of all stones; mixed calcium oxalate and hydroxyapatite make up 20% and brushite stones make up 2%. Uric acid and Struvite (magnesium ammonium phosphate), each

comprise approximately 7% of stones, and cysteine stones represent only about 1% [2]. Infection stones are composed primarily of magnesium ammonium phosphate hexahydrate (MAP) ($MgNH_4PO_4 \bullet 6H_2O$) [3]. Struvite stones are infection stones frequently present as renal staghorn calculus. The driving force behind Struvite stone is infection of the urine with urease producing bacteria, the most common bacteria associated with stones are *Proteus*, *Pseudomonas*, *Providencia*, *Klebsiella*, *Staphylococci*, and *Mycoplasma*. The high ammonium concentration derived from the urea-splitting organisms results in an alkaline urinary pH. The urinary pH of a patient with a MAP calculus rarely is <7.2 (normal urinary pH is 5.85). It is only at this elevated urinary pH (>7.19) MAP crystals precipitate. MAP crystals are soluble in the normal urinary pH range of 5-7 [4]. Infection stones occur most commonly in those prone to frequent urinary tract infections (UTI), Struvite stones occur more often in women than men by a ratio of 2:1 [5]. The presence of urease-producing organisms in only 48% of Struvite stones, while 32% of calcium oxalate stones were infected. It may therefore be inferred that an infection from urease-producing organisms is not always present in infected Struvite stones; furthermore, positive cultures can be obtained not only from Struvite stones, but also from calcium oxalate stones [6]. Nano bacteria have also been suggested to cause stone disease and be pathogenic for Urosepsis following kidney stone treatment. Nano bacteria are micro-organisms that are (10-100) times smaller than normal bacteria; they may be involved in the formation of calcium phosphate crystals, thus creating nidus for the formation of the stone. The risk of sepsis would therefore be correlated with the release of these micro-organisms from the stone during treatment. Endotoxins are another factor supposed to be involved in the pathogenesis of urinary infection from kidney stones. High levels of endotoxins are found both in infection stones (Struvite and carbonate apatite stones), and in non-infection stones. High concentrations of these (lipopolysaccharides) are thought to be released in the systemic circulation during stone treatment, inducing a systemic inflammatory response (SIRS) and this led to urosepsis [7]. Sepsis, SIRS and infection either documented or strongly suspected characterized by fever (>38 °C) or hypothermia (<36 °C). Tachycardia (>90 beats/min in patients not on B-blockers), tachypnea (respiration >20 breaths/min or $PaCO_2$ <4.3 kPa or a requirement for mechanical ventilation) White cell count >12000 cells/mm^3 [8]. The primary predictive risk factors of urosepsis are the following: patient conditions such as immunodepression and a deteriorated performance status, a urinary infection in progress or a history of recurrent infections, characteristics of the stone, anatomy of the urinary tract. In the hospital setting, the most common causes are the presence or manipulation of indwelling urinary catheters, urinary tract surgery (PCNL), and urinary tract obstruction (particularly that due to stones obstructing the ureter) [9]. Urine cultures are performed if there is a suspicion of infection-related calculi or if there are signs or symptoms of a UTI. A culture that is positive for urea-splitting organisms such as *Proteus, Pseudomonas*, *Klebsiella*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* would help explain the formation of a Struvite calculus. A positive culture also will warrant therapy with appropriate antibiotics before initiation of any surgical procedure to remove the stone [10]. Extracorporeal shock wave lithotripsy (ESWL) was introduced into medical practice in the 1980s, and since then has become one of the main treatment options in patients with renal and/or ureteral calculi [11]. Lithotripters have been developed with new sources for generating shock waves, such as electromagnetic and piezoelectric sources, Regarding the Piezoelectric type is a spherical dish is covered with about 3000 small ceramic elements, each of which expands rapidly when a high voltage is applied across them, this rapid

expansion generates a shock wave [11]. The European Association of Urology (EAU), in its urolithiasis guidelines, recommends ESWL as the preferred first-line therapy for all kidney stones smaller than 10 mm. Stones 1 cm or less in diameter, ESWL achieves stone-free rates of approximately 50% to 90% and effectiveness quotients of approximately 50% to 70%. Shock wave lithotripsy treatment success rates exceeding 70% have been reported for stones in the upper (71.8%) and middle (76.5%) calyces, lower pole stone clearance rates range lower, between 37% and 61% [12]. In patients with pre-SWL sterile urine, the procedure poses a risk of post-operative UTIs up to 14% in patients without prophylactic antibiotics [13]. Pre-ESWL prophylactic antibiotics have an important role in reducing post-SWL infections, however, previous studies reported conflicting results. The issue of administering prophylactic antibiotics remains controversial in patients with sterile urine undergoing ESWL [14].

The European Association of Urology (EAU) and the American Urological Association (AUA) guidelines proposed different protocols for prophylaxis. The AUA Best Practice Statement on Urological Surgery Antimicrobial Prophylaxis recommends routine antibiotic prophylaxis [15]. Conversely, the more published guidelines on urological infections by the EAU advocate for prophylaxis only in patients with urinary drainage tubes, ureteral stents or infected stones [16].

The purpose of this study was to evaluate possible risk factors for post ESWL bacteriuria and consequently to identify patients with higher danger for UTI or sepsis

Methods

Urine samples from 50 patients underwent ESWL, were collected by clean catch mid-stream urine collection method in sterile containers and promptly transported to the laboratory. The relevant data pertaining to history, examination and laboratory work up were recorded in predesigned forma, all the samples were subjected to urine culture. Those patients were attending and admitting to Al-Imamein Al-Kadhimein Medical City conducted with Al-Nahrain University College of Medicine during the period from October 2016 to January 2017. All patients had a urine culture without targeted Antibiotics performed before and after shock wave lithotripsy Loopful of the sample were inoculated on a blood agar and MacConkey agar aerobically for 18-24 hours at 37 °C. The identification of Enterobacteria family was performed according to the biochemical tests indicated in the scheme of Farmer and his co-workers [17]. Other bacterial species were identified by Gram stain and biochemical test related to each isolate. Statistical analysis was performed with Epi-Info 7 and Excel programs. Statistical significance was evaluated using the Fisher's exact test with $p < 0.05$ considered statistically significant.

Results

A total of 50 patients who underwent ESWL during the 2-months study period were enrolled in the study. Thirty-three 33 (66%) out of 50 were Men and 17 (34%) out of 50 were women with the mean±SD age of 45±14 years with a ratio of 1.9:1 (Table 1 and Figure 1 and 2). Out of 50 patients suffering were enrolled in this study, the Following patients were with difference underlying diseases as: 10 (20%) had hypertension and 5 (10%) had Diabetes mellitus. Out of 50 patients enrolled in the study, 41 (82%) patients had a renal stone, those patients were grouped according to the site of stone into three anatomical groups that 21 (42%) right sided renal stone, 20 (40%) left sided renal stone and 9 (18%) had a ureteral stone with only 5 of 9 patients underwent ESWL with previously placed Double-J stent. The size of treated stone was (0.64 cm±0.48) (Table 1). Pre- ESWL results revealed 14 (28%) were urine culture positive (Table 2), while 23 cases were Post-SWL urine culture positive Table (3).

Proteus microorganism is positive in both patients with history of previous UTI and history of previous surgery (Figures 3, 4 and 5).

Table 1. Demographic information of the patients

Age (years)	Frequency	Percent (%)
10-19	1	2
20-29	5	10
30-39	13	26
40-49	17	34
50-59	8	16
60-69	3	6
70-79	3	6
Total	50	100
Gender		
Male	33	66
Female	17	34
Total	50	100
The site and side of Renal stone		
Right	21	42
Left	20	40
Ureter	9	18
Total	50	100
The size of Renal stone		
<1cm	18	36
>1cm	32	64
Total	50	100
History of Urinary tract infection		
Yes	32	76
No	18	24
Total	50	100
History of previous surgery		
Yes	23	46
No	27	54
Total	50	100

Table 2. Demographic information of the patients

Pre-ESWL urine culture Results	Frequency	Percent (%)
Negative culture	36	72
Bacillus	1	2
Corynbacterium	1	2
E.Coli	4	8
Enterococcus	2	4
Klebsiella	1	2
Proteus	2	4
Staphylococcus Aureus	1	2
Streptococcus Agalactiae	2	4

Table 3. The results of Post-ESWL urine culture

Post-ESWL urine culture Results	Frequency	Percent (%)
Negative culture	27	54
Bacillus	1	2
Corynebacterium	1	2
E. coli	4	8
Enterococcus	3	6
Klebsiella	2	4
Proteus	7	14
Staphylococcus Aureus	5	10

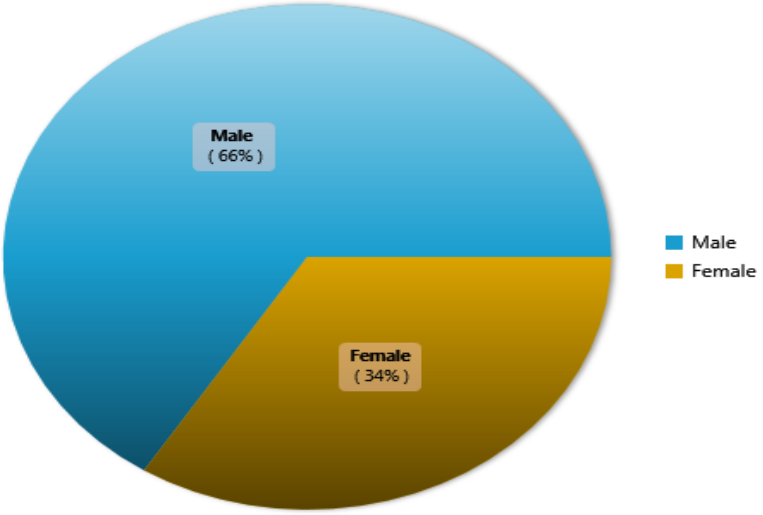

Figure 1. Sex distribution among patients of the study

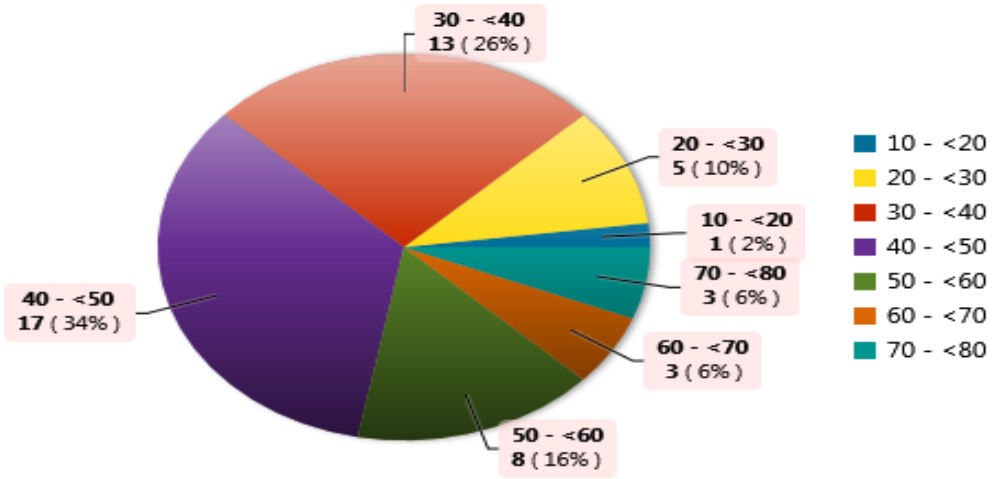

Figure 2. Age distribution among patients of the study

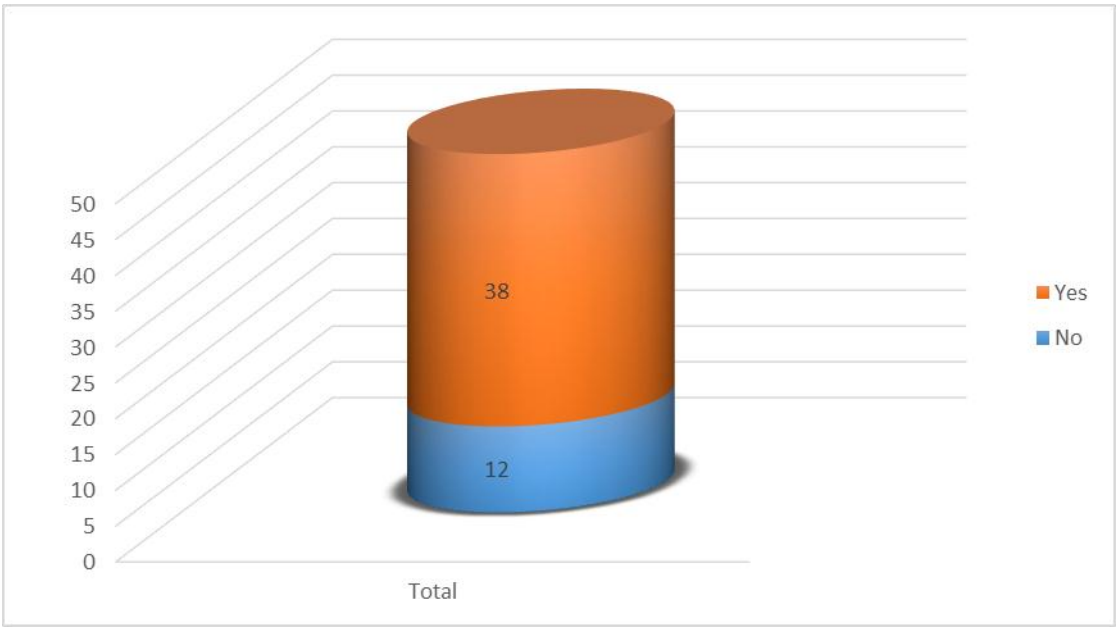

Figure 3. The prevalence of patients with history of UTI

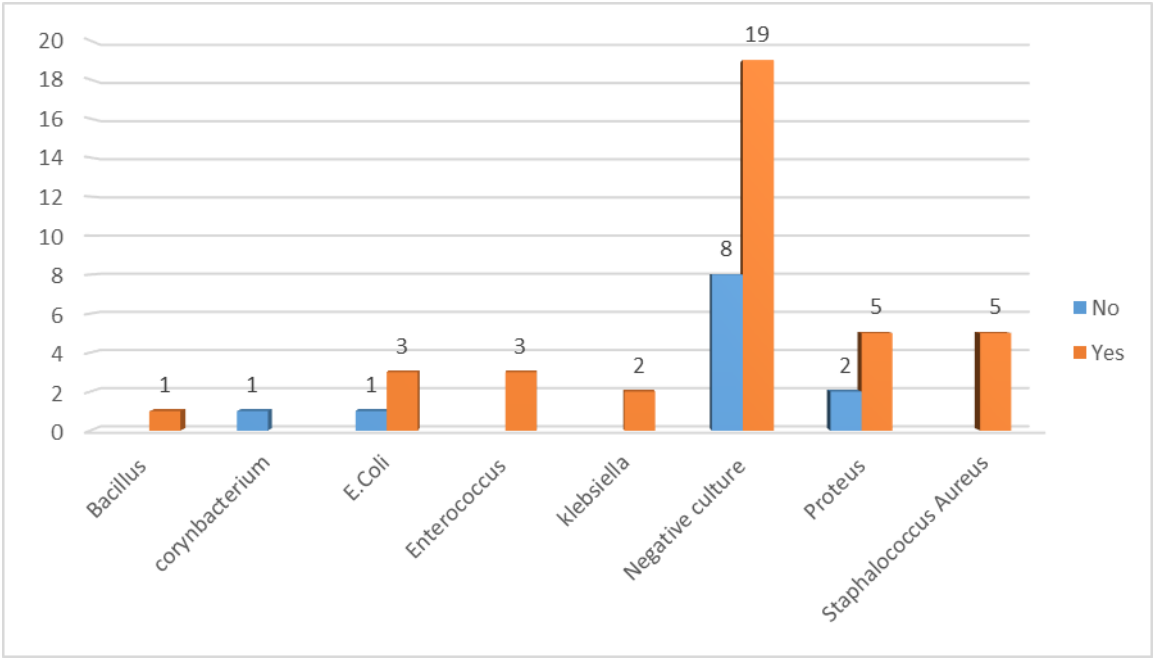

Figure 4. The prevalence of Post ESWL urine culture with the history of UTI

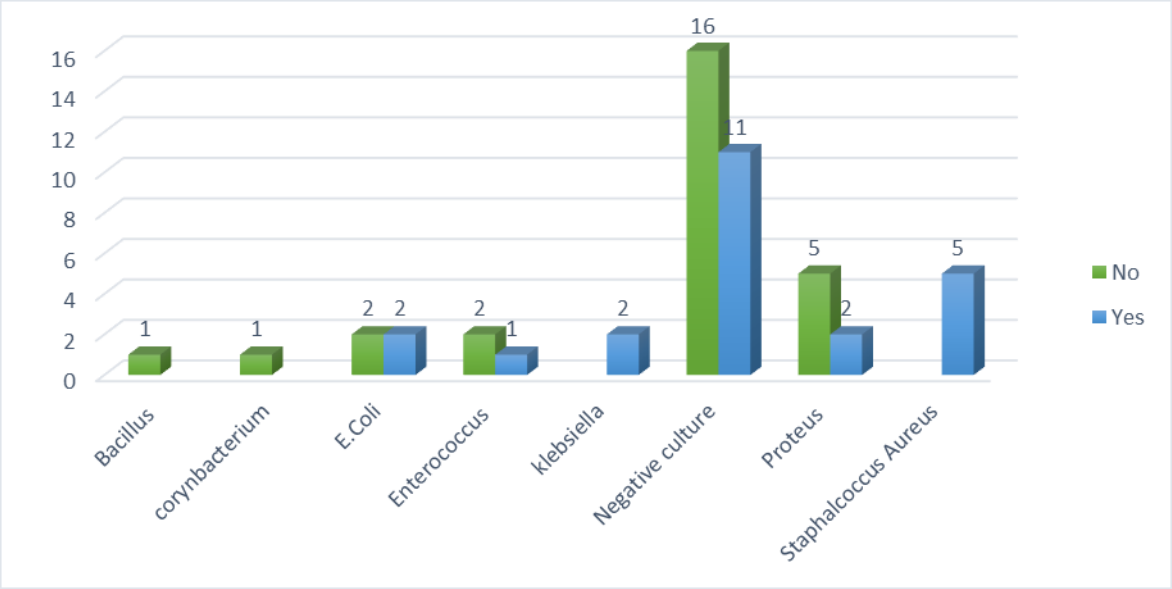

Figure 5. The prevalence of Post ESWL urine culture with history of previous surgery

Discussion

Clearly controversy exists regarding the need for antibiotic prophylaxis with ESWL as underlined by the differences in the AUA and EAU guidelines for antimicrobial prophylaxis. The AUA Best Practice Policy Statement on Urological Surgery Antimicrobial Prophylaxis states that antibiotic prophylaxis is indicated in all patients with a duration of therapy of 24 hours or less. This recommendation is based on a meta-analysis by Pearle and Roehrborn evaluating 8 randomized controlled trials and 6 clinical trials [(14)], their meta-analysis of these 8 RCTs demonstrated a reduction in the median probability of UTI after SWL from 5.7% in the no treatment arms to 2.1% in the antibiotic treatment arms. However, there are several key limitations with this meta-analysis and the RCTs that comprise it. Alternatively, the EAU Guidelines on Urological Infections only recommend prophylaxis in cases of "internal stent and treatment, due to the increased bacterial burden (e.g. indwelling catheter, nephrostomy tube, or infectious stones)" [(18)]. In this prospective single cohort study of 50 patients with Pre-& after ESWL urine culture without receiving antibiotics prophylaxis; in this research analyzed risk factors that could be related to a positive urine culture. The factors considered were gender, age, diabetes mellitus, arterial hypertension, and history of previous surgery (including Double J stent and Nephrostomy tube), personal history of UTI, stone size and stone location; Found that the patients having history of UTI and history of previous surgery with determining the patients had a history of Double J tube in case of ureteral stone and nephrostomy tube as a part of previous surgery is significant according to the statistical analysis of this study (P=0.04); this study support the EAU guidelines that no routine prophylaxis is necessary for patients undergoing ESWL with sterile urine cultures. However, that the patients with a history of UTI and a history of previous surgery (with ureteral stent or nephrostomy tube) recommended antibiotic prophylaxis prior to ESWL. Further supporting the notion that antibiotic prophylaxis is not required in all patients undergoing ESWL. In this prospective study revealed that the gender, age, number of stone attacks, the size of stone was an independent risk factor for significant urine culture. This current study had some limitations, that had no comparison group with prophylactic antibiotic treatment. It would

have been useful to determine whether the rates of bacteriuria and symptomatic urinary tract infection were or were not similar. Moreover, this study could not determine whether the presence of a Double-J stent before ESWL is an independent risk factor for bacteriuria after ESWL, although this is considered a risk factor by the AUA and EAU [13,14]. Perhaps it should increase the population to determine that fact. Another limitation is that it could not determine the risk factors associated with symptomatic infections and serious events. This analysis was not possible due to the low incidence of these events. Accordingly, future studies are needed to clarify the appropriate indications for targeted antibiotic prophylaxis before ESWL with Pre-& Post ESWL urine culture, and the ideal choice and duration of antibiotic treatment.

This prospective cohort study revealed the need for universal antibiotic prophylaxis before ESWL as UTIs and ASB are low incidence in patients with negative urine cultures treated with targeted antibiotic Prophylaxis. This suggested that prophylactic antibiotics in these patients is unnecessary with no benefit in reducing infectious complications and may pose the risk of increased bacterial resistance and side effects of antibiotics. This led us to conclude that antibiotic prophylaxis is not justified without defined risk factors such as positive urine culture before ESWL, an external bladder catheter or nephrostomy tube and a history of infectious stones or recurrent urinary tract infection.

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Authors contribution

Dr. Khaleel drafting the article, revising it critically for important intellectual content and made collection of urine sample from the patients Pre-& Post ESWL. Dr. Hassan made a conventional method of urine culture in Microbiology laboratory in the Collage of Medicine, Al-Nahrain university. Dr. Al-Anbary made a statistical analysis of the research and give a final approval of the version of the research to be submitted.

Conflict of interest

Authors declare no conflict of interest.

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Immunohistochemical Expressions of PAX8 in Ovarian Surface Epithelial Tumors

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Abstract

Background PAX8 is a nuclear transcription factor with limited expression in normal and neoplastic tissues, it has the potential to induce tumorigenesis and is expressed in a tissue during neoplastic transformation. PAX8 is a useful marker to distinguish gynecologic cancers from non-gynecologic malignancies.

Objective To assess the immunohistochemichal expression of PAX8 in ovarian surface epithelial tumors.

Methods This study included a total of 100 ovarian tissue paraffin blocks, 70 tissue paraffin blocks of ovarian tumors obtained from patients who underwent total abdominal hysterectomy and bilateral salpingo-oophorectomy and 30 tissue paraffin blocks were the control group included normal ovarian tissue and fallopian tubes tissue. From each paraffin block, 2 sections were taken, one was stained with the routine hematoxylin and eosin stain and the other section was stained immunohistochemically for PAX8.

Results PAX8 showed a high significant difference in its immunohistochemical expression between the control group and the case group with the highest expression in malignant ovarian surface epithelial tumors ($P < 0.001$). Also, PAX8 showed a highly significance in the expression of PAX8 in relation to histopathological types ($P < 0.001$) except for the mucinous tumors, which showed statistically non-significance ($P = 0.641$), also, a highly significant relation with the increment of tumor grade ($r = 0.769$, $P < 0.001$). PAX8 showed a non-significant relation with increase of the age ($r = 0.147$, $P= 0.225$), with tumor stage ($r = 0.433$, $P = 0.057$), with the presence of ascites ($P = 0.446$).

Conclusion The highly significant differences in the immunohistochemical expression of PAX8 in the ovarian surface epithelial tumors tissues compared to the control groups reflect its important role in ovarian tumorigenesis. Besides PAX8 can be used as an important marker for discriminating ovarian non-mucinous from mucinous tumors. Also, there is a possible role of PAX8 in the development and differentiation of ovarian malignant surface epithelial tumor.

Keywords Ovarian surface epithelial tumors, ovarian carcinoma, transcription factor, paired box antigen, PAX8, immunohistochemistry

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List of abbreviations: CIC = Cortical inclusion cysts, EMT = Epithelial-mesenchymal transition, FIGO = Federation of gynecology and obstetrics, H&E = Hematoxylin & Eosin staining, HGSC = High grade serous carcinoma, LGSC = Low grade serous carcinoma, OCCC = Ovarian clear cell carcinoma, STIC = Serous tubal intra-epithelial carcinoma, TCC = Transitional cell carcinoma, WT-1 = Wilms tumor 1 protein

Introduction

Ovarian cancer represents the 6th most commonly diagnosed cancer among women around the world, and each year it cause more deaths than any other cancer of the female reproductive system [(1)]. It is found to be the 2nd most common gynecological malignancy in the United States, and it is the leading cause of death in more than 13,000 each year in the United States. The risk that a woman can develops ovarian cancer is 1/71 [(2,3)]. In Iraq, ovarian tumors rank the 6th commonest cancer among females and

constituted 4.1% according to latest published Iraqi Cancer Board Registry in 2011 [4].

Surface epithelial tumors represent about 90% of primary ovarian tumors, which can be found solid, cystic or mixture of both, they can be benign, borderline or malignant. These tumors represent 15% of all epithelial ovarian cancers, with the serous and mucinous types making up the vast majority of cases [5]. The ovarian surface epithelium develops from the coelomic epithelium (mesothelium) that covering the embryonic gonad. This epithetlium is continuous with the coelomic epithelium that penetrates the underlying mesenchyme to form the Müllerian duct. This embryonic close proximity is reflected in the various directions of Müllerian differentiation, which manifested by the ovarian surface epithelium when it undergoes neoplasia, such as tubal-type epithelium in serous neoplasms, endometrial epithelium in endometrioid tumors, and endocervical epithelium in at least some mucinous neoplasms [6].

PAX8 is a nuclear transcription factor with limited expression in normal and neoplastic tissues in a cell lineage-dependent manner [7]. It is a member of the PAX gene family, consisting of nine well-described transcription factors (PAX1-9) [8]. PAX8 is important in embryogenesis of the thyroid, Müllerian, and renal/upper urinary tracts, it's also regulates Wilms tumor suppressor gene (WT1) expression [9,10]. In the pancreas, PAX proteins play a critical role in islet cell differentiation [11]. PAX8, initially detected in ovarian tumors and is characteristic for the epithelial histotypes (serous, clear cell, and endometrioid). So, positive expression of PAX8 represents a strong argument for the confirmation of the origin of ovarian carcinoma arising from the fimbrial area of Fallopian tubes or from an endometriosis foci. Moreover, PAX8 allows the differentiation between Müllerian and non-Müllerian origin which could be derived from a primary tumor in pancreas, colon or mammary glands [12]. While the PAX8 expression is useful in the diagnosis of ovarian cancers, it failed to show any prognostic role in an analysis of 148 serous ovarian carcinoma [13].

The objectives of this study were to assess the immunohistochemichal expressions of transcription factor (PAX8) in ovarian surface epithelial tumors (including benign, borderline and malignant) and to study its relationship with the age of the patient, presence or absence of ascites, tumor histopathological type, tumor grade and tumor stage.

Methods

A retrospective study included a total of 100 ovarian tissue paraffin blocks. Seventy tissue paraffin blocks were assigned as the case group included ovarian tumors obtained from patient who underwent total abdominal hysterectomy and bilateral salpingo-oophorectomy for malignant tumors, and ovarian cystectomy for benign tumors. These blocks were collected from Teaching laboratories of Medical City, Al-Yarmook Teaching Hospital and private laboratories from January 2015 to April 2017. Thirty tissue paraffin blocks were assigned as the control group included normal ovarian tissue, inclusion cysts, corpus luteal cysts, follicular cysts, endometriosis (chocolate cyst), and fallopian tubes tissue, these blocks were collected from Medical City, Teaching laboratories from November 2016 to January 2017.

The clinico-pathological parameters including (age of patient, ascites, tumor type, and tumor grade and tumor stage) were obtained from patients' admission case sheets and pathology reports.

From each paraffin block, 2 sections were taken, each of 5 µm thickness. One was stained with the routine Hematoxylin & Eosin (H&E) stain examined and the histopathological diagnosis, tumor histological type and grade according to FIGO system were revised by a pathologist [14]. The other section was deparaffinized and rehydrated at room temperature, Antigen retrieval by Antigen retrieval citrate buffers pH 6.0 [DAKO, Denmark] was carried out by microwave 1x10

min then allowed to cool for 20 mins. Mouse monoclonal PAX8 antibody [PAX8R1] (ab53490) (Abcam, UK (dilution 5 µg/ml) applied to sections and were incubated for an overnight. Expose mouse and rabbit specific HRP/DAB detection IHC kit (ab80436) (Abcam, UK) reagents was used [15]. Counterstaining of the sections by Mayer's Hematoxylin stain for 20-30 seconds then followed by mounting of the sections by using Roti®-Mount Aqua (ROTH, Germany) followed by glass coverslip. Technical negative control was done by the omission of PAX8 antibody.

Interpretation of immunohistochemistry staining and quality control for PAX8

Immunohistochemically stained slides were reviewed and positive staining was based on the presence of nuclear expression, the staining expressions were scored with assessment of both staining percentage and staining intensity under the light microscopy as follow:

A. The intensity: the intensity of positivity was scored as follows:

0: no staining,
1: weak,
2: moderate,
3: strong.

B. The percentage: The extent of positivity was scored according to the percentage of cells showing positive staining as follows:

- 0% is 0, negative
- 1-25% is 1+
- 26-50% is 2+
- 51-75% is 3+
- 76-100% positive cells is 4+.

These values [intensity and the percentage] then were multiplied, a final score with possible range of values from 0 to 12 was obtained. The scores = or > 1 were considered as positive [16].

Statistical analysis

Numerical data were presented as mean ± standard error, unpaired t-test was used to compare means between two groups while analysis of variance (ANOVA) was used in case if number of groups was more than two. Categorical data were presented as number and percentage. the comparison of number between different groups was done using Fisher exact test and chi square test. Pearson correlation was done between different parameters and presented as r (correlation coefficient) and p value (level of significance). P value < 0.05 was considered significant. The software used was Microsoft excel 2016 and SPSS (statistical package for social sciences) version 23.

Results

Among the 70 cases of ovarian surface epithelial tumors; 13 cases (65%) of benign tumors showed positive expression for PAX8, borderline tumors, 25 cases (83.3%) showed positive expression for PAX8, and the malignant tumors, 17 cases (85%) showed positive expression for PAX8 marker, while out of 30 cases of control group, only 2 cases (6.7%) showed positive expression of PAX8. According to that, there is a highly significant difference in the expression of PAX8 between the control group and the case group with the highest expression seen in malignant ovarian surface epithelial tumors (P < 0.001) (Table 1).

According to semiquantitive scoring system, the control group showed the lowest immunohistchemical expression of PAX8 (Mean=0.1, SE= 0.07), while the ovarian surface epithelial tumors scoring showed higher results, in which malignant ovarian surface epithelial tumors showed the highest immunohistchemical expression of PAX8 (Mean= 9.3, SE= 1.01) , the borderline tumors immunohistchemical expression of PAX8 was (Mean= 6.13, SE= 0.66) and the benign tumors showed the lowest immunohistchemical expression of PAX8 among the ovarian surface epithelial tumors (Mean= 4.5, SE= 0.94), according to that, there is a highly significant difference in the immunohistochemical expression of PAX8 according to the semiquantitive scoring between the control

group and the case group with the highest expression seen in the malignant ovarian surface epithelial tumors (P < 0.001) (Table 2).

Table 1. Frequency of the positive and negative expression of PAX8 in case and control groups

Marker	Expression	Control No. (%)	Benign No. (%)	Borderline No. (%)	Malignant No. (%)	P value
PAX8	Positive	2 (6.7)	13 (65.0)	25 (83.3)	17 (85.0)	< 0.001**
	Negative	28 (93.3)	7 (35.0)	5 (16.7)	3 (15.0)	

**: high statistically significant

Table 2. Scores of immunohistochemical expression of PAX8 in control and ovarian tumors

Marker	Control N=30 Mean±SE	Benign N=20 Mean±SE	Borderline N=30 Mean±SE	Malignant N=20 Mean±SE	P value
PAX8	0.1±0.07	4.5±0.94	6.13±0.66	9.3±1.01	< 0.001**

**: high statistically significant

According to the semiquantitive scoring PAX8 showed a high significant difference among benign, borderline and malignant ovarian surface epithelial tumors in relation to histopathological types (P < 0.001) except for the mucinous tumors which showed non-significant difference in the immunohistochemical expression of PAX8 according to the semiquantitive scoring (P=0.641). Also, a highly statistically significant difference in the immunohistochemical expression of PAX8 according to the semiquantitive scoring among serous, mucinous and others histological subtypes in relation to histological classification of ovarian surface epithelial tumors in to benign, borderline and malignant (P < 0.001) (Table 3).

Among the 70 cases of ovarian epithelial tumors collected during the study, papillary serous cystadeno-carcinoma and serous adenocarcinoma showed the highest immunohistochemical expression score of PAX8 (Mean= 11.6, SE= 0.4) (Figure 1), followed by other histopathological subtypes [endometrioid adenocarcinoma (Figure 2), malignant brenner tumors, clear cell carcinoma, and transitional cell carcinoma] (Mean= 11.0, SE= 0.63), while in the mucinous cystadenocarcinoma was with (Mean= 1.0, SE= 1.0) (Table 3).

PAX8 expression in borderline tumors was lower in serous tumors (Mean= 8.16, SE= 0.45) (Figure 3), mucinous (Mean= 0.43, SE= 0.3) and other histological subtypes (Mean= 6.5, SE= 0.5) (Figure 4). Benign serous tumors PAX8 expression was (Mean= 8.2, SE= 0.66) while benign mucinous cystadenoma and the other histological category showed the lowest expression of PAX8 (Table 3).

Regarding other clinicopathological parameters, PAX8 showed a highly significant relation with the increment of tumor grade (r = 0.769 and P < 0.001). PAX8 showed a non-significant relation with increase of the age (r = 0.147, P= 0.225), with tumor stage (r = 0.543, P = 0.013) (Table 4).

There was no significant association found with the presence of ascites (P = 0.446) (Table 5).

Table 3. Score of the expression of PAX8 in relation to histopathological type of ovarian surface epithelial tumors

Histopathology	Benign Mean±SE	Borderline Mean±SE	Malignant Mean±SE	P value
Serous	8.2±0.66	8.16±0.45	11.6±0.4	**< 0.001****
Mucinous	0.25±0.25	0.43±0.3	1.0±1.0	0.641 NS
Others	1.17±0.75	6.5±0.5	11.0±0.63	**< 0.001****
P value	**< 0.001****	**< 0.001****	**< 0.001****	

**: high statistically significant, NS: Non-statistically significant. Others = Endometrioid tumors, Brenner tumors, clear cell tumors, and transitional carcinoma

Table 4. Relation of PAX8 expression with age, tumor grading, tumor staging

Parameter	PAX8 score	
	r	P
Age (years)	0.147	0.225 NS
Tumor grade (FIGO)	0.769	**< 0.001****
Tumor stage (FIGO)	0.433	0.057 NS

**: high statistically significant, NS: Non-statistically significant.

Table 5. Relation of PAX8 expression scores with presence of ascites

Marker	Ascites		P value
	Positive N=33 Mean±SE	Negative N=37 Mean±SE	
PAX8 score	7.0±0.82	6.19±0.69	0.446 **NS**

NS: Non-statistically significant

Discussion

PAX8 plays an important role in the tumorigenic phenotype of ovarian cancer cells [8]. Among the 70 cases of ovarian epithelial tumors; 13 cases (65%) of benign tumors showed positive expression for PAX8, borderline tumors, 25 cases (83.3%) showed positive expression for PAX8, and the malignant tumors, 17 cases (85%) showed positive expression for PAX8 marker. For the control group; out of 30 cases, only 2 cases (6.7%) showed positive expression of PAX8. According to the results of this study, there is a highly significant difference in the expression of PAX8 between the control group and the case group with the highest expression seen in malignant ovarian surface epithelial tumors (P < 0.001).

The current study showed much lower level expression of PAX8 in control group than a study performed by Adler et al. which showed that PAX8 was expressed in 44% of the ovarian surface epithelial cells, while it was negative in 56% of normal ovarian tissues and all stromal tissues [17].

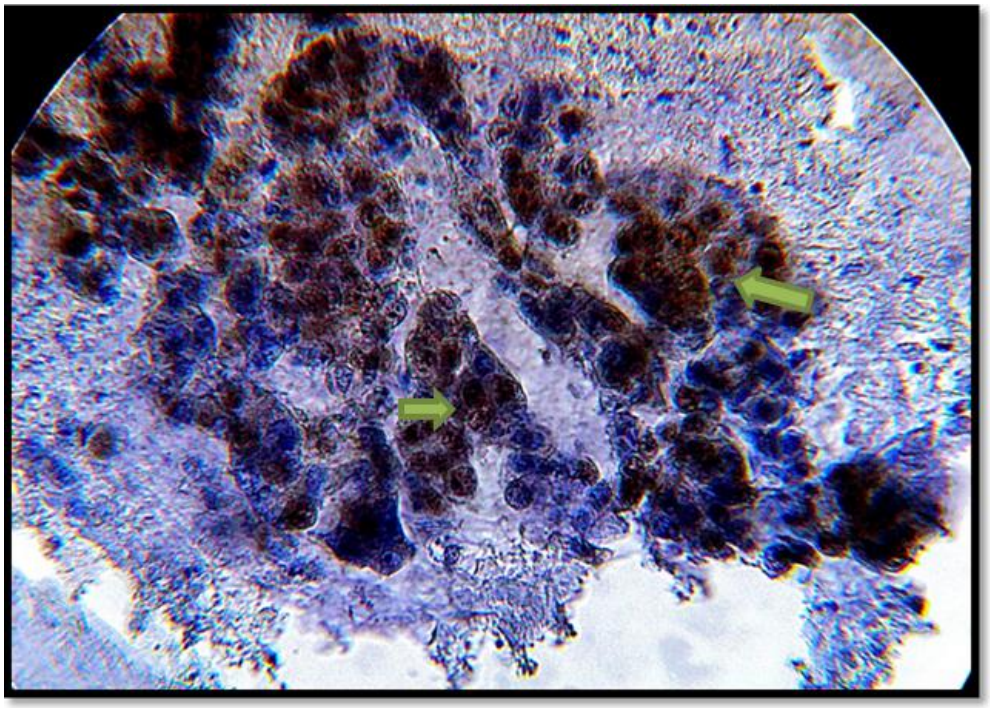

Figure 1. Serous adenocarcinoma (FIGO grade III) tissue section showing positive brown nuclear expression of PAX8 monoclonal antibody of 95% of epithelial cells with strong intensity and score of 12 (arrows) (40×)

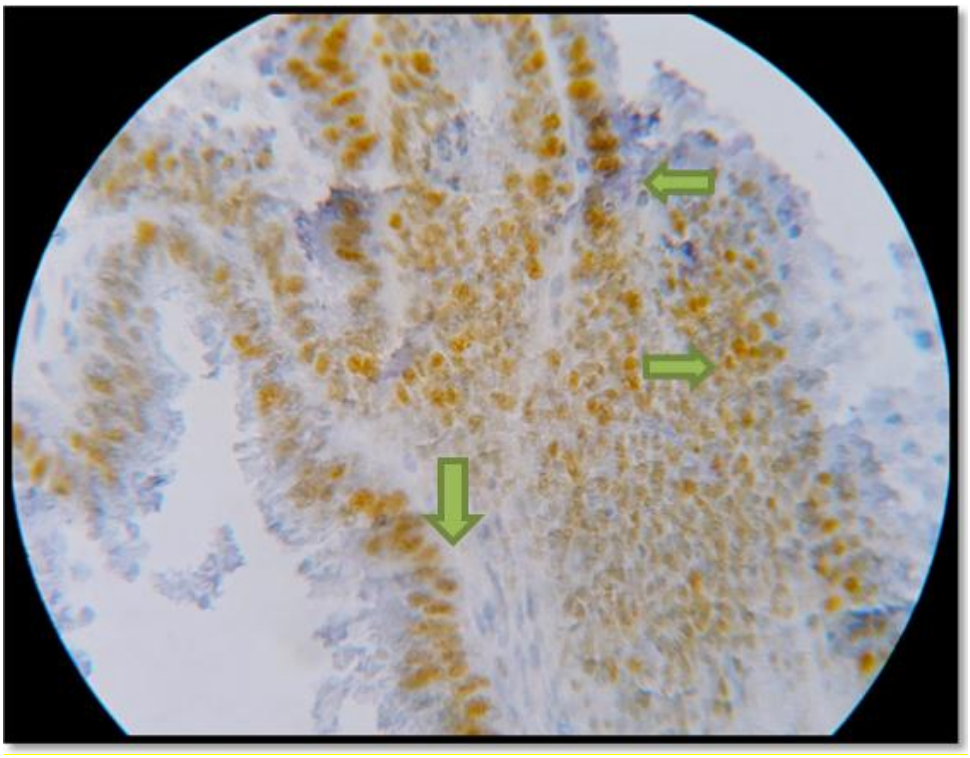

Figure 2. Endometrioid adenocarcinoma (FIGO grade I) tissue section showing positive brown nuclear expression of PAX8 monoclonal antibody of 85% of epithelial cells with moderate intensity and score of 8 (arrows) (40×)

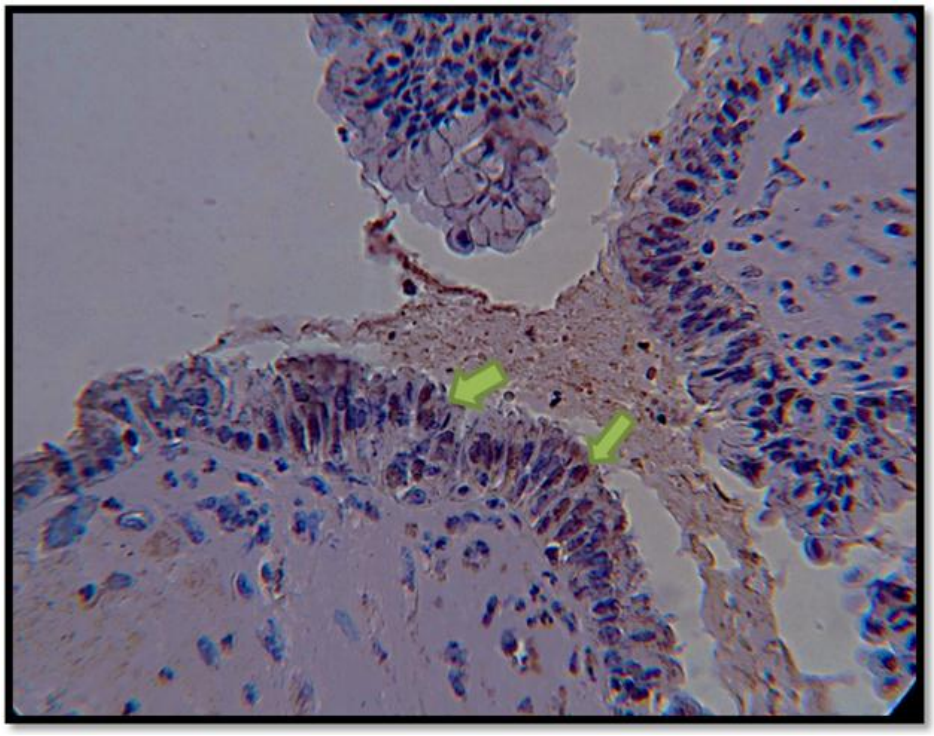

Figure 3. Borderline serous tumor tissue section showing positive brown nuclear expression of PAX8 monoclonal antibody of 45% of epithelial cells with moderate intensity and score of 4 (arrows) (40×)

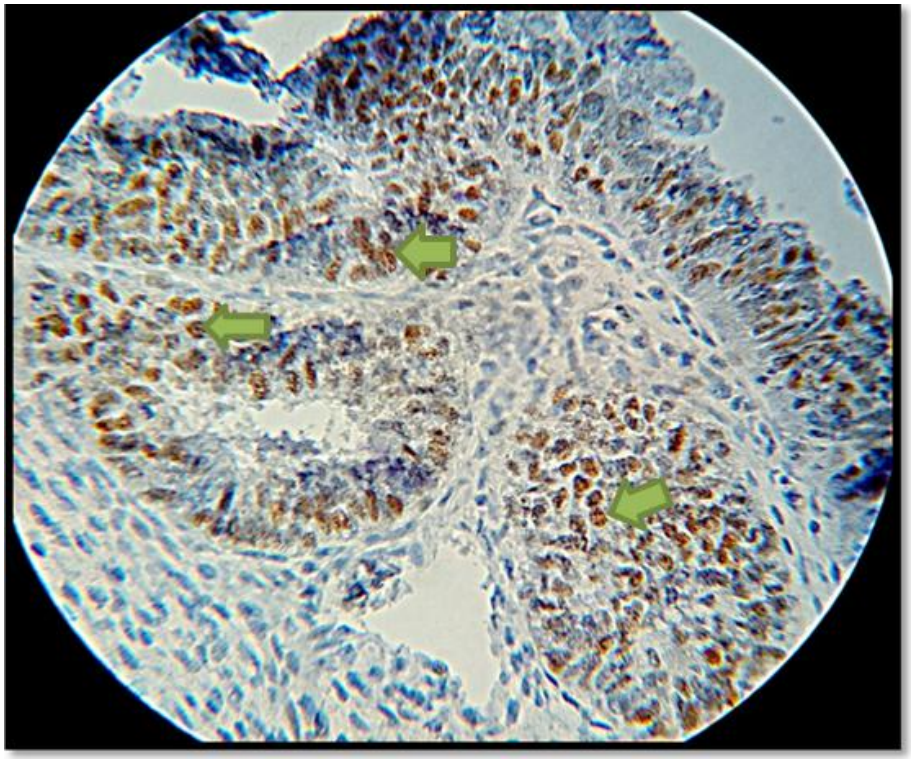

Figure 4. Borderline endometrioid tissue section showing positive brown nuclear expression of PAX8 monoclonal antibody of 55% of epithelial cells with moderate intensity and score of 6 (arrows) (40×)

This may be attributed to that in Adler et al. [17] study, PAX8 expression was evaluated in 27 histologically normal ovaries that contained ovarian surface epithelial cells present on the surface while in current study not all the 30 sections contained epithelial surface cells, so the variation in the level may be attributed to types of cases included in the study, geographical factor, difference in clone of marker used, and immonohistochemical technique. Parallel to our study results, normal ovarian surface epithelium was uniformly negative in the Bowen et al. [18] study analysis, which also demonstrate that PAX8 is expressed in the epithelial ovarian cancer cell line (OVCAR-3) and this support the hypothesis that PAX8 plays equivalent role in the development of epithelial ovarian cancer. In contrast to our study normal ovarian surface epithelium was positive in 62% of Turkish study by Ozcan et al. [20], which showed that PAX8 are constantly expressed in normal or non-neoplastic tissue of mullerian origin and parallel to this study results, Ozcan et al. [19] demonstrated that strong and diffuse staining of PAX8 was found in most cases of the primary ovarian epithelial tumors except for the mucinous type. The differences attributed to differences in sample size in which Ozcan et al study involved very large number samples, multiple organs were involved, difference in clone of marker used, and immonohistochemical technique.

In the current study among the 70 cases collected, papillary serous cystadenocarcinoma and serous adenocarcinoma showed positive expression of PAX8 with the highest immunohistochemical expression level for PAX8. Mucinous tumors showed the lowest immunohistochemical expression levels of PAX8 among the ovarian surface epithelial tumors. A highly significant association of PAX8 expression in relation to histopathological types ($P < 0.001$) except for the mucinous tumors which showed statistically non-significance ($P = 0.641$).

An American study performed by Nonaka et al. found that PAX8 serves as an important marker for discriminating ovarian nonmucinous surface epithelial carcinomas, in which PAX8 reaction was found in 87.1% ovarian surface epithelial carcinomas, the commonest was serous papillary carcinomas, followed by endometrioid carcinomas, clear cell carcinomas, and mucinous carcinomas [10]. The results of the immunohistochemistry stains of this study was 85%, which is parallel to Nonaka et al. [10] study, in which 87.1% of ovarian carcinomas positive for PAX8. Most of the tumors in the current study were of serous type, followed by ovarian non-mucinous tumors subtypes, which was parallel to Nonaka et al. [10] study. Serous malignant tumors showed highest expression levels of PAX8 followed by endometrioid carcinoma and clear cell carcinoma. The current study was parallel to Nonaka et al. [10] study, in which the mucinous carcinoma type showed the lower expression levels of PAX8. In Ozcan et al. [19] study PAX8 showed strong and diffuse staining in most cases of all histologic subtypes, except in mucinous tumors, this study agrees with the current one [19].

The current study showed a non-significant relation between the expression of PAX8 and increase of the age ($r = 0.147$ and $P = 0.225$). Due to the limited number of articles that consider the relation of PAX8 and age parameters we reviewed its relation in other organs, in which we found that the results of the current study go parallel to the Austrian study performed by Brunner et al. on the immunoexpression of PAX8 in endometrial cancer and its relation to high grade carcinoma, which found that no significant correlation was observed between PAX8 and patient age [21]. Also parallel to other American study performed by Long et al. on PAX8 expression in well-differentiated pancreatic endocrine tumors, and its correlation with clinicopathologic features showed that PAX8 expression was not correlated with patient age [11].

Regarding the relation of PAX8 expression semiquantitive scoring and tumor grade in the current study, there is high significant relation between the expression of PAX8 and increase of tumor grade ($r = 0.769$ and $P < 0.001$). Ozcan et al. mentioned that PAX8 was much more pronounced for high-grade carcinomas (99%) than for cystadenomas/borderline tumors

(96%), however they noted that the PAX8 expression is independent of tumor grade, at least for serous tumors [19]. Laury et al. mentioned that in the ovary about 92% of endometrioid carcinomas were positive for PAX8, and the higher the tumor grade, the more diffuse and stronger PAX8, however, they mentioned that there was no obvious relation with tumor grade in mullerian carcinomas as the majority of tumors of various subtypes were positive for PAX8 regardless tumor grade [9]. While a positive correlation was observed between PAX8 and histologic grade in both Brunner et al. [20] (P=0.02) and Mhawech-Fauceglia et al. [21] (P=0.002) in endometrial carcinoma.

Regarding the relation of PAX8 immunohistochemical expression and tumor stage, there was no significant relation between the PAX8 expression and increase of tumor stage (r = 0.433 and P = 0.057). The results of this study go parallel with the results of an American study performed by Mhawech-Fauceglia et al., which found that though PAX8 was expressed in 61% cases of 148 serous with late stage ovarian carcinoma, there was no association between PAX8 and tumor stage [22]. Also, was parallel to the Malaysian study performed by Rhodes et al. [23], which noticed that the expression of PAX8 in the ovarian serous and endometrioid subtypes was not associated with stage and they regard this to the small number of cases, and the majority being with late stage.

Regarding the relation of PAX8 expression and presence of ascites, among the 70 cases of ovarian surface epithelial tumors, there were 33 cases present with ascites and PAX8 expression score of (Mean = 7.0, SE = 0.82). According to that, there is non- significant relation between the expression of PAX8 and presence of ascites with P = 0.446. One study performed by Ayantunde and Parsons mentioned that ascites is an independent prognostic factor at presentation, and > 1/3 of women with ovarian cancer will develop ascites during their disease course, and this is not limited to a specific histological subtype [24]. Also, the American study performed by Mhawech-Fauceglia et al. found that there was no correlation between PAX8 expression level and tumor stage, and it failed to predict a value in disease outcome [21]. This support that there is no correlation between PAX8 expression levels and the presence of ascites.

In conclusion, the current study shows a highly significant differences in the immunohistochemical expression of PAX8 in ovarian surface epithelial tumors tissues compared to control groups, which in turn reflects the important role of PAX8 in ovarian carcinoma development. Also, PAX8 immunohistochemical expression was highest in ovarian epithelial tumors of serous type and lowest in the mucinous type, so that it can be used as an important marker for discriminating ovarian non-mucinous from mucinous tumors. The significant relation of PAX8 expression with higher tumor grade, may refer to the possible role of PAX8 in the development and differentiation of ovarian malignant surface epithelial tumor, which in turn suggests that PAX8 may promote the progression of ovarian cancer, through activation of the anti-apoptotic genes which promote increased cell proliferation through upregulation of epithelial-mesenchymal transition (EMT).

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Authors' contribution

Dr. Al-Shami collected the cases data, performed the routine H&E and the immunohistochemical technique and analyzed the results of the study. Dr. Qasim helped in study design and supervising the work. Dr. Hassan participated in the immunohistochemical scoring of the histopathological sections.

Conflict of interest

The authors declare no conflict of interest.

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Immunohistochemical Expression of CD133 in Ovarian Surface Epithelial Tumors

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Abstract

Background The identification of antigenic profile of the cancer stem cells might have relevant clinical implication as they're able to proliferate and self-renew, in turn sustaining tumor growth. CD133 is one of robust surface marker for cancer stem cells in various neoplastic human tissues, including ovaries. It is associated with the clinical outcome of patients.

Objective To assess the immunohistochemichal expression of cancer stem cell marker (CD133) in ovarian surface epithelial tumors.

Methods This study included a total of 100 ovarian tissue paraffin blocks, 70 tissue paraffin blocks included ovarian tumors obtained from patient who underwent total abdominal hysterectomy and bilateral salpingo-oophorectomy, while 30 tissue paraffin blocks assigned as the control group included normal ovarian tissue and fallopian tubes tissue. From each paraffin block, 2 sections were taken, one was stained with hematoxylin and eosin stain and the other section was stained immunohistochemically for CD133.

Results CD133 showed a high significant difference in its immunohistochemical expression between the control group and the case groups with the highest expression seen in malignant ovarian surface epithelial tumors ($P < 0.001$). CD133 expression was highly significantly associated with histopathological type ($P < 0.001$). CD133 also showed a significant relation to age ($r = 0.254$, $P= 0.034$), tumor grade ($r = 0.794$, $P < 0.001$), tumor stage ($r = 0.543$, $P= 0.013$) and presence of ascites ($P < 0.001$).

Conclusion CD133 expression revealed a highly significant differences in the ovarian surface epithelial tumors tissues compared to control group, which reflect its important role in ovarian carcinogenesis.

Keywords Ovarian surface epithelial tumors, ovarian carcinoma, cancer stem cells, cancer stem cell marker, CD133, immunohistochemistry

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List of abbreviations: CD133 = Cancer stem cell marker, CSCs = Cancer stem cells, EMT = Epithelial-mesenchymal transition, FIGO = Federation of gynecology and obstetrics, HGSC = High grade serous carcinoma, IL = Interleukin, LDH = Lactate dehydrogenase, LGSC = Low grade serous carcinoma, OCCC = Ovarian clear cell carcinoma, STIC = Serous tubal intra-epithelial carcinoma, TCC = Transitional cell carcinoma, TICs = Tumor initiating cells, TNF-α = Tumor necrosis factor - alpha, VEGF = Vascular endothelial growth factor

Introduction

Ovarian cancer is one of the most common causes of gynecologic neoplasm. The high mortality rate in women with ovarian cancer is due to its detection at advanced stages. Even though there have been improvements in surgical techniques and treatment options, five-year survival for ovarian cancer still remain at approximately 45% [(1)]. Ovarian cancer represents the sixth most commonly diagnosed cancer among women in the world, and causes more deaths per year than any other cancer of the female reproductive system [(2)]. In Iraq,

ovarian tumors rank the 6th commonest cancer among females and constituted 4.1% according to latest published Iraqi Cancer Board Registry in 2011 [3].

Surface epithelial tumors represent about 90% of primary ovarian cancers they may be solid, cystic or mixture of both, may be benign, borderline or malignant [4]. They are traditionally classified into four main histological subtypes: serous, endometrioid, clear cell and mucinous carcinoma. Serous ovarian carcinoma is account for ~70% of epithelial ovarian cancers from which, the high grade serous ovarian carcinoma (HGSOC) accounts for two-thirds of all ovarian cancer deaths making it, by far the most extensively studied ovarian carcinoma.5

Cancer stem cells (CSCs) are a class of pluripotent cells that have been observed in most types of solid and hematologic cancers. It has been shown to be involved in tumor development, cell proliferation, and metastatic dissemination, while possessing a capacity for sustained self-renewal, it exhibits resistance to chemotherapy and radiotherapy [6]. The first time in which the presence of cancer stem cells (CSCs) in ovarian cancer was confirmed is in a multilayered spheroid cells that had been isolated from patient ascites and subsequently verified in a mouse model using side population phenotype [7]. It has been found that epithelial–ovarian cancer stem cells (EOC stem cells) are the source of metastatic progenitor cells through a differentiation process involving epithelial-mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET) [8].

CD133 (prominin-1), a 5-transmembrane glycoprotein that has been used as a stem cell marker in various normal and neoplastic human tissues, including the ovary. CD133 expression as a putative marker for cancer stem cells in human malignant tumors, including ovarian cancer, may define a subpopulation of tumor-initiating cells and is associated with the clinical outcome of patients. However, its clinical significance in ovarian cancer remains uncertain at this time [9]. Ferrandina et al. first indicate that CD133 may be a marker of ovarian CSC through analyzing the expression of CD133 in 41 ovarian tumors, 8 normal ovaries, and 5 benign ovarian tumors. They found that primary ovarian cancer CD133+CK7+ cells had greater colony forming potential and had a higher proliferative potential than CD133−CK7+ cells [10].

This study aimed to assess the immunohistochemichal expression of cancer stem cell marker (CD133) in ovarian surface epithelial tumors (benign, borderline, malignant tumors) and its correlation to clinicopathological parameters, which may reflect CD133 important role in ovarian carcinogenesis, migration, invasion and tumorigenic ability of ovarian cancer cells.

Methods

A retrospective study was intended, which included a total of 100 ovarian tissue paraffin blocks. Seventy tissue paraffin blocks were assigned as the case group included ovarian tumors obtained from patient who underwent total abdominal hysterectomy and bilateral salpingo-oophorectomy for malignant tumors, and ovarian cystectomy for benign tumors. These blocks were collected from Teaching Laboratories of Medical City, Al-Yarmook Teaching Hospital and private laboratories from January 2015 to April 2017. Thirty tissue paraffin blocks were assigned as the control group included normal ovarian tissue, inclusion cysts, corpus leuteal cysts, follicular cysts, endometriosis (chocolate cyst), and fallopian tubes tissue, these blocks were collected from Medical City, Teaching Laboratories from November 2016 to January 2017.

The clinico-pathological parameters including (age of patient, ascites, tumor type, and tumor grade and tumor stage) were obtained from patients' admission case sheets and pathology reports.

From each paraffin block, 2 sections were taken, each of 5 µm thickness. One section was stained with the routine hematoxylin and eosin

stain and the histopathological diagnosis, tumor histological type and grade according to FIGO system were revised by a pathologist [11]. The other section was deparaffinized and rehydrated at room temperature, antigen retrieval by antigen retrieval citrate buffers pH 6.0 [DAKO, Denmark] was carried out by microwave 1x10 min then allowed to cool for 20 min. Mouse monoclonal CD133 (MBS415235) (MyBioSource, USA) (dilution 1:200) applied to sections and were incubated for an overnight. Expose mouse and rabbit specific HRP/DAB detection immunohistochemistry kit (ab80436) (Abcam, UK) reagents was used [12]. Counterstaining of the sections by Mayer's Hematoxylin stain for 20-30 seconds then followed by mounting of the sections by using Roti®-Mount Aqua (ROTH, Germany) followed by glass coverslip. Technical negative control was done by the omission of Anti- CD133 antibody.

Interpretation of immunohistochemistry staining and quality control for CD133

Apical or diffuse cytoplasmic and membranous brown staining of the ovarian epithelial tumor cells was taken as positive results [13,14]. A tissue section of colorectal adenocarcinoma was taken as a positive quality control tissue, while by omission of primary antibody technical negative control was obtained. The immunohistochemical expression of CD133 positivity was analyzed in a semi-quantitative scheme as following:

A. The intensity: the intensity of positivity was scored as follows:

0: no staining,
1: weak,
2: moderate,
3: strong.

B. The percentage: The extent of positivity was scored according to the percentage of cells showing positive staining as follows:

- <10% is 1;
- 11%-50% is 2;
- 51%-75% is 3;
- >75% is 4.

Then the intensity and the percentage of positivity scores were multiplied to obtain the final score, a range from 0 to 12 obtained. the scores >1 were considered as positive [14].

Statistical analysis

Numerical data were presented as mean ± standard error, unpaired t-test was used to compare means between two groups while analysis of variance (ANOVA) was used in case if number of groups was more than two. Categorical data were presented as number and percentage. the comparison of number between different groups was done using Fisher exact test and chi square test. Pearson correlation was done between different parameters and presented as r (correlation coefficient) and p value (level of significance). P value < 0.05 was considered significant.

The software used was Microsoft excel 2016 and SPSS (statistical package for social sciences) version 23.

Results

According to frequency of the positive and negative expression of cancer stem cell marker CD133 in case and control groups, there is a highly statistically significant difference in CD133 expression between the control group and the case group with highest expression in the malignant ovarian surface epithelial tumors (P < 0.001) (Table 1).

According to semiquantitive scoring system, the control group showed the lowest immunohistochemical expression of CD133 (Mean = 0.2, SE = 0.11). While the ovarian surface epithelial tumors scoring showed higher results, in which malignant ovarian surface epithelial tumors showed the highest immunohistochemical expression of CD133 (Mean = 10.25, SE = 0.55), the borderline tumors immunohistochemical expression of CD133 was (Mean = 2.87, SE = 0.25) and the benign tumors showed the lowest immunohistochemical expression of CD133 among the ovarian surface epithelial tumors (Mean = 0.25, SE = 0.12) (Table 2). According to that, there is a highly significant difference in

the immunohistochemical expression of CD133 between the case and control groups with the highest scores seen in malignant ovarian surface epithelial tumors (P < 0.001) (Table 2).

Table 1. Frequency of the positive and negative expression of cancer stem cell marker CD133 in case and control groups

Marker	Expression	Control No. (%)	Benign No. (%)	Borderline No. (%)	Malignant No. (%)	P value
CD133	**Positive**	3 (10.0)	1 (5.0)	29 (96.7)	20 (100)	**< 0.001****
	Negative	27 (90.0)	19 (95.0)	1 (3.3)	0 (0.0)	

**: high statistically significant

Table 2. Immunohistochemical expression of CD133 according to the semiquantitive scoring in case and control groups

Marker	Control N=30 Mean±SE	Benign N=20 Mean±SE	Borderline N=30 Mean±SE	Malignant N=20 Mean±SE	P value
CD133	0.2±0.11	0.25±0.12	2.87±0.25	10.25±0.55	**< 0.001****

**: high statistically significant

CD133 showed a highly significant difference in the expression in relation to histopathological type (P < 0.001) and in malignant ovarian epithelial tumors regardless their histopathological types (P < 0.001) (Table 3). Among the 70 cases of ovarian epithelial tumors collected during the study, papillary serous cystadeno-carcinoma and serous adenocarcinoma showed the highest immunohistochemical expression score of CD133 (Mean = 11.3, SE = 0.47) (Figure 1), followed by other histopathological subtypes [endometrioid adenocarcinoma (Figure 2), malignant Brenner tumors, clear cell carcinoma, and transitional cell carcinoma], then the mucinous cystadenocarcinoma was with (Mean = 6.75, SE = 1.11) (Figure 3).

CD133 expression in borderline tumors was lower in serous tumors (Mean = 3.05, SE = 0.33) (Figure 4), mucinous (Mean = 2.0 , SE = 0.0) (Figure 5), and other histological subtypes (Mean = 3.5 , SE = 0.96). Benign tumors showed the lowest CD133 expression while benign mucinous cystadenoma showed negative expression of CD133 (Figure 6, Table 3).

Regarding other clinicopathological parameters, CD133 showed a significant relation between its expression and increase of the age (r = 0.254, P = 0.034), highly significant relation with the increment of tumor grade (r = 0.794, P < 0.001). CD133 showed a significant relation between its expression and tumor stage (r = 0.543, P = 0.013) (Table 4).

Also, a significant relation of CD133 expression with the presence of ascites (P < 0.001) was found (Table 5).

Table 3. Score of the immunohistochemical expression of cancer stem cell marker CD133 in relation to histopathological type of ovarian surface epithelial tumors

Histopathology	Benign Mean±SE	Borderline Mean±SE	Malignant Mean±SE	P value
Serous	0.1±0.1	3.05±0.33	11.3±0.47	**< 0.001 ****
Mucinous	0.0±0.0	2.0±0.0	6.75±1.11	**< 0.001 ****
Others	0.67±0.33	3.5±0.96	10.83±0.75	**< 0.001 ****
P value	0.074 NS	0.140 NS	**0.001 ***	

**: high statistically significant, NS: Non-statistically significant. Others = Endometrioid tumors, Brenner tumors, clear cell tumors, and transitional carcinoma

Table 4. Relation of CD133 score with age, tumor grading, staging

Parameter	CD133 score	
	r	P
Age (years)	0.254	**0.034 ***
Tumor grade (FIGO)	0.794	**< 0.001****
Tumor stage (FIGO)	0.543	**0.013 ***

*: statistically significant **: high statistically significant

Table 5. Relation of CD133 immunohistochemical scores with presence of ascites

Marker	Ascites		P value
	Positive N=33 Mean±SE	Negative N=37 Mean±SE	
CD133 score	6.3±0.82	2.38±0.47	**< 0.001****

**: high statistically significant

Discussion

In the present study, all the malignant tumors showed 100% positive expression for CD133 marker, 96.7% of borderline tumors showed positive expression, and only 5.0% of benign tumors showed positive expression for CD133. For the control group; only 10.0% showed positive expression for CD133. Also, according to semiquantitive scoring system, the control group showed the lowest immunohistochemical expression of CD133. While the ovarian surface epithelial tumors scoring showed higher results, in which malignant ovarian surface epithelial tumors showed the highest expression of CD133. According to that, there is a highly significant difference in the expression of CD133 according to the semiquantitive scoring between the control and case groups with the highest expression in malignant ovarian surface epithelial tumors (P < 0.001). This study was parallel to Ferrandina et al. an Italian study in which there was significant difference in CD133 expression among control group, benign ovarian tumors compared to malignant ovarian tumors [10]. Also parallel to Korean study performed by Kim et al. which compared CD133 expression in benign, borderline, and malignant tumors, and found that the expression levels of CD133 was significantly increased in cancer, compared with benign tumors and borderline tumors with P = 0.00064 [15].

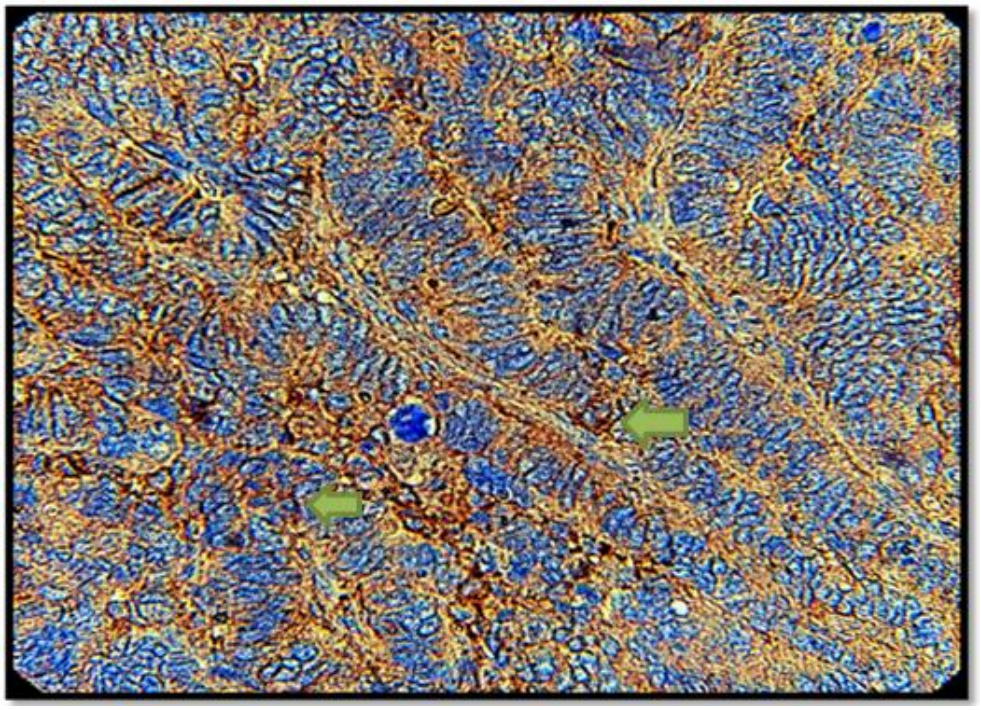

Figure 1. Serous adenocarcinoma (FIGO grade III) tissue section showing positive immunohistochemical diffuse brown cytoplasmic and membranous expression of CD133 monoclonal antibody of > 75% epithelial cells with strong intensity and score of 12 (arrows) (40×)

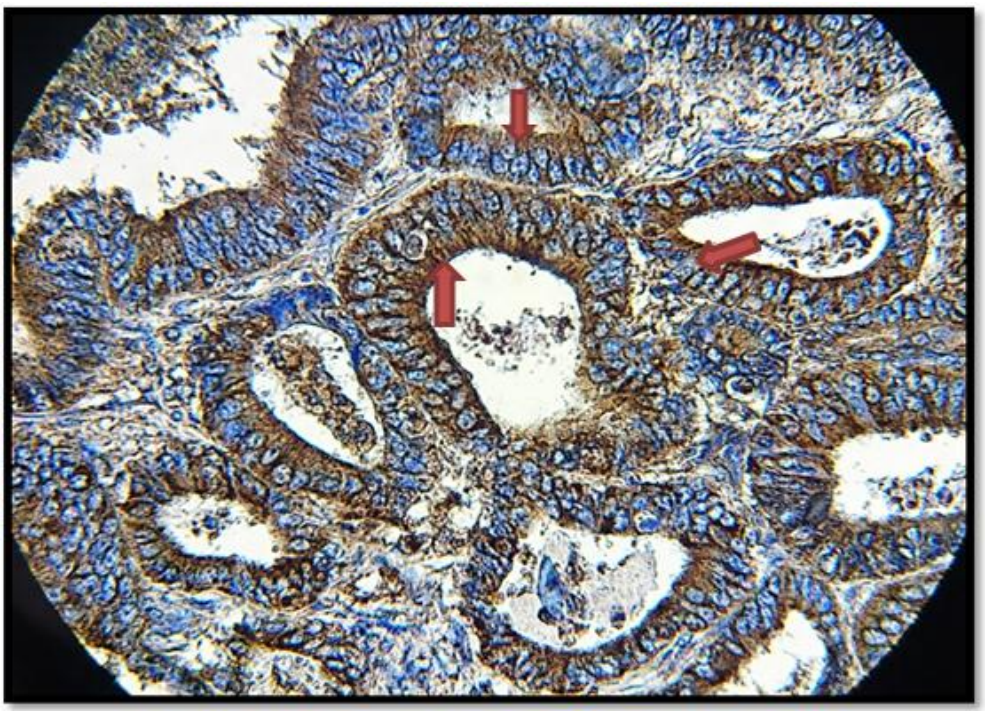

Figure 2. Endometrioid adenocarcinoma (FIGO grade I) tissue section showing positive immunohistochemical diffuse brown cytoplasmic and membranous expression of CD133 monoclonal antibody of 70% epithelial cells with strong intensity and score of 9 (arrows) (40×)

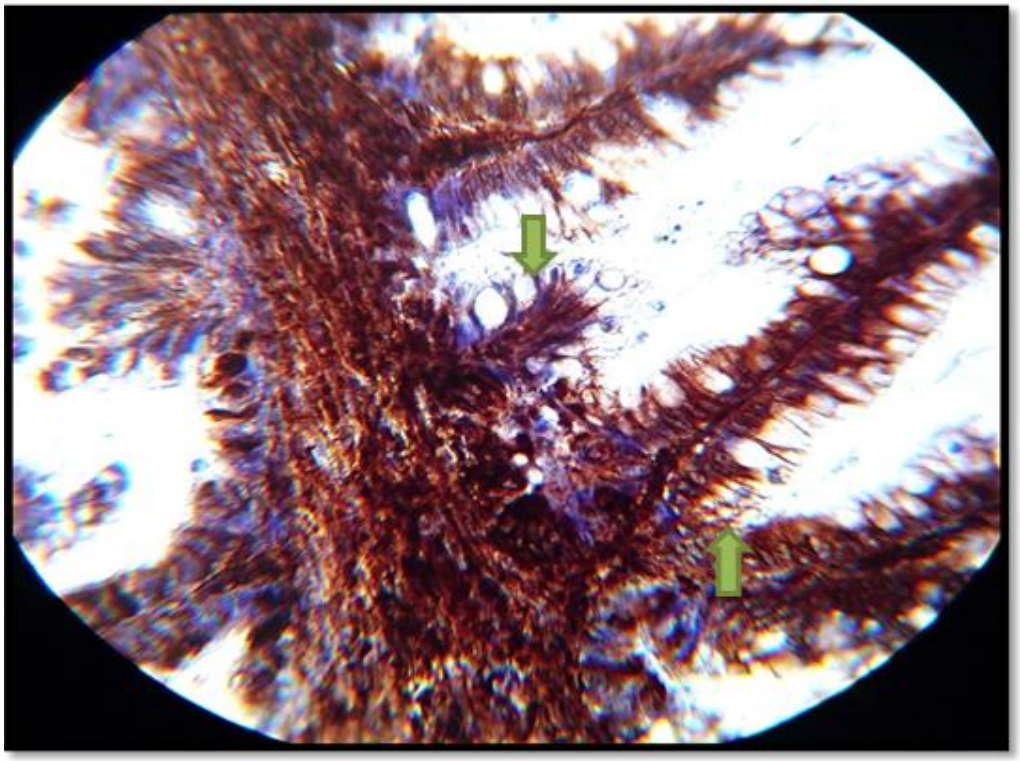

Figure 3. Mucinous adenocarcinoma (FIGO grade I) tissue section showing positive immunohistochemical diffuse brown cytoplasmic and membranous expression of CD133 monoclonal antibody of 60% epithelial and stromal cells with strong intensity and score of 9 (arrows) (40×)

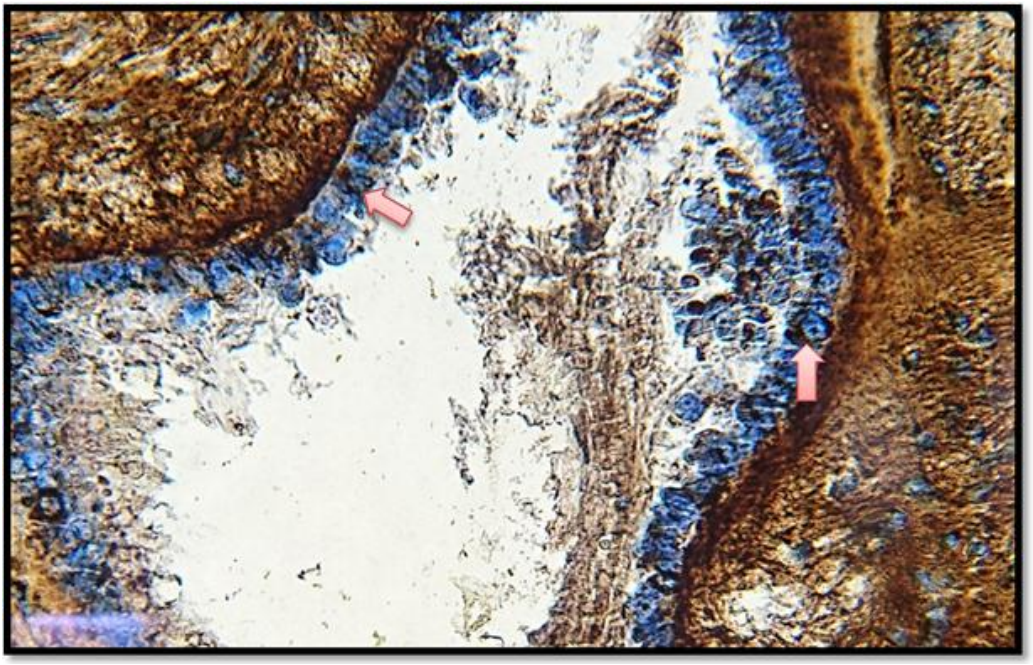

Figure 4. Borderline serous tumor tissue section showing positive immunohistochemical diffuse brown cytoplasmic and membranous expression of CD133 monoclonal antibody of 50% epithelial cells with moderate intensity and score of 4 (arrows) (40×)

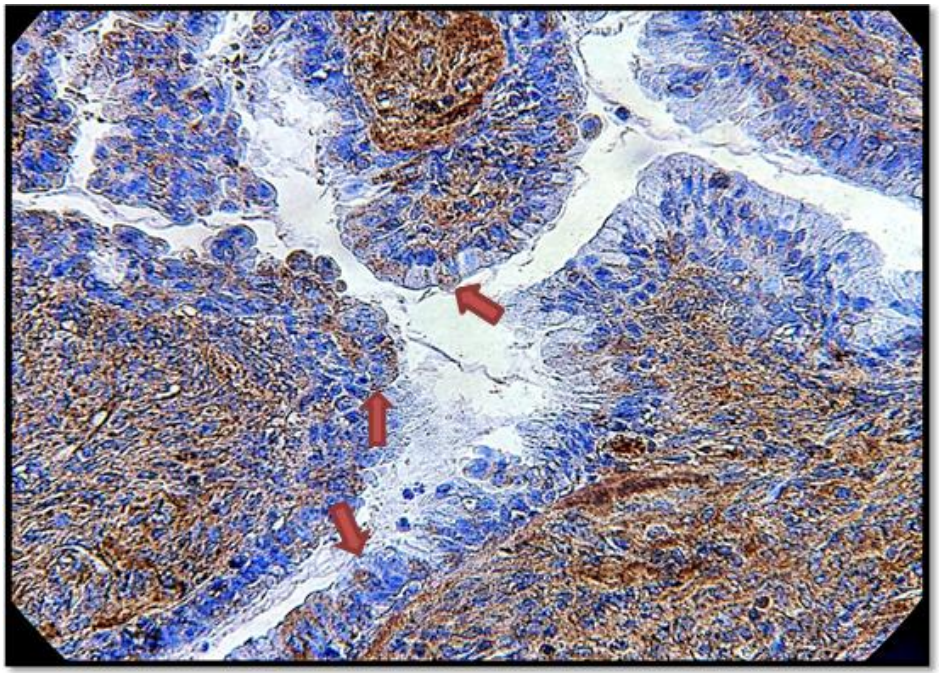

Figure 5. Borderline mucinous tumor tissue section showing positive immunohistochemical diffuse brown cytoplasmic expression of CD133 monoclonal antibody of 50% epithelial cells with moderate intensity and score of 4 (arrows) (40×)

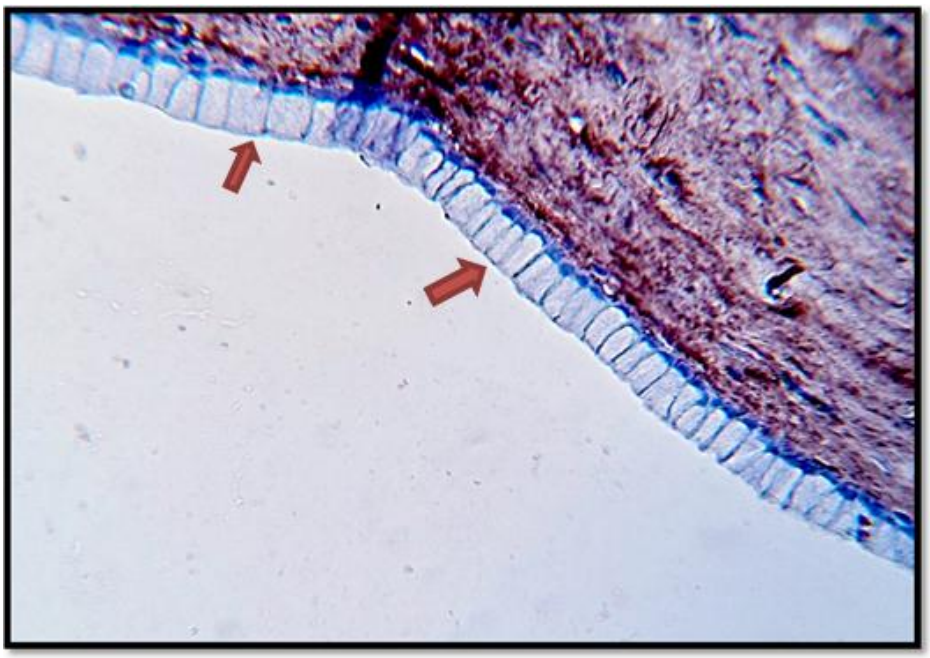

Figure 6. Benign mucinous cystadenoma tissue section showing negative immunohistochemical expression of CD133 monoclonal antibody of the epithelial (arrows) (40×)

Among the 70 cases collected during this study; papillary serous cystadenocarcinoma and serous adenocarcinoma showed the highest immunohistochemical expression of CD133 followed by other histopathological subtypes (endometrioid adenocarcinoma, malignant brenner tumors, clear cell carcinoma, and transitional cell carcinoma), then the mucinous cystadenocarcinoma, so there is a highly significant difference in the immunohistochemical expression of CD133 relation to histopathological type ($P < 0.001$) and highly significant relation between immunohistochemical expression of CD133 and malignant tumors regardless the histopathological types ($P < 0.001$). These results are in agreement with Zhang et al. a study, which demonstrated that CD133 expression was found in 32% HGSC, 27% LGSC, 9% endometrioid carcinoma, 32% consists of mucinous carcinoma, clear cell carcinoma, transitional cell carcinoma and others and found that there is significant correlation between expression of CD133 with histological type specially serous carcinoma ($P = 0.035$) [(9)]. Also parallel to a study performed by Ruscito et al. which showed that about 49.1% of primary ovarian high-grade serous adenocarcinomas were CD133+ [(16)].

Regarding the relation of CD133 immunohistochemical expression semiquantitive scoring and age during the current study, among the 70 cases of ovarian surface epithelial tumors, there is a statistically significant relation between the immunohistochemical expression of CD133 score and increase of the age ($r = 0.254$ and $P = 0.034$). Kim et al. found that the expression levels of CD133 didn't vary by age in benign tumors, borderline tumors, and cancer, however in the cancer samples, the expression levels were significantly increased among the oldest age group [(15)]. Also, the Chinese meta-analytic study performed by Zhou et al. on prognostic value of CD133 in ovarian cancer, mentioned that there is no correlation between CD133 expression and patients' age [(17)], the differences among the current study, Kim et al. and Zhou et al. may attributed to differences in the population sample size, time quality, different study technique.

Regarding the relation of CD133 immunohistochemical expression and tumor grade, among the 20 cases of ovarian surface epithelial malignant tumors, there is highly significant relation between the immunohistochemical expression of CD133 and the increment of tumor grade ($r= 0.794$ and $P < 0.001$). This data reflects the following; on the increment of CD133 expression there is increase of the loss of differentiation or increase of tumor grade, this suggest the high tumorogenicity of CD133. These findings were opposite to Zhou et al. study, which mentioned that there is no correlation between CD133 expression and tumor grade [(17)] the Romanian study performed by Onisim et al. on expression of CD133, found that CD133 expression in ovarian tumor cells was not significantly associated with the tumor grade ($p>0.05$) [(18)]. The differences among current study, Onisim et al. study and Zhou et al. study may be attributed to Sample size and quality, geographical areas differences, focusing on specific histological type, different study technique.

Regarding the relation of CD133 expression and tumor stage, among the 20 cases of ovarian surface epithelial malignant tumors, there is a statistically significant relation between the immunohistochemical expression of CD133 and increase of tumor stage ($r = 0.543$ and $P = 0.013$). This study was in agreement with Ricci et al. study, which mentioned that overexpression of CD133 may correlates with the tumor stage and with a reduced 2-year survival. However, CD133 were variably expressed resulting in absence of significant correlation, this may be attributed to their sample population, as the number of patients were too small [(19)]. Kim et al. showed that there is significant correlation of CD133 expression to ovarian tumor stage, in which CD133 was increased in stage IV compared with stage I [(15)].

Regarding the relation of CD133 immunohistochemical expression semiquantitive scoring and presence of ascites, there is statistically significant relation

between the expression of CD133 and presence of ascites with P < 0.001. The results of present study were in agreement with Zhang et al. study, which found that CD133 expression among the ovarian epithelial carcinoma cases, there is a significant correlation between expression of CD133 with presence of ascites (P = 0.010) [9]. While opposite to Onisim et al. study which found that CD133 expression in ovarian tumor cells was not significantly associated with the presence of malignant ascites (p>0.05) [18]. The differences between current study and Onisim et al. study may be attributed to sample size and quality, focusing on specific histological type, different study technique.

In conclusion CD133 revealed a highly significant differences in the expression of ovarian surface epithelial tumors tissues compared to control group, which reflect its important role in ovarian carcinogenesis through enhancement of migration, invasion and tumorigenic ability of ovarian cancer cells. The association of cancer stem cells marker CD133 expression with various ovarian epithelial histopathological types, higher tumor grade, and the association with the presence of ascites may indicate that CD133 expression may have a role in tumor cells proliferation and invasion which in turn show potential clinical value in the predicting disease progression or prognosis in ovarian epithelial cancer, supporting the proposed link between CD133 and cancer stem cells. The significant relation between the expression of CD133 and the increase of the age in patients with ovarian surface epithelial tumors may indicate that old age associates with later stage and higher-grade tumor, which in turn carry poor survival of these patients, this may attribute to the potential biologic and molecular difference among various age groups in ovarian epithelial tumors.

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Authors' contribution

Dr. Al-Shami collected the cases data, performed the routine H&E and the immunohistochemical technique and analyzed the results of the study. Dr. Qasim helped in study design and supervising the work. Dr. Alzubaidi participated in the collection of cases and revision of histopathological sections.

Conflict of interest

The authors declare no conflict of interest.

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Detection of Respiratory Syncytial Virus in Infants and Young Children with Chest Infection: A Comparison of Reverse Transcription-PCR Technique to Chromatographic Immunoassay and Enzyme Linked Immunosorbent Assay

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Abstract

Background Human respiratory syncytial virus (hRSV) is a major cause of viral lower respiratory tract infection among infants and young children less than 2 years old. Multiple methods are used for the laboratory diagnosis of hRSV infections, including chromatographic immunoassay, enzyme linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) technique for detection hRSV-antigens, hRSV-antibodies and hRSV-RNA, respectively.

Objective To compare the efficiency of three diagnostic methods in detection of hRSV in infants and young children with chest infection.

Methods This study included 100 hospitalized infants and young children (39 females and 61 males) aged from (1) month to (24) months, their mean age (6.87 ± 6.03) months, who required hospital admission at the Pediatric Department in Al-Imamein AL-Kadhimein Medical City Hospital, Central Teaching Pediatric Hospital, and Al-Kadhimiya Pediatric Hospital in Baghdad-Iraq. Samples were collected over a three-month winter period from January 2017 to April 2017. Fresh nasal swab specimens were collected and testes for hRSV antigens by using chromatographic immunoassay as a rapid test, in addition, nasopharyngeal/throat swabs specimens were processed for detection of hRSV-RNA by RT-PCR, both for direct detection. Also, ELISA was done to measure anti-hRSV IgM antibodies in serum for indirect detection of RSV infection.

Results hRSV was found to be positive in (27%), (56%) and (44%) of specimens by rapid chromatographic immunoassay, ELISA and RT-PCR technique, respectively. Comparing with RT-PCR, the sensitivity of rapid test was (59.09%) ranged from (44.41) to (72.31) and the specificity was (98.21%) ranged from (90.55) to (99.91) with likelihood ratio equal to (33.09), while the sensitivity of ELISA test was (75.61%) ranged from (60.66) to (86.17) and specificity was (59.62%) ranged from (46.07) to (71.84) with likelihood ratio equals to (1.87).

Conclusion The RT-PCR technique was more sensitive than antigen or antibody detection methods for the diagnosis of hRSV

Keywords hRSV, rapid chromatographic immunoassay, ELISA, RT-PCR

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List of abbreviations: ELISA = Enzyme linked immunosorbent assay, hRSV = Human respiratory syncytial virus, RT-PCR = Reverse transcription polymerase chain reaction

Introduction

Human respiratory syncytial virus (hRSV) is one of the major causes of viral respiratory tract diseases in infants and young children, with high rates of morbidity and mortality in infants and in the elderly [(1)]. Severe hRSV bronchiolitis and pneumonia requiring hospitalization typically occur in infant less than 6 months of age [(2)]. hRSV infections are usually symptomatic varying from a mild common cold to a life threatening

that are characterized by worsening cough, tachypnea, dyspnea, hyperinflation, atelectasis, hypoxemia and increasing respiratory rate. It accounts for approximately 50% of all pneumonia and up to 90% of the reported cases of bronchiolitis in infancy [3]. Worldwide variation in the prevalence of respiratory viruses was reported to be between 2% and 78.6% [4]. There are an estimated over 30 million cases per year, globally. However, in children younger than 5 years of age, the hRSV can causes very sever disease with more than 3.4 million hospital admissions and 66,000 to 199,000 deaths, most of whom occur in developed world [5-7].

The virus is a globally ubiquitous respiratory pathogen of the genus *Pneumovirus*, within the family *Paramyxoviridae* and order *Mononegavirales*. hRSV has a single stranded, negative sense RNA genome [8]. The virus can spread by close contact with aerosols of infectious respiratory secretions and from medical staff who are often instrumental in its transmission [9,10]. In temperate climates the infection occurs as yearly winter epidemics. The first infection is usually the most severe but milder re-infections are common throughout life [11].

The laboratory diagnosis of hRSV infections is mostly done by rapid test [12,13] and ELISA [14]. The diagnosis can also be done by RT-PCR technique [15]. There are no approved vaccines against RSV infection; therefore, the management of severe infection of hRSV consists of supportive measures, such as oxygenation and maintenance of adequate hydration [16]. Palivizumab, a monoclonal antibody approved against hRSV for prophylaxis to prevent and safe to treat hRSV-related hospitalizations in high-risk children [1].

This study aimed to determine the frequency of hRSV in three hospitals in Baghdad city and to evaluate rapid test and ELISA in relation to RT-PCR in diagnosis of hRSV.

Methods

Subjects

In this descriptive cross-sectional study, 100 infants and young children were enrolled (39 females and 61 males) aged from (1) month to (24) months, their mean age was (6.87 ± 6.03) months, who required hospital admission at the Pediatric Department in Al-Imamein AL-Kadhimein Medical City Hospital, Central Teaching Pediatric Hospital, and Al-Kadhimiya Pediatric Hospital in Baghdad-Iraq. Samples collection was carried out from January to April, 2017. The study was approved by the Institutional Review Board (IRB) at Al-Nahrain College of Medicine and an informed consent was obtained from either parents of the child before they had been included in this study.

Specimens collection

Fresh nasal swabs were collected, stored in the refrigerator (2-8 °C) and processed as soon as possible within 8 hours after collection for rapid detection of hRSV-Ag. Nasopharyngeal/throat swabs were collected and combined in universal transport medium (UTM) tube (Cat. No. 80346C, Copan, Italy). Each sample was liquated in cryotube (Nunc-Kamstrup, Denmark) and stored at (-80 °C) until testing by RT- PCR for detection of hRSV-RNA. Two ml of blood were collected in serum separator tube (SST) and allowed to clot for 20 min. at room temperature before centrifugation at 1000xg for 15 min. Then serum samples were liquated, immediately frozen and stored at (-20 °C) until screening by ELISA (Cat. No. CSB-E13790h, Cusabio, China) for anti-RSV IgM antibodies.

Detection of hRSV-Ag by Rapid Chromatographic Immunoassay

All (100) samples were tested for hRSV antigen in fresh nasal swab samples by one step card test, CerTest RSV Kit (CerTest Biotec, Spain). The procedure was done following the manufacturer's instructions. Nasal swab samples were prepared by placed into the testing tube, which supplied with the kit and shaking well. Four drops were dispensed into the samples (S) circulated window. After incubation at room temperature for 10 min, the results were read by monitoring of colored

development: Negative test; only one green line appears in the control line region. Positive test: In addition to the green control line, a red color line also appears in the test region.

Detection of anti-hRSV IgM antibodies by ELISA

Ninety-three samples were retested for anti-hRSV IgM by ELISA Kit (Cusabio, China). The micro titer plate was coated with antigen, indirect ELISA was used to capture anti-hRSV IgM from serum samples. The procedure was done following manufacturer's instructions. For sample preparation, serum samples were diluted by adding 10 µl of the serum sample to 100 µl of sample diluent which supplied with the kit. Blank, positive and negative control was included when the Kit was run. The optical densities (O.D.) of each well were measured at wave length (450 nm and reference filter 630 nm). The O.D. of the negative control less than 0.05 was calculated as 0.05 according to the manufacture. Then the O.D. of the sample was divided by O.D. of the negative control. Anti-RSV IgM antibody (≥2.1) considered as positive, while Anti-RSV IgM antibody (<2.1) considered as negative.

Detection of hRSV-RNA by RT-PCR

All 100 nasopharyngeal/throat swab samples were tested for the presence of hRSV-RNA by hRSV 298/550 IC Kit (Sacace Biotechnologies, Italy). To avoid possible contamination with exogenous sequences during extraction or amplification, all nucleic acid extraction, amplification, and detection steps were performed in separate laboratories. Negative and positive controls were extracted, reverse transcribed, and amplified in each batch of samples tested by PCR.

Nucleic acid extraction

Nasopharyngeal/throat processed samples were removed from deep freeze (-80 °C) and thawed. After that, they were centrifuged at 10000g/min for 5 min; the supernatant was discarded except 100 µl of the solution was left to be used in re-suspension of the pellet for RNA extraction. The Ribo-Sorb nucleic acid extraction kit (Sacace Biotechnologies, Italy) was used for isolation and purification of RNA/DNA from samples. The procedure was done following manufacturer's instructions. Extracted RNA with purity in between (1.7-1.9) at absorption wavelength 260/280 was included in this study, otherwise; RNA extraction of the sample was repeated. The RNA extracts were reverse transcribed to cDNA according to the manufacturer's instructions. Each obtained cDNA sample was diluted (1:2) with Tris-EDTA buffer solution and stored at (-20 °C) for a week until cDNA amplification by PCR, otherwise they were stored at (-80 °C) for longer periods storage.

cDNA amplification

DNA amplification reactions were carried out on target region L-gene by PCR according to manufacturer's instructions in three steps: first initial denaturation at 95 °C for 5 min (1 cycle) and then the DNA amplification by sequentially heated for denaturation of DNA template at 95°C for 45 sec., annealing at 56 °C for 45 sec. and extension at 72 °C for 45 sec. (42 cycles) and then final extension at 72 °C for 5 min (1 cycle).

Interpretation of the results: Ten µl of PCR products were subjected to electrophoresis in agarose (2%) in the presence of ethidium bromide and visualized under UV transilluminator. The band size was assessed by direct comparison with a 100-bp DNA marker. Analysis of PCR results is based on the presence or absence of specific bands of amplified DNA in agarose gel. The sample is considered to be positive for hRSV-RNA if the band of 298 bp is observed on agarose gel. The presence of a 550 bp fragment indicated positive result for internal control (IC) specific amplified DNA fragments

Statistical analysis

Data were analyzed using SPSS program (Statistical Package for the Social Sciences), versions 21 program for windows software package release 2013. Descriptive statistics were presented as frequencies, means and standard deviation (SD). Validity and predictability of different screening tests were

assessed in relation to gold standard test by calculating sensitivity, specificity, predictive value of positive and negative test results.

Results

A total of 100 infants and young children with chest infection were included in this study. The male to female ratio was 1.5:1, their age ranging between 0-24 months, most of the admitted patients were below 6 months. The clinical characteristics of enrolled patients were obtained from their hospital records; 40 (40%) had pneumonia, 37 (37%) had bronchiolitis and 23 (23%) had other chest infection such as; cough, fever, shortness of breath, pertussis-like cough, wheezing and cyanosis, no case was recorded with croup or bronchitis, as shown in table (1).

Table 1. Demographic and clinical characteristics of the study population

Characteristics	No. of patients (%)
Gender	
Males (M)	61 (61%)
Females (F)	39 (39%)
M:F ratio	1.5 : 1
Total	100 (100%)
Age groups (months)	
[0-6]	62 (62%)
[7-12]	27 (27%)
[13-24]	11 (11%)
Total	100 (100%)
Respiratory manifestations	
Croup	0 (0%)
Bronchitis	0 (0%)
Bronchiolitis	37 (37%)
Pneumonia	40 (40%)
Other chest infection	23 (23%)
Total	100 (100%)

This study showed that hRSV was positive in 27%, 55.91% and 44% by Rapid test, ELISA and RT-PCR technique, respectively as shown in table (2) and figure (1).

Out of 100 samples, 27 (27%) were hRSV-Ag positive by rapid test and 26 of these were positive for hRSV-RNA by RT-PCR, therefore, they were considered to be true positives, but one sample was negative for RT-PCR, therefore, it was considered to be false positive. Eighteen samples were PCR positive while negative for Rapid test for the purpose of defining the test characteristics were considered false negative. Given these definitions, the overall sensitivity of rapid test was 59.09% and specificity was 98.21%. The positive predictive value was 96.3% with confidence interval (CI) ranged between (81.72-99.81), and the negative predictive value was 75.34%, CI = 64.36-83.8). Also, anti-hRSV IgM Ab was identified by ELISA in only 93 samples; 52 (55.9%) out of 93 were ELISA positive sera, 31 of these sera were hRSV-RNA positive, therefore, they were considered to be true positives, while 21 of these sera was negative by RT-PCR, therefore, they were considered to be false positive. Ten of these samples were positive for hRSV-RNA, while negative in ELISA test, therefore, were considered to be false negative. Accordingly,

the overall sensitivity of ELISA test was 75.61% and specificity was 59.62%. The positive predictive value was 59.62%, CI = (46.07-71.84), and the negative predictive value was 75.61%, CI = (60.66-86.17), as show in table (3).

Table 2. Results of hRSV detection in infants and young children with chest infection by three diagnostic methods: rapid test, ELISA and RT-PCR technique

Methods of Diagnosis	No. of Positive (%)	No. of Negative (%)	Total (%)
Rapid test	27 (27.0%)	73 (73.0%)	100 (100%)
ELISA	52 (55.91%)	41 (44.09%)	93 (100%)
RT-PCR	44 (44.0%)	56 (56.0%)	100 (100%)

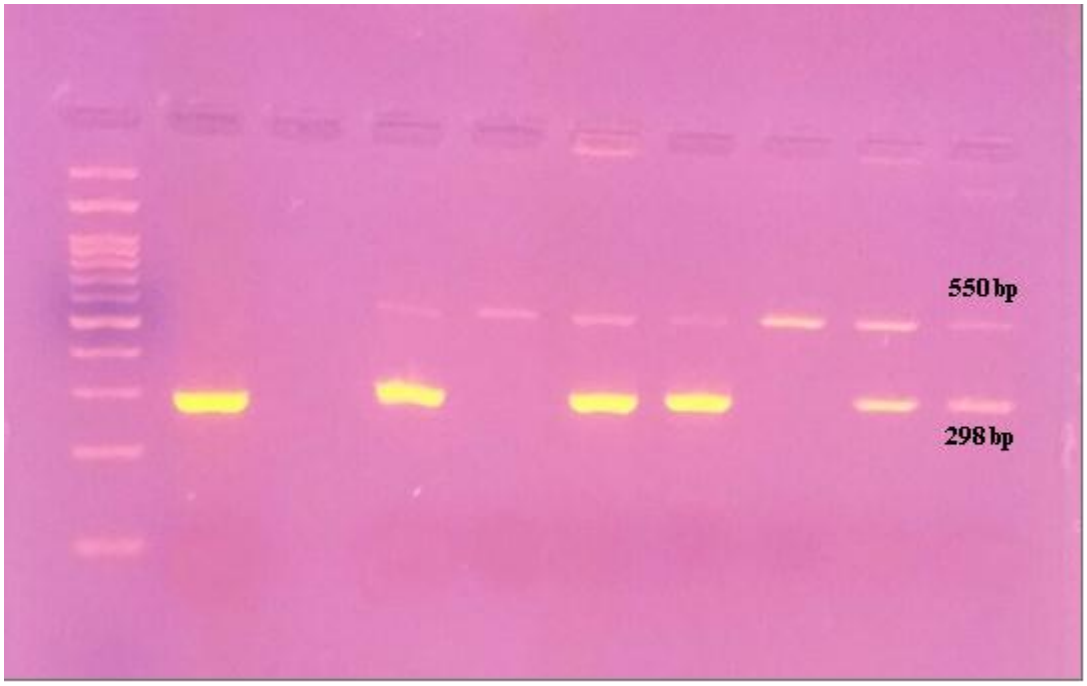

Figure 1. Gel-electrophoresis of PCR products of hRSV from Nasopharyngeal/throat swab sample using 2% agarose in TBE buffer. Lane 1, DNA marker (100-3000 bp ladder); Lane 2, hRSV cDNA (C+) serves as positive control for amplification; Lane 3, DNA- buffer (C-) serves as negative control for amplification; Lane 4, 6, 7,9,10 PCR products from patient positive for hRSV-RNA; Lane 5, 8 PCR products from patient negative for hRSV-RNA

Table 3. Evaluation of Rapid test and ELISA sensitivity & specificity confirmed by RT-PCR

Methods of Diagnosis		RT-PCR (No.=100) Positive (%)	RT-PCR (No.=100) Negative (%)	Total (%)
Rapid test (No.=100)	Positive	26 (59.1%)	1(1.8%)	27 (27%)
	Negative	18 (40.9%)	55 (98.2%)	73 (73%)
	Total	44 (100%)	56 (100%)	١٠٠ (100%)
Statistics		X^2= 41.0531, P<0.001		
Sensitivity		59.09 (95% CI, 44.41 to 72.31)		
Specificity		98.21 (95% CI, 90.55 to 99.91)		
Positive Predictive Value		96.3 (95% CI, 81.72 to 99.81)		
Negative Predictive Value		75.34 (95% CI, 64.36 to 83.8)		
Likelihood Ratio		33.09		
ELISA (No=93)	Positive	31 (75.61%)	21 (40.38%)	52 (55.91%)
	Negative	10 (24.39%)	31 (59.62%)	41 (44.09%)
	Total	41 (100%)	52 (100%)	93 (100%)
Statistics		X^2= 11.54, P<0.001		
Sensitivity		75.61 (95% CI, 60.66 to 86.17)		
Specificity		59.62 (95% CI, 46.07 to 71.84)		
Positive Predictive Value		59.62 (95% CI, 46.07 to 71.84)		
Negative Predictive Value		75.61 (95% CI, 60.66 to 86.17)		
Likelihood Ratio		1.872		

Discussion

hRSV is considered as one of the most important respiratory viruses in infants and young children throughout the world. In this study, the frequency of hRSV infection in infants and young children ≤ 2 years of age with chest infection was (27%), (55.91%) and (44%) by three diagnostic tests; rapid test, ELISA and RT-PCR, respectively. These findings indicate that hRSV is an important cause of respiratory tract infection in infants and young children less than two years old.

Comparison of Rapid test and RT-PCR for diagnosis hRSV

Detection of hRSV-RNA by PCR is currently the most sensitive and specific method for detecting infection in infants and young children ≤ 2 years old [2,17]. In the present study, RT-PCR was used to screen for the presence of hRSV-RNA in all 100 nasopharyngeal/throat swabs, while Rapid test was used to screen for the presence of hRSV-Ag in nasal swabs. Eighteen samples gave positive result on RT-PCR that were negative on Rapid test were considered to be false negative, table (3), The possible explanation for these results, is the small amount of viral antigen usually present in nasal swabs collected from patients infected with hRSV comparing with nasopharyngeal/throat swabs; also this result may be due to that the current antigen detection assays may lack sufficient sensitivity to detect hRSV antigen, another explanation is related to the fact that sample was collected after hRSV shedding [18]. In addition, it has been demonstrated that a nasal wash or a nasopharyngeal aspirate is more sensitive for the detection of hRSV than nasal swab specimen [19]. Therefore, RT-PCR may be required to detect and diagnose hRSV in infants and young children [17,18].

A study conducted by Gregson et al. [20], found that 115 (49%) out of 236 samples were positive for hRSV-Ag by Direct Fluorescent-Antigen (DFA). Of these, 106 (44.9%) were positive for hRSV-Ag by using Respi-test. On the other hand, Iranian study detected hRSV-Ag in 9 (5.7%) out of 160 samples by using Respi-strips method [21]. Currently the available antigen detection kit (Certest) in pediatric specimens has sensitivities of 44.41 to 72.31% and specificities of 90.55 to 99.91% as compared to PCR [19]. The current study showed that the sensitivity of Rapid test was (59.09%) ranged from (44.41% to 72.31%),

while the specificity of Rapid test was (98.21%) ranged from (90.55% to 99.91%), with positive likelihood ratio 33.09. There are a number of factors that affect the sensitivity and specificity of an assay for viral detection, these include; Low viral copy number in the clinical sample, quality of the specimen and reagents, laboratory technician experience, transport conditions of the sample, suitability of the assay to specific populations (e.g., rapid antigen test in elderly versus young people), inter- and intra-laboratory standardization, and prevalence of the virus in the community [17,22].

Comparison of ELISA and RT-PCR for diagnosis hRSV

In this study, 93 sera were screened for anti-hRSV-IgM antibodies by ELISA, 52 (55.91%) of these 93 were sera positive and 21 out of these 52 were negative on RT-PCR and considered to be false positive, which might be explained by sera samples were collected after viral RNA had cleared or the time before evaluation was longer for the hospitalized patients [23]. Another explanation on PCR negative result is that almost all children are negative by RT-PCR after 14-21 days, similar results reported by [24], who found that anti-hRSV IgM antibody was detected in higher prevalence than hRSV-RNA (81.9% vs. 1%). In addition, IgM antibody positive result might represent cross-reactive antibodies causing false positive anti-hRSV results. This may be due to serologic cross-reactions between the hRSV and other respiratory viruses [25]. Serology is not 100% sensitive and not always able to accurately determine the stage of infection since false positive and false negative results are regularly observed [22]. In the present study, 10 patients out of 41 (24.39%) was RSV-RNA positive, but did not show a rise in anti-hRSV IgM antibody level we considered to be false negative. This study showed that the sensitivity of ELISA was (75.61%) ranged from (60.66% to 86.17%), while the specificity was (59.62%) ranged from (46.07% to 71.84%), with positive likelihood ratio 1.872, therefore, ELISA technique was mostly performed to obtain seroepidemiologic information and for research purposes [26].

The present study confirms that rapid test and ELISA technique were less sensitive comparing with RT-PCR for detection of hRSV in infants and young children ≤ 2 years of age with chest infection, and indicate that the golden standard RT-PCR is more sensitive, and specific method for detection of hRSV in comparison to serological tests.

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Authors' contribution

All authors contributed to this manuscript. Dr. Al-Shuwaikh: design, interpreted and arranged this manuscript. Ali: performed all the laboratory work, implementation and progress of this study. Dr. Arif: helped in clinical aspect and collection of samples.

Conflict of interest

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Identification Common Cause of Neonatal Sepsis by Analytical Profile Index System (API)

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Abstract

Background Neonatal Sepsis is a bacterial infection of the blood in a neonate and an infant younger than 4 weeks of age. The analytical profile index or API is a classification of bacteria based on experiments, allowing fast identification. This system is developed for quick identification of clinically relevant bacteria.

Objective To identify the pattern of organisms in neonatal sepsis using API system in Baghdad City hospital, Al-Imamein Al-Kadhimein Medical City and Central Pediatrics Teaching Hospital.

Methods In this cross-sectional study, blood samples from 100 neonatal patients were inoculated into a blood culture bottle and incubated at 37 °C under aerobic conditions, subculture was done after 24 h of incubation, the growth was identified by phenotypic characteristics, gram's stain, and API system.

Results Positive blood cultures were detected in 82 (82%), according to API system the most prominent bacterial isolates from blood culture in neonates with early-onset sepsis were non-coagulase *Staphylococcus* (20.4%) *Staphylococcus aureus* (18.1 %), *Acinetobacter bumanii* (13.6%), *Pseudomonas aeruginosa* (11.36 %), *Streptococcus agalactiae* (11.3 %), while in late-onset sepsis the most common bacteria were *Staphylococcus aureus* (21.0%), non-coagulase Staphylococcus (13.1%), *Citrobacter freundii* and *Pseudomonas aeruginosa* (10.5 %) respectively.

Conclusion The API 20E may be useful for the identification of the bacterial species rarely described as pathogens in neonatal sepsis will help us to study the clinical burden resulting from the emergence of these species as causes for this neonatal infection.

Keywords Early-onset sepsis, late-onset sepsis, blood culture, API system

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List of abbreviations: EOS = Early-onset sepsis, LOS = Late-onset sepsis, API = Analytical profile index, PROM = Premature rupture of membranes, UTI = Urinary tract infection

Introduction

Neonatal sepsis is a bacterial infection of the blood in a neonate and an infant younger than 4 weeks of age. Babies with sepsis were listless, overly sleepy, floppy, weak, and pale [(1)]. Epidemiologists divided Neonatal Sepsis into two types of an early-onset sepsis (EOS) and late-onset sepsis (LOS). Of newborns with EOS sepsis, 85% present within 24 hours, 5% present at 24-48 hours, and a smaller percentage present within 48-72 hours [(2)]. LOS is sepsis occurring after 72 h in

NICU infants and 7 days of life in term infants, has been variably defined as occurring up to the age of <90 or 120 days, and may be caused by vertically or horizontally acquired pathogens [3]. It was found that neonatal deaths account for a third of global child mortality and that infection are a major cause of neonatal mortality [4]. The pathogens responsible of neonatal sepsis has also changed dramatically, Since the mid-20th century the infectious agents that cause neonatal sepsis have changed from Staphylococcus aureus and Escherichia coli which were the most common bacterial pathogens among neonates in the United States for decades to group B *Streptococcus* (GBS) as the most common gram-positive organism that caused early-onset sepsis [5]. At 1990s, *GBS* and *Escherichia coli* kept on being related with neonatal infections but coagulase-negative *Staphylococcus epidermidis* is presently more often watched. Additional organisms, for example, *Listeria monocytogenes*, *Chlamydia pneumoniae*, *Haemophilus influenza*, *Enterobacter aerogenes*, and types of *Bacteroides* and *Clostridium* spp. have likewise been recognized in neonatal sepsis [6]. The analytical profile index or API is a classification of bacteria based on experiments, allowing fast identification. This system is developed for quick identification of clinically relevant bacteria.

The goal of this investigation to identify the pattern of organisms in neonatal sepsis using API system in Baghdad City hospital, Al-Imamein Al-Kadhimein Medical City and Central Pediatrics Teaching Hospital.

Methods

About (1.5-3 ml) of venous blood was obtained from 100 neonates who admitted to neonatal care unites of Baghdad City hospital, Al-Imamein Al-Kadhimein Medical City and Central Pediatrics Teaching Hospital during the period from January 2017 to March 2017. Clinical manifestations including poor feeding, lethargy, temperature instability, respiratory distress, abdominal distention and seizures were determined by consultation of a pediatric specialist and verification of the information in the medical record. Inclusion criteria for selecting children were the age group (from zero time to 30 days), and diagnosed clinically with sepsis while exclusion criteria neonates with obvious congenital anomalies and neonates with neonatal respiratory distress syndrome (NRDS).

Pre-term including live born infant delivered before 37 weeks from the last menstrual period. Term infants are an infant who delivered after 37 weeks of gestation. Post-term infants are those born after 24 weeks of gestation [7].

A consent letter was signed by each neonate parents and the study were approved by the Research Ethical Committee College Medicine of Al-Nahrain University. This cross-sectional study was conducted in the Microbiology Department at College Medicine of Al-Nahrain University. The following data were collected: gestational age, birthweight gender and whether the baby was born inside the hospital or outside the hospital and then transferred to the nursery.

Samples were immediately transferred to Brain heart infusion medium vial which prepared specially for bacterial cultivation, and incubated in 37 °C, then samples subcultured in MacConkey and blood aerobically while chocolate agar incubated under CO_2 conditions, the initial reading was recorded after 24 hours and the results continued to be recorded for 72 hours. The result is considered negative after this time if there is no growth. API 20E system for *Enterobacteriaceae*, API Staph System for identifying 23 species of *Staphylococci*, API 20 Strep System according to the procedure suggested by the manufacturing company (bio-Merieux) were used to recognize the species level.

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 6 software, percentages were used for the comparison between samples of the study. Data analysis was done using Chi-square for the comparison of categorical data.

Results

The participants neonates were grouped in to two categories according to type of onset, the first was early onset group included children in age groups from (0-7 days), while other late onset group included children from (8-30 days). The characteristics of the study population are shown in table (1).

Table 1. Demographic character of the study neonates

Variable		Mean±SD (Range)
Age /days		1 30 9.86 ±8.76
		%
Gender	Female	43
	Male	57
Gestational age	Pre-term	47
	Full-term	53
Mode of delivery	cesarean section	57
	Vaginal	43
Place of delivery	Hospital in born	74
	Out born	26
Birth weight	<2.5 kg	54
	>2.5 kg	46
Presentation (days)	≤7 (EOS)	51
	>7 (LOS)	49

EOS: Early-onset sepsis, LOS: Late-onset spesis

According to the mother's clinical presentation for neonatal sepsis patients, this result demonstrated that the higher percentage of neonatal sepsis found in those mothers who had history of premature rupture of membranes (PROM) 72 (72%), UTI 79 (79%) and previous abortion (37%), while less percentage found in women who had fever 22 (22%) and Hemorrhage 10 (10%) (Table 2).

Table 2. Maternal risk factors for neonatal sepsis

Clinical presentation	% of mother
Premature rupture of membranes (PROM)	72 (72%)
Mother fever	22 (22%)
UTI	79 (79%)
Hemorrhage	10 (10%)
Previous abortion	34 (37%)

Conventional methods for diagnosis of neonatal sepsis

Blood culture

One hundred samples of the patient's blood were implanted in the culture media that attended to this purpose, positive blood cultures were detected in 82 (82%), gram

negative was the major causative pathogen 43 (43%) followed by gram positive 38 (38%) and one sample showed growth of candida species, the patients with negative blood culture were 18 (18%). Table (3) explains the result of blood culture.

Table 3. Pathogens isolated from neonatal sepsis by blood culture

Blood culture	Percentage
Gram negative	43%
Gram positive	38%
Candida species	1%
Total positive	82%
No growth	18%

Bacterial distributions by API system strips

A total of 81 bacterial positive culture were examined by the different API strips system which were tailored to certain groups of microbes to identify the bacterial isolate. The distribution of bacterial isolates in EOS and LOS were represented in table (4).

Table 4. Distribution of bacterial species according to API system

Bacteria	EOS	%	LOS	%
Acinetobacter bumanii	6	13.6%	1	2.6 %
Pseudomonas aeruginosa	5	11.36 %	4	10.5 %
E. coli	4	9 %	0	0.0%
Serratia marcescens	3	6.81 %	2	5.26 %
Citrobacter freundii	2	4.45%	4	10.5 %
Pantonia	2	4.45 %	0	0.0%
Klebsiella pneumonia	1	2.27 %	3	7.89 %
Aeromonas hydrophilia	1	2.27%	0	0.0%
Pseudomonas cepacian (Burkholderia cepacian)	1	2.27%	1	2.6 %
Enterobacter	0	0.0%	1	2.6 %
Serratia phymuthica	0	0.0%	2	5.26 %
Staphylococcus aureus	8	18.1 %	8	21.0 %
Staphylococcus xylosus	3	6.81%	2	5.26 %
Staphylococcus lentus	3	6.81 %	1	2.6 %
Staphylococcus auricularis	1	2.27 %	0	0.0%
Staphylococcus cohnti	1	2.27 %	0	0.0%
Staphylococcus heamolyticus	1	2.27 %	2	5.26 %
Streptococcus agalactiae	5	11.3 %	1	2.6 %
Streptococcus pneumonia	1	2.27 %	0	0.0%
Veridanis streptococcus	0	0.0%	1	2.6 %
Total	48	100%	33	100%

Discussion

Based on the current data, the percentage of neonatal sepsis was 82% out of 100 patients enrolled in this study, this observation disagreement with a study done in Iraq by Albahadle and Abdul Abass [8] where sepsis was constituted 89.76% of the studied neonate, another study conducted by Ibrahim and Rahma [9] Stated that neonatal sepsis was 12.4% However, a study by Al-Hamadani found that the sepsis was 58 % [10].

This discrepancy in such results may be due to different reasons such as blood culture technique, administration of antibiotic in mother, difficulty in sample collection, development of much more antibiotics resistant bacterial strains.

In current study, (86.0%) of neonates were male and (75.4%) of them were female male to female ratio was (1:1.4) This result comes incompatible with those obtained in others studies of Albahadle, and Abdul Abass [8] who found that male was (40.16%) and the female was 59.84%, Ibrahim found (68.7%) neonates were males and (31.2%) were females [11], while another study by Ibrahim and Rahma [9] found the (60.3%) were males and (39.7%) were the females, which may be attributed to neonatal admission in our societies, medical attention is predominantly male to female children, and it is noteworthy to mention that many studies in our country found that regarding gender admission, boys more than girls [12] furthermore genetic, socioeconomic factors also play a role in the development of infections and may partially explain the observed differences [13].

For the successful administration of neonatal sepsis, information about bacteriological profiles, which play an essential part in the management and antibiotic administration , so for the purpose and for rapid identification we conducted in this study analytical profile index (API) system , were included API-20E, , API-Staph, and API- Strep , The most prominent bacterial isolates from blood culture in neonates with EOS were *Non-coagulase Staphylococcus* (20.4%) *Staphylococcus aureus* (18.1 %), *Acinetobacter bumanii* (13.6%), *Pseudomonas aeruginosa* (11.36%), *Streptococcus agalactiae* (11.3 %), while in LOS the most common bacteria were *Staphylococcus aureus* (21.0%), *Non- coagulase Staphylococcus* (13.1%), *Citrobacter freundii* and *Pseudomonas aeruginosa* (10.5%) respectively.

Pattern of organisms in this study do not agree with most studies at the level of our country [14,15] but it is interesting this study was recorded to our knowledge and to the first of some types of bacteria that caused the neonatal sepsis in Iraq from neonatal intensive care unit (NICU), which includes *Aeromonas hydrophilia* and *Pantoea agglomerans*. Hochedez et al. (2010) reported Bacteremia Caused by *Aeromonas hydrophila* Complex in the Caribbean Islands [16].

Others case report by Padmaja et al. in 2011, Okumura et al. in 2013 [17,18] reported a case report study sepsis caused by *Aeromonas hydrophila*, during literature search in this study 15 reports of Aeromonas Blood stream infection (BSI) in 14 articles published in English language found [19-31], seven were pediatric patients (including two neonates) [20-22,29-31].

In our study, one cases were diagnosed with sepsis caused by *Aeromonas hydrophila,* the baby had episodes of hypotension, seizures, apnea, bradycardia, diarrhea, and temperature instability and the patient was died.

Sepsis caused by *Aeromonas hydrophila* remains uncommon life-threatening conditions among the spectrum of infections occurring in neonates, with high mortality [31].

In this study, other two unique neonatal sepsis cases were reported caused by *Pantoea agglomerans*. It is an opportunistic pathogen and very rarely causes disease in healthy individuals [32] disease with *Pantoea* species related to exogenous source, *Pantoea agglomerans* the most widely recognized human pathogen mainly septicemia due to contaminated blood products, parenteral

nutrition, intravenous fluid and the anesthetic agent [33].

These two cases represented by neonates were referred to neonatal intensive care unit 48 (early onset sepsis) an hour after delivery presented with fever, tachypnea and Respiratory distress. The weight of the children was less than 2.5 kg, the ages of their mothers range from 19-20 years came from a rural area, she gave a history of PROM approximately 8 to 10 hours before delivery. One of them noticed a foul-smelling discharge after rupture of membrane. One of the children died two days after being admitted to the hospital, second one was responded well to antibiotic.

Results in current study similar to many cases reported through the world [34,35] and its quietly similar to results obtained by Senanayake et al in-Sri Lanka who reported that out of 55 blood cultures, 14 were positive for *P. agglomerans* [36].

Other interesting results reached in this study 7 (8.6%) bacterial isolates were identified as Serratia species, 5 isolates belong to *Serratia marcescens* and *Serratia phymuthica*.

Noteworthy five patients out of seven infected with Serratia species were die, many researchers reported that Serratia species and mainly *Serratia marcescens* is a well-recognized pathogen of severe nosocomial infections with highly mortality rate as found by Mahdi in isolation and molecular identification of a *Serratia spp*. from suspected neonatal sepsis in intensive care unit (ICU) of Basra Province, Iraq) [37].

The possible explanation for increase mortality in neonatal sepsis infected with *Serratia spp.* ability to produce a beta-lactamase that confers resistance to broad-spectrum beta-lactam antibiotics, which often complicates therapy [38,39].

In conclusion, the most common cause of neonatal sepsis in current study was *Staphylococcus aureus* and *coagulase negative Staphylococcus* of gram-positive bacteria while *Pseudomonas aeruginosa* and *Acinetobacter bumanii* were the most common gram-negative bacteria. *Achromobacter xylosoxidans*, *Pantonia agglomerans* and *Aeromonas hydrophilia* should be included as one of the most important causes of neonatal sepsis. These findings suggest that the API 20E may be useful for the identification of the bacterial species rarely described as pathogens in neonatal sepsis will help us to study the clinical burden resulting from the emergence of these species as causes for this neonatal infection.

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Authors' contribution

Al-Mossawi: Samples collection, molecular diagnosis, design and acquisition of data. Dr. Hassan: Drafting the article and revising it critically for important intellectual content and interpretation of results. Dr. Al-bashier: DNA extractions and conventional methods diagnosis. Dr. Al-Omrani: Patients selections and statistical analysis.

Conflict of interest

There is no conflict of interest.

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Patients Attitude Towards Presence of Undergraduate Medical Students During Consultation in 2016

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Abstract

Background Bedside teaching enables students and trainees to acquire many clinical skills and professional behaviors during process of learning.

Objective To investigate views of patients towards presence of undergraduate medical students during consultation.

Methods This is a cross-sectional study conducted in Al-Imamein Al-Kadhimein Medical City, Baghdad-Iraq; during period of February through July, 2016. It involved patients admitted to internal medicine, surgery, and gynecology and obstetrics departments of hospital during time of data collection. The study relied on performing face to face interviews with participants and a total of 400 individuals were enrolled in it. To be included in the study, patient's age should be 18 years or older. Seriously ill, confused or cognitively impaired individuals were not involved. SPSS program was used for computerized statistical analyses. Categorical variables were compared using Chi-square X^2 tests, and Continuous variables were compared by Student t - test or analysis of variance (ANOVA) test. P -values less than 0.05 & 0.01 was considered to be statistically significant and highly significant, respectively.

Results Those who allowed presence of students represented 80.3% of participants. Factors associated significantly with patients' decision were age (p=0.034) and hospital department (p=0.009). Among those with positive attitude, 145 (45.2%) thought students' number should not exceed five, while remaining did not care. Answers of patients about their view for allowing students to perform examination revealed that 169 (52.6%) accepted all times, 105 (32.7%) linked acceptance by students' sex, and remainders rejected all times.

Conclusion The overall attitude of our patients towards students' involvement in consultation was positive and comparable to that reported in previous studies.

Keywords Patients' Attitude, Bedside Teaching, Medical Consultation, Iraq

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List of abbreviations: None.

Introduction

Bedside teaching is one of conventional pillars of medical education. It enables students to acquire many clinical skills and professional behaviors during their process of learning [1-2]. This method needs great interest and intense adherence by the students and teaching staff, in addition to willingness for cooperation by patients who undertake a key role in this process [3-4]. The readiness of patients for collaboration and contribution in the medical training process is considered now a fundamental prerequisite to provide the best teaching environment at various levels of patient's care [5-7].

Modern health services users are active partners in determining their care, which may lead to difficulties in training if the patients refuse to participate. With raised emphasis on issues related to patients' rights and informed consent, it becomes necessary to obtain patients' agreement regarding the presence of students during medical consultation [8-10]. Previous researches have revealed that patients tolerated well the presence of students during consultation in various medical situations, starting from emergency units and ending with outpatient departments [11-17]. Nevertheless, the studies have showed also that patients' attitudes from students' involvement were strongly influenced by many factors like sociodemographic characteristics and the clinical situation of each condition [16-20].

Medical education in Iraq is recognized by its traditional nature as there are big number of students and hospital-based learning. Clinical teaching takes place in the third to sixth years of medical training [21-22]. Till now, no study has been conducted to enquire about the acceptability of medical students by patients. In the absence of such studies and in conjunction with national efforts for quality improvement in both medical education and health care standards, it became necessary to explore the opinion of patients, what they felt and the degree of their comfort upon presence of undergraduate students during consultations.

Consequently, these facts prompted to conduct this study that aims to assess views of patients towards the presence of undergraduate medical students during consultation with their doctors at a university teaching hospital in Iraq; and to identify factors that may potentially influence their decision to allow or refuse medical students' participation.

Methods

This is a cross-sectional study with an analytic element, carried out in Al-Imamein Al-Kadhimein Medical City, Baghdad-Iraq; during period of February through July 2016. It involved patients admitted to different departments and wards of the hospital during the time of data collection. The sampling method was systematic random sampling, i.e. sample was selected according to a random starting point and a fixed periodic interval. A sample size of 400 patients was chosen as it gives 95% confidence level with less than 5% margin of error. To be included in the study, patient's age should be 18 years or older. Seriously ill, confused or cognitively impaired individuals were not involved. Informed consents were obtained from all participants. Ethical approval was obtained from Al-Nahrain medical school.

Study instrument

The study relied on conducting face to face interviews using paper-based, predesigned questionnaire that was used in previous similar research [23]. Minor changes were made on original version in a way did not affect its validity and it was translated to Arabic to make it more suitable for local circumstances of current study. The research tool consisted of three main sections: First section contained questions about socio-demographic characteristics (e.g. age, sex, education, and residence) and clinical departments of patients (internal medicine, surgery, or gynecology and obstetrics wards); second section contained one question asking if patients allow the presence of medical students during their consultation with hospital doctor. If answer was "Yes", patients were requested to continue and answer the remaining questions but if answer was "No", then interview will end at this point. The third section contained set of questions seeking the volunteers' attitudes towards the presence of students during medical consultations (e.g. patients believe whether they can learn more about their problems or get more attention when students are present, their opinion about need of having some time alone with doctor, and importance of doctor's presence with students at all times during consultation).

Statistical analysis

The SPSS program, version 20, was used for computerized statistical analyses. The results were expressed as mean ± SD (standard deviation), or frequency & percentage. Categorical variables were compared using Chi-square X^2 tests, and Continuous variables were compared by Student t - test or analysis of variance (ANOVA) test. P -values less than 0.05 and 0.01 was considered to be statistically significant and highly significant, respectively.

Results

Most of patients were women in fourth to fifth decade of age and lived in urban areas. More than half of the study sample was recruited from internal medicine departments (Table 1).

Table 1. Basic Characteristics of study sample

Character	Value
Number	400
Age; (mean ± SD)	42.0 ± 17.1 yr
Female Sex; n (%)	264 (66%)
Years of education; (mean ± SD)	6.61 ± 5.21 yr
Urban Residency; n (%)	329 (82.3%)
Hospital Department; n (%)	
- Internal medicine	207 (51.8%)
- Obstetrics & gynecology	117 (29.3%)
- Surgeries	76 (19.0%)

When patients asked for their opinion towards presence of medical students, the vast majority (80.3%) said they allowed their existence during consultation (Figure 1).

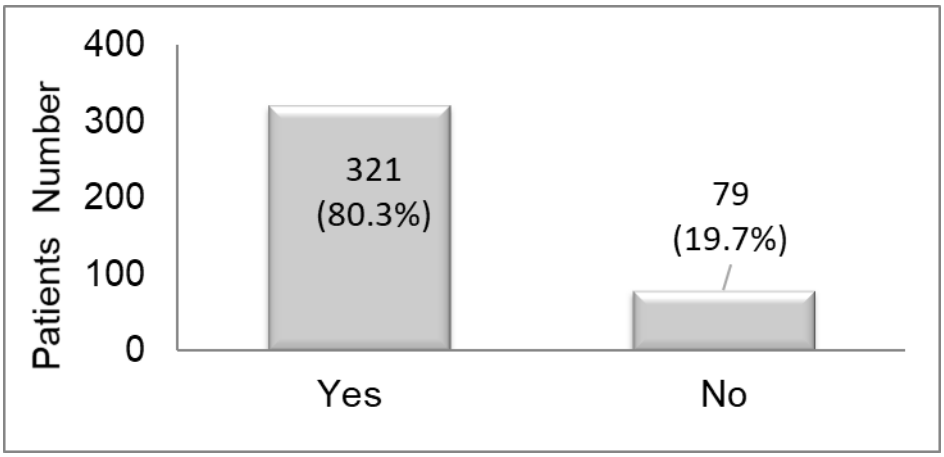

Figure 1. Patients' acceptance for presence of medical students during consultation

Factors that were found to affect significantly their decision were age (more in older people) and department in hospital (lower in obstetric and gynecological department). Table 2 shows these relations in more detail.

Those who accepted the presence of students during consultation were asked for their

opinion for suitable number of students that should be present during consultation. The answer for this question revealed that 145 (45.2%) thought the number of students should not exceed five, while the remaining 176 (54.8%) did not care. Another question was asked to those patients if they allowed the students to perform physical examination to them. Here, more than half said they always accepted, 169 (52.6%); around one-third linked their acceptance by presence of students from same sex, 105 (32.7%); while the remaining 47 (14.6%) patients did not permit at all.

Table 2. Relationship between patients' agreement for presence of students and study sample characteristics

Character		Results	P-value
Age (yr) (mean ± SD)	**Accepted**	42.9 ± 17.4	0.034
	Refused	38.3 ± 15.5	
Sex* n (%)	**Men**	111 (81.6%)	0.622
	Women	210 (79.5%)	
Education Years (mean ± SD)	**Accepted**	6.58 ± 5.18	0.780
	Refused	6.76 ± 5.35	
Residence * n (%)	**Urban**	265 (80.5%)	0.748
	Rural	56 (78.9%)	
Hospital department* n (%)	**Internal Medicine**	176 (85.0%)	0.009
	Surgeries	62 (81.6%)	
	Obst. & Gyne	83 (70.9%)	

* The results of these variables concerned those who answered 'yes' only

Analysis for possible influential factors on opinions (Table 3) discloses the following: Regarding relationship with students' number, the age of patients appeared to have high significant role. As age increases, those who care for presence of a limited number of students decline. Sex of patients and departments in hospital in which they found affected significantly their opinion also. Men and those who existed in internal medicine departments were more likely to never mind the presence of large number of students. Patients' residency & their education level did not exhibit any significant association.

Concerning permission for students to perform examination, the age exhibits again high significant association. More than three-quarters of those with age equal or more than 60 years allowed students from both sexes to examine them. For younger patients the decision of acceptance tends to depend more on presence of students from same sex. The sex of patients and hospital departments has demonstrated also high significant association here. More than 80% of men allowed students from both sexes to examine them; while nearly half of women allowed examination for female students only. Nearly 70% and 55% of those in internal medicine & surgical wards allowed examination for all students, respectively. However, this percentage decline markedly in obstetrics and gynecology where sex of students plays an important role. As with opinion for students' number, the residency & education of patients did not show significant association.

The last table (Table 4) explores more in depth the perceptions & considerations of those who accepted the presence of medical students. The written answers in this table belong to those who answered with yes on relevant questions.

Table 3. Relationship between patients' opinion for suitable number of students /allowing students to do examination and sample characteristics

	Character	Suitable number of students n (%) ≤ 5	≥ 6	P-value	Allowing students to do examination n (%) Yes	Depend*	No	P-value
Age (yr)	18 - 39	83 (55.0%)	68 (45.0%)	<0.001	56 (37.1%)	67 (44.4%)	28 (18.5%)	<0.001
	40 - 59	44 (43.1%)	58 (56.9%)		61 (59.8%)	27 (26.5%)	14 (13.7%)	
	≥ 60	18 (26.5%)	50 (73.5%)		52 (76.5%)	11 (16.2%)	5 (7.4%)	
Sex	Men	40 (36.0%)	71 (64.0%)	0.017	90 (81.1%)	11 (9.9%)	10 (9.0%)	<0.001
	Women	105 (50.0%)	105 (50.0%)		79 (37.6%)	94 (44.8%)	14 (17.6%)	
Residency	Urban	116 (43.8%)	149 (56.2%)	0.274	147 (55.5%)	83 (31.3%)	35 (13.2%)	0.070
	Rural	29 (51.8%)	27 (48.2%)		22 (39.3%)	22 (39.3%)	12 (21.4%)	
Hospital Department	Int. Medicine	70 (39.8%)	106 (60.2%)	0.039	122 (69.3%)	41 (23.3%)	13 (7.4%)	<0.001
	Surgeries	28 (45.2%)	34 (54.8%)		34 (54.8%)	20 (32.3%)	8 (12.9%)	
	Obstetrics and Gynecology	47 (56.6%)	36 (43.4%)		13 (15.7%)	44 (53.0%)	26 (31.3%)	

* Acceptance depends on presence of student from same sex

Table 4. Patients' perspectives concerning presence of medical students

Questions	Agreement*
Do you believe you will have more attention if medical students are present?	200 (62.3%)
Do you believe you can learn more on your problem if students are present?	219 (68.2%)
Do you think it is important to have time alone with the doctor during consultation?	243 (75.7%)
Do you think it is good and acceptable for students to see your medical records?	313 (97.5%)
Do you consider it essential for the doctor to be present with students at all times?	174 (54.2%)

* Number and percentages in this table include only those who answered with 'Yes'

Discussion

Participation of patients in medical teaching is an important tool of medical education. This study contributes to the understanding of Iraqi patients' attitudes towards the involvement of medical students in clinical teaching, as reported by those seen at Al-Imamein Al-Kadhimein Medical City.

Overall, the degree of acceptance of medical students by patients was high (≈80%) and falls within range that was reported in many other studies in the Arab World and other countries

[23-26]. The study conducted by Sayed-Hassan RM & colleagues at Damascus University Teaching Hospitals [25] found that 67.8% of patients approved the presence of medical students during the medical consultation. Another study conducted at University Charity Teaching Hospital in Sudan estimated the acceptance rate to be 95.2% [26]. An article, which assessed acceptability of medical education among patients and their companions in Brazil, found that 85% of participants would allow a student to be present during consultations [23].

This high allowance was attributed in previous studies to patients' willingness to participate in education process, the additional time that doctors will give to them, and availability of opportunity to talk about their illness. Moreover, it was demonstrated that patients learn more about their condition when physician teach students [3,25,27-28]. The current study succeeded in finding common ground with these explanations as around two-thirds of those who accepted existence of students believed that they have more attention and care if medical students are present during consultation; and nearly 70% of them believed that they can learn more and have a better understanding of their problem in the presence of medical students.

Certain factors seem to have a marked influence on attitude of patients towards involvement of medical students during consultations. Concerning sociodemographic factors, the main feature that can be noted is that mean age of those who accepted the presence of students is higher than those who refused. Nevertheless, no one of other factors like sex, education and residence succeeded in achieving such significant association. Socio-demographic and individual variables were found in previous studies to have positive association with patients' attitudes [3,16,24,29]. The results, however, were inconsistent and sometimes contradictory with each other indicating the effects of local conditions and circumstance related to each study. In Nigeria, Onotai et al. [29] also reached that older patients tend to show more positive attitude and willingness to accept students in their care. Another study conducted by Ben Salah et al. in Tunisia [3] concluded that female patients accepted more the role of medical students in reading their medical files, being present in outpatient clinic, attending ward rounds and surgical intervention and taking medical history. Lastly, Ghobain et al. study [16]) in Saudi Arabia reported that those in middle age group (45-64 years) and those with low education level have lower acceptance of medical students' participation in their healthcare.

The lower rate of acceptance of presence of students in gynecology/obstetrics departments was expected due to nature of work in them, which requires exposure of hidden parts of bodies and dealing with women recently recover from delivery and labor. These findings were consistent with results of Shann et al. [12], Mavis B et al. [13], and Hartz et al. [30] which reported that approval of students' involvement in obstetrics and gynecology as well as in Genito-Urinary departments were lower than in others.

As the degree of student participation increased (from observation to records review to examination and procedures), the rejection rate increased. This is most probably related to privacy issues which have also been noted in study conducted by Ben Salah and colleagues in Tunisia [3]. Another reported reason for objection towards students' involvement in physical examination was low confidence in medical students' skills to do a proper examination that can detect findings [26,31]. More than half of patients in the current study considered it is essential for tutor/doctor to be present with the students at all times; and this finding matches with the results of Sayed-Hassan et al. [25], who concluded that the patient's feeling of safety and comfort relies on presence of a supervisor.

The current study revealed also that patients' reaction towards allowing medical students to perform examination depend on certain

characteristics of patients themselves (such as sex and age). Female patients showed nearly equal acceptance rate to male patients when asked about situations where there was a minimal direct contact with students (e.g. observation and checking records). However, when asking for examinations, they were less likely than male patients to accept students, mainly of different gender. This finding agrees with that previously obtained in other Muslim countries [3,25] and even in Muslim women living in non- Muslim countries [32]; where cultural and religious issues might affect attitude of patients toward male students. This attitude may lead to poorer clinical experience of male students [25]. Similarly, Male students have been reported in other study conducted in obstetrics and gynecology clinics [33] to be more likely to experience gender bias from patients.

One of the noticeable finding of this study is the view of patients for the suitable number of patients that should be present in consultation. The results, which revealed that female patients and those who admitted to obstetrics and gynecology departments preferred more limited number of students seems logical. The presence of large number may aggravate the feeling of embracement and confusion that they already have. However, the influential effect of patients' age on opinion for suitable students' number may need further reflection. Those with older age tolerated more the higher numbers than younger. No clear explanation was found; however, it has been reported in literatures that elderly patients tend to hold fewer negative views for young students than younger patients who are more likely to feel vulnerable from students [18].

The important strength of this study is its originality. Up to our knowledge, this study is the first one in Iraq that aims to assess patients' acceptability of medical students in a teaching hospital, in a country where medical education is based on bedside teaching. Social desirability bias (tendency of survey respondents to answer questions in a manner that will be viewed favorably by interviewer) may be a limitation to our study. In fact, patients were surveyed while they still hospitalized which could influence response rate and answers. However, data collectors were alerts for this issue and have informed patients that their answers would not affect the quality of care provided.

As a conclusion, this study revealed that patients in Al-Imamein Al-Kadhimein Medical City showed overall positive attitudes towards the involvement of undergraduate medical students in consultation which was comparable to that reported in previous studies. The reasons for this positive attitude can be attributed among other causes to patients believe that they will have more attention and care if medical student is present and/or they can learn more and have a better understanding of their problem.

This study explores an important and sensitive aspect in medical education. It highlights the need for patient's education and giving information regarding importance of students' involvement in consultation. Modern medical teaching programs must take into consideration advantage of positive attitude, emphasize patient role as educator and use alternative learning methods in situations where patient's consent for student involvement was not obtained in order to guarantee optimal care and safety to patients and good medical education to future physicians. Patients, students as well as clinical teachers need further learning about the ethics of patients' involvement in medical teaching.

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Conflict of interest

The author has declared that no competing interests exist.

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Molecular Detection of Cytomegalovirus in A Sample of Iraqi Patients with Acute Leukemia and Stem Cell Transplantation

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Abstract

Background Acute leukemia and hematopoietic stem cell transplantation are risk factors for opportunistic infection and reactivation of many latent infection like cytomegalovirus.

Objective Detection and quantification of cytomegalovirus viremia in patients with acute leukemia after induction chemotherapy and post allogeneic stem cell transplantation patients.

Methods A prospective study enrolled 61 patients with acute leukemia. Forty-eight of them evaluated while induction chemotherapy (group I), while the other 13 within 1-year post bone marrow transplantation (BMT) (group II). In addition, 30 apparently healthy individuals were recruited as (control group), blood samples were collected from all groups. Viral DNA was extracted from 1 ml plasma samples, and then, cytomegalovirus DNA was detected and quantitatively assessed by Taqman quantitative real-time PCR.

Results Twelve (25%) out of 48 patients in group I, 2 (15.4%) out of the 13 patients in group II, and 2 (6.7%) out of 30 in the control group had positive cytomegalovirus viremia. The mean cytomegalovirus viremia was 5.192x102, 2.71x102 and 1.60x102 copies/ml for group I, group II and controls respectively, p=0.056.

Conclusion There is a relatively high prevalence of cytomegalovirus viremia in Iraqi patients with acute leukemia after chemotherapy and post BMT. Real-time PCR assay is helpful for early diagnosis of cytomegalovirus viremia in leukemic patients and used to monitor post BMT patients at risk for cytomegalovirus disease.

Keywords HCMV, acute leukemia, stem cell transplantation, real-time PCR

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List of abbreviations: ALL = acute lymphoblastic leukemia, AML = acute myeloblastic leukemia, CBC = Complete blood count, DM = diabetes mellitus, HBV =hepatitis B virus, HCMV = human cytomegalovirus, HCV = hepatitis C virus, HT = hypertension, QRT-PCR = Quantitative real time polymerase chain reaction, SCT = stem cell transplantation

Introduction

Acute leukemia is aggressive disease in which malignant transformation occurs in hemopoietic stem cell or early progenitors [(1)]. Acute leukemia has two broad classifications: acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML) [(2)]. In acute leukemia, normal hematopoiesis is replaced by immature cells and deregulated proliferation of leukocytes [(3)]. Chemotherapy and stem cell transplantation (SCT) are the established therapeutic options for these patients. These methods cause an increased

risk of infections [4]. Patients undergone SCT are more susceptible because their immune system is depressed by immunosuppressive therapy and the immune reconstitution is not fully developed. This can lead to severe infection [5].

Cytomegalovirus (HCMV) belongs to the human herpes viruses, beta herpes viruses. It is a major pathogen causing significant mortality in immunocompromised hosts [6,7]. It has double-stranded DNA (dsDNA) genome, which is longer than all other human herpes viruses [8]. HCMV can infect a wide range of cells within its host, including various hematopoietic cell types and connective tissue and parenchymal cells of any organ [9]. HCMV seropositivity is common in the general population, with a prevalence ranging from 30-97%. After the primary infection, HCMV establishes a life-long latency in various organs [10-12]. HCMV has a wide spectrum of clinical presentation. It can present generally as asymptomatic and persistent infections in healthy persons. However, it can also lead to serious disorders among transplant recipients, immunodeficient patients, patients on immunosuppressive treatment [13], and patients with hematological malignancies [14].

Most of the recent studies showed high incidence of HCMV infection in leukemia patients [15,16]. In addition, HCMV infection is a major infectious complication after allogeneic hematopoietic cell transplantation (allo-HSCT) [17-19]. Early HCMV reactivation remains associated with increased transplant-related mortality [19]. A previous study in Iraq revealed a 28% prevalence of HCMV in ALL patients. However, the best of our Knowledge, there is no similar study regarding HCMV in overall acute leukemia [20].

This study aimed to detection of HCMV in patients with acute leukemia after chemotherapy (induction) courses and post allogeneic stem cell transplantation patients within the first year, and to determine copy number of HCMV in these groups and compare with apparently healthily individuals.

Methods

Study population

A prospective study conducted from 1st of December 2016 to 1st of June 2017. Sixty-one (61) patients with acute leukemia were enrolled in this study. Forty eight (78.7%) of them had received an induction course of chemotherapy within one month of diagnosis as group I. Those are comprised as 18/48 (37.50%) patients with ALL and 30/48 (62.50%) with AML. They collected from Hematology Ward at Al-Imamein Al-Kadhimein Medical City and Baghdad Teaching Hospital, Medical Complex. The rest thirteen (21.3%) acute leukemia patients (5 ALL and 8 AML) had assessed after bone marrow transplantation within the first year of diagnosis as group II and collected from the Bone Marrow Transplantation Center in the Medical Complex, Private Nursing House. Thirty apparently healthy individuals from volunteers and donors in the blood bank who served as control group. A consent letter was obtained from all patients and controls enrolled in the study. This study approved by the Institutional Review Board of the College of Medicine, Al-Nahrain University. Clinical and laboratory data were obtained from all patients from records and controls by direct interview. Blood sample were collected in EDTA tube form study groups and 1 ml of plasma was separated and preserved in deep freeze for viral DNA extraction.

Viral DNA extraction

For viral DNA extraction from the plasma samples; Geneius™ Viral Nucleic Acid Extraction Kit III (Geneaid, Taiwan) was used. One ml plasma was used in viral DNA extraction, according to the manufacturer protocol.

Real Time PCR for measuring HCMV viremia

For the quantitative detection of HCMV; HCMV dtec-qPCR Test F-100 Kit (Genetic PCR Solutions TM, Spain) a Real-Time test, which is based on the principle of the so-called - "TaqMan" probe was utilized. Fifteen µl of Master Mix were added into PCR tubes, and 5

µl of the (sample DNA, positive or negative controls, or standards) were added to the master mix. The final reaction volume was 20 µl. All components were kept at room temperature during the PCR preparation. Real time PCR instrument used in this work was STRATAGENE MxPro QPCR (Agilent Technologies, USA). The thermal protocol for HCMV dtec-qPCR kit is composed of a two hold steps, and one amplification cycle. The real time data is collected at the second step of the amplification cycle as demonstrated in table (1).

At the end of the thermal protocol, the Real Time PCR (MxPro QPCR) instrument software automatically calculates the baseline cycles and the threshold. The standard curve is plotted using the data obtained from the defined standards, with the (Y) axis is the Ct-Threshold Cycle, and the (X) axis is the viral DNA copy number. According to the manufacturer instructions, HCMV DNA copies were calculated depending on to the following formula:

$$\text{copy/ml} = \frac{\text{SC x EV}}{\text{IV}}$$

SC = Sample Concentration (copy/µL)
EV = Elution Volume
IV= Isolation Volume (ml)

Table 1. HCMV real time PCR amplification profile

Step		Time	Temperature
Activation 1		15 min	95 °C
40 cycles	**Denaturation**	15 sec	95 °C
	Hybridization/Extension and data collection 2	60 sec	60 °C

*1- step one; **2- step two

Statistical analysis

Microsoft excel 2016 and SPSS (statistical package for social sciences) version 23 was used for statistical analysis. Most of the data were numerical so presented as mean ± standard deviation, and comparison between means of study groups was done by using independent student t-test. Categorical data were presented as frequency and percentage; fisher exact test, and chi-square test and Mann Whitney test were used for comparison between frequencies of study groups. P value less than 0.05 was considered as significant.

According to test of normality (Shapiro-wilk) most of data, were not normally distributed for this we used the Mann Whitney test, WBC < 0.001, Neutrophil < 0.001, lymphocyte < 0.001, Hb < 0.057, platelets < 0.001.

Results

The ratio of males was the predominant, 56.3% (27/48), 76.9% (10/13) and 56.7% (17/30) in group I, II and control respectively, while the mean age was 37.27 ± 15.66, (range of 14-70 years); 29.77 ± 14.45 (range of 12-56 years) and 30.87± 10.58, (range of 14-53 years) respectively. Statistically, there was no significant difference (p=0.076) between the mean age of the patients and control indicating that they were of a comparable age.

Quantitative real time PCR (QRT-PCR) run gave positive viremia in (25.0%) 12 out of 48 in group I and (15.4%) 2 out of 13 in group II as well as (6.7%) 2 out of 30 in control, however, these results were statistically not significant (p=0.056) in the mean of the copy numbers in all groups. Table (2) shows that the mean copy number in group I was (519.17 ± 236.44), group II was (271.0 ± 24.04) and the mean of copy number in control was (160.0 ± 4.24).

Table 2. Comparison of HCMV copy number in different study groups by ANOVA

Parameter	Group I copy/ml	Group II copy/ml	Control copy/ml	P value
HCMV copy no.	0.379×10^3 0.409×10^3 0.384×10^3 0.511×10^3 0.518×10^3 0.670×10^3	0.288×10^3	0.163×10^3	0.056
	0.405×10^3 0.207×10^3 0.881×10^3 0.427×10^3 1.045×10^3 0.394×10^3	0.254×10^3	0.157×10^3	
Mean	519.17	271.0	160.0	
SD	236.44	24.04	4.24	
Range	$(0.207\text{-}1.045)\times10^3$	$(0.254\text{-}0.288)\times10^3$	$(0.157\text{-}0.163)\times10^3$	

*Cut-off level of this method was 0.150×103 copies/ml. HCMV viremia was defined by positive HCMV -specific RT-PCR in plasma [21]

According to type of leukemia in group I and II, 2 (8.7%) patients with ALL and 12 (31.6%) patients with AML positive for HCMV. There was a significant association between HCMV viremia and type of leukemia (p value = 0.012) as in table (3).

Table 3. Comparison of the results of HCMV viremia according to type of acute leukemia in group I and II

HCMV	ALL (group I + II) N=23 No. (%)	AML (group I + II) N=38 No. (%)	P value
Positive	2 (8.7)	12(31.6)	0.012
negative	21 (91.3)	26 (68.4)	

Relationship between the HCMV viremia and the demographic data

Data of this study revealed that there was no significant difference in the age of the three groups and HCMV viremia. Group I (p value = 0.582), age group (20-39) was the most frequently reported in all groups. Regarding gender, males were predominant in group I. HCMV was positively expressed more in male where 7 out of 12 patients. In group II patients HCMV viremia was detected in 2 male patients, whereas two females in control group. No statistically significant difference was found among sex in the 3 groups.

Relationship between the HCMV viremia and history of blood transfusion and co-infection

There was no significant association between HCMV positive viremia and blood transfusion (p value = 0.601, 0.656) in group I and group II respectively. Similar results were reported in relation to co-infection with HBV as there is no statistical significance in both groups (p value =

0.08, 0.512). Only one patient was positive for HCMV and HBV together.

Relationship between the HCMV and the hematological parameters

Statistically significant difference according to hematological parameters between the HCMV positive and HCMV negative patients (group I and group II) was found as shown in table (4).

Table 4. Relationship between HCMV positivity group I and group II with hematological parameters

Parameter		HCMV Negative N=47	HCMV Positive N=14	P value
WBC (*10^3/µl)	Mean	10.46	2.44	0.004
	SD	19.11	1.59	
	Median	4.80	2.42	
	Range	0.2-86.43	0.26-4.7	
Neutrophils (*10^3/µl)	Mean	4.26	0.90	0.01
	SD	6.56	0.70	
	Median	1.55	0.71	
	Range	0.2-30.57	0.01-2.06	
Lymphocytes (*10^3/µl)	Mean	3.71	0.90	0.015
	SD	8.10	0.44	
	Median	1.42	1.05	
	Range	0.01-40.39	0.24-1.4	
Hemoglobin (g/dl)	Mean	9.76	7.94	0.045
	SD	3.13	1.43	
	Median	9.30	7.75	
	Range	4.2-16.4	4.7-10.6	
Platelets (*10^3/µl)	Mean	124.36	64.64	0.03
	SD	95.63	73.10	
	Median	96.00	36.50	
	Range	7-355	12-260	

*Leukopenia (WBC count < 4.0×103/µl) [22].
*Neutropenia (neutrophil count <1.5 ×103/µl) [23].
*Lymphopenia (Lymphocytes < 1.0×103/µl) [24].
*Anemia (hemoglobin < 12 g/dl) [25].
*Thrombocytopenia (platelets<150×103/µl) [26].

Discussion

This study revealed that 12 out of 48 (25%) of acute leukemia patients were positive for HCMV viremia, distributed as 10 out of 30 (33.3%) of AML positive to HCMV, which is comparable to other studies such as Capria et al. 2010 [27], in which 35% (21/59) patients in complete remission after chemotherapy were HCMV positive. On the other hand, out of 18 ALL patients, only 2 (11.11%) were HCMV positive. This result is comparable to that of Han et al. 2007 [28] who reported HCMV viremia in 11.1% of ALL cases and Jain et al. 2016 [29] who reported it to be in 10% of children with ALL by PCR.

Out of those patients who were studied after bone marrow transplantation only two out of 13 (15.4%) were HCMV positive, which disagreed with other studies, accomplished by Guenounou et al. 2016 [30] who reported it to be 49/136 (36%) and Poiré et al. 2017 [31] who reported that 84/125 (68.3%). Important

explanation is patient under prophylaxis drug, the serostatus for HCMV of donors and recipients before transplantation, restricted accessibility for all patients and may be due to the small number of cases in the present study compared to previous studies. In the control group; only 2 out of 30 (6.66%) was positive for HCMV, this result is within range in comparison to other studies included study in Ouagadougou, Burkina Faso which was 5.1% by Traore et al. 2016 [32], and HCMV showed 10% seropositive in donors of blood bank of Mosul city by Al-Dabbagh et al. 2011 [33].

HCMV viremia and type of leukemia

The results of this study showed a significant association between HCMV viremia and type of acute leukemia; (P = 0.012). 12 AML and 2 ALL in acute leukemia (group I) and PBMT (group II); which is contrary to that of Dixon et al. 2017 [34] who showed that high HCMV positivity directly associated with ALL rather than AML. Also, another study by Han et al. 2007 [28] showed that HCMV expressed in 11.1% of ALL cases, while only 4% in AML. Many reasons standing behind this result including, the use of aggressive chemotherapy regimen in AML patients, or hospital acquired infection from para-medical personnel or form care giving career, in addition to another possible source for the primary infection which might be through blood transfusion where they transfused more regarding platelet and blood product transfusion. Another, possible explanation is that the AML samples are larger than ALL in this study and the different in number of chemotherapy courses between these studies.

Viremia and demographic data

There was no significant association between HCMV viremia with age and sex of the patients and control groups, a result which is supported by other reports from Loutfy et al. 2017 [35] and Loutfy et al. 2006 [36].

Relation HCMV viremia with blood transfusion and Co-infection

Regarding the correlation of HCMV positivity with the blood transfusion, it appeared that there was no significant relation. This result was consistent with that obtained by Pennap et al. 2016 [37] and Ojide et al. 2012 [38]. Another possible source for the primary infection might be that the blood donor was infected with active asymptomatic HCMV infection and may be viremia at the time of donation. A study reported that 3% of normal blood donors can be viremic during the time of donation and HCMV has been isolated from peripheral blood of healthy blood donors [39]. This study found no statistical significance association between HCMV viremia and HBV, compared to another study showing that the HCMV infection is common in chronic HBV patients, who can be regarded as patients at high risk for HCMV disease and those result was obtained by Bayram et al. 2009 [40].

HCMV viremia and hematological parameters

Regarding correlation between the hematological parameters and HCMV viremia in both groups, there was significant association between the mean WBCs count HCMV positive patients was lower than that in HCMV negative, (2.44 ± 1.59) x 10^3/µl vs. (10.46 ± 19.11) x 10^3/µl cells, respectively, p = 0.004, those findings were comparable with the results obtained by Loutfy et al. 2017 [36] and Jang et al. 2011 [41]. These studies clarified that the majority of patients positive to HCMV DNA was associated with leucopenia (p = 0.03, 0.012 respectively).

Intensive cytotoxic chemotherapy can cause severe and sometimes prolonged neutropenia, which may cause potentially fatal infection. Severe prolonged neutropenia is most likely to occur in the pre-engraftment phase of hematopoietic cell transplantation (HCT; particularly allogeneic) and in patients undergoing induction chemotherapy for acute leukemia. The mean neutrophil was higher in HCMV negative patients as compared to the HCMV positive patients, the differences in mean values of these parameters were statistically insignificant (p = 0.01) and those results in agreement with Loutfy et al. 2017 [35] and Jang et al. 2011 [41]. Who reported that HCMV viremia was mainly expressed in patients who had neutropenia.

In addition, the mean lymphocytes count in HCMV positive patients was lower than in HCMV negative patients, (0.90 ± 0.44) x 10^3/µl vs. (3.71 ± 8.10) x 10^3/µl, respectively, (p = 0.015), this result is comparable to that of Loutfy et al. 2017 [35], Jain et al. 2016 [29], which conformed that HCMV viremia is significantly associated with Lymphopenia.

The mean Hemoglobin level of HCMV positive (7.94 ± 1.43) g/dl was lower than (9.76 ± 3.13) g/dl of the HCMV negative, p = 0.045. Our result disagreed with Loutfy et al. 2017 [35]. Leukemic patients of different classifications are associated with anemia [42]; it may also be a result of patient's antineoplastic therapy or progressive disease and hemolysis. Acute infections with HCMV may lead to severe hematologic disorders. HCMV infection can also be associated with hemolytic anemia [43,44]. Platelets count was different also, and lower in HCMV viremic patients, in which it was significantly higher in HCMV negative patients, (64.64 ± 73.10) x 10^3/µl vs. (124.36 ± 95.63) x 10^3/µl cells, respectively, p = 0.03. This result agreed with Loutfy et al. 2017 [35]. It may also be early evidence of HCMV infection; Symptomatic thrombocytopenia may be early evidence of haematological disorders caused by HCMV infection [45].

This study concluded that patients with acute leukemia after chemotherapy or post BMT are at high risk of HCMV infection or reactivation.

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Authors' contribution

Al-Toban: Collection of specimens, DNA extraction, and real time-PCR run, writing of the manuscript and references. Dr. Al-Marsomy: Supervision. Dr. Al Tameemi: Consultant hematologist helped in selection of patients, providing of patients and writing of the manuscript. Dr. Al-Obaidi: helped in real time-PCR run. Dr. Mohammed: Consultant hematologist help in providing of patients. Dr. Al-Saeed: Consultant hematologist help in providing of patients. Dr. Al-Shemary: Consultant hematologist help in providing of patients.

Conflict of interest

The authors declare no conflict of interest.

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