Iraqi Journal of Medical Sciences

IRAQI JMS

المجلة العراقية للعلوم الطبية

Volume 16, Number 2, 2018

April - June

P- ISSN 1681-6579

E- ISSN 2224-4719

Volume 16 (2) 2018
DOI: 10.22578/IJMS.16.2.

P-ISSN 1681-6579
E- ISSN 2224-4719

IRAQI JOURNAL OF MEDICAL SCIENCES

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Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

Crimean-Congo Hemorrhagic Fever in the Middle East: History and Facts

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Abstract

Crimean-Congo Hemorrhagic Fever (CCHF) is the highest widespread, tick-borne viral hemorrhagic fever that affect humans. This virus is endemic in many areas in the world, such as Africa, Asia, and Europe. Nowadays, the incidence of CCHF is increasing rapidly in several countries of the middle-east, with several outbreaks and sporadic cases in human of CCHF, that are reported in several countries in this region.

Keywords CCHF, Middle east, Iraq

Citation Al-Obaidi AB. Crimean-Congo hemorrhagic fever in the middle east: history and facts. Iraqi JMS. 2018; 16(2): 111-113. doi: 10.22578/IJMS.16.2.1

List of abbreviation: CCHF = Crimean-Congo hemorrhagic fever, ssRNA = Single stranded RNA

Introduction

Crimean–Congo haemorrhagic fever virus (CCHF), genus Nairovirus, family Bunyaviridae, is an enveloped- with a negative-sense ssRNA genome, causes zoonotic disease in many countries of Africa, Asia, Middle East and southeastern Europe [1-3].

The distribution of CCHF virus coincides with the distribution of its vector; ticks of genus Hyalomma, makes the spread of these infected ticks into new unaffected areas to facilitate the spread of CCHF virus. The virus circulating in a tick-vertebrate-tick life cycle. These ticks infest on wide spectrum of wildlife animals, and livestock animals, e.g. cattle, sheep, and goat [3,4].

Viremia in these livestock is of short lived and low intensity. The livestock animals play a very important role in life cycle of ticks, and in amplifying and transmitting the virus and are, therefore, the focus of veterinary public health, however, animals do not develop clinical signs, while the virus causes significant human illness [4-7].

Disease characteristics

Infection acquired in humans from bites of tick, or from contact with the infected blood or tissues of livestock or human patients. After a 3-7 days incubation period, the patient can develop severe disease, with a pre-hemorrhagic phase, a hemorrhagic phase, and then a convalescence period. The hemorrhagic manifestations can range from small petechiae to large-hematomas. Bleeding could be from the nose, gastrointestinal tract, respiratory tract, urinary tract, and uterus, with a mortality rate range from 5% to 80% [3,8,9].

The severity of this infection in humans focus the impact of this zoonotic disease on public health. Serological screening of animal serums for CCHF virus-specific antibodies is very important. Because the virus prevalence in animals is good indicator of the circulating local

virus, these sero-prevalence studies allow the identification of high-risk areas of human infection [10].

Slaughterhouse workers, stockmen, and veterinarians, should be made aware of the disease. They should work on limiting or avoiding exposure of the naked skin to infected blood and tissues, and to avoid tick bites. In general, treatment of livestock could reduce tick density among these animals, and then reduce the of tick bite risks in these animal handlers [10]. The infectivity of CCHF virus can be destroyed by boiling low concentrations of formalin and lipid solvents [4].

Migration and Middle East outbreaks

There are 7 CCHF viral genotypes: Africa-1, Africa-2, Africa-3, Asia-1, Asia-2, Euro-1, and Euro-2. According to the region in which they were first recognized and still circulate. However, more than one genotype can be found through multiple countries. This might suggest the migratory pattern of the virus and the appearance of outbreaks [11]. CCHF described for the first time in 1944 in the Crimean Peninsula-Russia, and later in the Democratic Republic of the Congo in 1956, which later on gave its name Crimean-Congo. Firstly, this virus was widely reported in Africa, however, in recent years, outbreaks in the Middle East, Asia and Eurasia have become more common [2].

Studies suggested that CCHF virus is a migrating pathogen, however it is not clear this migration is to what extent. Mild et al. in 2010 have analyzed the worldwide migration pattern of CCHF virus, for the first time, their study found that Turkey could be the source of migration to Europe, both to the east and the west, and the United Arab Emirates is the source of migration to the Middle East [12].

In 2002 and 2003, twelve patients in Turkey had proved CCHF virus infection that resemble the virus genotypes found in Russia and Kosovo very closely, and a different genotype from those that caused the outbreak of CCHF in Iran in the year 2002. These data proved that the disease in the affected areas in Turkey was not introduced from Iran whether by a livestock or from an infected tick [13].

In 2009, Chinikar et al. reviewed the history of CCHF in Iran, showed that the disease was detected in Iran since 1970. However, the mortality rate was about 20% in 2000 and then dropped remarkably to 6% in 2007 [14]. Iranian molecular studies demonstrated that the Iranian CCHF virus strains were very similar to the Pakistani Strain [15].

A study in Iraq's Sulaimani province in 2016, showed that CCHF is a rare viral infection in the Sulaimani province, and no proven reported cases, which is mainly due to the ticks' eradication which was done by the veterinary and agricultural authorities. However, other preventive measures and strategies could be carried out and always monitored by local Sulaimani authorities [16].

The World Health Organization (WHO) reported sporadic human cases and some outbreaks of CCHF virus in the Eastern Mediterranean area, mainly in Iran, Saudi Arabia, Iraq, Oman, Pakistan, Kuwait, and the United Arab Emirates. In these countries, CCHF was increasing in the recent years, and new cases are reported in new areas in this region, with a more geographic extension of this viral disease, which might be linked to the spread of infected ticks by the migratory birds and the trade in livestock. The rise in temperature and decrease in rainfall in the Eastern Mediterranean Region can lead to increase in the distribution of the habitats that are suitable for Hyalomma-ticks, which results in increase in the CCHF virus infection rates [17].

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Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

Effect of Stick Sweet Cherry Extracts (Prunus (SP)) on Some Biochemical Markers in Albino Mice and Biological Activities in Different Types of Bacteria

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Abstract

Background The sweet cherry (Prunus avium L) has a wide variety of secondary metabolites, which has biochemical and biological activity and used as potential source of a drug. There is no data published on stick cherry extract effect on enzymes activities, kidney function test, inflammatory marker and minerals. As well as on antimicrobial activity of stick cherry extract for inhibition the growth of pathogenic bacteria of different types.

Objective To illustrate the effect of ethanolic stick sweet cherry extract on serum enzyme activities; lactate dehydrogenase (LDH), aspartate transaminase (AST), creatine kinase (CK) and the concentration of urea, uric acid, creatinine, C-reactive protein (CRP) also potassium, calcium in serum of albino mice as well as to investigate antimicrobial activity of the extract to inhibit the growth of different types of pathogenic bacteria.

Methods A total of 28 albino mice were classified into three groups, the first control group (G1) consist of 8 animals treated with 0.2 ml/day distilled water, second group (G2) comprised of 10 animals treated with 30 mg/Kg/day of stick cherry extract, third group (G3) formed of 10 animals treated with 100 mg/Kg/day of extract.

Results There was statistically significant reducing effect in serum enzyme activities of LDH, CK ($P<0.001$) at 30 and 100 mg/Kg/day, and significant change in serum AST at low and high concentration. There is remarkable change in concentration of urea, uric acid, creatinine and increase in potassium and calcium ($P<0.001$). The result of antimicrobial activity of stick sweet cherry extracts show inhibition the growth of pathogenic bacteria, different types which involves; (*Pseudomonas aeruginosa, Escherishia coli, Staphylococcus aureus, Proteus vulgarweris, Serratiam arcescens*). This indicates that the chloroform extract was active against the most pathogenic bacteria at all the concentration used and was more than ethanol extracts.

Conclusion Orally administration of sweet stick cherries extracts to albino mice animals caused a potential difference in different biochemical parameters, enzyme activities, LDH, AST, CK, concentration of urea, uric acid and creatinine, C-reactive protein. Those are risk factors for different diseases, inflammatory, oxidative stress, heart disease. This could be minimized or prevented by polyphenols of cherries stick extract.

Keywords Prunus, Creatine kinase, Sweet cherry, CRP, Potassium, *Escherichia coli*

Citation Saleem MNMN. Effect of Stick Sweet Cherry Extracts (Prunus (SP)) on some biochemical markers in Albino Mice and Biological Activities in Different Types of Bacteria. Iraqi JMS. 2018; 16(2): 114-124. doi: 10.22578/IJMS.16.2.2

List of abbreviations: AST = Aspartate transaminase, CRP = C-reactive protein, CK = Creatine Kinase, LDH = Lactate dehydrogenase

Introduction

Numerous bioactive chemical compounds of plants are found in dietary sources such as fruits, vegetable, herbs, the plants medicinal value is related to their phytochemical components, which produce definite physiological actions on human body. The most important of these components are a wide variety of secondary metabolites such as alkaloids, flavonoids, tannins, terpenoides, and phenolic compounds which have been found in vitro to have antimicrobial properties [(1)]. The sweet cherry (*Prunu savium L*) are among the most delicious fruit, it is also known popularly as the "super fruit" because of its low in calories, high

antioxidants, which have significant health benefits and provide good immunity against numerous diseases [2,3]. Pharmacological and biochemical effects exhibited by anthocyanins tart cherry have been recommended as nutritional supplements or chemopreventive agents [4]. The biological action mechanism predominantly is thought to result from enzyme inhibition, antioxidant activity, scavenge free radicals [5]. Cherry contain sensible amounts of anthocyanins in addition to other bioflavonoids such chlorogenic acid, gallic acid, p-coumaric acid kaempferol, quercetin, hydroxycinnamates, cyanidins; all are potent antioxidants, and have characteristic anti-inflammatory via inhibition of cyclooxygenase activities [6]. The skin phenolic compounds are contributed to sensory and fruits organoleptic qualities such as astringency and taste [7]. Cherry are rich in dietary fibers, generous intake of dietary fiber reduces risk of developing the following diseases: coronary heart disease, lowers blood pressure and appears to improve immune function [5,8]. Numerous other studies show that other cherries phenolic compounds reducing atherogenicity [9]. Cherries contain several polyphenols antioxidants, anthocyanins and cyanidin that possess many biological activities, anticancer, antidiabetic, and anti-inflammation, antiobese properties [10,11]. Also decreased risk for atherosclerosis, and other metabolic syndrome of heart disease [6,12], and other diseases or protective effects on neuronal cells [13]. Anthocyanin of cherry pigment and bioflavonoid, after consumption transferred to human body, helps to generate essential amino acid and play vital in protection of body cells [10,11]. Cherries contain oxidized form vitamin C dehydroascorbic acid [14], and it contain both hydro soluble, vitamins B and liposoluble vitamins E, K and also characterized by presence of higher content of beta carotene vitamin A, folate and to a lower extent zeaxantine and lutein. Cherries also contain minerals such as calcium, magnesium, phosphorous, potassium and iron [15,16]. Consumption of cherry in healthy women has been observed to reduce uric acid levels circulation and improve symptoms gout that include reduction in symptoms associated with gout, and many inflammatory diseases and provide good immunity against numerous diseases by regulation of circulating inflammatory markers [17]. Inflammatory substance in blood, C-reactive protein, associated with an increased heart disease risk. It was suggested modulatory selective effect of cherries on C-reactive protein (CRP), such effects may be essential for prevention and management inflammatory diseases [18]. Its production also occurs in macrophages, neurons, kidneys, in atherosclerotic lesions by smooth muscle cells and adipose tissue and pulmonary alveoli [19]. CRP is a critical component of the immune system a complex protein that our body make when faced with a major infection or trauma, it depends on genetics as well as lifestyle habits [19].

Herbals, fruits, and spices because of their antimicrobial effect they are of interest due to their possible use as alternatives to food preservatives, apart from being the primary source of food some nutrients essential, fruits and vegetables also contain a variety of bioactive components, which might have other health beneficial [7]. Previously it was observed that the kind and amount of the leaves of *Prunus Laurocerasus L* cherry leaves extracts have a significant effect against tested fungi of different types by using Six different extracts (4 solvent extracts and 2 water extracts) were used to determine the antifungal effect by disc diffusion and micro dilution methods [20].

Despite the high number of publications that document plants extracts of antimicrobial activity against different species of fungi, none of reports have concerned with stick sweet cherry extract (*Prunu savium*).

To our knowledge there is no studies on the effect of stick sweet cherry extract on enzymes activities lactate dehydrogenase (LDH), aspartate transaminase (AST), Creatine kinase (CK), and concentrations of urea, uric acid,

creatinine, potassium and calcium and CRP as well as on the inhibition of different types of pathogenic bacteria growth to find antimicrobial activity which involves; (*Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Serratia marcescens*). Therefore, the aim of the recent work was to investigate the effect of stick cherries extract on this biochemical parameter in blood serum of white albino mice evaluation of antimicrobial activity to inhibit growth of pathogenic bacteria of different types.

Methods

Extraction of stick cherry

Stick sweet cheery (*Prunus avium L*) were collected, purified and air dried at room temperature then grinding to powder, prepared for extraction. The powder was extracted by weighting of 35 gm of crushed stick cherries and adding 400 ml of 70% ethanol in soxhulate at boiling degree for seven hours and with slow continuous mixing and then filtrate the extract in the rotary evaporation until getting a thick solution. At room temperature drying the solution for 2-3 days until it become a crushed dried and was dissolved in distilled water to prepared two different concentrations 30 mg/Kg, 100 mg/Kg for the measurement of biochemical parameter.

Laboratory animals

From Animal House Production Unit of Biotechnology Division, University of Technology, albino mice were obtained, A total of 28 healthy albino mice weighting (15-35 gm) aged of 2-3 months were used in this study. The animals were divided into three main groups: The first group (G1) contained 8 animals, served as the control which received distilled water, while the second group (G2) contained 10 animals, which treated with an oral administration of 0.2 ml/day of stick cherry extract at 30 mg/kg/day concentration for a period of 21 days, and the third group (G3) contained 10 animals, which treated with an oral administration of 0.2 ml/day of stick sweet cherry extract at 100 mg/Kg/day for 21 days. After three weeks of receiving of the extract, the mice were sacrificed in the morning by decapitation. The blood sample were collected by cardiac puncture with disposable syringe, withdraw into plain tube and the taken blood was left for 15 minutes at room temperature for clotting, centrifugation and separated serum were used for the measurement of enzymes activities, LDH, AST, CK, urea, uric acid, creatinine, potassium, calcium, CRP at the same day of collection. Biochemical assay Serum enzyme activities of AST, CK, was determined by spectrophotometer using kit method. Urea, uric acid, creatinine, potassium, calcium and CRP levels were measured by spectrophotometer.

Extraction method of stick cherry extract for evaluation of antimicrobial activity

For extraction of stick cherry powder two solvents were used, chloroform and 70% ethanol solvent by using soxhlet, in experimental study for evaluation of antimicrobial activity of stick sweet cherry extract to find the inhibitory effect of pathogenic bacteria growth. The dried extract was diluted with distilled water for ethanol, chloroform extract used to yield the final concentration (5, 10, 25, and 50, 100) mg/ml of both solvent to test for the antimicrobial activity of plant extract.

Plant extract antibacterial activity was determined by using an agar-well diffusion assay [21]. Bacterial samples obtained from (Biotechnology Branch/University of Technology) where testing was conducted used for the assays. Five strains bacterial were used in present study, they were *Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Staphylococcus aureus, Serratia marcescens*. The suspension cell culture was adjusted by comparing against 0.4-0.5 McFarland scale standard. For the investigation of the antimicrobial activity, the suspensions (0.1 ml) of target strain were spread on the plates. To allow reproduction of the results or further detailed analysis of the strains used the isolates

were kept in culture. The antibacterial activity of the crude plant extracts was cultures were grown on nutrient broth and then inoculated onto Muller - Hinton agar for testing. Following the initial incubation, organisms were suspended in saline solution and their concentration equilibrated. Each sample was transferred onto Muller - Hinton agar by using a sterile cotton swab. The well diameter was 8 mm and distilled water were applied as control for ethanol extract samples and ethanol was applied as control for chloroform extract samples. Plates were incubated over night at 37 °C, for 24 hrs, the inhibition zone was appearing around well measured and recorded the results. The plate crude extract showing the activity were considered as antibacterial activity, the dried extract was diluted with distilled water for the sample of ethanol extract and with ethanol for the sample of chloroform extract to yield the final concentration.

Statistical analysis

All the result was expressed as the mean ±SD. Statistically all data grouped were evaluated with SPSS/10.5 software. Hypothesis of testing method included one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test; P values of less than 0.05 were considered to indicate statistical significance.

Results

The present investigation tested the efficacy of the oral administration of 0.2 ml/day stick sweet cherry extract for period of three weeks on the activities of enzymes, LDH, AST, CK, and kidney function assessment, urea, uric acid, creatinine, as well as potassium, calcium and CRP in serum of albino mice at two different doses, 30 mg/Kg/day and 100 mg/Kg/day, as illustrated in tables 1, 2 and 3. The effect of stick sweet cherry extract on the activities of, LDH, AST, and CK in serum of albino mice, were represented in table 1, the mean±SD values of serum LDH, AST and CK activities were very highly significantly decreased in treated animals at 30 mg/Kg/day and as compared to untreated animal group, ($P<0.001$, $P<0.001$, $P<0.001$) respectively. Significant reduction in the activities of LDH, AST and CK ($P<0.001$, $P<0.01$, $P<0.001$) at concentration 100 mg/Kg/day in treated animal with extract as compared to untreated respectively.

Table 2 illustrated the effect of oral administration of stick sweet cherry extract on concentration of urea, uric acid, creatinine, it shows significant reduction in concentration of urea, uric acid and creatinine ($P<0.001$) at both extract concentration 30 mg/Kg/day and 100 mg/Kg /day respectively in comparison with untreated animal.

Table 1. Effect of stick cherry extract on the activities of, LDH, AST, CK in serum of mice

Parameters (U/L)	Untreated Group (n=8) (G1)	Treated extract with 30 mg/Kg/day (n=10) (G2)	Treated extract with 100 mg/Kg/day (n=10) (G3)
LDH	933.66±59	717.54 ±48*	581±43*
AST	34.45±5.2	27.5±2.7**	23.5±2.4*
CK	105±9	70.0±4.5*	50.0±3.2*

*Very highly significant decrease at $P<0.001$, **highly significant decrease at $P<0.01$ (parameters as means ±SD)

Table 2. Effect of stick cherry extract on, uric acid, urea, creatinine in mice serum

Parameters (mg/dL)	Untreated Group (n=8) (G1)	Treated extract with 30 mg/Kg/day (n=10) (G2)	Treated extract with 100 mg/Kg/day (n=10) (G3)
Urea	39.6±4.3	28.3±3.2*	24.4±1.7*
Uric acid	5.5 ±0.96	3.33±0.3 8*	2.9±0.30*
Creatinine	15.1±0.8	9.1±0.7*	6.4±0.4*

*Very highly significant decrease at P<0.001, (parameters as means ±SD)

Table 3 revealed the effect of oral administration stick sweet cherry extract on potassium, calcium, and CRP, there was statistically significant decrease in the concentration of potassium, calcium, and CRP of treated group (P<0.001, P<0.001) at both extract concentration 30 mg/Kg/day and 100 mg/Kg /day respectively in comparison with untreated animal.

Table 3. Effect of stick sweet cherry extraction, Potassium, Calcium, and C-reactive protein in serum of albino mice

Parameters	Untreated Group (n=8) (G1)	Treated extract with 30 mg/Kg/day (n=10) (G2)	Treated extract with 100 mg/Kg/day (n=10) (G3)
Potassium (mmol/L)	4.7±0.38	7.6 8±0.29*	9.4 ±0.24*
Calcium (mg/dL)	9.02±0.2	11.56±032*	12.4±0.42*
C-reactive protein (mg/L)	8.4 ±1.28	5.3±1.1**	3. 8±0.87**

*Very highly significant increase at P<0.001, **very highly significant decrease at P<0.001, (parameters as means ±SD)

The inhibition zone of chloroform of stick cherry extract (Prunus) was shown in table 4. The inhibition zone of stick sweet cherry (Prunus) ethanol extract against pathogenic bacteria in table 5.

Table 4. The inhibition zone of chloroform of stick cherry extract (Prunus)

	Diameter of inhibition zone (mm)				
Concentration mg/ml	***Escherichia coli***	***Pseudomonas aeruginosa***	***Proteus vulgaris***	***Staphylococcus auruus***	***Serratia marcescens***
5	6	4	9	4	0
10	8	12	16	12	0
25	9	14	16	14	9
50	11	14	16	14	9
100	14	14	16	17	9

The diameter of well (8mm) the upper results without the diameter of well. (0) means no inhibition zone

Table 5. The Inhibition zone of ethanol stick sweet cherry extract (Prunus)

Diameter of inhibition zone (mm)					
Concentration mg/ml	***Escherichia coli***	***Pseudomonas aeruginosa***	***Proteus vulgaris***	***Staphylococcus auruus***	***Serratia marcescens***
5	0	0	4	0	0
10	0	0	4	0	4
25	0	0	5	0	4
50	8	8	8	0	8
100	9	9	8	7	12

The diameter of well (8mm) the upper results without the diameter of well. (0) means no inhibition zone

Result clearly demonstrated that the stick cherry chloroform extract was active against the most types of pathogenic bacteria types at all the concentration used and was more activity formed than ethanol extract as in figure 1 and figure 2.

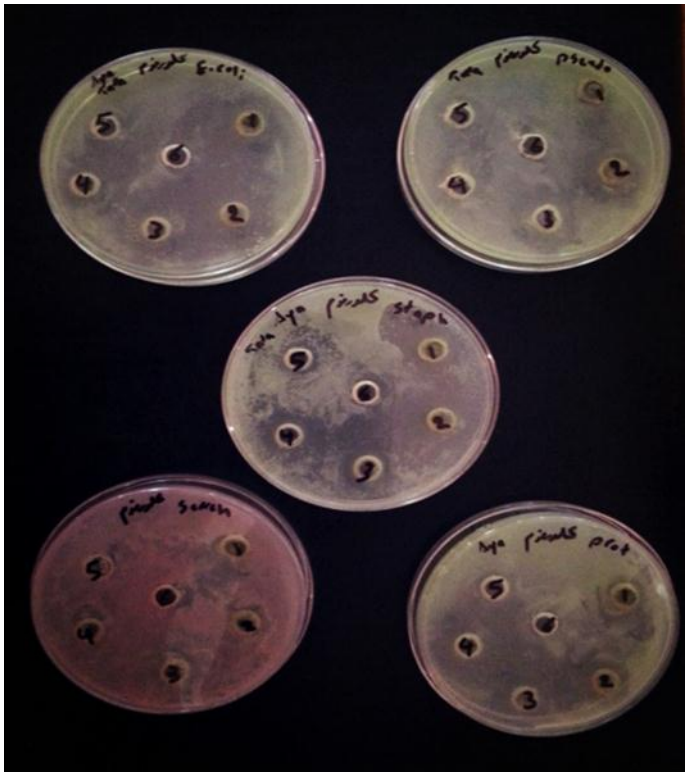

Figure 1. The inhibition zone of chloroform stick sweet cherry extracts (prunus)) against pathogenic bacteria. Plate in the upper right *Pseudomonas aeruginosa*, plate in the upper left *E. coli*, plate in the center *Staphylococcus aureus*, plate in the lower right *Proteus vulgaris*, plate in the lower left *Serratia marcescens*. Well No.1: The concentration of prunus chloroform extract 100 mg/ml. Well No. 2: The concentration of prunus chloroform extract 50 mg/ml. Well No. 3: The concentration of prunus chloroform extract 25 mg/ml. Well No. 4: The concentration of prunus chloroform extract 10 mg/ml. Well No. 5: The concentration of prunus chloroform extract 5 mg/ml. Well No. 6: The control Ethanol

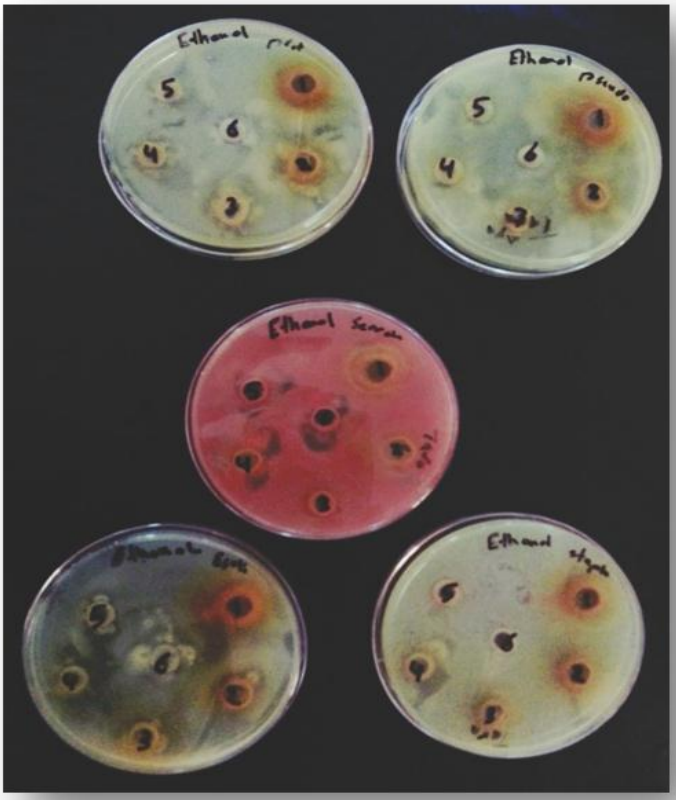

Figure 2. The inhibition zone of stick sweet cherry (Prunus) Ethanol extract against pathogenic bacteria. Plate in the upper right *Pseudomonas aeruginosa*, plate in the upper left *E. coli*, plate in the center *Staphylococcus aureus*, plate in the lower right *Proteus vulgaris*, plate in the lower left *Serratia marcescens*. Well No.1: The concentration of prunus ethanol extract 100 mg/ml. Well No. 2: The concentration of prunus ethanol extract 50 mg/ml. Well No. 3: The concentration of prunus ethanol extract 25 mg/ml. Well No. 4: The concentration of prunus ethanol extract 10 mg/ml. Well No. 5: The concentration of prunus ethanol extract 5 mg/ml. Well No. 6: The control distilled water

Discussion

Cherries are loaded with diseases fighting antioxidant, and contain a great number of anthocyanins, constituents that possess strong antioxidant, anti-inflammatory activities, and anti-aging properties [(22)]. The present study illustrated significant reduction in the activities of LDH, AST and CK at concentration 30, and 100 mg/Kg/day in treated animal with extract as compared to untreated table 1. This change in serum enzyme activity is due to polyphenol bioactive compounds in cherry that may offer protection against heart disease and metabolic syndrome due enhancement in blood vessels health [(16)]. The observed decreased value in LDH activity was 1.30 at extract concentration 30 mg/Kg/day and 1.60 at concentration 100 mg/Kg/day times lower than upper normal limit. The reduced value in AST activity was 1.25 at concentration of 30 mg/Kg/day and 1.46 at concentration 100 mg/Kg/day times lower than upper normal limit and the value for CK activity was 1.5 at extract concentration 30 mg/Kg/day and 2.1 at concentration 100 mg/Kg/day times lower than the upper normal limit. This may be explained that in tart cherries bioactive compounds found beneficially inhibit certain enzymes activities [(2)] while others boosting [(23)] and enhance primary antioxidants [(24)]. Anthocyanins scavenge free radicals directly, in a number of ways: protect the body against oxidative damage by binding

to DNA and activate detoxification and antioxidant enzyme systems in the body. Anthocyanins cherry have been shown to protect brain cells and blood vessels against oxidative stress, implying that consumption cherry may help to prevent formation of plaque atherosclerotic and diseases of neurodegenerative [12,14].

Result in table 2 show statistically significant reduction in concentrations of urea, uric acid and creatinine at both concentration 30 mg/Kg/day and 100 mg/Kg/day of stick cherry extract in comparison with untreated animal, this could be attributed to the presence of antioxidants and phytochemical compound, it was demonstrated that cherries constituents; anthocyanins flavanols, phenols, inhibiting inflammation, and provide protection against cell injury [11,14,25]. The observed decreased value of urea in serum level at 30 mg/Kg/day and 100 mg/Kg/day were 1.4 and 1.6 times lower than the upper normal limit respectively. The observed reduced value of creatinine in serum level at 30 and 100 mg/Kg/day were 1.66 and 2.35 times lower than the upper normal limit respectively. Some studies suggest that cherries fruits have anti-inflammatory benefits that may relieve the pain of arthritis and gout, associated with higher risks disease of cardiovascular and mortality [26,27]. The observed decreased value of serum uric acid level at 30 mg/Kg/day and 100 mg/Kg/day was1.65 and 1.9 times lower than the upper normal limit respectively. In previous studies it has been observed that consumption of cherry fruit lowers serum uric acid level and plasma creatinine in healthy human subjects and animals [17,28], this is in agreement with our result on stick cherries but there is no report on stick sweet cherry. It was reported that sweet cherries selectively and significantly were found to reduce a number of biomarkers associated with inflammatory diseases [29]. These findings suggesting that cherries may possess the capacity of lowering urate production through reducing tubular reabsorption and/or increasing the glomerular filtration rate [29]. In an animal study, intake of diet rich cherry in rats with hyperuricemia significantly decreased the levels of serum uric acid by inhibiting of xanthine oxidase and xanthine dehydrogenase of hepatic activity (28), cherries may also have anti-inflammatory properties against the series of inflammatory responses triggered by urate monosodium crystals [26,30].

Table 3 demonstrates significant reduction in the concentration of potassium, calcium, and CRP of treated group at both concentration 30 mg/Kg/day and 100 mg/Kg/day in comparison with untreated animal, this is due to differences in human genetic, could lead to differences in metabolic clearance and absorption between individuals, in addition to differences in intestinal microorganism populations [31]. The observed increased value for potassium in serum level at 30 mg/Kg/day, 100 mg/Kg/day was 1.63 and 2 times higher than the upper normal limit respectively. Increasing of potassium intake has a direct effect on preventing cerebrovascular accident, independent of its effect on blood pressure through a variety of mechanism [32]. The observed increased value for calcium in serum level at 30 and 100 mg/Kg/day was 1.28 and 1.37 times higher than the upper normal limit respectively. Low absorption or high excretion of calcium, causes osteoporosis because the maintenance of the plasma calcium concentration is more important to the organism, for neuromuscular [33]. The present data on C-reactive protein shows significant reduction in treated animal with extract at both does 30 mg/Kg/day and 100 mg/Kg/day compared with untreated group table 3. The reduction of CRP has been observed in treated animals with cherry extract also this been linked to atherosclerosis and heart disease [30]. In atherosclerosis, or cholesterol plaguing of the arteries, is known to have an inflammatory component that is thought to cause a change in CRP levels in the blood, and this is depending on genetics as well as lifestyle habits [19]. The compounds found in cherries modulate numerous pathways to protect against other conditions associated with inflammation including cancer, cardiovascular disease, metabolic syndrome [2,34]. Change in CRP may be useful for the detection of systemic inflammatory processes to assess treatment of

bacterial infections with antibiotics to differentiate between active and inactive forms of disease infection [19].

In conclusion, orally administration of sweet stick cherries extract to animals caused a potential difference in the enzyme activities, LDH, AST, CK, concentration of urea, uric acid and creatinine C-reactive protein those are risk factors for different diseases, which lead to increased inflammation, and oxidative stress and which may be prevented by the polyphenols and phytochemical compound or minimized in stick cherries extract. To test the clinical relevance of our findings, future studies need to be performed with large number of animal.

In the previous study, the in vitro antifungal activities of the P. laurocerasus L. leaf extracts against the fungi and their activity potentials were qualitatively and quantitatively assessed by the presence or absence of inhibition zones and zone diameters, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) [20]. Several studies have been conducted to understand the mechanism of action of different plant extracts and essential oils [35]. There is no report on the inhibition of pathogenic bacteria growth by stick sweet cherry extracts, therefore the antimicrobial activity of stick cherry extract was evaluated for inhibition the growth of pathogenic bacteria. Result clearly demonstrated that the stick cherry chloroform extract was active against the most types of pathogenic bacteria types at all the concentration used and was more activity formed than ethanol extract. Highest inhibition found that the chloroform and ethanol illustrated the more activity at concentration 100 mg/ml.

The highest inhibition zone for *Escherichia coli* was 14 mm when chloroform extract was used 100 mg/ml and the lower was 6mm as in table 4 and figure 1. For ethanol extract the highest inhibition zone in the same bacteria (*E. coli*) was 9 mm at (100 mg/ml extract) table 5 and figure 2. For *Pseudomonas aeruginosa* the highest inhibition yields when we using chloroform extract in the concentration (25, 50, 100) mg/ml the diameter of inhibition zone was 14 mm and the lower inhibition zone at the concentration (5, 10) mg/ml with diameter 4 mm, 12 mm respectively, while for ethanol extract the highest inhibition was (50,100) mg/ml diameter 8 mm, 9 mm respectively, but there was no inhibition zone at the concentration (5, 10, 25) mg/ml. Proteus vulgaris the highest inhibition for chloroform extract was 16 mm at the concentration (10, 25, 50, 100) mg/ml and the lower at concentration was (5) mg/ml with diameter 9 mm, while for ethanol extract the highest inhibition zone at the concentration (50, 100) mg/ml with diameter 8mm And the lower (5, 10) mg/ml with diameter 4 mm. *Staphylococcus aureus* give the highest inhibition zone for chloroform with extract (25, 50, 100) mg/ml diameter 14, 14, 17 mm respectively, and the lower (5) mg/ml with diameter 4 mm , while give for ethanol extract highest inhibition zone at the concentration (100) mg/ml with diameter 7 mm and there was no inhibition zone at the concentration (5, 10, 25, 50) mg/ml. *Serratia marcescens* gives the highest inhibition zone for chloroform extract at the concentration (25, 50, 100) mg/ml was 9 mm and at the lower concentration (5, 10) mg/ml there is no inhibition zone. And give for ethanol extract at the concentration (100) mg/ml 12 mm, and there was no inhibition zone at the concentration (5) mg/ml.

In the literature it was reported that the bioactive phytochemical compounds penetrate inside the cell, where they interfere with cellular metabolism [35], and also that they react with active sites of enzymes or act as a H^+ carrier, disturb the cellular membrane depleting adenosine triphosphate pool. It was found that antifungal resistance may depend on strain and source of isolation, species, genus [36], as well as on the active components in the leaf cherry extracts. Moreover, leaf composition and the solvents used in extract, origin and cultivars of plant, climate and harvesting time may affect the consequent antifungal properties [22,36]. Phytochemical compound in stick sweet cherry extract and the solvents used extracts, may affect the antibacterial properties, plants and fruits could

be that alternative because most of them are with few side effects and safe if any, they cost less and affect a wide range of resistant microorganisms.

Acknowledgments

The author grateful to Department of Applied Sciences and Biotechnology Division and very thankful to assistant professor Dr. Amani Abdul Wahhab Abdul Razzak for providing their kind support.

Conflict of interest

None declare.

Funding

Self-funding.

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Received May 16th 2017
Accepted Oct. 22nd 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

Intramedullary Nailing Versus Fixed Angled Blade Plating for Treatment of Subtrochanteric Femoral Fracture

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Abstract

Background The subtrochanteric fractures represent 7-44% of the fractures of proximal femur caused by low energy trauma in elderly patients or high energy trauma in younger age group. Different surgical options used to treat and fix this fractures that could be Intramedullary devices like intramedullary nail or extramedullary devices like fixed angle blade plate.

Objective Comparing the result of close reduction and internal fixation with intramedullary nail to the open reduction and internal fixation with 95-angled blade plate for treatment of subtrochanteric fractures.

Methods Prospective multicenter study was done in Al-Imamein Al-kadhimein Medical City and Al-Wasity Teaching Hospital for thirty patients with close and open (Gustilo type-1) subtrochanteric femoral fractures between December-2014 and September-2016. Eighteen patients (8 closed + 10 open fractures) treated with Intramedullary nail (IMN) were compared to twelve patients (9 closed + 3 open fractures) treated with open reduction and 95-angled blade plate fixation (BP).

Results There were significant statistical differences between the two groups. The IMN group show better outcome regarding the mean union rate time (IMN were 16 weeks while in the BP were 22 weeks). Mean hospitalization stay (IMN were 82 hours while in BP were 110 hours) and rate of infection (IMN 0% while in BP were 16%), but no statistical difference regarding the mean operation time (IMN were 1.59 hour while in BP were 1.43 hour) and functional outcome (HHS in IMN were 82 while in BP were 79).

Conclusion Closed reduction and internal fixation with Intramedullary Nail is preferable (for Close and Open Gustilo's type-1 Subtrochanteric fractures) when compared to the open reduction and Internal Fixation with 95-angled Blade Plate.

Keywords Subtrochanteric femur fractures, closed reduction intramedullary nail fixation, open reduction internal fixation, fixed angle blade plate.

Citation Joda AI, Aldookhi AA, Abd Ali AS. Intramedullary nailing versus fixed angled blade plating for treatment of subtrochanteric femoral fracture. Iraqi JMS. 2018; 16(2): 125-132. doi: 10.22578/IJMS.16.2.3

List of abbreviations: ATLS = Advance Trauma Life Support, AAOS = American Academy of Orthopedic Surgery, BP = Blade plate, IMN = Intramedullary nail, MVA = Motor vehicle accident, OTA = Orthopedic Trauma Association, ORIF = Open reduction and internal fixation, PFN = Proximal femoral nail

Introduction

Subtrochanteric fractures involve the segment of the proximal femur from the lesser trochanter to the isthmus. The major fracture involves a zone between the inferior border of the lesser trochanter and the junction of the proximal and middle one third of the femur (approximately a 5-cm segment) (Figure 1) [(1)]. Fractures in this area may extend proximally into the trochanteric area or neck and distally into the shaft.

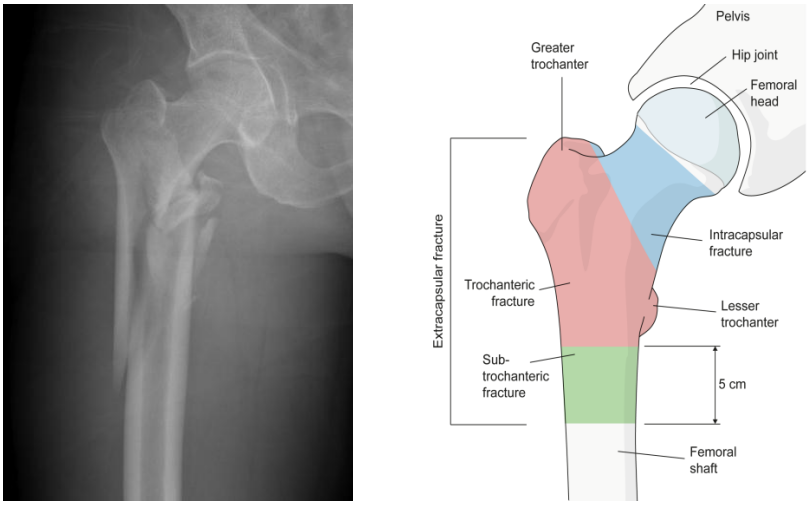
Subtrochanteric femoral fractures are common and account for 7 to 44% of all proximal femoral fractures, depending on the classification used [(2)].

Compression, tensile, and torsional stresses of the subtrochanteric region have challenged orthopaedic surgeons with problems of mal-union and non-union [3]. Moreover, subtrochanteric fracture causes more blood loss than neck femur or intertrochanteric femur fracture [4].

Extramedullary as well as intramedullary fixation techniques have been used to fix such fractures. Extramedullary fixation devices are used for more than a century but they have been associated with extensive surgical dissection, periosteum and soft tissue damage [5].

Superiority of intramedullary devices had been shown by biomechanical studies in comminuted subtrochanteric femur fractures [6]. However, intramedullary fixation in subtrochanteric fractures is not without complication. Various authors have reported improper reduction with resultant mal-union or non-union after intramedullary nailing of comminuted fractures [7].

The superiority or equality between the two fixation methods cannot be established due to dearth of literature on comparison of the two fixation methods.



A B

Figure 1. A) Typical subtrochanteric fracture extending to lesser trochanter in high energy trauma, B) Subtrochanyeric region [8]

Epidemiology of subtrochanteric fracture

Subtrochanteric femur fractures account for approximately 25% of all hip fractures and have a bimodal age and gender distribution. They are seen in either young men as a result of high-energy injuries (often highly comminuted and significantly displaced) or in elderly osteoporotic women as a result of low-energy falls (typically long spiral fractures) (Figure-4) [9].

The high-energy cases often have concomitant injuries involving thoracoabdominal and head injuries in 10% to 30% of patients and associated noncontiguous long bone, spine, and pelvic injuries in up to 50% of patients. Mortality rates as high as 21% have also been described [9,10].

These fractures may also occur because of a stress riser in the lateral cortex of the proximal femur secondary to placing cannulated screws too distally during treatment of femoral neck fractures or drilling too inferiorly when performing core decompression or bone grafting for avascular necrosis of the hip [11].

Other causes include gunshot wounds and the more recently described “atypical” proximal femur fracture as a result of prolonged bisphosphonate therapy [12].

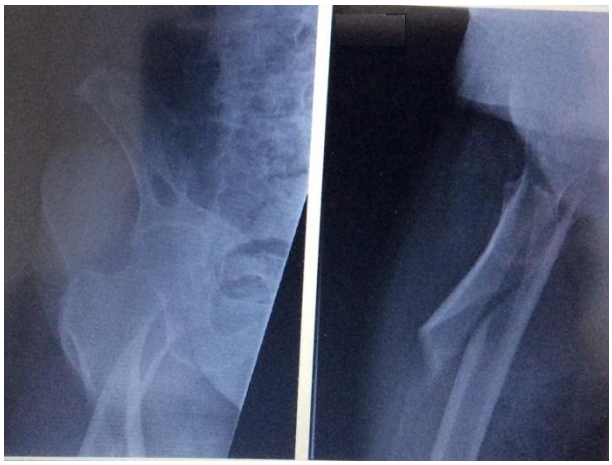


Figure 2. Typical long spiral fracture in elderly patient [9]

This study aimed to compare the result of closed reduction and internal fixation with intramedullary nailing to open reduction and internal fixation with 95 Blade plate for subtrochanteric (close and open type-1 Gustilo) fracture regarding union rate, hospitalization stay, operative time, infection rate, alignment and functional outcome.

Methods

Prospective comparative multicenter study was done at Al-Imamein Alkadhimein Medical city and Al-Wasity Teaching Hospital through the period from Dec. 2014 to September 2016 (total duration 1 year and 9 months) for thirty patients admitted to the hospitals with clinical and radiographic evidence of subtrochanteric femur fracture (fracture within 5 cm from distal border of lesser trochanter of femoral bone).
Eighteen patients (8 closed+10 open fractures) has been treated with Intramedullay Nail were matched to twelve patients (9 closed +3 open fractures) were treated with Open reduction and internal fixation with 95 angled Blade plate.

Inclusion criteria

- Closed subtrochanteric fracture.
- Open type of subtrochanteric fracture (Gustillo type 1) treated within 72 hours.
- Skeletally mature (closed proximal femoral and trochanteric physes).

Exclusion criteria

- Multiple injured patients.
- Ipsilateral femoral shaft and neck fracture.
- Previous operation or fracture on the same bone.
- Old burn or scar tissue near the fracture site.
- Vascular injury (acute or chronic).
- Sever peripheral vascular disease, cardiopulmonary instability, and uremia and cerebrovascular accident patients.
- Pathological fracture.

The patients were allocated into two group, Group 1; were treated with closed reduction and internal fixation with Intramedullary Nail (IMN), Group 2; were treated with Open reduction and internal fixation with 95 angled Blade plate (BP).
Patient's demographic detail, type of fracture, mechanism of injury, associated injuries, local and systemic complications were recorded.
Informed consent and permission for study protocol were taken from the patients.

Results

The Study population consists of 18 patients (8 closed fractures, 10 open fractures) in group 1 (treated with IMN) and 12 patients (9 closed fractures, 3 open fractures) in group 2 (treated with BP).

The mean age of the patients in group 1 was 50 years, while the mean age of the patients in group 2 was 55 years.

In both groups the mean age for males were 52 (range between 50-54 year) and the mean age of female were 55 (range between 50-60 years).

Regarding the affected side there are no significant deference in both groups, p>0.05.

Regarding the mechanism of injury: the major cause in both group was high energy trauma like road traffic accident 50% and fall from height 30% followed by low energy trauma 20%.

When we compare the closed fracture treated with IMN to closed fracture treated with Open reduction and internal fixation (ORIF) with BP, statistical significant difference in union time and mean hospitalization Stay as the p-value <0.05, while no significant statistical difference regarding mean operative time and infection rate (Table 1).

Table 1. Results of treatment for close subtrochanteric fractures

Results of treatment	Group 1/IMN Number=8	Group 2/ORIF with BP Number=9	P value
Mean union time (weeks)	16.38	22	S
Number of non-union	0	1	NS
Mean operative time (hours)	1.52	1.86	NS
Mean Hospitalization stay (hours)	70	96	S
Infection rate	0	1	NS
Alignment	0	1	NS

S=Significant p value ≤ 0.05, NS=Not significance p value >0.05

While when we compare the result of treatment of open fractures treated with IMN (group 1) to open fractures treated with ORIF with BP fixation (Group 2), the significant statistical difference was in the infection rate, mean time for fracture union and mean hospitalization stay as p value <0.05, while no significant difference regarding the mean operative time (Table 2).

Table 2. Results of treatments for open subtrochanteric fractures

Results of treatment	Group 1/IMN Number=10	Group 2/BP Number=3	P value
Mean union time (weeks)	16	22.67	S
Number of non-union	0	1	S
Mean operative time (hours)	1.66	2	NS
Mean Hospitalization stay (hours)	94	124	S
Infection rate	0	1	S
Alignment	0	0	NS

S=Significant p value ≤ 0.05, NS=Not significance p value >0.05

When we compare the two group (including close and open cases) group 1 treatment with IMN, group 2 treatment with ORIF with BP, there is significant statistical difference between the two groups regarding the Union rate time, hospitalization stay and rate of infection, but no statistical difference regarding the mean operation time and functional outcome (using Harris Hip Score (HHS)) (Table 3).

Table 3. The overall result of the patient's treatments

Results of treatment	Group 1/IMN Number=10	Group 2/BP Number=3	P value
Mean union time (weeks)	16.2	22.17	S
Number of nonunion	0	2	S
Mean operative Time(hours)	1.59	1.43	NS
Infection rate	0	2	S
Mean time for hospital stay (hours)	82	110	S
Alignment	0	2	S
Functional outcome, Harris Hip Score	82	79	NS

S=Significant p value ≤ 0.05, NS=Not significance p value >0.05

One of the complications that happen during follow up of the patients as one case of group 2 closed fracture develop femoral neck fracture after 13 weeks from the surgery while begin full weight bearing.

We had two cases in group 2 develop infection; one was a closed fracture had developed postoperative superficial wound infection treated with antibiotics and local care of the wound and the other had deep infection postoperatively that require removal of the implant after the fracture union.

One of the cases in closed fracture treated with open reduction and blade plate fixation develop varus mal-alignments.

No patient had shortening more than 2 cm in both group.

No statistical differences (p-value 0.813) regarding the functional outcome as we calculate the HHS at 6 months postoperatively and shown that the mean of HHS for IMN-group 82 and the mean HHS for Blade plate group were 79 (Table 4).

Table 4. Functional outcome using Harris Hip Score at 24 weeks postoperatively

Harris Hip Score	Results	IMN group N=18	BP group N=12
90-100	excellent	3	2
80-89	good	8	3
70-79	fair	6	5
<69	poor	1	2

Discussion

There are no roles for conservative management for subtrochanteric fractures as shown by DeLee et al. [13].

Current study shows the mean age of both groups between 40-60 years (graph-1). The incidence of fracture in the current study are more common in male than female, this can be attributed to socioenviromental factors that the females in our society are less involved in high activity level and hard work. The same result of the study done by Sridhar and Neelakrishman that they shown the male were more prone to sustain proximal femoral fracture and he attribute it to the fact that male Indian were more active and more mobile than females who are confined to house hold activities [14]. The main cause of fractures in younger age group was a road traffic accident while in elderly patients, low energy trauma like fall on ground.

Kuzyk et al. [15] meta-analysis suggest for better outcome when compare between the fixation technique to separate the young age group

with high-energy trauma from elderly patients with low energy trauma.

Mean union time in close intramedullary group was earlier (16.38 weeks) than close fractures Blade plate (22 weeks). Also, the mean union time of open fractures intramedullary group was more earlier (16.1 weeks) than open fractures of angled blade plate group (22.67 weeks), and the overall union time for intramedullary group earlier than blade plate groups and this relation was statistically significant (P value< 0.05). The union rate was markedly delayed in cases that develop infections (32 weeks).

Pelet et al. radiological assessments for subtrochanteric fracture mean union time was 4.2 months for intramedullary group and 6.3 months for plate group [16].

We had two cases of nonunion both in Blade plate group, one was closed fracture and the other was open fractures and no reported cases of non-union in this study for intramedullary Nail. We think that the fracture treated by open way (Blade plate group) may impair the vascularity of the bone and affect the union process. Celebi et al. [17] reported better results when using fixed angle blade plate with minimum invasive technique.

Mean hospitalization stay for open fractures longer than close fractures probably related to the care for the open wound and postoperative injectable antibiotics in hospital, while the intramedullary nail group mean hospital stay (close fractures 70 hrs, open fractures 94 hrs) shorter than Blade plate group (closed fractures 96 hrs, open fractures 110 hrs).

Because in our study, we exclude the concomitant head, chest or multiple organ injuries, the average time between the admission to the orthopedic surgical ward and the surgery were between 24-48 hours, as the most of authors recommend early surgical fixation within 24 hours [18]. While if there were associated head and chest injuries, early surgical femoral fixation increases the morbidity and mortality as shown by series of study done by Pape et al. [19], Jaick et al. [20] and Townsend et al. [21].

Despite the use of prophylactic antibiotic and local care of wound, there were two infected cases (16%) in Blade plate group. While no infection in IMN group (statistically significant) and correlate with study done by Miedel et al. [22] in 2005 as the infection rate was 8 % and high revision rate 16% in extramedullary device in comparison with IMN.

In our study, one case (8%) develop varus deformity in Blade plate group that happen when the patient begins weight bearing, these may be explained by biomechanical properties of the plate as it loads bearing device. Patel et al. [23] show in his study the rate of varus malalignment 5% and one of them infected that required a revision surgery.

No statistical difference between both techniques (Intramedullary and Extramedullary) regarding the functional outcome using HHS (p value <0.05), but it is greatly affected (poor HHS) in infected and nonunion cases, there was only minimal changes in Harris hip score after the interval of 6 months [24].

Roy and Subramanyam [25] in 2014 was shown that the HHS mean was 80.7 for the subtrochanteric cases treated by Intramedullary Nail. Patel et al. [23] in his study in India 2016 shown the mean of Harris hip score for intramedullary Nail was 81.3 and the extramedullary fixation was 85.

Comparative study between intramedullary nail and fixed angle blade plate have been reported [26,27].

The advantage of the IMN over the BP [28], is that it is stronger biomechanically, the device was load shearing (not load bearing) which allow fracture compression, less exposure of the fracture site, less blood loss and excellent rate of union. The distal locking screw were maintained the rotation and length control so earlier weight bearing achieved. However, the surgery had technical difficulties related to the entry site, reduction of the fractures, free hand technique for distal locking screws and long learning curve for surgeon experience.

The main disadvantage related to the ORIF with Blade plate were in large surgical exposure, sever damage to the soft tissue, more blood loss and nonunion. and due to the effect of load sharing on the plate that may result in fatigue breakage, but the plate fixation still

preferable in fractures that extend to proximal trochanter, fractures of lateral wall and narrowing of the femoral medullary canal as the main advantage in these methods of fixation was the preservation of the blood supply to the medial fragments if the dissection and fixation were done by biological method [29].

The outcome of our study was in agreement with the general trend toward the use of IMN fixation method for Subtrochanteric fracture. In addition, the overall results of IMN were better than Blade plate fixation as showed by Parker et al. [30].

Limitation of this study were in small sample size, not double blind so there are surgeon biases, no special type of classification used in our study so we are not compare specific type of subtrochanteric fractures and the surgical experience of the surgeon affect the outcome.

Closed reduction and internal fixation with Intramedullary Nail (under the aid of fluoroscopic control) is preferable (for closed and open Gustilo's type-1 Subtrochanteric femoral fractures) when compared to the open reduction and Internal Fixation with 95-angled Blade Plate.

We recommend further studies like, studies with large sample size, studies including more severe types of open fractures (like Gustilo's type-2) and studies that comparing the Intramedullary nail to other modalities of Extramedullary devices like locked plate or Dynamic condylar screws.

Acknowledgments

The authors thank Al-Imamein Al-Kadhimein Medical city and Al-Wasity Teaching Hospital staff for providing all operative facilities.

Authors contribution

The patients were operated on and followed for their outcome by dr. Joda and Dr. Abd Ali. Research conduction and statistical analysis done by dr. Joda and Aldookhi.

Conflict of interest

Current study enrolled a small sample size, not double blinded, so there are surgeon biases, no special type of classification used in our study so authors are not compare specific type of subtrochanteric fractures and the surgical experience of the surgeon affect the outcome.

Funding

The authors offer all funding research.

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Received May 29th 2017
Accepted Nov. 2nd 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

Vaginal Progesterone Pessary for Preterm Labor Prevention in Women with a Short Cervix Early in The Second Trimester

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Abstract

Background The prevention of preterm birth is a major health care priority.

Objective To evaluate the efficacy of vaginal progesterone pessary in reducing the rate of preterm delivery and subsequent neonatal morbidity and mortality events in pregnant women with a short cervical length early in the 2nd trimester.

Methods Women with a singleton pregnancy without a history of preterm labor nor a history of second trimester miscarriage, underwent cervical length measurement at 14+0 to 15+6 weeks of gestation. Women found to have a cervical length less than 30 mm received vaginal progesterone pessary (400 mg per pessary) on daily basis, or no treatment. Primary outcome was preterm delivery rate before 37 weeks gestation. Secondary outcome includes neonatal morbidity and mortality events.

Results From the 7725 pregnant women screened between the period from April 2015 to January 2017, 613 were found to have a cervical length less than 30 mm and only 518 pregnant women met the inclusion criteria and agreed to participate in this study. However, only 492 were followed up till the time of delivery. From those 252 women administered 400 mg vaginal progesterone pessary once daily at night and the remaining 240 women did not receive any form of progesterone and served as control. There was a significant reduction in preterm delivery rate less than 37 weeks gestation among women receiving progesterone vaginal pessary compared to the control group 11 (4.4%) vs 38 (15.8%), p value < 0.001. Regarding neonatal outcome, there were significant reduction in the frequency of respiratory distress syndrome, low birth weight neonates and admissions to neonatal intensive care unit in women taking vaginal progesterone pessary compared to the control. While other neonatal morbidity and mortality events, incidence of neonatal congenital anomalies were not significantly different between the two groups.

Conclusion Vaginal progesterone pessaries in women with a cervical length less than 30 mm early in the second trimester are found to be effective in reducing the rate of preterm birth and some of the prematurity related morbidity events.

Keywords preterm labor, vaginal progesterone pessaries, short cervix, premature delivery

Citation Khazaali EAA. Vaginal progesterone pessary for preterm labor prevention in women with a short cervix early in the second trimester. Iraqi JMS. 2018; 16(2): 133-143. doi: 10.22578/IJMS.16.2.4

List of abbreviations: BMI = Body mass index, BPD = Bronchopulmonary dysplasia, CNS = Central nervous system, 17-OHPC = 17 hydroxy progesterone caproate, LMP = Last menstrual period, PTB = Preterm birth, RDS = Respiratory distress syndrome, TVU = Transvaginal ultrasound

Introduction

The definition of preterm birth (PTB) is delivery of a baby before 37 completed weeks of pregnancy [1].

About one-quarter of PTB are iatrogenic usually for pre-eclampsia, fetal growth retardation or maternal disease. The remainder is due to spontaneous preterm labor and delivery [2].

There was steady improvement in the survival rates of preterm babies over the past two decades, mainly due to the introduction of surfactant therapy, wider spread use of antenatal steroids and improvement in neonatal respiratory management [2].

PTB is a leading cause of neonatal and infant mortality as well as short- and long-term disability. In developed countries, rates for PTB range between 6% and 12% and are generally higher in developing countries [3,4].

Premature delivery in an under-resourced setting, place the baby at extremely high risk of death in the early neonatal period. The lower the gestational age at birth, the greater the need for more expensive interventions and support to improve the chances of infants' survival. In developing countries, the absence of skilled maternity care, leads to high rates of neonatal morbidity and mortality for premature babies. Despite increasing incidence of prematurity in both developed and developing countries, no significant advances have been made in the prevention or treatment of preterm delivery [3,4].

It has been found that progesterone plays a major role in the maintenance of pregnancy and in the majority of mammals, labor is preceded by a decline in circulating progesterone levels [5].

In the human, there is no systemic withdrawal of progesterone prior to labor, although there is an increase in the expressions of genes formerly repressed by progesterone [6].

It has been widely thought that progesterone inhibits contractions principally by repressing contraction-associated proteins such as oxytocin, gap-junction proteins, prostaglandin receptors and prostaglandin-metabolizing enzymes [7,8].

Preterm labor cannot be considered as a single disease entity, but it is a syndrome or symptom that may have one or more causes [3].

Because of multifactorial (social, behavioral and biological) causes in preterm delivery, efforts in prevention measures have not been successful so far [9].

Early identification of woman at risk and the use of prophylactic therapies are one of the most important strategies to reduce perinatal morbidity and mortality associated with PTB. Initial identification of women at high risk of preterm labor is based on their past obstetric history [10].

A single previous preterm delivery increases the risk of preterm delivery in the next pregnancy by four folds compared with a previous delivery at term. However, the large majority of spontaneous preterm delivery occurs in nulliparous women, and progesterone has not been widely assessed in those women [11].

Although women with of previous history PTB and those with multiple gestation are at the highest risk of preterm delivery [12,13], the majority of spontaneous PTB occur in women with low risk [14].

There is good evidence that support the use of transvaginal sonographic measurement of cervical length to predict the risk of preterm labor in both low- and high-risk pregnancies and in symptomatic women [15].

Currently, there are two strategies in common use: a single measurement of cervical length usually at the time of the routine ultrasound scan at 18-22 weeks of gestation or a serial measurement of cervical length throughout the 2nd and early 3rd trimester of pregnancy. There is a direct relationship between cervical length and preterm delivery risk at any given gestational age, example, a cervical length of 15 mm or less at 20-24 weeks predicts a 50% risk of preterm delivery prior to 34 weeks in a low risk population. However, identification of a risk of preterm labor as late as 23 weeks may be too late for any potential prophylactic therapies to be beneficial [16].

There is currently no effective method for the prediction of preterm labor in prime gravid women with no other significant risk factors for preterm delivery. However, it is possible to identify a subgroup of women who can be identified as being at risk of PTB depending on the use of screening tests e.g. measurement of cervical length, detection of fetal fibronectin in vaginal secretions and the presence of abnormalities of the genital tract.

Commonly used therapies include cervical cerclage, non-steroidal anti-inflammatory drugs (NSAIDs) and progesterone [2].

The weight of both clinical evidence and basic science currently points to progesterone being potentially beneficial in women at high risk of preterm delivery, except those with multiple gestation and there appear to be few if any side effects [2].

The efficiency and safety of progestogens are related to individual pharmacologic properties of each drug within this class of medication and characteristics of the population that is treated [17].

Research among exposed women and controls showed no difference with respect to the occurrence of abnormalities of the central nervous system (CNS), limbs and joints, urogenital tract and circulatory tract between treated and untreated programs, even when 17 hydroxy progesterone caproate (17-OHPC) was administered in early pregnancy [18].

Manuck et al. [19] demonstrated a variable response to 17-OHPC exposure, based on the progesterone receptor genotype.

The recommendation of the American College of Obstetrics and Gynecology about the use of antenatal progesterone to prevent preterm delivery is that: its use should be restricted to women incidentally found to have a short cervix (less than 15 mm), or to women with a documented history of prior spontaneous preterm delivery at less than 37 weeks [20].

It has been shown that the administration of progesterone decreases both the number of episodes of uterine contractions and the incidence of preterm birth in women at high risk for preterm delivery [21].

The objective of this study was to evaluate the efficacy of vaginal progesterone pessary in reducing preterm delivery rate and subsequent neonatal morbidity and mortality events in pregnant women with a short cervical length early in the 2nd trimester.

Methods

The present study was conducted from April 2015 to January 2017 in the ultrasound outpatient department at Al-Imamein Al-Kadhimein Medical City and in 5 ultrasound private clinics operated by an ultrasound specialist, and it was approved by the Institutional Review of the Iraqi Board of the participating center.

A verbal informed consent was taken from all the eligible participants before study entrance.

This trial enrolled 518 low risk asymptomatic women with a singleton pregnancy who were nulliparous or multiparous without a history of spontaneous PTB less than 37 weeks gestation nor 2nd trimester miscarriage and who were found to have a cervical length < 30 mm on transvaginal ultrasound scan at 14+0 to 15+6 weeks gestation.

Gestational age was estimated based on the last menstrual period (LMP), which was reported by the participants and was confirmed by ultrasound or ultrasound alone when LMP was unknown.

After emptying the urinary bladder, transvaginal measurement of cervical length was performed. The name of the ultrasound machine used is Voluson E6 ultrasound machine GE health care, the transvaginal probe used is IC5-9-D Micro Convex Endocavitary Probe. The cervical length was measured from the internal os to the external os along the endocervical canal.

All participating sonographers were experienced.

For each participant, baseline demographic data including maternal age, body mass index (BMI), level of education were collected. BMI was calculated around the time of the transvaginal scan, using the following formula: weight in kilograms (Kg) divided by the height in meters squared (Kg/m^2). Obstetric and medical history were taken and physical examination was done.

Exclusion criteria include women less than 18 years of age, women with a history of previous preterm delivery and 2nd trimester miscarriage, vaginal bleeding, fetal congenital malformation or suspected chromosomal abnormalities (from the increased nuckal translucency thickness measurement above 2.9 mm), cervical or abdominal cerclage in situ or planned cerclage,

uterine anomalies e.g. bicornuate uterus or septate uterus, current or recent progesterone therapy within the previous 4 weeks, allergy to any ingredient of the pessaries, cervical dilatation, chronic maternal medical condition e.g. diabetes mellitus, chronic hypertension, liver disease, psychiatric disorders, epilepsy, porphyrias, known or suspected progesterone-sensitive tumors e.g. breast cancer, deep venous thrombosis, pulmonary embolism, thrombophlebitis, heart attack or stroke.

All the participants were randomly allocated to receive either vaginal or rectal progesterone pessaries (trade name: cyclogest, company's name: Actavis, Barnstaple EX328NS, UK) 400 mg daily or no treatment till 36+6 weeks gestation.

Progesterone pessaries were self-administered vaginally using the 400 mg formulation, on daily basis at night. Those who had vaginal infection e.g. moniliasis and in those with recurrent cystitis the pessaries were applied rectally. However, in the presence of colitis or fecal incontinence, it was applied vaginally.

The treatment was initiated between 14+0 and 15+6 weeks of gestation and continued until 36+6 weeks gestational age, rupture of membranes or delivery, whichever occurred first.

All the participants were followed-up every 3 weeks and were interviewed to determine the occurrence of any adverse events and to ensure about the compliance with the treatment in those who receive the progesterone pessaries.

Women who developed 2nd trimester miscarriage (one) and those who were lost to be followed up (twenty-three) were excluded from the study as well as those who needed an emergency cerculage (two) which was done if cervical dilatation exceeds 1 cm, in the presence of an intact membranes and in the absence of uterine contractions, significant vaginal bleeding and clinical or subclinical (increased c-reactive protein, increased white blood cell count) evidence of chorioamnionits.

Progesterone pessaries were stored below 25 °C in a dry place.

Maternal outcome was preterm birth prior to 37 weeks gestation.

Neonatal outcome was recorded after delivery and up to the first 28 days of life and it included fetal death, neonatal death, or neonatal morbidity in the form of grade III or IV intraventricular hemorrhage, periventricular leukomalacia, respiratory distress syndrome (RDS), bronchopulmonary dysplasia (BPD), proven sepsis, necrotizing enterocolitis.

According to radiological appearance, intraventricular hemorrhage was classified as followed: [22]

Grade three intraventricular hemorrhage: the blood filling and distending the ventricular system. Dilated ventricles which are more than 50% full of blood

Grade four intraventricular hemorrhage: parenchymal involvement of hemorrhage, also known as periventricular venous infarction.

Periventricular leukomalacia is a form of white-matter brain injury, characterized by the necrosis (more often coagulation) of white matter near the lateral ventricles [23].

RDS was defined as need for artificial ventilation and an x-ray meeting RDS criteria [24].

BPD was defined as need for supplemental O_2 during at first 28 days after birth [25].

Analysis was by an intention to treat.

Statistical analysis

With using the Microsoft excel 2016 and GraphPad Prism version 6 software, most of data was categorical and presented as frequency and percentage, the comparison of these data between the two groups of study was done Using chi square test and Fisher exact test. Only age and body mass index were presented as mean ± standard deviation and comparison done by an unpaired t-test. P value less than 0.05 was considered significant.

Results

From the 7725 women scanned, 613 found to have a cervical length less than 30 mm; and from the 613 women, 575 met the inclusion criteria. However, only, 518 pregnant women agreed to participate in this study.

From the 518 women who participated in this study, 492 were followed-up till the time of delivery.

Of the remaining 26 women, 23 women were lost to follow-up and 3 were excluded from the study because one of them miscarried at 17+3 weeks of gestation and two needed emergency cerclage for cervical dilatation one at 17+6 weeks gestation and the other at 21+2 weeks gestation.

From the 492 participants who were followed up; 252 women administered 400 mg progesterone pessary once daily at night and the remaining 240 women did not receive any form of progestogens and served as control.

Thus, this study included two groups; pregnant women receiving vaginal or rectal progesterone pessaries, the study group n=252 and the control group n=240.

The two study groups were matched regarding maternal age (26.34±3.62 for women taking cyclogest and 26.44±3.63 for control) p value = 0.759. Likewise, for the body mass index, no significant difference was found between the two groups (28.49±2.34 for women taking cyclogest and 28.6±2.28 for control) p value = 0.577 (table 1).

Table 1. Comparison of maternal age and body mass index between the two groups

Parameter	Women taking progesterone pessary N=252 Mean±SD (Range)	Control N= 240 Mean±SD (Range)	P value*
Age (yr)	26.34±3.62 (19-34)	26.44±3.63 (19-35)	0.759
BMI (kg/m^2)	28.49±2.34 (24-32)	28.6±2.28 (25-32)	0.577

* unpaired ttest

There was no significant difference between the two groups in other maternal related data including obstetric history, method of delivery, history of infertility ± assisted reproductive technologies, cervical length and higher professional education as shown in table (2).

The frequency of preterm delivery was significantly less in women receiving cyclogest in comparison with control (11 vs 38), p value <0.001 as shown in table (3).

Table (4) shows a significant lower frequency of respiratory distress syndrome in neonates born to women taking progesterone pessary than those born to the controls; (9 vs 32), p value < 0.001. With regard to the frequency of other neonatal morbidity and mortality events (apart from respiratory distress syndrome), no significant difference was shown between the two study groups (2 vs 7), p value = 0.099, as illustrated in table (4) as well.

Table (5) demonstrated the presence of significant lower frequency of low birth weight neonates born from women taking progesterone pessary than those born to the controls (5 vs 26), p value < 0.001. Moreover, there was no significant difference regarding the frequency of congenital anomalies in the neonates between the two study groups (one case of ventricular septal defect in women taking progesterone pessary vs one case of cleft lip in control group), p value = 1.000, furthermore, a significant lower frequency of admission of neonates to neonatal intensive care unit (NICU) was shown between women taking progesterone pessary and control group (10 vs 34), p value < 0.001.

Table 2. Comparison of other maternal related data between the two groups

Parameter		Women taking progesterone pessary N=252 No. (%)	Control N= 240 No. (%)	P value*
Obstetric history	Nulliparous	95 (37.7)	93 (38.7)	0.853
	Multiparous	157 (62.3)	147 (61.3)	
Method of delivery	Vaginal	215 (85.3)	207 (86.3)	0.797
	Cesarean section	37 (14.7)	33 (13.7)	
History of infertility ± ART	Positive	0 (0.0)	0 (0.0)	1.000
	Negative	252 (100)	240 (100)	
Cervical length	< 20 mm	42 (16.7)	39 (16.2)	0.904
	20-30 mm	210 (83.3)	201 (83.8)	
Higher professional education	Present	97 (38.5)	90 (37.5)	0.853
	Absent	155 (61.5)	150 (62.5)	

* Fishers' exact test, ART: Assisted reproductive technologies

Table 3. Comparison of preterm delivery between 2 groups

Parameter	Women taking progesterone pessary N=252 No. (%)	Control N= 240 No. (%)	P value*
Preterm delivery	11 (4.4)	38 (15.8)	< 0.001
Term delivery	241 (95.6)	202 (84.2)	

* Fishers' exact test

Table 4. Comparison of respiratory distress syndrome and other neonatal morbidity and mortality events between 2 groups

Parameter		Women taking progesterone pessary N=252 No. (%)	Control N= 240 No. (%)	P value*
Respiratory distress syndrome	Present	9 (3.6)	32 (13.3)	< 0.001
	Absent	243 (96.4)	208 (86.7)	
Other morbidity or mortality events	Present	2 (0.8)	7 (2.9)	0.099
	Absent	250 (99.2)	233 (97.1)	

* Fishers' exact test

Table 5. Comparison of neonates' parameters between the two groups

Parameter		Women taking progesterone pessary N=252 No. (%)	Control N= 240 No. (%)	P value*
Low birth weight neonates	Present	5 (2.0)	26 (10.8)	< 0.001
	Absent	247 (98.0)	214 (89.2)	
Neonates with congenital anomalies	Present	1 (0.4)	1 (0.4)	1.000
	Absent	251 (99.6)	239 (99.6)	
Admission to NICU	Present	10 (4.0)	34 (14.2)	< 0.001
	Absent	242 (96.0)	206 (85.8)	

* Fishers' exact test, NICU: Neonatal intensive care unit

Concerning incidence of treatment related adverse events, no significant difference was found between women taking cyclogest and control groups (5 vs 0), p value = 0.062 as shown in table (6). Only five patients from the 252 experienced minor side effects in the form of soreness and flatulence with rectal administration and leakage of the pessary with rectal and vaginal administration.

Table 6. Comparison of incidence of treatment related adverse events between the two groups

Treatment adverse events	Women taking progesterone pessary N=252 No. (%)	Control N= 240 No. (%)	P value*
Present	5 (1.98)	0 (0.0)	0.062
Absent	247 (98.02)	240 (100)	

* Fishers' exact test

Discussion

The prevention of preterm birth is a major health care priority [26].

The only class of medication to demonstrate significant reductions repeatedly in the rate of early preterm birth are progestogens, natural progesterone or the synthetic 17-hydroxy progesterone caproate (17-OHPC) [27,28].

The rationale behind the use of progesterone supplementations in reducing the rate of preterm birth is the following fact: although there is no significant change in progesterone concentration in the maternal circulation in the weeks preceding labor, the onset of labor both at term and preterm is associated with a functional withdrawal of progesterone activity [8,29,30].

The vaginal route of drug delivery results in a greater concentration of supplemental progesterone within the uterus and cervix compared to serum (a first uterine pass effect) [31].

In 2003, Da Fonseca et al. [32] reported a lower rate of preterm delivery in women at high risk and who receive a 100 mg vaginal suppository daily, 13.8% before 37 weeks compared with the placebo group 28% before 37 weeks.

In a similar study, Meis et al. [33] used weekly injections of 250 mg of 17 α-hydroxy progesterone caproate between 16 and 36 and this reduced the preterm delivery rate from 55 to 36% before 37 weeks. In this study, the neonates of mothers treated with progesterone had lower morbidity.

In the current study, there was further reduction in the rate of preterm delivery in the progesterone treated group than in the control group (4.4% versus 15.8%) as well as the findings of our study were translated into a significant reduction in RDS, low birth weight

infants and overall neonatal morbidity and mortality events in women who were taken cyclogest vaginal pessary compared to control group.

In agreement with the findings of the current study, a randomized clinical trial of vaginal progesterone capsules to prevent preterm delivery in women with a short cervix (defined as 15 mm or less) on transvaginal ultrasound at 20-25 weeks' gestation, reported a 44% reduction in the rate of preterm delivery (19.2% vs 34.4%) [34]. However, in this study, the reduction in the rate of preterm delivery was 72%. This probably can be explained by earlier gestational age of intervention, which was at 15 weeks gestation as well by the longer cervical length of the women enrolled in our study (less than 30 mm). In addition to the above findings our trial was associated with a significant improvement in neonatal outcome, which was not the case in the study done at 2007 [34].

The above difference in the findings might be explained by the following fact: the risk of preterm birth based on cervical length varies according to the population in which the measurement is obtained and the gestational age in which a short cervix is identified [14,35].

In line with finding of the current study, a large trial done at 2009 [36] measured cervical length at enrollment and at 28 weeks gestation in asymptomatic singletons, there was significantly smaller difference in the measurement of cervical length at these time points and significantly longer cervices at 28 weeks gestation in women who were treated with progesterone.

It has been found that natural progesterone exposure, significantly decrease contraction frequency [32,37].

Another trial that showed findings similar to those found in the current study is that done by Maher et al., who demonstrated a significant reduction in preterm birth at < 34 weeks with supplemental vaginal progesterone (16.6% vs 25.7%) [38], although the reduction in this study is more marked.

Hassan et al. [39] and Romero et al. [40] demonstrated that progesterone treatment indicated for a sonographic short cervix based on a universal screening strategy by TVU scanning can reduce the rate of preterm birth.

In the current study, we extended the upper limit of cervical length to less than 30 mm to explore whether vaginal progesterone pessaries would have a beneficial effect above the limit of cervical lengths included in the previous trials and therefore expand its therapeutic range.

As well our treatment protocol began at earlier gestation (15 weeks) than in the previous studies and continued till the time of delivery or up to 36 weeks + 6 days because it is possible that earlier treatment may confer more beneficial effects and this what has been shown in our results in comparison with the findings of the previous trials.

However, the findings of this study disagree with that shown by Grobman et al. [41] who conducted a randomized trial among nulliparous women with a singleton gestation and mid trimester cervical length < 30 mm, weekly injections with 17 α-hydroxy progesterone caproate or placebo did not alter the frequency of preterm birth less than 37 weeks of gestation or neonatal outcome. Other investigators observed that vaginal progesterone but not 17 α-hydroxy progesterone caproate was associated with beneficial effects [42].

The reason for this discrepancy in the findings between this trial and other trials including ours, may be related to the type, dose and route of administration of progesterone.

With regard to the risk of congenital anomaly in the progesterone treated group, our findings did not show any increase.

There is one concern exist is a possible increased risk of hypospadias in male offspring exposed to exogenous progestins [43] even if this risk is real, it is limited to exposure prior to 11 weeks gestation.

In the current study, there was no report of significant side effects in the progesterone treated group. However, long-term side effects on mothers and infants should be considered in further investigation.

Adverse effects of progesterone suppositories were not mentioned [32,34,44].

The main implication of this study for clinical practice is that universal screening of women with transvaginal ultrasound to measure cervical length early in the second trimester to identify women at risk of preterm labor can now be coupled with an intervention, the administration of vaginal progesterone to reduce the rate of preterm labor and improve neonatal outcome.

The limitation of this trial is that it is not double blind and not placebo controlled.

This study concluded that vaginal progesterone pessaries in women with a cervical length less than 30 mm early in the second trimester is effective in reducing the rate of preterm birth and some of the prematurity related morbidity and mortality events.

Acknowledgments

The author would like to thank all patients who did agree to participate in this study as well as special thanks to the ultrasound specialist who had a vital role in the current work.

Conflict of interest

The author declares no conflict of interest.

Funding

Self-funding.

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Received May 31st 2017
Accepted Oct. 22nd 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

Value of Multi-detector CT Angiography in Chronic Ischemia of Lower Limbs in Comparison with the Doppler ultrasound

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Abstract

Background Peripheral arterial disease (PAD) is one of the most common cardiovascular diseases in developed countries and is an emerging problem in developing countries. Duplex ultrasonography (DUS) has been used as the initial imaging modality in mild symptomatic PAD. Multi-slice helical CT angiography of arteries of the thigh represents a reliable means for the detection of relevant stenoses in patients with peripheral occlusive artery disease.

Objective To assess value of multi-detector computed tomography angiography (MDCTA) and to compare it with DUS to diagnose chronic ischemia of lower limbs.

Methods A prospective comparative study was conducted on 30 patients with chronic lower limbs ischemia of both limbs during the period from September 2015 to September 2016 at the Department of Diagnostic Radiology of Al-Imamein Al-Kadhimein Medical City, Baghdad, Iraq. DUS was done for all the patients and then MDCTA was done.

Results Thirty patients (20 males and 10 females) with a mean age of 57.1 ± 8.5 (range: 33 –80) years were included in this study. MDCTA detects 69 lesions (41 occluded segments and 28 stenotic segments) and DUS detects 58 lesions (35 occluded segments and 43 stenotic segments). In MDCTA, 8 patients (26.7%) had lesion in only one arterial segment, 13 patients (43.3%) had two segment lesions, 3 patients (10%) had three segment lesions, 4 patients (13.3%) with four lesions and only two patients (6.7%) had lesions in five arterial segments. Regarding the findings of the DUS one segment lesion was detected in 13 patients (43.3%), two segment lesions in 11 (36.7%), three segment lesions in 2 (6.7%), four segment lesions in 3 (10.0%) and only five segment lesions in only one patient (3.3). Furthermore, the measure of agreement between both MDCTA and DUS in the number of lesions detected revealed a good agreement between both tests, (Kappa = 0.81) with a percent agreement of (86.6%).

Conclusion Multi-detector CT angiography is a fast, accurate, safe and a minimally-invasive imaging modality which may be used in cases of PAD for diagnosis, grading and for preoperative assessment of lower limb arterial disease.

Keywords Multi-detector CT Angiography, chronic ischemia of lower limbs, doppler ultrasound

Citation Kadhim MA, Eisa YA, Mohammed SJ. Value of multi-detector CT angiography in chronic ischemia of lower limbs in comparison with the doppler ultrasound. Iraqi JMS. 2018; 16(2): 144-151. doi: 10.22578/IJMS.16.2.5

List of abbreviations: ATA = Anterior tibial artery, CLI = Chronic limb ischemia, CFA = Common femoral artery, CT = Computed tomography, DFA = Deep femoral artery, DUS = Duplex ultrasonography, MRI = Magnetic resonance angiography, MDCTA = Multi-detector computed tomography angiography, PAD = Peripheral arterial disease, PeA = Peroneal artery, POPA = Popliteal artery, PTA = Posterior tibial artery, SFA = Superficial femoral artery, TCA = Trans-catheter angiography

Introduction

Peripheral arterial disease (PAD) is one of the most common cardiovascular diseases in developed countries [(1)] and is an emerging issue in developing countries [(2,3)]. It is a manifestation of systemic atherosclerosis that commonly affects the lower extremities and is defined as any pathologic process causing obstruction to blood flow in the arteries [(4)]. Chronic limb ischemia (CLI) is the end result of PAD. Among aging people, and with elevating incidence of both diabetes and chronic kidney disease, chronic ischemic limb is likely to be more prevalent [(5)]. Existence of PAD is an extensive atherosclerosis marker [(6)]. The diagnosis of PAD and the subsequent treatment decisions rely on clinical exam and non-invasive imaging [(7)]. Clinical symptoms depend most of all upon the degree of vascular stenosis/occlusion, the location of lesions in

particular vascular segments, the degree of advancement of collateral circulation [5,8-10]. In the early stages, PAD is mostly silent. With the progression of disease, it may manifest as intermittent claudication, pain at rest [11]. Most individuals with lower extremity PAD are asymptomatic and do not experience recognizable ischemic symptoms until late in the disease progression [12]. Imaging is necessary for planning interventions in patients with lower extremity PAD [4].

Trans-catheter angiography (TCA) is considered as the "gold standard" for the assessment of occlusive vascular diseases of the aorta and lower extremity arteries [13]. However, this method is known to be invasive and has a definite morbidity. Computed tomography (CT) scan has enormously improved during last decade [14,15]. Duplex ultrasonography (DUS) has been used as the initial imaging modality in mild symptomatic PAD [16]. Color Doppler allows the rapid identification of normal and abnormal segments of vessel [17]. Multi-slice helical CT angiography of arteries of the thigh represents a reliable means for the detection of relevant stenosis in patients with peripheral occlusive artery disease [18]. It has the advantage of visualizing the arterial lumen and arterial wall calcifications [19]. Also, it aids in good assessment of unusual lesions and identification for larger number of arterial segments, specially lesions with occlusive pathologies [20-23]. The CTA is accurate in about 87% in visualizing >50% stenotic lesions and visualizing total obstruction in about 96%, and to be 92% to 97% sensitive and of 93% to 97% regarding specificity [24]. The advantage of CTA is that it is noninvasive (unlike TCA), disadvantages are exposure to radiations, needs for potentially nephrotoxic contrast agents [25].

This study aimed to assess the value of multi-detector computed tomography angiography (MDCTA) and to compare it with DUS in reaching the diagnosis of lower limb chronic ischemia.

Methods

This was a prospective comparative study done from September 2015 through September 2016 in Radiology Department of Al-Imamein Al-Kadhimein Medical City, Baghdad,Iraq. Thirty patients (20 males and 10 females) with chronic ischemic lower limbs were included regardless of their age or gender. Data were collected form patients referred from medical and cardiovascular surgery wards.

Inclusion criteria were patients with symptomatic chronic ischemic limbs.

Exclusion criteria were patient having previous interventional radiological procedures, arterial stenting or grafting, a history of significant lower limb trauma, with raised renal indices, an acute lower limb ischemia and patients with history of allergy to iodinated contrast medium.

Examination protocols

Doppler ultra-sonography examinations

Patients were examined using HD11XE (Philips medical system, Netherland). The examination was done beginning at the common femoral artery (CFA), superficial femoral artery (SFA), deep femoral artery (DFA), popliteal artery (POPA), anterior tibial artery (ATA), posterior tibial artery (PTA) and peroneal artery (PeA) were examined using a 7.5 MHz probe. The diagnostic segment was diagnosed according to the diameter reduction less than or equal/ more than 50%.

Multi-detector CT angiography

Both limbs of the patient were examined in the CT unit using (somatome definition 64 slices, Siemens medical system, Germany). CT angiography was performed following target injection of 100-120 ml of contrast medium at a flow rate of 3-3.5 ml/s by using bolus tracking. The contrast medium used low osmolar non-ionic contrast medium (Iohexol 350 mg I/ml). MDCTA was performed by using a thin section slice of 0.6 mm.

The criteria of analysis

1) Assessment of collateral vessels; 2) opacification or non-opacification of the

examined part; 3) Presence or absence of stenotic segments; 4) presence or absence of an occlusion with estimating its length; 5) The arterial tree was then divided into 7 segments CFA, SFA, DFA, POPA, ATA, PTA and PeA.

Statistical analysis

Patients' data were entered and analyzed using the statistical package for social sciences (SPSS). Measure of Agreement (Kappa) (as shown in table 1) was used to assess the performance and agreement of CT angiography and duplex ultrasonography and the percent agreement was calculated, the significance level was assessed using Pearson's chi square test. Level of significance, a P value ≤ 0.05 was considered as statistically significant.

Table 1. Lower limb ischemia Benchmark scales to Kappa's value

Kappa value	Interpretation
< 0.20	Poor
0.21 – 0.40	Fair
0.41 – 0.60	Moderate
0.61 – 0.80	Good
0.81 – 1.00	Very good, Almost perfect

Results

Thirty patients were enrolled in this study, with a mean age of 57.1 ± 8.5 (range: 33-80) years. Males were 20 represented two thirds of the studied group, (66.7%) and females were 10 represented the remaining (33.3%).

According to the findings of the CT angiography and DUS, there were a total of 69 (16.4%) and 58 (13.8%) lesions detected, respectively, in both lower limbs of the 30 patients, which indicated the higher number of lesions visualized using CT angiography than DUS, however, the difference was statistically non-significant, (P= 0.34). whilst, according to the CTA 8 patients (26.7%) had a single arterial segment lesion, 13 patients (43.3%) had two segmental lesions, 3 patients (10%) had three lesions, 4 patients (13.3%) with four lesions and only two patients (6.7%) had five artery segmental lesions. In regards to DUS findings single lesion was found in 13 patients (43.3%), two lesions in 11 (36.7%), three lesions in 2 (6.7%), four lesions in 3 (10.0%) and just five lesions in a single patient (3.3%), (Table 2). Moreover, the measure of agreement between both CTA and DUS in the number of lesions found revealed a good agreement between the two tests, (Kappa = 0.81) with agreement percentage of (86.6%).

Additionally, the distribution of the visualized lesions according to the type of lesion visualized and the arterial segment affected is shown in (Table 3), where the findings of CT angiography revealed that out of the 69 arterial lesions visualized to have 41 segments (68.3%) were occluded and the remaining 28 segments (46.6%) were found to be stenosed. The duplex ultrasonography revealed 35 (58.3%) occluded and 23 (38.3%) stenosed arterial segments out of the 58 total lesions detected by this test, these findings are summarized in table 3.

Table 2. Number and proportions of patients according to the number of affected segments detected by CT angiography and Doppler ultrasonography

Number of affected segments	CT Angiography		Duplex ultrasonography	
	No. of patients	%	No. of patients	%
One	8	26.7%	13	43.3%
Two	13	43.3%	11	36.7%
Three	3	10.0%	2	6.7%
Four	4	13.3%	3	10.0%
Five	2	6.7%	1	3.3%
Total	30	100%	30	100%
Measure of Agreement for number of lesions detected				
Kappa		0.81		
Percent agreement		86.6%		
P.value < 0.001, significant at ≤ 0.05				

Table 3. Distribution of lower limbs arterial lesions detected by CT angiography and doppler ultrasound according to the type of lesion and artery segment

Artery	Occlusion				Stenosis			
	CT angiography		Doppler Ultrasound		CT angiography		Doppler Ultrasound	
	No.	%	No.	%	No.	%	No.	%
CFA	2	6.7	2	6.7	4	13.3	3	10
DFA	0	0	0	0	2	6.7	1	3.3
SFA	8	26.7	6	20	5	16.7	4	13.3
POPA	7	23.3	7	23.3	4	13.3	3	10
PTA	8	26.7	7	23.3	4	13.3	5	16.7
ATA	10	33.3	8	26.7	5	16.7	4	13.3
Per. A	6	20	5	16.7	4	13.3	3	10
Total	41	68.3	35	58.3	28	46.6	23	38.3

The Measure of agreement between CT angiography and duplex ultrasonography regarding the type of lesions detected showed that there was a good agreement between both tests in detection of both occlusion and stenosis of the examined arterial segments, with higher percent agreement in occlusive lesions than stenosis , [(Kappa = 0.82), (percent agreement = 85.7%) and (Kappa = 0.75), (percent agreement=78.5%) , respectively, and for the detection of all lesions (Kappa =0.79) with agreement percentage of (82.7%) as shown in table 4.

The following figures (1 and 2) show selected images of some patients presented with lower limb ischemia participated in the current study.

Table 4. Measure of agreement between CT Angiography and Doppler ultrasonography in detection of type of arterial lesions

	Measure of Agreement (Kappa)		
	Kappa	Percent agreement	P value*
In occlusion	0.82	85.70%	< 0.001
In Stenosis	0.75	78.50%	0.003
For all lesions	0.79	82.70%	< 0.001

* P. value is significant at ≤ 0.05

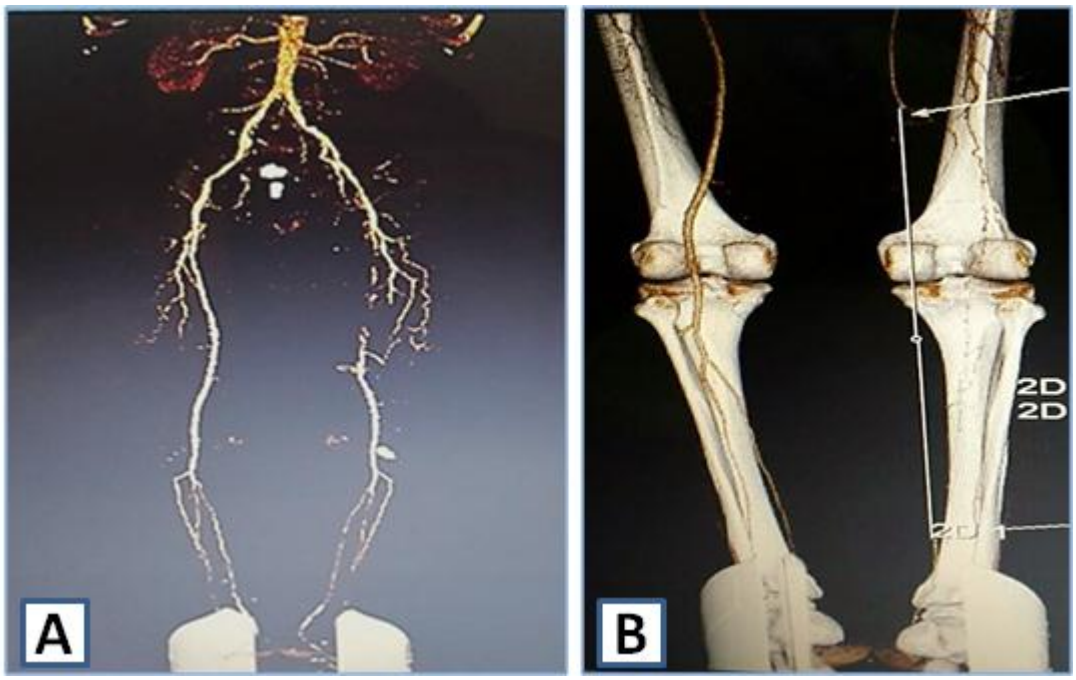

Figure 1. A: MDCTA of 60 years old male patient showing occlusion of the Left superficial femoral artery. B: MDCTA (posterior view) of 54 years old male patients presented with lower limb ischemia shows occlusion of left side distal superficial femoral, popliteal and posterior tibial arteries

Discussion

Chronic ischemic lower limb is a disease manifested via a wide range of clinical presentations, starting from being asymptomatic, through intermittent claudication, to critical limb ischemia [26]. Management strategies are ruled by disease severity. Imaging is vital for planning the intervention of PAD specifically the lower limbs [4,27].

Non-invasive imaging procedures, including DUS, magnetic resonance angiography (MRA), and MDCTA are available for grading lower limb arterial disease [6,7]. The DUS has been proved as a high specific and sensitive test for identification of significant hemodynamic lesions with more than 50% stenosis or occlusion [28]. CT angiography continues to be attractive due to the continuous fast technical improvement, thinner slices, higher spatial resolution, short acquisition time and availability of scanning of the whole vascular tree in a limited time with a reduction in the quantity of contrast medium [29-33]. CT angiography assessing the extent of PAD and provides plan and guide for vascular interventions [34].

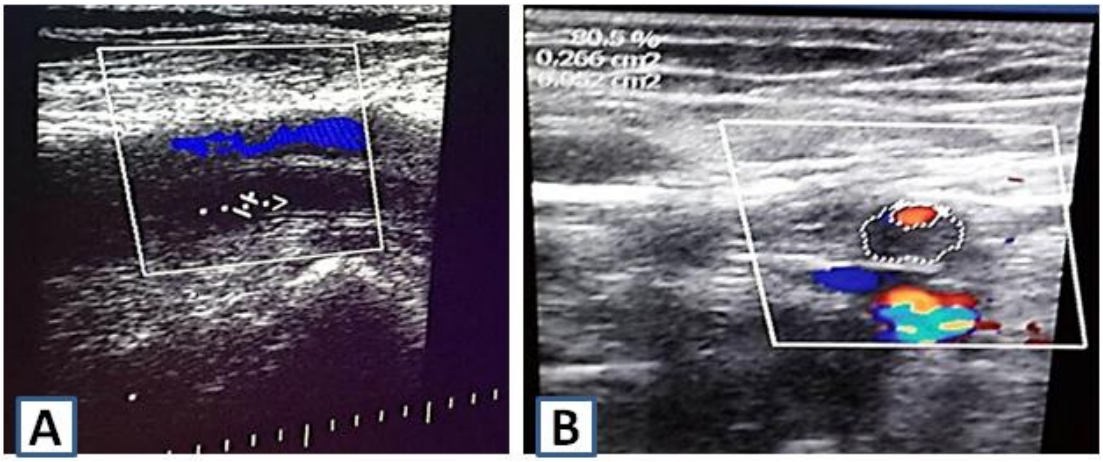

Figure 2. A: Doppler US of the Left superficial femoral artery shows no flow in color Doppler and no spectral wave in a 60 years old male patient showing occlusion of the artery. B: Color Doppler shows area of stenosis of the left femoral artery in a 54 years old male patient with lower limb ischemia

Various imaging techniques are used in the diagnosis of PAD. The usual is DUS and conventional angiography. The gold standard conventional angiography is responsible for complications in 1 to 2% of patients. For this reason, non-invasive techniques have been recently developed [32].

In the present study, the MDCTA findings revealed a total of 41 occluded and 28 stenosed segments with a total lesions of 69, these findings show no great difference compared to that of DUS examination where DUS detected 58 lesions of all examined arterial segments, these lesions included 35 occlusions and 23 stenosis and according to the number of affected segments detected by MDCTA and DUS findings indicated good agreement between both tests with larger number of lesions detected on CT angiography than DUS. The cause behind this slightly lower number of lesions visualized in DUS may be due to deep anatomic position and small vascular diameter of some arterial segments which may compromise intonations seen in the peroneal artery and this show the additional value and complementary role of the CT angiography as a diagnostic tool for lower limb peripheral arterial lesions [27,28,34].

The findings in regard to the good performance and good agreement rate between MDCTA and DUS go with that reported in previous study of Pollak et al. [35] who compared MDCTA vs DUS and found that overall, the technique for imaging vessel stenosis by using DUS is less sensitive than MDCTA and need longer time for evaluation of two lower extremities this considered the greatest limitation of DUS. Another study by Algazzar et al. [30], revealed results differ from ours in term of no statistically difference between MDCTA and DUS, and this difference might be due to population difference, patients' inclusion criteria, and the difference in age groups of the patients in both studies.

Some studies limit the use of DUS in evaluating lower limb arterial pathologies as the procedure is totally operator dependent, it requires highly trained person, it also lacks the arterial imaging capability of MDCTA that the vascular surgeons need for preoperative planning and assessment, it can document only a small arterial segmental lesion in each image [27,28].

This study concluded that MDCTA is a fast, accurate, safe and a minimally-invasive imaging modality, which may be used in cases of peripheral vascular diseases for diagnosis, grading and for preoperative assessment of lower limb arterial disease. The limiting factors that prevent the widespread usage of MDCTA

are the limited number of multidetector row CT machines and the limited experienced stuff that can perform such a recent examination. Interpretation of the images by a radiologist with experience in vascular imaging combined with experience in multi-detector row CT imaging is mandatory.

DUS is a reliable non-invasive method of investigating the lower limb arterial system. It has an advantage over MDCTA that it provides us with hemodynamic data proximal, distal and at the site of obstruction. The limiting factor for color DUS imaging is that this examination is totally operator dependent. It requires highly trained personnel, which is not always available. It also lacks the arterial imaging capabilities of MDCTA that surgeons need for preoperative planning. It can only document a small arterial segment in each image. This leads us to the conclusion that MDCTA may replace color DUS in many cases.

Acknowledgments

Authors would like to thank the medical staff in the Radiology Department of Al-Imamein Al-Kadhimein Medical City for offering the opportunities of this study. Thanks a lot, to all patients who agreed to participate in this study.

Authors contribution

All the three authors were collaborated together in collecting data and writing the thesis.

Conflict of interest

No conflict of interest is present.

Funding

The funding of this research is all personal.

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Received Jun. 15th 2017
Accepted Dec. 25th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

Assessment of Spinal Cord Compression in Patients with Cervical Spondylosis, A Clinical Prospective Study of 25 Patients

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Abstract

Background Cervical spondylosis myelopathy (CSM) is a common cause of disability in older persons. Because spondylosis is a universal finding as the patients aged.

Objective To assess the demographic and management of cervical spondylosis in a sample of Iraqi patients.

Methods This is a prospective clinical study, carried out over the period from January 2013 to December 2014, and included 25 patients with cervical spondylosis myeloradiculopathy referred for surgical intervention in Neurosurgical Department in the Al-Imamein Al-Kadhimein Medical City. General examination and full neurological assessment were performed, as well as relevant investigations, particularly; radiological assessments. All of the patients were subjected to surgery, with 17 patients (68%) were treated with laminectomy and foraminotomy, while the remaining 8 patients (32%) were treated with laminectomy only.

Results They were 14 males and 11 females, slightly affects males more than females, aged 38 to 82 years it is more common in 5th and 6th decades of life, and in rural than urban areas. Myeloradiculopathic features were the most common presenting ones with C5-C7, which were the most affected levels. General examination and full neurological assessment were performed, as well as relevant investigations, particularly; radiological assessments. All of the patients were subjected to surgery, with 17 patients (68%) were treated with laminectomy and foraminotomy, while the remaining 8 patients (32%) were treated with laminectomy only. More than half of the patients were showed slight improvement in their complaints, while significant improvement occurred in more than 28 % of patients. Despite that, 20% of patients showed no improvement in their symptomatology, however; no reported deterioration was noticed in the study.

Conclusion Myeloradiculopathic feature were the most common presenting features with C5-C7 was the most level affected. Laminectomy with foraminotomy was surgery of choice in tow third of patients, with remaining one third underwent laminectomy only.

Keywords Cervical spondylosis, spinal cord compression

Citation Jaizany AJA, Nema IS, Hassan YM. Assessment of spinal cord compression in patients with cervical spondylosis, a clinical prospective study of 25 patients. Iraqi JMS. 2018; 16(2): 152-158. doi: 10.22578/IJMS.16.2.6

List of abbreviations: CT scan = Computerized tomography scan, MRI = Magnetic resonance imaging

Introduction

Cervical spondylosis is a non-specific term describing the morphological manifestations of progressive degeneration of the spine. It is a disorder caused by abnormal wear on the cartilage and bones of the neck (cervical vertebrae) with degeneration and mineral deposits in the cushions between the vertebrae (cervical disks) and there is subsequent impingement of neural elements in a narrow cervical canal [(1)]. It

includes degenerative changes in the facet joints, longitudinal ligaments, and ligamentum flavum. The changes of neural compression resulting in radiculopathy or compression of the spinal cord resulting in myelopathy. Both the neural and spinal cord compression will result in radiculomyelopathy [(1)]. Due to aging intervertebral disc undergoes progressive desiccation, becomes more compressible and less elastic and secondary changes arise [(2)]. The primary event is a progressive decrease in the degree of hydration resulting in loss of disc height, disc fibrosis and annular weakening [(3)].

There are several predisposing factors, which may cause acceleration of these changes including: Occupations, Previous injury with fracture or disc prolapsed, Segmentation defects [(4)].

Common clinical syndromes associated with cervical spondylosis include the following: cervical pain, cervical radiculopathy, cervical myelopathy.

The diagnosis of cervical spondylosis is based on observation of the aforementioned symptoms, physical examination, x-rays, computerized tomography (CT) scan and Magnetic resonance imaging (MRI) [(2)].

Evaluating the efficacy of any particular treatment strategy for Cervical spondylosis myelopathy (CSM) is difficult because reports show that as many as 18 percent of patients with CSM will improve spontaneously, 40 percent will stabilize and approximately 40 percent will deteriorate if no treatment is given [(5)].

The objective of this research was to study the incidence, clinical features and presentations of the cervical spondylotic myelopathy in Iraq.

Methods

This is a prospective clinical study, carried out during the period from January, 2014 throughout December 2014, and included 25 patients with cervical spondylosis referred for surgical intervention Neurosurgical Department in the Al-Imamein Al-Kadhimein Medical City. Including 25 patients they were14 males and 11 females, 38 to 82 years of age. All patients were admitted to hospital and opened case sheets and detailed history was taken including general and neurological. In addition, general as well as full neurological assessment was performed, relevant investigations, particularly radiological especially MRI to give detail about the pathology which involved the spine and caused spinal cord compression or roots compression, neurophysiological studies to exclude other causes such as peripheral neuropathy, motor neuron disease, and multiple sclerosis were followed up. Patients operated in sitting position cervical collar remained throughout the position and even during intubation, and the collar remained post-operative for long period. Steroid started preoperatively and intra operative then gradually tapered, prophylactic antibiotics also considered. Patients were subjected to surgery, with 17 patients (68%) those with radiculopathic features and Myeloradiculopathic features treated by laminectomy and foraminotomy, while the remaining 8 patients (32%) those of myelopathic features treated by laminectomy only. Midline posterior cervical incision, subcutaneous, ligamentum nuchae and muscles are all opened with good hemostats, cautery, forceps arteries, self-retaining retractors were used, subperiosteal gauze dissection for muscles with spade, spinous processes were removed by shear, laminae were opened by fine manipulation with Roenguers to exposed the dura and thickened ligaments, ligament removed by tenatom knife carefully to avoid dural injury, if we need to open the foramina we must extended more laterally in decompression, good hemostasis and wound closed in layers and dressing cervical collar used immediately postoperative. Posterior decompression was performed in all patients and follow them in during a period of staying in the hospital for 10 days, most of them get slight improvement others remained same, no deterioration in their neurological state were found post-operatively.

Results

Distribution of cervical spondylosis according to age and sex is shown in table 1; (14 males and 11 females), the commonest age group affected by the disease was 40-59 years. followed by the group 60-70 years), while the percent decline for the age group before 40 years and those above 70 years. It is also found that in all age group, males and females affected equally.

Table 1. Distribution of patients with cervical spondylosis according to age and sex

Age (yr)	Sex		Total
	Male No. (%)	Female No. (%)	No. (%)
<40	1 (7.2)	1 (9.9)	2 (8.0)
40-49	5 (36.0)	4 (39.6)	9 (36.0)
50-59	4 (28.8)	5 (49.5)	9 (36.0)
60-69	3 (12.6)	1 (9.9)	4 (16.0)
≥70	1 (7.2)	0 (0.0)	1 (4.0)
Total	14 (100)	11 (100)	25 (100)

Distribution of patients with cervical spondylosis according to the residency is illustrated in table 2; the majority of studied patients come from urban areas comprises 64%, the remaining 9 patients were rural inhabitant.

Table 2. Distribution of patients with cervical spondylosis according to the residency

Residency	No. (%)
Urban	16 (64.0)
Rural	9 (36.0)
Total	25 (100)

Table 3 displays presentation of patients with cervical spondylosis, 12 patients (48%) complained of myeloradiculopathic symptoms, 8 patients (32%) complained of myelopathic symptoms, while the remaining 5 patients (20%) had mixed features from those with radiculopathic features.

Table 3. Presentation of patients with cervical spondylosis

Presentation	No. (%)
Myeloradiculopathy	12 (48.0)
Myelopathy	8 (32.0)
Radiculopathy	5 (20.0)
Total	25 (100)

Among patients with myelopathy, it is found that 4 patients (50%) have involvement of upper limbs alone. While, 2 out of total 8 patients (25%) had Paraparesis and 2 patients (25%) had quadriparesis. Neither paraplegic

nor quadriplegic was documented; this is shown clearly in table 4.

Table 5 shows those patients with radiculopathy, 3 out of 5 patients (60%) complaining of pain and paresthesia, while 1 patients (20%) presented with motor and sensory loss, while complete motor paralysis occur in 1 patient (20%).

Table 4. Deficit among myelopathy

Deficit	No. (%)
Upper extremities weakness	4 (50.0)
Quadriparesis	2 (25.0)
Paraparesis	2 (25.0)
Quadriplegia or paraplegia	0 (0.0)
Total	8 (100)

Table 5. Deficit among radiculopathy

Clinical features	No. (%)
Pain and paresthesia	3 (60.0)
Motor and sensory loss	1 (20.0)
Complete motor paralysis	1 (20.0)
Total	5 (100)

Myeloradiculopathic were the most presenting features of cervical spondylosis and table 6 shows mixed features.

Levels of cervical spondylosis is shown in table 7, the commonest level affected was C5-C7 15 patients then (60%), followed by C3-C5 5 patients 20% and C3-C7 5 patients 20%. It was found no patient with spondylotic changes in C1-C2 involved in the study.

Table 8 shows the surgical treatment of patients with cervical spondylosis, all the 12 patients with myeloradiculopathy and the 5 patients with radiculopathy were treated by laminectomy and foraminotomy (68 %), while the remaining the 8 patients with myelopathy treated by laminectomy alone (32%).

Table 6. Deficit among myeloradiculopathy

Deficit	No. (%)
Upper extremities weakness	4 (30.8)
Quadriparesis	2 (15.4)
Paraparesis	2 (15.4)
Pain and paresthesia	3 (23.0)
Motor weakness and sensory loss	1 (7.7)
Complete motor paralysis of the segmental involvement	1 (7.7)
Total	13 (100)

Table 7. Levels of cervical spondylosis

Level	No. (%)
C1-C2	0 (30.8)
C3-C5	5 (20.0)
C5-C7	15 (60.0)
C3-C7	5 (20.0)
Total	25 (100)

Table 8. Treatment of patients with cervical spondylosis

Surgical treatment	No. (%)
Laminectomy with foraminotomy	17 (68.0)
Laminectomy	8 (32.0)
Total	25 (100)

Table 9 presented the outcome of surgical treatment in this study, where 28% of patients showed significant improvement in their symptoms, while 52% showed slight improvement. No improvement was seen in 20% of patients, whereas no deterioration in symptoms noted in any of patients in our study. the figures with regard to type of pathological deficit (myelopathy, radiculopathy or mixed) were somewhat comparable to the overall figures. The parameter for degree of improvement is the British Medical Research Council Classification the motor power M0-M5, and the sensory function S0-S4.

Table 9. Outcome of surgical treatment

Outcome	Myelopathy No. (%)	Radiculopathy No. (%)	Mixed No. (%)	Total No. (%)
Significant improvement	2 (25.0)	2 (40.0)	3 (25.0)	7 (28.0)
Slight improvement	4 (50.0)	2 (40.0)	7 (58.3)	13 (52.0)
No improvement	2 (25.0)	1 (20.0)	2 (16.7)	5 (20.0)
Deterioration	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	8 (100)	5 (100)	12 (100)	25 (100)

Discussion

CSM is the most common spinal cord disorder in the person more than 50 years of age. As the numbers of older persons and the advance investigations, myelopathy will most likely increase. The lesion is progressive, but when early diagnosis and surgical treatment applied progression can be prevented.

Regarding age and sex in the current study, it was found that CSM nearly affected males and females, but more commonly between 40-60 years (60%), the result agrees with that of Benzel [3].

CSM more common among the rural areas, as compared with Epstein et al. result [6].

In the current study, myeloradiculopathy was the most common presenting feature in 50% of patients which goes with Stringer et al. study [4].

Among myelopathic patients, upper extremities involvement more than lower extremities in this study, and this goes with Nakajima et al study [6].

Pain and paresthesia were the most common presenting features among radiculopathic patients (60%), followed by motor weakness

and complete motor (20%) which is similar to the finding of Morgan et al. study [7] who said compression of cervical nerve roots lead to ischemic changes that cause sensory dysfunction (e.g. radicular pain) and/or motor dysfunction (e.g. weakness) and C6 root is most commonly affected because the predominant degeneration at the C5-C6 interspaces.

Posterior decompression (laminectomy) was performed in all our patients. Most patients presented with delayed diagnosis and presented with severe deficit. The treatment in those to arrest the progression of the lesion. Those with severe deficit attributed to a fact that those patients diagnosed early and refused operation and later on accept the surgery. Laminectomy with foraminotomy was used in the current study because most patients present with myeloradiculopathy, posterior decompression without foraminotomy for those with myelopathy only, Benzel principle of spinal surgery: posterior approach [8] said once frank myelopathy occurs surgical intervention is necessary, the primary goal to decompress the cord. Stoops and King [9] mentioned a variety of factors determine success of surgery include severe preoperative neurological signal changes within spinal cord seen on radiographic study. LaRocca and Macnab [10] said whatever the surgical approach used; improvement can be expected if symptoms have been present for less than 2 years.

The complications associated with dorsal approaches are predictable and preventable and although ventral operations for CSM have recently been reported to be superior to dorsal approaches a ventral approach has been limited to three levels.

Significant neurological and non-neurological complications associated with ventral approaches, in addition to long-term complications related to fusion, such as increased spinal laxity, hypermobility, and degeneration of the adjacent vertebral segments, affect outcome. Therefore, they must be taken into account when using any ventral decompressive approach [9]. In addition to long-term complications related to fusion, such as increased spinal laxity, hypermobility, and degeneration of the adjacent vertebral segments, affect outcome. Therefore, they must be taken into account when using any ventral decompressive approach [9], ventral approach has been limited to three level

The level of cervical spondylosis in this study was C5-C7 as the most commonly affected (60%) which is similar to results of Epstein et al. study [5] and Morgan et al study [7].

In the present study, the outcome of surgery where 28% of patients showed significant improvement in their symptoms, while 52% showed slight improvement. No improvement was seen in 20% of patients, whereas no deterioration in symptoms noted in any of patients in this study compared with the Kawakita et al. and Benzel studies [12,13]. The course of the lesion may be slow and prolonged, and the patients may either remain asymptomatic or have mild cervical pain, long periods of non-progressive disability are typical, and in a few cases, the patient's condition progressively deteriorated.

This study concluded that:

1) CSM slightly affects males more than females, it is more common in 5th and 6th decades of life and is more common in rural compared to urban people.
2) Myeloradiculopathic features were the most common presenting features with C5-C7 was the most level affected.
3) Laminectomy with foraminotomy was surgery of choice in tow third of patients, with remaining one third underwent laminectomy only.
4) More than half of patients showed slight improvement in their complaints, while significant improvement occurred in more than 28% of patients. Despite that 20% of patients showed no improvement in their symptomatology, however no reported deterioration occurred in any patient in this study.

Acknowledgments

The authors would like to express our thanks and gratitude to medical and paramedical staff of neurosurgical department of Al-Imamein Al-Kadhimein Medical City in Baghdad and Al-Basra Teaching Hospital.

Authors contribution

Dr. Jaizany: provide 9 cases, Dr. Hassan: 8 case, Dr. Nema 8 cases and analysis of the result.

Conflict of interest

The authors declare no conflict of interest.

Funding

Personal.

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Received Jun. 15th 2017
Accepted Oct. 25th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

Cyperus Rotundus Tubers Extract Inhibits Stem Cell Markers Expression in Cervical and Human Glioblastoma Cancer Cell Lines

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Abstract

Background Cancer stem cell markers known for their ability to induce tumor initiation, angiogenic activity, therapy resistance and metastasis formation. Plant extract potentially was used to treat cancer and/or target its genes. Cyperus rotundus tubers extract has been used in ancient as a folk medicine for its antibacterial, anti-diabetic and for other maladies for its antioxidant properties that have been estimated in modern medicine.

Objective To determine the effects of total oligofalvonoids (TOF) extracted from Cyperus rotundus tubers against cervical cancer cells line (HeLa) and human glioblastoma (AMGM) cell line.

Methods Cytotoxicity of TOF extract against both cancer cell lines was determined after 24 hr of exposure and the best concentration of inhibition was 350 µg/ml. Total RNA extracted from both cell lines after treated with TOF and the expression levels of cancer stem cell markers OCT3/4 as well as matrix metalloproteinasese MMP2 and MMP9 have been measured using quantitative real time polymerase chain reaction.

Results Cytotoxicity of TOF extract with concentration (350 µg/ml) shown to reduce the growth of cancer cell lines after 24 hr of exposure. The expression level of OCT3/4 was highly significantly reduced in both AMGM and HeLa cells after treated with TOF and the fold change reduced from (15 to 0.03) and (10 to 0.09), respectively. On the other hand, the expression levels of MMP2 and MMP9 were significantly decreased in AMGM and HeLa cells treated with TOF extract with decreasing in fold change from (10.2 to 0.02) for MMP2 in AMGM cells and (1.85 to 0.5) for MMP9. And fold change of MMP2 expression in HeLa cells was decreased after treated with TOF from (11.1 to 0.01) and for MMP9 (11.43 to 0.08).

Conclusion The result indicates that the inhibition of cancer stem cell markers OCT3/4 and MMP2 and MMP9 may provide a novel strategy to treat cancer using a natural plant extract.

Keywords Cancer stem cell markers, AMGM cells, Hela cells

Citation Abdulghany ZS, Mahmood NA, Tawfeeq AT, Yassen NY. Cyperus Rotundus Tubers extract inhibits stem cell markers expression in cervical and human glioblastoma cancer cell lines. Iraqi JMS. 2018; 16(2): 159-165. doi: 10.22578/IJMS.16.2.7

List of abbreviations: AMGM = human glioblastoma cell line, AMN3 = Mice mammary adenocarcinoma cell line, CSC = Cancer stem cell, ct = threshold cycle, DMSO = Dimethysulphoxide, ECM = Extracellular matrix, ER-alpha = Estrogen alpha receptor, HeLa cells = Human cervical cancer cell line, MCF-7 = Human breast adenocarcinoma cell line (estrogen, progesterone receptors +, HER2-), MDA-MB-231 = Antiproliferative human breast cancer cell line (triple negative), MMP = Matrix metalloproteinase (group of enzymes), MTT= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye, mRNA = Messenger ribonucleic acid, OCT3/4 = Octamer-binding transcription factor, PCR = Polymerase chain reaction, qPCR = Quantitative real time PCR, RMPI1640 = Roswell Park Memorial Institute, TOF = Total oligomeric flavonoids

Introduction

Cancer is one of the most common diseases worldwide, and ranks the second most common cause of death following cardiovascular diseases. Chemotherapy is able to kill some cancer cells especially the more rapidly replicating tumor cells, but they were nonspecific, characterized by low therapeutic index and associated with a wide range of side effects. The anticancer field still searching for herbal treatments to avoid these side effects; the primary tumor and death related cancer result from tumor spread and metastasis [1]. Progression of metastasis starts after cancer cell detachment from the primary tumor, basement membrane degradation and cancer cell invasion into the

surrounding stroma then transport through the vascular or lymphatic system spread to distant organs [2]. The invasion depends how cancer cells degrades the extracellular matrix (ECM), which is composed of collagen, proteoglycans, fibronectin, laminin and other glycoproteins and when intact acts as a barrier to block cancer cell invasion [3].

From last century, cancer research studies have been used traditional plants in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents [4]. In Asian countries, the rhizomes of Cyperus rotundus (C. rotundus) was used as traditional folk medicines for the treatment of stomach and bowel disorders, and inflammatory diseases, have been widely, investigated [5]. Then the new researches have demonstrated its important using as anti-microbial, anti-malarial, anti-oxidant, and anti-diabetic compounds isolated and identified from C. rotundus [6]. Phytochemical studies mentioned that C. rotundus contains alkaloids, flavonoids, tannins, starch, glycosides and furochromones, and many novel sesquiterpenoids [7].

Matrix metalloproteins (MMPs) play role in cancer migration, invasion, metastasis and angiogenesis [8]. Activities of MMPs are regulated at levels of mRNA transcription and stability control in that they will control cell fate and alter developmental and pathological outcomes [9]. Both MMP-2 (gelatinase A) and MMP-9 (gelatinase B) belong to the gelatinase subfamily, which is a group of proteolytic enzymes distinguished by their fibronectin-like gelatin-binding domain [10]. Also, their expressions have been increased in most types of cancer for example, breast, prostate, colon and others [10,11].

The nuclear transcription factor OCT3/4 is a novel marker with high sensitivity and specificity expressed in embryonic cells, germ cells, and stem cells. Also, it is an important regulator of tissue specific gene expression, and a critical amount of OCT3/4 is required to maintain stem cell replication [12].

To study the effect of total oligoflavoniods (TOF) inducing apoptosis to cancer cell line, this project was designed to determine the effect of TOF on HeLa and AMGM cancer cell lines and on the expression of OCT3/4, MMP2 and MMP9 genes expression before and after treatment by measuring the fold change using real time qPCR.

Methods

Plant extract preparation

Plant has been collected in previous study as presented in the research of [13]. Briefly, tubers part of C. rotundus, were harvested in summer especially between May and late September. Botanical identification was carried out by Prof. Dr. Ali H.E. Al Mosawi, Head of Iraqi Herbarium and Professor in plant taxonomy, Biology Department, College of Science, Baghdad University in identification the genus and species of the herb as a Cyperus rotundus.

The cytotoxicity of the plant also study and the optimal inhibition concentration toward cancer cell line also described previously in study of [14], in short TOF were tested against two different cancer cell liens AMGM and AMN3, were tested and cultured in the presence of TOF extracts at different concentrations extended from 50 µg/ml to 500 µg/ml with 50 µg/ml increment each time for 24 hr. Cells viability was determined by MTT assay and calculated as a percentage of control untreated cells. And the results of previous study [14] mentioned that TOF inhibited both AMGM and AMN3 cancer cells proliferation by 67.09% and 52.41% at concentration of 350 µg/ml during incubation time of 24 hr, then we continued from this point.

Cell line culture maintenance and seeding

Two types of human cancer cell lines have been selected, human cerebral glionlastoma-multiform (AMGM) and human cervical cancer cell line (HeLa), these cell lines were kindly supplied by the Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetics Research Center (ICCMGR). These cell lines were propagated and

maintained on RPMI1640 medium (US biological, USA) using the protocol [15]. To this media, 10% fetal bovine serum (Cellgro, USA) and 1% Pencillin/ Strptomycin (Cellgro, USA) were added and then incubated at 37 °C in a humidified incubator (Memmert, Germany) with 5% CO_2. The monolayer cell culture formed in the culturing flasks, which can be observed under an inverted microscope (Lycia). The cells were subcaultered after they had achieved 80-90% confluency and trypsinized to detached from the flask and to be reday for separating and seprading on the petri disch 5 ml. Three replicates of the TOF optimal concentration 350 µg/ml was used and another three replicates for the control untreated cells. After exposure to TOF for 24 hrs, the cells were collected and centrifuged. The pellet was stored in deep freezer (GFL, Germany) at -80 °C to be ready for next step. These steps were carried out according to [15] guidelines.

RNA extraction

Transcript levels of interested genes were examined by quantitative real-time PCR. Total RNA extraction has been carried out depending on the manual protocol using AccuZol™ total RNA Extraction Solution (Bioneer, South Korea). The concnetrations were recorded using nanodrop spectrophotometry (Quawell, UK).

Primer selection

Primers were designed for each gene using NCBI/ primer-BLAST and its specificity for each gene investigated were verified using BLAST and single peak disassociation curve. Optimum annealing temperature was optimized over range of different temperature extended from 50ºC to 62 °C using conventional PCR (Sure Cycler 8800 Thermal Cycler, Agilent technologies, USA). One step KAPA real time PCR kit has been used to determine the expression of OCT3/4, MMP2 and MMP9 in HeLa and AMGM cell lines before and after treated with TOF extract.

The sequences of specific primers used for determination of OCT3/4, MMP2 and MMP9 genes as follow: for OCT3/4 Forword: ATGTGGTCCGAGTGTGGTTC Reverse: ACAGTGCAGTGAAGTGAGGG. For MMP2, Forward: AAGGACAGCCCTGCAAGTTT Reverse: GTTCCCACCAACAGTGGACA and for MMP9, Forward: GGTGATTGACGACGCCTTTG Reverse: GGACCACAACTCGTCATCGT. Beta-actin gene was used as a housekeeping gene for normalizing the results [16].

Perform Real time-PCR

For quantitative reverse transcriptase PCR (qRT-PCR), one-step SYBR green kit was used (one-step SYBR Fast, KAPA Biosystems, USA) using the primers of each gene in reveres transcription (RT) step for cDNA synthesis and for amplification. Thermal profile consists of 42 °C for 5min to synthesize cDNA, 95 °C for 3min to deactivate reverse transcriptase, followed by 40 cycles at 94 °C for 15 seconds, 30 seconds at 60 °C, 59 °C, and 58 °C for 20 seconds to anneal primers (according to each gene optimum). Fold expression for each gene was determined using ΔΔCt method in comparison with β-actin gene as a housekeeping gene [17].

Statistical analysis

The statistical significance was determined using the unpaired t-test. Probability less than 0.05 was considered as indicative of significance as compared to the control group. The data collected from triplicate for each gene. The expression of mRNA was assessed by evaluating threshold cycle (Ct) values. The Ct values were normalized with the expression levels of Beta-actin and the relative quantity of mRNA specific to each of the target genes was calculated using the 2−ΔΔCTmethod according to [17] methods.

Results

Quantitative real time PCR assay was analyzed the mRNA expression of OCT3/4, MMP2 and MMP9 genes in AMGM and HeLa cell lines before and after treatment with TOF extract. The calculation of gene expression fold change was carried out using relative quantification method. This method depends on

normalization of Ct values calculating the ΔCt which is the difference between the mean Ct value of target gene expression and that of beta-actin. Then to calculate the gene expression folds in relation to the housekeeping genes the result of 2-ΔCt of each exposure cells in relation to that of control group untreated cell lines [17].

Expression of OCT3/4, MMP2 and MMP9 in treated AMGM cell line with TOF

Expression level of OCT3/4, MMP2 and MMP9 genes have been detected in AMGM (human glioblastoma cell line) before and after treatment with TOF extract using quantitative real time PCR. The results demonstrated that the expression levels of OCT3/4, MMP2 and MMP9 genes were reduced in AMGM cell line when treated with TOF extract compared with untreated cells. The fold changes in expression of OCT3/4 was reduced from 15 to 0.03 with highly significant probability (P= 0.0008). While, the fold changes in MMP2 gene was reduced from 10 to 0.02 with high significant probability (P= 0.0001). On the other hand, MMP9 fold expression changed from 1.8 to 0.5 after treated with TOF extract with significant probability (P= 0.0028) as presented in Table 1.

Expression of OCT3/4, MMP2 and MMP9 in treated HeLa cell line with TOF

Expression levels of OCT3/4, MMP2 and MMP9 gene have been detected in HeLa cell line before and after treated with TOF using quantitative real time PCR. The results that obtained determined that OCT3/4, MMP2 and MMP9 genes reduced expression in HeLa cell lines after treated with TOF. The fold changes in expression of OCT3/4 was 10 before treatment and reduced to 0.09 after treated cells with TOF, while fold changes of MMP2 gene was 11times and reduced to 0.01 after treatment and MMP9 gene expression level was 11 times and reduced to 0.08 after treatment with TOF with high significant probability 0.0001 for all the genes, these results presented in table 1.

Table 1. Fold change in genes expression before and after treated AMGM and HeLa cancer cell lines with TOF extract

Cell lines	Genes	Fold expression before treatment	fold expression after treatment with TOF	P value (p<0.05)	Up / Down expression after treatment with TOF
AMGM	OCT3/4	15.7	0.03	0.0008 *	DOWN
	MMP2	10.2	0.02	0.0001 *	
	MMP9	1.85	0.5	0.0028 *	
HeLa	OCT3/4	10.3	0.09	0.0001 *	DOWN
	MMP2	11.11	0.01	0.0001 *	
	MMP9	11.43	0.08	0.0001 *	

* means there are statistically significant (p≤0.05)

Discussion

According to emphasize the use of herbal medicines in the treatment of cancer, in this study the effect of TOF on the expression of genes OCT3/4, MMP2 and MMP9 in AMGM and HeLa cancer cell lines were determined and the data showed that TOF extract has effect on expression of genes by decreasing genes expression that may be related to reduction in the rate of cell division, and this loss leads to the development of cancerous tissue. The results can be used as an example

of the use of herbal medicines in anticancer studies [18]. Complementary medicine therapies can be beneficial in cancer control and anti-tumor compounds suitable for further projects to be occurred.

Several studies mentioned the use of medicinal plant in cancer treatments one of these are the using of Bangladeshi medicinal plants (Emblica officinalis, Aegle marmelos, Vernonia anthelmintica, Oroxylum indicum, Argemone mexicana) as antiproliferative human breast tumor cell lines MDA-MB-231, this leads to the increase of ER-alpha mRNA accumulation (a marker of neoplastic status) [19].

While in recent study [20] the Iranian medicinal plant N. binaloudensis hexane extract effect on the expression of adenosine deaminase and ornithine decarboxylase 1 genes in two breast cancer cell lines (MCF-7, MDA-MB-231). They found that the extract play as antiproliferative of breast cancer cell lines and decrease in the expression of ornithine decarboxylase 1 and adenosine deaminase genes (these enzymes participates in purines metabolism and has role in development of immune system and maturation of mammalian cells) reduction was 4.9 fold - 3.5 fold in MCF-7 cell line and 3.6 fold - 2.6 fold in MDA-MB-231 cell line, respectively.

From different studies that mentioned the role of active compounds present in herbs have role in cancer treatment, a study of [21] mentioned that two new sesquiterpenes were isolated from the soluble fraction of rhizomes of Cyperus rotundus L. were evaluated for their cytotoxic activities against human ovarian cancer cells and endometrial adenocarcinoma cells (Ishikawa). The effects of C. rotundus on cell proliferation and apoptosis induction in murine and human leukemia cells were also examined in other cell lines. Besides, the main phenolic (orientin) compound in the methanol extract was isolated by chromatographic methods and was determined by spectroscopic data analysis and by a comparison with the literature [22].

Several studies have shown that consumption of certain foods and herbs can inhibit the growth of cancer cells. Dixon and his team workers studied the effects of curcumin as anti-metastatic breast cancer [23]. Curcumin inhibits the transcriptional network in stages and thus prevents the cell proliferation [24]. In another study of cell cycle arrest and growth of curcumin on gastric cancer cells was observed [25]. All these studies prove the importunacy of herbs and their active compounds in cancer treatment and managements of diseases.

In conclusion, C. rotundus shows anti-cancer effects in AMGM and HeLa cells. The effect was mediated through the inhibition of cell proliferation of these cell lines suggesting that it can complement current chemotherapeutic treatment. This study confirms the demonstrating the potential applications of C. rotundus as an anti-cancer drug and thus highlight further research on cancer drug discovery.

Acknowledgments

The authors regret to advice of the passing of Dr. Zaid A. Munium prior to publication. And we would like to thank all the technician staff in the lab. who helped us in this project.

Authors contribution

Dr. Abdulghany: did the actual lab work (RNA extraction, real time PCR running times), statistical analysis and writing of draft. Dr. Mahmood: conducted the seeding and maintenance of cell lines plus the molecular work and writing the discussion. Dr. Tawfeeq: extraction of plant, drafting the article and revising it critically for important intellectual content. Dr. Yassen: giving the advice in writing style and the correcting the theory of the work.

Conflict of interest

No potential conflicts of interest.

Funding

The author received no specific grant from any funding agency for preparing this project.

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Received Jul. 2nd 2017
Accepted Nov. 14th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

The Value of Magnetic Resonance Imaging in the Evaluation of Peri-Anal Fistula

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Abstract

Background Perianal fistula accounts for 0.01% of general population and is frequently managed inadequately resulting in a significant morbidity. Magnetic resonance imaging (MRI) plays an essential role in the preoperative assessment of the disease, therefore improving post-operative surgical outcome.

Objective To study the role of MRI in the evaluation of perianal fistula and to show the value of using contrast enhanced MR study in the determination of the precise tract pathway, extensions and other associated pathologies.

Methods A cross sectional analytic study was conducted on a total of 32 patients with perianal fistulas in the MRI Department of Al-Imamein Al-Kadhimein Medical City, Baghdad, Iraq during the period from November 2015 to December 2016. Patients underwent MRI examination using axial and coronal T2 weighted images with and without fat suppression and T1 fat suppressed sequences before and after contrast administration. The type of fistula, location of the internal opening, associated abscesses and/or sinus tracts and horseshoe extension were evaluated using different MR sequences.

Results The most common type of fistula encountered was the inter-sphincteric type, which was seen in 21 patients (66%), of those patients 16 fistulas (50%) were grade I and 5 fistulas (16%) were grade II. Trans-sphincteric fistulas were seen in 9 patients (28%), 2 of them (6%) were grade III and 7 fistulas (22%) were grade IV. Two patients (6%) had extra-sphincteric type. T2 weighted TSE, T2 TSE with fat suppression and T1 weighted fat suppressed post contrast sequences all show significant correlation with surgical results (with p-value less than 0.05) and the highest significance was obtained by the post contrast sequence (p-value of 0.00001). The highest accuracy in the diagnosis of fistula in ano was with the use of T1 enhanced fat suppressed sequence (98.8%) followed by 87% for the T2 fat suppression sequence and only 57% for the T2 weighted TSE sequence.

Conclusion MRI is an essential, noninvasive tool in the preoperative assessment of perianal fistulous tracks, with the axial and coronal post contrast fat suppression T1 providing the highest accuracy and clinical significance with surgical data and therefore giving a highly promising decrement in the incidence of post-operative complications.

Keywords MRI, perianal fistula

Citation Jawad AM, kadhim MA, Al-Jobouri ZK, Hussain MAA. The value of magnetic resonance imaging in the evaluation of peri-anal fistula. Iraqi JMS. 2018; 16(2): 166-176. doi: 10.22578/IJMS.16.2.8

List of abbreviations: CT = Computed tomography, MRI = Magnetic resonance imaging, STIR = Short T1 inversion recovery, TE = Time to echo (Echo time), TR = Time to repeat (Repetition time), TSE = Turbo spin echo

Introduction

Perianal fistula is an abnormal tract between an opening in the anal canal and the skin surface, and can result in significant morbidity. The prevalence of this condition is approximately 10 in 100,000, affecting males two to four times as frequently as females [(1)]. Anorectal fistulas have been the subject of medical literature for over 2,500 years [(2)]. The use of a seton (horsehair) in the treatment of anal fistulas was described by Hippocrates [(3)]. Actually, the assessment of

fistulous extension is the most important clinical indication for imaging in fistula in ano. This is due to the fact that the perianal fistula can affect the anal sphincter complex; the subsequent surgical intervention may result in impaired continence [4]. Therefore, identifying the relationship of the fistula to the anal sphincter is essential. It is also important to clarify the exact extensions of the fistulous tract and the presence or absence of associated abscesses, as missing them can lead to incomplete surgical management and thus leading to its recurrence, which is one of the main problems following surgery. Recurrence is inevitable if the internal opening was not correctly identified; this is because in such cases the original source of sepsis will not be eliminated, so identification of these parameters with proper imaging methods pre-operatively will decrease the percentage of recurrence [5-8].

In recent years, magnetic resonance imaging (MRI) has emerged as the leading imaging modality for preoperative classification of perianal fistulas. The first studies on cryptoglandular fistulas were performed with body-coil MRI and the true potential of MRI in detection of fistulas became evident [9,10]. MRI is now considered by many to be the gold standard in assessing and classifying anal fistulas and is equal or superior to examination under anesthesia [10-12]. MRI provides accurate information on the anatomical plane in which the fistula is located as well as on the relationship between the fistula track and anal sphincters, pelvic floor and the levator ani muscle [13], with accuracies reported up to 93% in classifying fistulas and 96% in delineating abscesses [14,15]. MRI examinations performed with body phased-array coils require no special patient preparation and are well tolerated. Advantages of the body phased-array coils include a larger field of view, which prevents fistula extensions from being overlooked. An important advantage of MRI is the multiplanar capability. The imaged volume should extend to the levators, include the whole presacral space and the entire perineum, which are common sites for extensions [16]. Preoperative MRI has shown to reveal additional and clinically relevant information, thereby reducing recurrence rates after fistula surgery [9]. MRI can be used to evaluate the activity of fistulas, which is a significant factor for determining the therapeutic strategy [17,18]. Although active fistulas appear hyperintense on T2-weighted images (T2WI), in some cases, hyperintensity of fistulas may be related to edema [19]. Increased enhancement on T1-weighted images (T1WI), after intravenous administration of gadolinium-based contrast material, is generally considered indicative of active inflammation [19,20]. Theoretically higher-field-strength MRI provides a better signal-to-noise ratio, which can be used to achieve increased temporal resolution, decreased imaging time, and increased spatial resolution. The increased spatial resolution has the potential to improve lesion visibility, although comparative studies with 1.5-T or 3.0-T have not been reported on [21].

Classification of perianal fistula

Two main classification systems are used in classifying and grading ano rectal fistula; the classification proposed by Parks et al. [22] in 1976, which was made for surgical use, and the St. James University Hospital classification [6], which was established based on MRI examination.

The aims of this study are to evaluate the role of MRI in the evaluation of perianal fistula and to show the value of using contrast enhanced MR study in the determination of the precise tract pathway, extensions and other associated pathologies.

Table 1. Parks classification of anorectal fistula [22]

Type of fistula	Description
Inter-sphincteric	Limited to intersphincteric plane, not piercing external sphincter or levator muscles
Trans-sphincteric	Passes through the external sphincter
Supra-sphincteric	Track courses upward within the intersphincteric plane to pass over Puborectalis muscles and descends through levator muscles to the ischioanal fossa
Extra-sphincteric	Course is totally outside the external sphincter

Table 2. St. James University Hospital MRI classification system of perianal fistula [6]

Grade	Description
1	Simple linear inter-sphincteric fistula
2	Intersphincteric fistula with intersphincteric abscess or secondary fistulous track
3	Tran-sphincteric fistula
4	Trans-sphincteric fistula with abscess or secondary track within the ischioanal or ischiorectal fossa
5	Supralevator and translevator disease

Methods

This cross sectional analytic study was conducted in the MRI Department of Al-Imamein Al-Kadhimein Medical City, Baghdad, Iraq during the period from November 2015 to December 2016. Forty-one patients with age range of 16-76 years with clinical suspicion of perianal fistula were referred from the surgical outpatient clinic. All of the patients presented with signs and symptoms of one or more of the following: perianal discharge, pain, perianal swelling and induration, they found to have one or more external openings during their clinical examination. Those patients were examined by MRI and 9 of them were excluded from this study because they showed no fistulous tracts (5 of them had pure peri-anal abscesses and 4 had peri-anal sinuses), the remaining 32 patients included in the study had perianal fistulas, they were 25 males and 7 females.

Exclusion criteria

No visible external orifice, patients with only perianal abscesses or sinuses, but had no evidence of fistula and patients with general contraindications for MRI (e.g. Patients with metallic shells or cardiac pacemakers and claustrophobic patients).

Imaging technique

All patients were evaluated by contrast enhanced pelvic MRI. The MRI examinations were performed with Achieva 3 Tesla MR Scanner Philips medical system, Netherland. Examination was performed using body surface coil, each patient was placed in a supine position. Distal rectum, anal canal, the internal and external sphincters, ischioanal fossa, levator muscle, supralevator space and the subcutaneous tissue in the perineal region

were all included in the imaging volume. The following sequences were done for all patients:

1. T1 weighted fat suppression images in oblique axial and coronal planes with the following parameters: Repetition time (TR)= 400-600 mSec, Echo time (TE)= 5-10 mSec, slice thickness (4-5 mm) gap (0.5-1 mm), field of view (370-430 mm) and a flip angle of 90 degrees.
2. T2 weighted images with and without fat suppression in oblique axial and coronal planes with the following parameters: TR= 4000-5000 mSec, TE 100-130 mSec, slice thickness (4-5 mm) gap (0.5 -1 mm), field of view (370-430 mm) and a flip angle of 90 degrees.
3. Repeated T1 weighted fat suppression images in oblique axial and coronal planes after intravenous manual injection of 0.1 mmol/kg body weight of gadolinium-based contrast agent.

Since the anal canal is tilted 45 degrees anteriorly in the sagittal plane so it was necessary to obtain oblique axial and coronal images that are oriented perpendiculars and parallel to the anal canal respectively. Therefore, we used a sagittal T2 single shot image with centerline along anal canal serving as a localizer for the subsequent sequences (figure 1 a and b).

Image analysis

The following parameters were evaluated with the mentioned MRI sequences: the type of the fistula, location of internal opening, associated abscesses and/or sinus tracts, horseshoe extension as well as any associated inflammatory changes. The type of the fistulous tract was analyzed and graded according to the St. James University Hospital MRI classification system [6]. The transphincteric fistula was further sub classified into low or high levels according to the degree of involvement of the external sphincter (this is important because of different management between low and high levels fistula from the surgical point of view). In low type fistula, the lower third of the anal sphincter is involved while the involvement of the upper two thirds indicate a high fistulous type. The location of the internal opening was identified in the axial images using the anal clock as shown in figure 1c.

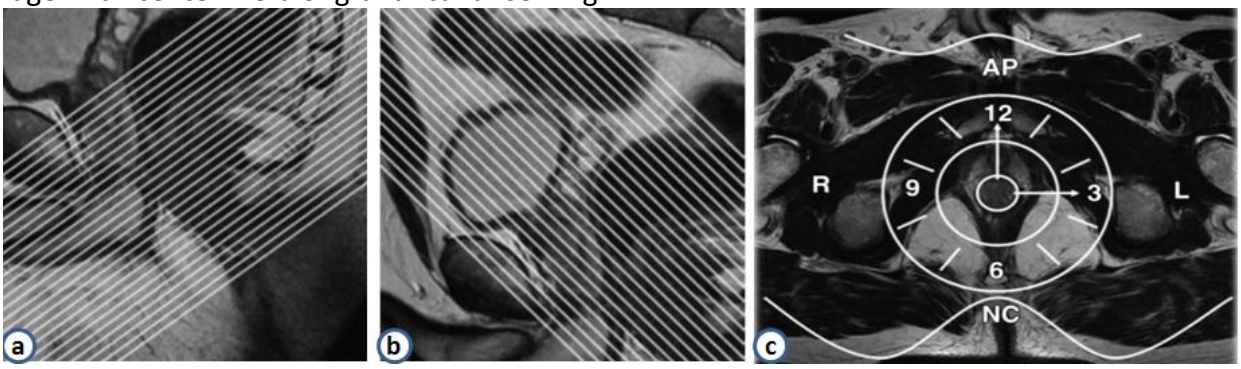

Figure 1. Single shot T2 weighted pelvic MRI sagittal section display the planning for acquisition of oblique axial (a) and oblique coronal (b) scans at an angle of 45o to the midline such that the images are orthogonal and parallel to the anal canal. (c) Axial T2-weighted MRI of the perineum showing the anal clock. NC = natal cleft, AP = anterior perineum, R = right aspect of the anal canal and L = left aspect of the anal canal [5]

The fistulous track was differentiated from abscess by using criteria of Laniado et al. [23], in which fistulas were defined as being fluid filled tubular structures with a diameter smaller than 10 mm and abscesses were larger than 10 mm. Air pockets within the fluid collection favor abscesses. Horseshoe fistula was considered if it crosses the midline to the contralateral side. Surgical confirmation of the diagnosis was obtained for all the patients except two where

the diagnosis was based on MRI results. Those 2 patients had extra sphincteric fistulas (they were known case of Crohn's disease). Surgery was not done for these 2 cases due to high risk of complications, therefore MR imaging characteristics and fixed anatomical landmarks were used in identifying and classifying these fistulas.

Statistical analysis

The collected data were tabulated and analyzed using Microsoft Excel 2010. The categorical data were presented as frequency and percentage tables. P-value was used to attain the significance of each MRI sequence in identifying perianal fistula and its associated findings in correlation to surgical results (p-value less than 0.05 was considered a statistically significant correlation). Regression data analysis was used to calculate p-value for each MRI sequence. The overall accuracy rate was also calculated for each MRI sequence.

Results

Thirty-two patients had perianal fistulas confirmed by their MRI examinations and subsequent surgical results were included in this study, they were 25 males (78%) and 7 females (22%) with male: female ratio of 3.5:1. The most common type of fistula encountered was the inter-sphincteric type, it was seen in 21 patients (66%) (16 fistulas (50%) were grade I and 5 fistulas (16%) were grade II). Trans-sphincteric fistulas were seen in 9 patients (28%) (2 of them (6%) were grade III and 7 fistulas (22%) were grade IV, of all the trans-sphincteric type fistulas, 3 were low in type and 6 of them were high type fistulas). Two patients (6%) had extra-sphincteric type (grade V according to MRI grading system). No supra-sphincteric fistulas were encountered in this study. These findings were shown in fig.2 A and B.

Associated abscesses were found in 10 cases and were all equally detected on T2 fat suppression and post contrast fat suppressed sequences with detection rates of 100 %, 8 of these 10 cases were seen on T2 TSE (80%) and was detected on T1 TSE in 7 cases (70%) only. Associated secondary tracts were seen in 11 cases, best detected by post contrast fat suppressed sequence (100%). Approximately half of them were detected on T2 fat suppression (54%), whereas only 27% and 18% was their detection rate on T2 TSE and T1 TSE.

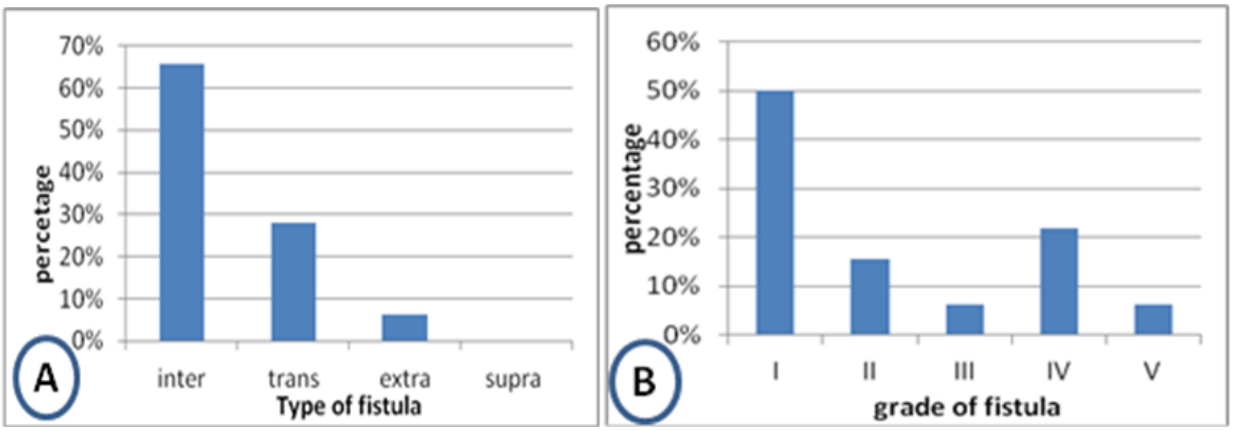

Figure 2. A: frequency and percentage of different types of perianal fistulas according to Parks classification. B: Frequency and Percentage of different grades of perianal fistula according to MRI grading system

The internal opening was seen in 29 patients and missed in 1 case that had been proven to be present according to surgical results later on. The two cases of the extrasphincteric type already do not have an internal opening as it bears no relationship to either of the

sphincters or the anal canal. The internal openings were clearly depicted by post contrast fat suppression sequence (96.6%), on T2 fat suppression sequence (90%), less accurately seen by T2 TSE (56%), and detected on T1 TSE in only (16.6%). Most common locations were found to be at the 6 O'clock (in 48.2% of cases) followed by 5 o'clock in 13.7%, as shown in table (3).

Table 3. Distribution of locations of internal opening on MRI axial images in patients with perianal fistula (No.=29)

Location	No. of cases	Percentage
6 o'clock	14	48.2
5 o'clock	4	13.7
12 o'clock	3	10.3
1 o'clock	3	10.3
2 o'clock	2	6.8
7 o'clock	2	6.8
8 o'clock	1	3.4
Total	29	100

Horseshoe fistulous extension were seen in 3 cases and were all detected on post contrast fat suppression and T2 fat suppression (100 %), 2 of them were seen in T2 TSE (66.6%) and only one-horse shoe extension was seen in T1 TSE (33.3%).

Regression analysis was used to evaluate the significance of each MR sequence and p-value was obtained for each one of them. T1 TSE showed no significant correlation with the surgical results in depicting pathologies with a p-value of 0.169 and accuracy rate of 36%; however, its role was important in demonstrating anatomy of the perianal region. T2 TSE showed a significant correlation with a p-value of 0.007, but it showed a diagnostic accuracy of 57%. A more significant correlation was obtained by using T2 fat suppression axial and coronal sequences with a p-value of 0.001 and it showed accuracy rate of 87%. The contrast enhanced axial and coronal fat suppressed T1 showed the strongest significance with the surgical results and revealed a p-value of 0.00001 and it had the highest accuracy rate of 98.8%, as shown in table (4). Fig. 3, 4, 5 and 6 show some selected images from this study.

Table 4. Significance and accuracy rates of different MRI sequences in the depiction of perianal fistula and its associated findings in correlation with surgical results (No.=32)

Parameters	No.	T1 TSE	T2 TSE	T2 fat sat	T1 PC fat sat
Primary tract	32	16 (50%)	19 (59%)	29 (90.6%)	32 (100%)
Associated abscesses	10	7 (70%)	8 (80%)	10 (100%)	10 (100%)
Secondary tract	11	2 (18%)	3 (27%)	6 (54.5%)	11(100%)
Internal opening	30	5 (16.6%)	17 (56%)	27 (90%)	29 (96.6%)
Horseshoe extension	3	1 (33.3%)	2 (66.6%)	3 (100%)	3 (100%)
P value		0.169	0.007	0.001	0.00001
Accuracy rate		36%	57%	87%	98.8%

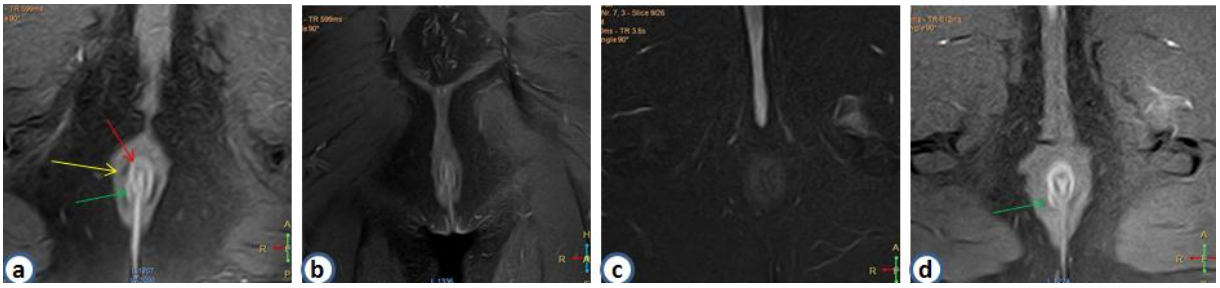

Figure 3. Axial (a) and coronal (b) fat suppressed PC T1 of 43 years old male presented with Rt. sided perianal discharge shows a grade I simple intersphincteric fistula (green arrow) with internal opening at 9 o'clock, the external (yellow arrow) and the internal (red arrow) anal sphincters. C and d are images of another 44 years old male patient presented with Rt. sided perianal discharge. A grade I intersphincteric fistula was not clearly depicted on the axial T2 fat suppression (c), but certainly seen on axial fat suppression PC T1 (d) (green arrow)

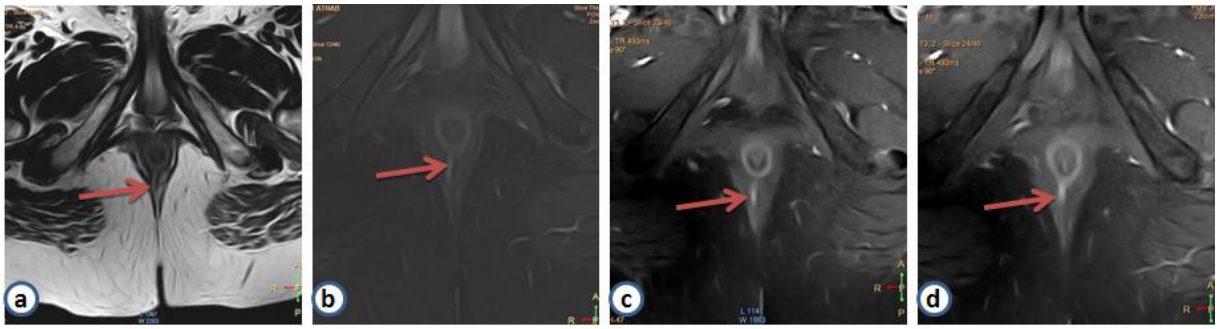

Figure 4. 38 years old male patient presented with signs and symptoms of perianal fistula. A grade III trans-sphincteric fistula (a) T2 axial, (b) T2 fat suppression axial, (c) and (d) T1 post contrast with fat suppression of the same patient showing a simple trans-sphincteric fistula (arrow) with internal opening at 6 o'clock

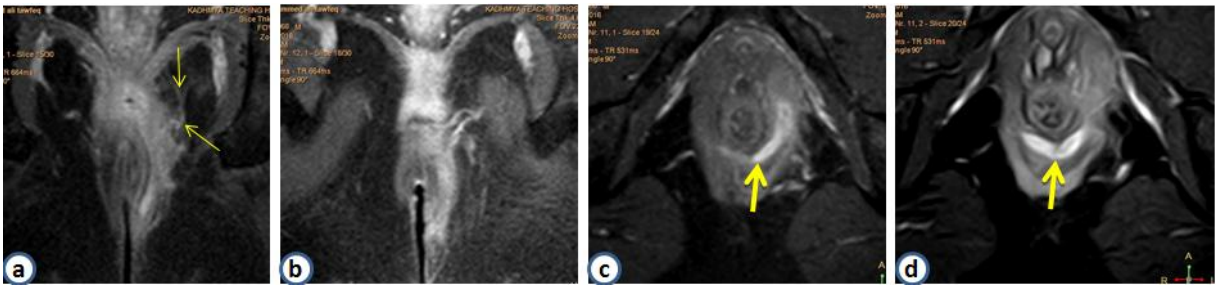

Figure 5. A 48 years old poorly controlled diabetic patient presented with left paramedian perianal discharge. (a-d) are coronal and axial contrast enhanced fat suppressed T1 showing a left sided high type tras-sphencteric fistula that crosses the midline (horseshoe extension "thick arrows") and showing two side branches (thin arrow) … This was a grade IV fistula

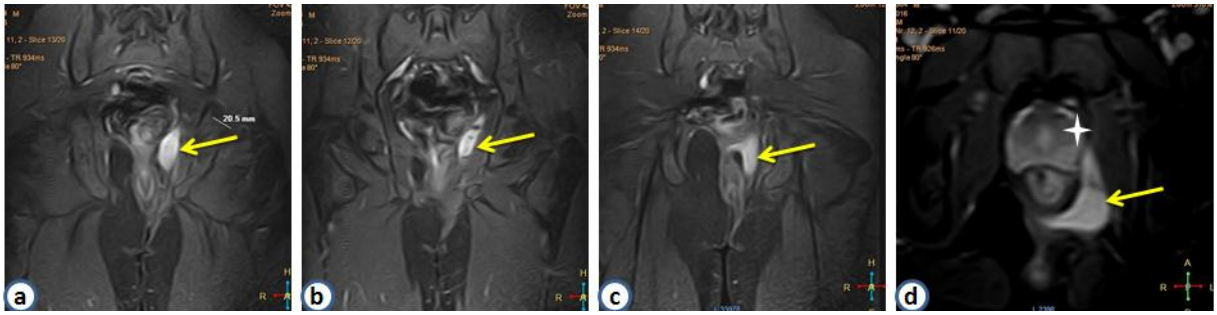

Figure 6. A 32 years patient who is a known case of crohn's disease showing a series of consecutive slices of coronal fat suppressed contrast enhanced T1 (a-c) and axial T1 enhanced fat suppressed sequence(d). There is a wide extrasphincteric fistulous tract (thick arrow) extending from the pelvis crossing the left levator anus muscle (star) and occupying the upper aspect of the ischioanal fossa, it also sends a side branch across the midline (horseshoe extension) and continues as abscess inferiorly (d). This was grade V fistula. There is enhancement of the wall of the lower rectum denoting associated proctitis

Discussion

The perianal fistulas have been described for centuries and were known to Hippocrates. This condition received more attention when Frederick Salmon started the St. Mark's Hospital in London in 1835, which was dedicated exclusively to patients suffering from perianal fistulas and other rectal diseases [13]. Halligan et al. [19] stated that the perianal fistula predominantly affects young adults, and males are more commonly involved than females. This was in agreement with the results of this study, which showed the mean age of the affected patients was 35 years with a male: female ratio of 3.5:1. Our results were also similar to those reported by Sainio et al. [1] and Lunniss et al. [24].

According to Parks classification [22], we found that the intersphincteric fistula was the most common type encountered, it was seen in 21 patients out of a total of 32 (66%) and the trans-sphincteric fistula was the second most common type seen in 9 patients (28%), these results were approximately similar to that of de Miguel Criado et al. [5], their study were done 44 patients with fistulas, 60% were intersphincteric and 33% were trans sphincteric in types. Moreover, we further characterized the trans sphincteric fistulas into high and low types according to the degree of involvement of external sphincter [25], this is important because of different management between high and low levels fistula, thus affecting patient's outcomes.

In this study, the grades of fistula were identified by using the St James's University Hospital classification. Grade I was the commonest category seen (50%), this was higher than that of Mohamed et al. [26], which was 37.5% in their study, but was similar to Khan et al. [27] who found that grade I fistulas were also seen in 50%. In contrast to Adurthy et al. [28] who found that type III was the most common and encountered in 46% in their study that included 26 patients and carried out in India, this difference may be attributed to the increased number of diabetic patients that led to more aggressive disease progression and later presentation.

The exact location of the primary tract whether in the ischioanal fossa or intersphincteric plane was most easily seen on axial images. The location of the internal opening according to the anal clock was also seen in this plane. We can differentiate trans-sphincteric from intersphincteric fistula by the presence or absence of external anal sphincter disruption. Coronal plane on the other hand is better in depiction of levator plane, thus allowing confident differentiation of supralevator from infralevator extension of the fistulas. Therefore, both axial and coronal sequences are required to provide all the important

details needed. These were also in agreement with the findings of Stoker et al. [29].

Associated perianal abscesses were found in 10 cases and were all equally detected on T2 fat suppression and post contrast fat suppressed sequences with detection rates of 100%. This finding was in agreement to what had been obtained by Mohamed et al. [26] with detection rates of 100% in these sequences. Regarding the associated peri-anal secondary tracts, they were seen in 11 cases, best detected by post contrast fat suppressed sequence (100%). This was comparable to the findings of Mohamed et al. [26] and Khan et al. [27] in which their detection rates were 100% and 94.4% respectively. It has been shown that MRI is more successful at showing secondary tracks than both digital examination and surgery [29].

Concerning the internal opening detection, out of 30 patients studied, the internal opening was seen in 29 patients and missed in 1 patient, which was evident during subsequent surgery, this may be attributed to the various anatomical or functional conditions such as small or narrow opening or it may be intermittently closed at the time of MRI examination. Similar findings were seen in a previous study done by Yildirim et al. [30], three internal openings were missed in their study which was performed on 26 patients.

The detection rate of the internal opening in this study was 96% on fat suppressed post contrast T1 and 90% on T2 fat suppressed sequence, These findings were comparable to that of Singh et al. [31], 46 internal openings were accurately detected out of 48 Cases (detection rate of 96% on fat suppressed enhanced T1), our results were also close to that of Mohamed et al. [26] where the detection rates in were 100% and 91% on T1 enhanced fat suppression and T2 fat suppression sequences respectively, however our detection rate and was slightly lower than the results of Khan et al. [27] where the detection rates was 100% on T1 post contrast sequences. We found that the most common location of the internal opening was in the region of 6 O'clock (seen in 48%), this was slightly lower than that seen by Mohamed et al. [26] (50%) and Yildirim et al. [30] (57%).

Horseshoe fistulous extension were seen in 3 cases and were all detected on post contrast fat suppressed T1 and T2 fat suppression sequences with a detection rate of 100% in each sequence, this was identical to the results obtained by Yildirim et al. [30]. (Detection rates of 100% in each sequence) and Mohamed et al. [26] (with detection rates of 95% in T2 fat suppression and 100% in fat suppressed enhanced T1).

We had seen that T2 fat suppression and T1 fat suppressed enhanced image had exactly the same detection rate (100%) regarding recognition of peri-anal abscesses and track horseshoe extensions, these results were similar to that of Singh et al. [31] were the detection rate was 100% for both parameters. Our results were also comparable to the results of Mohamed et al. [26] where both sequences were equally (100%) detect peri-anal abscesses, but there was slight increase in the detection of horseshoe extension seen with enhanced fat suppressed T1 compared with the T2 fat suppression sequence. However, we had concluded that T1 fat suppressed enhanced sequences had higher detection rate than T2 fat suppressed images regarding primary tracts, secondary extensions and internal openings, but still both sequences gave significant correlation with surgical results with p-values of less than 0.05. However, the accuracy rate of the T2 fat suppressed sequence was 87% in contrast to the 98% achieved by the post contrast sequence. The post contrast study was even more significant than fat suppressed T2 (p-values of 0.00001 and 0.001 respectively). These were in agreement with the study of Mohamed et al. [26] who attained a significant correlation for these two sequences (with a p values of less than 0.05) and even higher significance in using contrast MRI (with a p-value of less than 0.001). Their results showed a diagnostic accuracy of 99.4% for post contrast fat suppressed T1 TSE. Our results were also comparable to the results of Mazroa et al. [32] and Daabis et al. [33], they found the diagnostic accuracy of post contrast sequence in the detection of perianal fistula to be 97.3% and 95% respectively.

Finally, this study had shown that the T2 fat suppression had no added diagnostic information to that shown by the contrast enhanced MR imaging. This was in agreement with the results of Spencer et al. [20], however radiologists who are not routinely deal with MR interpretations of peri-anal fistulas, may benefit from both sequences in interpreting their data.

This study concluded that MRI is an essential, noninvasive tool in the preoperative assessment of perianal fistulous tracks and clarifying the presence or absence of other associated complications. Using St James's University Hospital MRI-based grading system provides more detailed information regarding the primary track as well as its secondary extensions and associated abscesses. This will help in deciding the suitable way of management and hence decreasing the incidence of recurrence and improving the patients' outcome. Axial and coronal contrast enhanced fat suppressed T1 showed highly significant and accurate correlation with surgical findings and was superior to other MRI sequences in showing hidden tracks and internal orifice locations.

Acknowledgments

Deep thanks to the great operators at the MRI department of Al-Imamein Al-kadhimein Medical City for their collaboration and technical help provision. Great thanks to all patients who agreed to participate in this study.

Authors contribution

Dr. Kadhim: conception and design, interpretation of results. Dr. Jawad: collection of data, assembly and interpretation of results, manuscript writing. Dr. Al-Jobouri: participation in data collection and statistical analysis. Hussain: Arranging the protocols for the MRI examinations. All authors have approved the final article.

Conflict of interest

Authors declare no conflict of interests.

Funding

No external funding sources.

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Received Jul. 5th 2017
Accepted Dec. 28th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

Evaluation of Phospho-AKt Immunohistochemical Expression in Patients with Laryngeal Squamous Cell Carcinoma

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Abstract

Background Akt, is a serine/threonine protein kinase which act as an important regulator of cell proliferation and survival. The Akt complex is upregulated by phosphorylation producing phospho-Akt, which trigger a continued cell proliferation and survival and inhibit apoptosis, thereby promote cell survival.

Objective To evaluate the immunohistochemical expression of phosphorylated Akt (Phospho-Akt) in laryngeal squamous cell carcinoma (SCC) and to be correlated with different clinicopathological parameters.

Methods Phospho-Akt expression was investigated Immunohistochemically in 49 formalin-fixed paraffin embedded laryngeal SCC tissue sections collected from Teaching laboratories - Baghdad Medical City.

Results Phospho-Akt positive immunostaining appears in 57% of samples. Akt activation present in advanced stages of tumors with p value 0.02.

Conclusion The current findings may provide evidence that aberrant expression of Akt contributes to the pathogenesis (mechanism of disease development) of laryngeal SCC.

Keywords Akt, phospho-Akt, immunohistochemistry, laryngeal SCC, larynx

Citation Wanas NS, Mehdi LY, Alzubaidi LKA. Evaluation of phospho-AKt immunohistochemical expression in patients with laryngeal squamous cell carcinoma. Iraqi JMS. 2018; 16(2): 177-181. doi: 10.22578/IJMS.16.2.9

List of abbreviations: DAB = diaminobenzidine, MSCC = Moderately differentiated squamous cell carcinoma, PBS = Phosphate buffered saline, PSSC = Poorly differentiated squamous cell carcinoma, PTEN = Phosphatase and tensin homolog, SCC = Squamous cell carcinoma, WSCC = Well differentiated squamous cell carcinoma

Introduction

Globally, Cancer of larynx is the second most common respiratory cancer after lung cancer [(1)]. In Iraq, laryngeal cancer constitutes 24.8% of head and neck cancer and 2.47% of all cancers [(2)].

Mortality rate of laryngeal cancer is about two times higher in developing than developed countries [(2)]. This increase is likely attributed to increased exposure to risk factors like smoking, drinking, population aging and increased exposure to industrial carcinogens. In Iraq, laryngeal cancer peak at age 80 years with 21.7 deaths per 100,000 men in 2010. It causes death at the lowest scale at age 30-34 years [(3)]. The highest rate of death for women was less than that of men, which were 2.8/100,000 women [(3)].

Akt or Protein Kinase B (PKB) is a serine/threonine protein kinase that functions as an important regulator of cell proliferation and survival [(4)]. It is comprised in cellular survival pathways, by suppressing apoptotic processes [(5,6).] The PI3K/Akt kinase pathway is a

central regulator of cell metabolism, proliferation, and survival and is dysregulated by oncogenic events in a substantial fraction of tumors. Constitutive activation of growth factor receptors, mutation of PI3K, and inactivation of the phosphatase and tensin homolog (PTEN) cause the activation of PI3K signaling in many tumors [7]. Since PI3K has a multitude of downstream targets, including Akt, mutations of which are oncogenic and occasionally present in human tumors. In seriously proliferating tumor cell, the Akt complex is turned on by phosphorylation, which will trigger a continued cell proliferation and cell survival and inhibit apoptosis [6]. Since Akt phosphorylation can hinder apoptosis, and by that promote cell survival, it has been involved as a major factor in many types of cancer [8-11].

These changes lead to constitutionally active survival signaling and that increase the insensitivity of tumor cells to apoptosis induction [6]. Therefore, the present study is aimed to figure out the role of Akt activation in laryngeal squamous cell carcinoma (SCC) pathogenesis.

Methods

Tissue samples

Forty-nine tumor specimens of laryngeal SCC were obtained from archived formalin fixed paraffin-embedded tissue samples of surgically resected tumors in National Center for Educational Laboratories in City of Medicine (Baghdad-Iraq). Ethical agreements were obtained from Baghdad directorate of health. Tumors were classified into three grades: well differentiated squamous cell carcinoma (WSCC), moderately differentiated squamous cell carcinoma (MSCC), and poorly differentiated squamous cell carcinoma (PSCC). Clinicopathological parameters of the larynx cancer patients were shown as age, gender, tumor grade and stage.

Immunohistochemical analysis

Immunohistochemical staining for Phospho-Akt in paraffin embedded tissue sections was performed using indirect biotin-avidin system. Slides deparaffinization processed in three changes of xylene for five minutes each. Slides were transferred to ascending grades of ethyl alcohol, followed by washing with distilled water. To unmask the antigenic epitope, antigen retrieval was performed by placing the slides in glass coplin jar filled with sodium citrate buffer (pH 6.0) in a microwave oven at 90 °C for 20 minutes. Slides were allowed to cool at room temperature. 0.3% of hydrogen peroxide were added then incubated for 30 minutes to block the activity of endogenous peroxidase. Slides were next Incubated with rabbit polyclonal phospho-Akt (Ser 473) antibody (Thermo Scientific, USA) in a humidified chamber over night at 4 °C. The slides were rinsed three times with PBS then incubated with a biotinylated secondary antibody for 30 minutes. The reaction product was developed using diaminobenzidine (DAB) as chromogen and observed under the microscope for development of brown color. The color reaction was stopped by dipping the slides in distilled water. Sections were then counterstained with Harris-haematoxylin. Slides were dehydrated through three changes of 99% alcohol for 5 minutes each and mounted using DPX slide mounting medium. Adjacent normal appearing epithelium within the tissue sections served as a positive internal control. Representative areas of each tissue sections were selected and were counted in 5 fields at x400 magnification in each section. Sections were considered immunopositive when more than 10% of the tumor cells had clear evidence of immunostaining [12]. For negative controls, similar procedure was followed without primary antibody.

Statistical analysis

The chi-square test was used to determine the correlation of Akt phosphorylation with different clinicopathologic parameters [13].

Results

The clinical and pathological parameters of 49 cases of laryngeal carcinoma are shown in table 1. Out of these, 14 (28.5%) were well

differentiated SCC, 20 (41%) were moderately differentiated SCC and 15 (30.5%) were poorly differentiated SCC. Stages of tumor were distributed as following: 31 (64%) with stages I&II, and 18 (36%) with stages III&IV. Out of 49 cases, 39 (80%) cases were fifty years old and above and 10 cases (20%) were less than fifty years old.

Phospho-Akt expression

The relationship between phosph-Akt expression and clinicopathological characteristics of tumor samples is shown in table (1). Phospho-Akt immunostaining was positive in 28 (57%) out of 49 samples. Among fifty years old and above patients, 23 (59%) cases were phospho-Akt positive out of 39 cases. In relation with patients' gender, out of 45 males, 26 (92%) show phospho-Akt positive staining and 19 males (90%) were negative (Table 1). However, no significant correlation was observed between both age, gender and phospho-Akt overexpression. Immunostaining for phospho-Akt was both nuclear and cytoplasmic staining (Figure 1A&B). Over-expression of phospho-Akt was 9 (32%), 11 (39%) and 8 (29%) out of 14 cases well differentiate SCC, 20 cases moderately differentiated SCC and 15 cases poorly differentiated SCC respectively. However, the observed differences failed to achieve the level of statistical significance. Our data showed that phosph-Akt overexpression was observed in 14 (50%) cases in advanced stages of tumor (stage III &IV). In earlier stages (stage I&II) of laryngeal carcinoma, phospho-Akt was not rare since it occurred in 14 (50%) of cases. Our marker showed a statistically significant correlation with tumor stage with p value 0.02 (table 1).

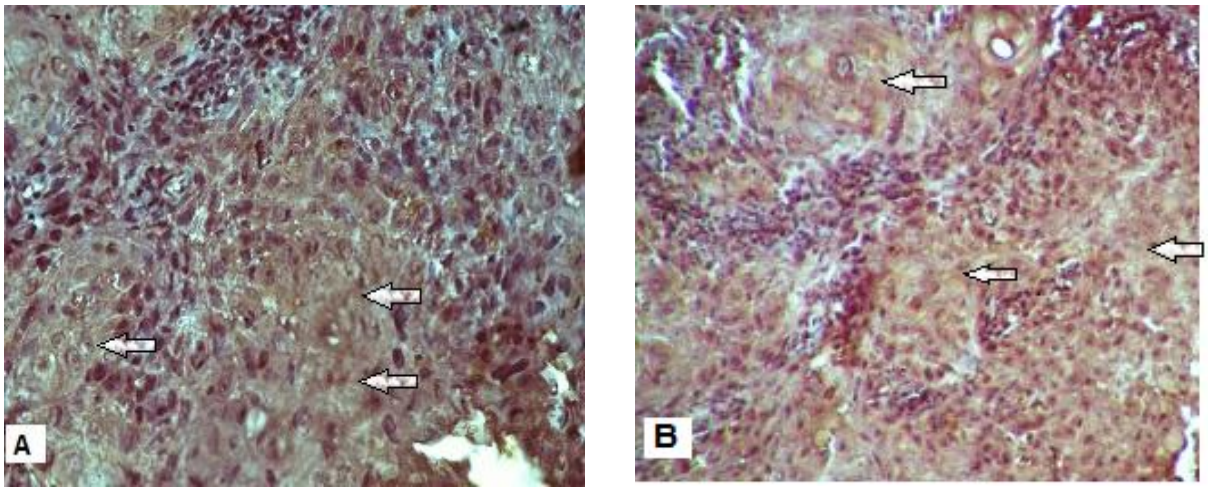

Figure 1. Representative photographs of Immunohistochemical staining for phospho-Akt expression. A&B: laryngeal Tumor showing strong positive immunostaining (both cytoplasmic and nuclear) for phospho-Akt at Ser 473 in well differentiated and poorly differentiated squamous cell carcinoma respectively (400x). Counter stain: hematoxylin

Discussion

Many approaches had been applied to avoid laryngectomy for patients with high stages of laryngeal SCC using chemotherapy and radiotherapy. However, these treatments are highly toxic, therefore, tumor understanding on molecular basis is essential in the treatment of laryngeal carcinoma. The present study was to determine the possible relevance of phospho-Akt overexpression in clinicopathological features of laryngeal SCC and its role in tumor development in order to provide basis for cancer understanding and treatment.

Table 1. Phospho-Akt expression and its association with clinicopathological parameters in SCC of the larynx

Clinicopathological parameters		Total n=49	Phosho-Akt positive n =28 (57%)	Phospho-Akt negative n =21 (43%)	P value
Age (yr)	≥ 50 < 50	39 (80.0%) 10 (20.0%)	23 (82.0%) 5 (18.0%)	16 (76.0%) 5 (24.0%)	0.61 (NS)
Gender	Male Female	45 (92.0%) 4 (8.0%)	26 (92.0%) 2 (8.0%)	19 (90.0%) 2 (10.0%)	0.76 (NS)
Tumor grade	W M P	14 (28.5%) 20 (41.0%) 15 (30.5%)	9 (32.0%) 11 (39.0%) 8 (29.0%)	5 (24.0%) 9 (43.0%) 7 (33.0%)	0.81 (NS)
Stage	I & II III&IV	31 (64.0%) 18 (36.0%)	14 (50.0%) 14 (50.0%)	17 (81.0%) 4 (19.0%)	0.02 (S)

Data are number (percentage) of patients; W well differentiated, M moderately differentiated, P poorly differentiated; NS: non significant; S: significant (p value ≤ 0.05)

In this study, it was found that phospho-Akt overexpression was not uncommon event since it occurred in 57% of examined cases. A significant correlation was remarked with the tumor stage (p value 0.02) as shown in table (1).

The act of Akt activation in cancer progression has been assessed in many kinds of tumors like ovarian cancer [8], head and neck cancer [9], tongue cancer [10], and prostate cancer [11]. Many studies of Akt expression in prostate cancer tissues revealed that tumor progression in western populations was significantly correlated with Akt upregulation [15,16]. Le Page et al. explore the localization and expression of the Akt three isomers, proposing a distinct act of Akt-1 expression as a prognostic marker in prostate cancer [17]. In laryngeal cancer, fewer studies were done on Akt marker. Yu et al. studied the possible prognostic significance of Akt in oropharyngeal SCC patients from the United States and found that Akt activation was associated with adverse patients' outcome indicating that Akt is a promising molecular target [18]. Another study from Netherland used antibodies against phospho-Akt revealed low levels of Akt phosphorylation in laryngeal cancer and it was significantly correlated with lymph node metastases [19], a result that doesn't match our present study. Assessment on the role of Akt overexpression was based particularly on data from developed countries. A study from Iraq on Akt expression in oral cancer revealed positive expression of Akt in 38 out of 40 cases and a statistical significant correlation was found with tumor stage [20]. The present study showed Phospho-Akt expression was both cytoplasmic and nuclear (figure 1). Although no significant statistical correlation was found between age of patients and Akt phosphorylation, phospho-Akt positive immune staining was found in 23 (82%) out of 39 patients who were fifty years old and above. For our knowledge this is the first study from Iraq to evaluate the role of phospho-Akt in laryngeal cancer. According to our scoring parameters, upregulation of Akt was noted in 14 (78%) out of 18 cases with advanced tumor stages (stage III&IV). Suggesting that tumor with phospho-Akt overexpression may be more threatening and aberrant expression of Akt may contribute to the pathogenesis of laryngeal SCC.

Acknowledgments

The authors would like to thank Mr. Hazim Alkhafagi, Chairman of Laboratories Section in Baghdad Medical City for all facilities he provided during samples collection, also, to thank Dr. A.K. Mandal from Maulana Azad Medical College India – New Delhi who

provided expertise and review that assisted the interpretations of this research.

Authors contribution

Dr. Wanas: processed the laboratory research work and interpretation of results. Dr. Mehdi: sample collection and article preparation; Alzubaidi: collected data from available registered records.

Conflict of interest

The authors declare no conflict of interest.

Funding

The research funding was carried out by the authors.

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Received Aug. 3rd 2017
Accepted Mar. 14th 2018

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

The Effect of The Enzyme Replacement Therapy on The Kidney Function Tests and Serum Electrolyte Levels in Children with Gaucher Disease

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Abstract

Background Gaucher disease (GD) is an inherited autosomal recessive disease. It is most common in the Ashkenazi Jewish population. Many biomarkers might be involved in the etiology, pathogenesis, diagnosis and prognosis of GD in children. Most of them are related to complications due to an involvement of many organs such as liver, spleen and bones by this lysosomal storage disease that caused by a lack of the enzyme glucocerebrosidase.

Objective To investigate the role of kidney function test and electrolytes (urea, creatinine, sodium and potassium) level in the monitoring of the response for the treatment used for patients with GD in follow-up manner.

Methods A case control study was done on 67 children (32 males & 35 females), age range from 2-14 years (mean±SD; 5.3±2.9). The levels of sodium, potassium, urea and creatinine were measured in the samples of patients who were categorized as newly diagnosed untreated patients (n=9), patients receiving enzyme replacement therapy (ERT) for 3-6 months (n=18), 6-12 months (n=20) and patients receiving ERT for more than one year (n=20) and compared with twenty age-matched control subjects (9 males & 11 females) age range from 2-14 years (mean±SD; 5.55±3.05).

Results The data indicated that the level of urea in GD patients (23.39±4.71 mg/dl) was significantly higher than that of age-matched controls (17.5±3.05 mg/dl). Non-significant differences were illustrated in the levels of sodium, potassium and creatinine. Negative significant ($p<0.05$) correlations were obtained between the levels of urea ($r= -0.752$; $p<0.001$) and creatinine ($r= -0.536$; $p<0.001$) with the period of receiving ERT. Additionally, ANOVA test also revealed significant ($p<0.05$) differences among the patients' subgroups in the levels of urea and creatinine. Results obtained from Receiver Operating Characteristic (ROC) curve revealed that urea and creatinine showed a high area under the curve (AUC), sensitivity and specificity (0.939, 77.8% and 85% for urea and 0.978, 100% and 80% for creatinine respectively) in newly diagnosed GD patients in a comparison with control.

Conclusion The possibility of using urea and creatinine in the diagnosis and monitoring the effect of ERT on the GD patients.

Keywords Gaucher disease, macrophage, renal function test, urea, creatinine, sodium, potassium, enzyme replacement therapy, imiglucerase, β-glucocerebrosidase, glucocerebroside

Citation Abdulhussein HA, Al-Obaidi FH, Arif HS. The effect of the enzyme replacement therapy on the kidney function tests and serum electrolyte levels in children with Gaucher disease. Iraqi JMS. 2018; 16(2): 182-190. doi: 10.22578/IJMS.16.2.10

List of abbreviations: ERT= enzyme replacement therapy, GD=Gaucher disease, ROC= Receiver Operating Characteristic, AUC= area under the curve, TB= tuberculosis, ANOVA= Analysis of variance.

Introduction

Gaucher Disease (GD) is considered as the most common autosomal recessive lysosomal storage disease that is caused by a deficiency in the enzyme β-glucocerebrosidase leading to an accumulation in its substrate that called glucocerebroside, which is derived chiefly from membrane components of hematopoietic cells [1,2]. The accumulation of this substrate in lysosomes of macrophages cause a formation of pathological macrophages that called Gaucher cells, which

considered as the main histological hallmark of the disease and are located in many organs [3,4]. The clinical manifestations and symptoms of this disease also ranged from asymptomatic individuals to massive hepatomegaly and splenomegaly in addition to hypersplenism and severe bone, and, occasionally, lung complications [5]. Neuronopathic involvements mainly occur in Gaucher disease types II and III leading to a wide variety of neurological manifestations in addition to visceral involvement [5,6].

The treatment of choice for symptomatic Gaucher patients is enzyme replacement therapy (ERT) with imiglucerase (Cerezyme®) [7]. ERT showed to have a valuable effect on the GD symptoms and significantly cause a reduction in the organomegaly, in addition to an elevation in the levels of hemoglobin and platelet counts, and also improve children growth in with Gaucher patients [8]. On the other hand, the effect of ERT on bone or lung complications still less clear than other manifestations [9].

This study tried to focus on the renal complications in GD patients since that there are a few case reports in the literature on the renal complications in association with GD. When renal abnormalities coexist with GD, a large variation in the clinical and laboratory manifestations were noticed including proteinuria, tubular defects or renal failure [3]. Additionally, autopsy of asymptomatic patients with GD showed Gaucher cells in the kidneys. It is still unclear whether the abnormalities in renal functions in these patients arise from their storage disease or whether during the course of a lifetime with GD, some patients seem to develop only one kind of kidney manifestation or another [7,10].

To realize the effects of GD and ERT on the renal function, we undertook a case control study on patients with GD. The aim was to evaluate the effect of the disease and the treatment on some renal function parameters noninvasively.

Methods

A case control study was done on 67 children (32 males and 35 females), age range from 2-14 years (mean± SD; 5.3±2.9) who had GD recruited from Pediatric Department and Unit of Rare Diseases at Al-Imamein Al-Kadhimein Medical City, Gastroenterology and Hepatology Teaching Hospital, Children Welfare Hospital Consultation Clinic and Central Child's Teaching Hospital.

The levels of sodium, potassium, urea and creatinine were measured in the samples of 67 Gaucher patients who were categorized as newly diagnosed untreated patients (n=9), patients receiving ERT for 3-6 months (n=18), 6-12 months (n=20) and patients receiving ERT for more than one year (n=20) and compared with twenty age-matched control subjects (9 males and 11 females); age range from 2-14 years (mean± SD; 5.55± 3.05). A Control group with exclusion criteria that include patients with chronic infections, those suspected to have tuberculosis (TB) and patients with chronic inflammatory conditions as chronic arthritis in addition to other factors that affect enzyme activity.

The local Ethical Committee of the College of Medicine, University of Al-Nahrain, Baghdad, Iraq, approved this study. In addition, an informed written consent of participation in the study was signed by the parents or the legal guardians of the investigated subjects according to the Helsinki principles.

Results

The data indicated that the mean±standard deviation (SD) levels of urea in whole Gaucher patients (23.39±4.71 mg/dl) was significantly higher ($p<0.05$) than that of age-matched controls (17.5±3.05 mg/dl). On the other hand, non-significant differences were illustrated in the levels of sodium, potassium and creatinine (table 1).

These parameters were remarkably associated with the period of receiving treatment with ERT that indicated by the decrease in the level of urea and creatinine with the increase in the duration of treatment as demonstrated by

table (4) and also this finding confirmed by the negative significant ($p<0.05$) correlations between the levels of urea ($r= -0.752$; $p<0.001$) and creatinine ($r= -0.536$; $p<0.001$) with the period of receiving treatment as demonstrated in table 2.

On the other hand a non-significant difference in the levels of sodium and potassium among all the studied groups were illustrated in table (3).

Table 1. The comparison of the studied parameters between Gaucher patients and control group by independent t-test

Parameter	Control N=20 mean±SD	Patients N=67 mean±SD	P value
Sodium (mEq/l)	137.95±1.23	137.74±1.67	0.615
Potassium (mEq/l)	4.13±0.2	4.12±0.35	0.982
Urea (mg/dl)	17.5±3.05	23.39±4.71	<0.0001
Creatinine (mg/dl)	0.27±0.09	0.3±0.08	0.163

Table 2. Correlation between the studied parameters in whole Gaucher patients

		Potassium	Urea	Creatinine	Duration of treatment
Sodium	r	0.057	-0.128	-0.339	0.189
	p	0.647	0.303	0.005	0.125
Potassium	r		0.007	-0.045	-0.091
	p		0.957	0.716	0.464
Urea	r			0.376	-0.734
	p			0.007	<0.001
Creatinine	r				-0.605
	p				<0.001

r: Pearson correlation coefficient, P: Significance

Additionally, ANOVA test also revealed the effect of ERT that is indicated by the significant ($p<0.05$) differences among the patients' subgroups in the levels of urea and creatinine (table 5). Results obtained from Receiver Operating Characteristic (ROC) curve revealed that urea and creatinine showed a high area under the curve (AUC), sensitivity and specificity (0.939, 77.8% and 85% for urea and 0.978, 100% and 80% for creatinine respectively) in newly diagnosed GD patients in a comparison with control and variable values of area under the curve (AUC), sensitivity and specificity in whole GD and other GD patient subgroups as demonstrated in tables (6-10).

Table 3. Sodium and Potassium levels in control, patients with 3-5 months, 6-12

Parameter	Group	mean±SD	P^a	P^b	P^c	P^d	P^e	P^f	P^g	P^h	P^i	P^j
Sodium (mEq /l)	Control n = 20	137.95 ±1.23										
	Newly diagnosed n = 9	137.0± 1.87										
	3-5 months treatment n = 18	137.61±1.46	0.115	0.443	0.826	0.920	0.36	0.13	0.24	0.39	0.6	0.79
	6-12 months treatment n = 20	138.05± 1.61										
	>1 year treatment n = 20	137.9±1.83										
Potassium (mEq /l)	Control n= 20	4.13 ±0.2										
	Newly diagnosed n = 9	4.26±0.48										
	3-5 months treatment n = 18	4.1±0.34	0.309	0.783	0.824	0.46	0.34	0.49	0.203	0.69	0.72	0.43
	6-12 months treatment n = 20	4.15±0.34										
	>1 year treatment n = 20	4.06±0.31										

Pa value between newly diagnosed patients and control
Pb value between patients with 3-5 months treatment and control
Pc value between patients with 6-12 months treatment patients and control
Pd value between patients with >1 year treatment and control.
Pe value between newly diagnosed patients and patients with 3-5 months treatment
Pf value between newly diagnosed patients and patients with 6-12 months treatment
Pg value between newly diagnosed patients and patients with >1 year treatment
Ph value between patients with 3-5 months and patients with 6-12 months treatment
Pi value between patients with 3-5 months and patients with >1 year treatment
Pj value between patients with 6-12 months and patients with >1 year treatment

Table 4. Urea and Creatinine levels in control, patients with 3-5 months, 6-12 months and >1 year treatment groups

Parameter	Group	mean±SD	P^a	P^b	P^c	P^d	P^e	P^f	P^g	P^h	P^i	P^j
Urea (mg/dl)	Control n = 20	17.5±3.05										
	Newly diagnosed n = 9	26.51±5.17										
	3-5 months treatment n = 18	27.06± 3.78	<0.001	<0.001	<0.001	0.3	0.76	0.04	<0.001	0.002	<0.001	<0.001
	6-12 months treatment n = 20	23.7±2.08										
	>1 year treatment n = 20	18.38±2.17										
Creatinine (mg/dl)	Control n= 20	0.27±0.086										
	Newly diagnosed n = 9	0.46±0.038										
	3-5 months treatment n = 18	0.30±0.029	<0.001	0.148	0.65	0.31	<0.001	<0.001	<0.001	0.093	0.002	0.56
	6-12 months treatment n = 20	0.28±0.045										
	>1 year treatment n = 20	0.25± 0.06										

Pa value between newly diagnosed patients and control
Pb value between patients with 3-5 months treatment and control
Pc value between patients with 6-12 months treatment patients and control
Pd value between patients with >1 year treatment and control.
Pe value between newly diagnosed patients and patients with 3-5 months treatment
Pf value between newly diagnosed patients and patients with 6-12 months treatment
Pg value between newly diagnosed patients and patients with >1 year treatment
Ph value between patients with 3-5 months and patients with 6-12 months treatment
Pi value between patients with 3-5 months and patients with >1 year treatment
Pj value between patients with 6-12 months and patients with >1 year treatment

Table 5. The comparison of the studied parameters between Gaucher patients' subgroups by ANOVA test

Parameter	Control n=20 mean±SD	Newly diagnosed n = 9 mean±SD	3-5 months treatment n = 18 mean±SD	6-12 months treatment n = 20 mean±SD	>1 year treatment n = 20 mean±SD	P value
Sodium (mEq/l)	137.95±1.23	137±1.87	137.61±1.46	138.05±1.61	137.9±1.83	0.442
Potassium (mEq/l)	4.13±0.2	4.26±0.48	4.1±0.34	4.15±0.34	4.06±0.31	0.575
Urea (mg/dl)	17.5±3.05	26.51±5.17	27.06±3.78	23.7±2.08	18.38±2.17	<0.001
Creatinine (mg/dl)	0.27±0.09	0.46±0.04	0.30±0.03	0.28±0.05	0.25±0.06	<0.001

Table 6. ROC curve results for all studied parameters in patients comparing with control

Parameters	AUC	Sensitivity (%)	Specificity (%)	Cut-off value
Sodium (mEq/l)	0.563	70.0	48.0	137.5
Potassium (mEq/l)	0.53	55.0	58.0	4.15
Urea (mg/dl)	0.865	82.0	80.0	18.5
Creatinine (mg/dl)	0.615	76.0	60.0	0.235

Table 7. ROC curve results for all studied parameters in newly diagnosed patients comparing with control

Parameters	AUC	Sensitivity (%)	Specificity (%)	Cut-off value
Sodium (mEq/l)	0.717	70.0	78.0	137.5
Potassium (mEq/l)	0.6	55.6	90.0	4.35
Urea (mg/dl)	0.939	77.8	85.0	22.15
Creatinine (mg/dl)	0.978	100	80.0	0.35

Table 8. ROC curve results for all studied parameters in newly diagnosed patients comparing with patients with 3-6 months treatment

Parameters	AUC	Sensitivity (%)	Specificity (%)	Cut-off value
Sodium (mEq/l)	0.664	83.0	56.0	136.5
Potassium (mEq/l)	0.611	55.6	83.3	4.35
Urea (mg/dl)	0.506	88.9	33.3	22.65
Creatinine (mg/dl)	1	100	100	0.365

Table 9. ROC curve results for all studied parameters in patients with 3-6 months treatment comparing with patients with 6-12 months treatment

Parameters	AUC	Sensitivity (%)	Specificity (%)	Cut-off value
Sodium (mEq/l)	0.586	65.0	61.1	137.5
Potassium (mEq/l)	0.551	35.0	83.3	4.35
Urea (mg/dl)	0.797	61.1	95.0	25.5
Creatinine (mg/dl)	0.657	83.3	50.0	0.275

Table 10. ROC curve results for all studied parameters in patients with 6-12 months treatment comparing with patients with >1 year treatment

Parameters	AUC	Sensitivity (%)	Specificity (%)	Cut-off value
Sodium (mEq/l)	0.519	65.0	35.0	137.5
Potassium (mEq/l)	0.568	45.0	70.0	4.15
Urea (mg/dl)	0.945	90.0	85.0	21.15
Creatinine (mg/dl)	0.703	100	65.0	0.205

Discussion

Generally, most of the previous studies stated that the Gaucher patients didn't have a renal involvement and the level of serum urea and creatinine in addition to electrolytes such as sodium and potassium were normal [3,11] with some exceptions [7,12] that occurs rarely. This clinical manifestation of organ dysfunction directly related to massive Gaucher cells infiltration [7,10] such as appeared in the present study in which a significant increase in the serum urea were observed. On the other hand, non-significant differences in the levels of creatinine, sodium and potassium were reported in Gaucher patients in comparison with control subjects as demonstrated in table (1).

Sodium and potassium

According to results obtained in table (3), it was demonstrated that there was a non-significant difference in the levels of sodium and potassium among all the studied groups that may indicate a non-significant effect of the GD and also the treatment with ERT on the level of these electrolytes in agreement with previous studies [3,11]. The present study also revealed that the level of sodium and potassium didn't affect by treatment as revealed by a non-significant result obtained by ANOVA test in table [5] and also confirmed by the non-significant correlation with the period of treatment as shown in table (2). Also, ROC curve results illustrated that these two electrolytes cannot be considered as biomarkers for GD due to low AUC, sensitivity and specificity in all patients in comparison with controls.

Urea

In contrast to tests for the assessment of sodium, potassium and also creatinine, urea showed a different pattern in the present study, which also differ from the most previous studies [3,11] in that urea levels showed a significant higher level compared to controls as demonstrated in table (1). Additionally table (4) also revealed that the levels of urea in newly diagnosed untreated patients, patients treated with ERT for 3-6 months and patients treated for 6-12 months were significantly higher than that of control while the level of urea return to a normal level, which is comparable to that of control after receiving the treatment for more than year and also noticed that there was a non-significant

differences between newly diagnosed untreated patient and patients received treatment for 3-6 months.

These results may indicate that the treatment with ERT for 6-12 months may cause a significant reduction in the renal complication and cause a urea level restoration to the normal levels after more than one year of treatment. This suggestion also confirmed by the significant difference among all patients' subgroups as demonstrated by ANOVA test in table (5) and also confirmed by the significant negative correlation between urea level and the period of treatment that demonstrated in table (2) and ROC curve results obtained in tables (6-10), which indicate that urea level can be considered as an excellent biomarker for GD patients either treated or untreated with ERT.

As mentioned previously, the assumed explanation of these abnormalities is the infiltration of glomeruli by Gaucher cells [7].

Creatinine

In contrast to urea, the levels of creatinine in all studied groups were normal and showed a non-significant difference between Gaucher patients and control and the only interesting finding was the significant difference between the newly diagnosed untreated patients and controls after that the level of creatinine return to a level comparable to that of control in patients receiving treatment which may indicate that the level of creatinine is more sensitive to treatments and respond more quickly than urea which is also revealed by a significant differences between untreated and treated patients as shown in table (4).

ANOVA test results in table (5) also prove the above finding of significant differences between all patient subgroups. In addition to that, the significant negative correlation between the creatinine levels and the period of treatment that illustrated in table (2) also ensure the effect of ERT on the renal complication together with ROC curve results which revealed that creatinine can be considered as an excellent biomarker for GD especially for newly diagnosed untreated patients and also to monitor the treatment in the first 3-6 months of treatment (tables 6-10).

Finally, according to the previous data and data provided in this study, it was concluded that kidney involvement in Gaucher patients may be monitored by urea and creatinine levels which provide a complementary picture about the all steps of kidney restoration to normal function given that creatinine is more sensitive for treatment in the first 6 months of treatment while the significant differences in urea level become more obvious after the 6 months of treatment.

In conclusion, kidney involvement in Gaucher patients can be monitored by urea and creatinine levels which provide a complementary picture about the all steps of kidney restoration to normal function given that creatinine is more sensitive for treatment in the first 6 months of treatment while the significant differences in urea level become more obvious after the 6 months of treatment. Sodium and potassium levels showed to be non-significantly affected by the disease and treatment with ERT.

Acknowledgments

Authors are grateful to staff of the Department of Chemistry and Biochemistry, College of Medicine, Al-Nahrain University for their facilities in performing this study, Department and Unit of Rare Diseases at Al Imamein Al-Kadhimein Medical City, Gastroenterology and Hepatology Teaching Hospital, Children Welfare Hospital Consultation Clinic, Central Child's Teaching Hospital and College of Education, Ministry of Science and Technology.

Authors contribution

Abdulhussein: Performing laboratory measurements. Al-Obaidi: Writing of the manuscript and statistical analysis. Dr Arif: Providing patient samples and diagnosing autistic patient cases.

Conflict of interest

Authors have no conflict of interests.

Funding

The work was not supported or funded by any drug company.

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E-mail: hibaabdulhussein@yahoo.com
Received Aug. 15th 2017
Accepted Nov. 16th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

Amyloid Precursor Protein Immunohistochemical Changes in The Newborn Mice Frontal and Parietal Cerebral Cortices Affected by Prenatal Exposure to Ketamine

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Abstract

Background Ketamine is N-methyl-D-aspartate (NMDA) receptors blocking drug, it affects the cerebral cortex and play an essential role in learning and memory. Amyloid β (Aβ) is a cleavage product of a large, trans-membrane protein, termed amyloid precursor protein (APP); it may have a role in controlling synaptic activity.

Objective To investigate the immunohistochemical beta APP reaction in newborn mice frontal and parietal cerebral cortices after prenatal exposure to therapeutic doses of ketamine as an attempt for scientific judgments of making better understanding for effects of ketamine on developing brain, which may help to reduce adverse effects.

Methods Thirty pregnant mice were divided into two groups named experimental and control groups (15 mice for each groups). The experimental group animals were injected intraperitoneally with 50 mg/kg ketamine, the control group animals were injected with intraperitoneal distilled water. Paraffin sections of newborn mice frontal and parietal cortices were stained immunohistochmically with anti-APP antibodies.

Results The immunohistochemical labeling in the experimental group showed scattered clumps of brown staining distributed randomly in the cerebral cortex. The brown stained deposits vary also in shape and size, the larger and more intense staining was seen in the more superficial layers of the frontal cortex. The statistical analysis found non-significant differences in staining pattern between frontal and parietal cortices of control group, while significant differences were found between frontal and parietal cortices in experimental group.

Conclusion The immunohistochemical APP reactivity showed different intensities and different morphology in the frontal and parietal cortices in the all experimental group animals were that injected with ketamine in this study. These differences could be related to the requirement of this substance in repair and differentiation of the developing NMDA dependent interneuron impaired by prenatal ketamine exposure.

Keywords Frontal cortex, parietal cortex, amyloid precursor proteins, ketamine, prenatal, immnuohistochemistry

Citation Najm MS, Mubarak HJ, Mohammed LH. Amyloid precursor protein immunohistochemical changes in the newborn mice frontal and parietal cerebral cortices affected by prenatal exposure to ketamine. Iraqi JMS. 2018; 16(2): 191-200. doi: 10.22578/IJMS.16.2.11

List of abbreviations: Ab = Beta amyloid, AD = Alzheimer's disease, APP = Amyloid precursor protein, CNS = Central nervous system, DAB = Diaminobenzidine, IHC = Immunohistochemistry, IP = Intrapritonial administration, NIH = National institute of health, NMDA = N-methyl-D-aspartate, SPSS = Statistical package of social sciences

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 an evidence suggesting that any changes in NMDA receptor function have an impact on learning and memory abilities [(4)].

The trial studies in animals have shown that exposure of the anesthetic agents during developmental periods can lead to neuronal apoptosis or neurodegeneration [(5)].

The laboratory models intend anesthetic interactions with neurodegenerative mechanisms, such as those linked to the onset and progression of Alzheimer's disease (AD) [5].
The theories of neurodegeneration in AD mainly focus on the toxic effects of aggregated amyloid β (Aβ) peptide oligomers that result from intramembranous proteolysis of the transmembrane protein amyloid precursor protein (APP) [6].
There are Several lines of evidence indicate that Aβ may have a role in controlling synaptic activity [7].
This study aimed to investigate the immunohistochemical beta APP expression in newborn mice frontal and parietal cerebral cortices after prenatal exposure to therapeutic doses of ketamine as an attempt for scientific judgments of making better understanding for effects of ketamine on developing brain which may help to reduce adverse effects.

Methods

The animals used in this study were obtained from Laboratory Animal House at College of Medicine, Al-Nahrain University. A total of (30) pregnant adult female mice (mus musculus) aged (8-12) weeks were used in this study. Weight of the animals was between (20- 40 gm).
Checks the mouse estrous cycle. The mating occurs after putting two females per one male in one cage.
After mating, pregnancy was confirmed the following morning by finding vaginal plugs and this was considered as day 0 of gestation [8].
All animals were treated according to National Institute of Health (NIH) Guidelines for the Care and Use of laboratory animals [8].
The 30 pregnant mice were divided into two groups; I namely experimental group and II namely control group (15 Mice for each group).
The pregnant mice of experimental group were injected intraperitoneally with a single shot of 50 mg/kg ketamine hydrochloride [9] on the 5th, 10th, 15th, and 20th days of pregnancy.
The control group mice were injected intraperitoneally with distilled water on the same gestational days.
Each female mouse delivered (6-10) neonates; after delivery neonates were selected randomly.
The one-day old neonate was decapitated after delivery and removed the skin of head, but the skull shield was not removed.
The newborn brain was fixed in 10% formalin, the fixed tissues were then submitted for routine paraffin embedding process including dehydration, clearing, infiltration, and embedding [10]. Sections of 5 µm thickness of cerebral tissue were taken and properly laid down on the surface of hot water (40 °C). The sections were then collected on clean strong positive slides (AFCO). From each paraffin tissue block. Each slide was contained 4 sections and was taken for immunohistochemical staining.
The immunohistochemical staining kits, provided by Abcam®, contained rabbit polyclonal antibody to Amyloid Precursor Protein clone (ab15272) and Rabbit specific DAB, (ab64261) detection kit from Abcam®.
The sections were examined under light microscopy (Genex, USA) to localize and determine the pattern of immunohistochemical expression of Anti-Amyloid Precursor Protein and then images were captured by camera (5 mega pixels, Genex, USA) equipped with in the genex light microscope.
Statistical Package for the Social Sciences (SPSS) version 22 company by (IBM) was a software used for statistical evaluation and in dependent (t) test was used for analysis of mean values of strong positive Pixel Count Algorithm obtained by the application Aperio Image Scope software on Amyloid Precursor Protein immunohistochemical reactivity in the frontal and parietal cortices of neonates' mice in both experimental and control groups.

Results

The immunohistochemical labeling in experimental group showed scattered clumps of brown staining distributed randomly in the

cerebral cortex, these clumps were localized in the extracellular matrix and having different intensities. The brown depositions varied also in size in superficial layer, however; larger and more intense accumulations of the immunohistochemical reactivity were seen in the more superficial layers of the frontal cortex (Figure 1).

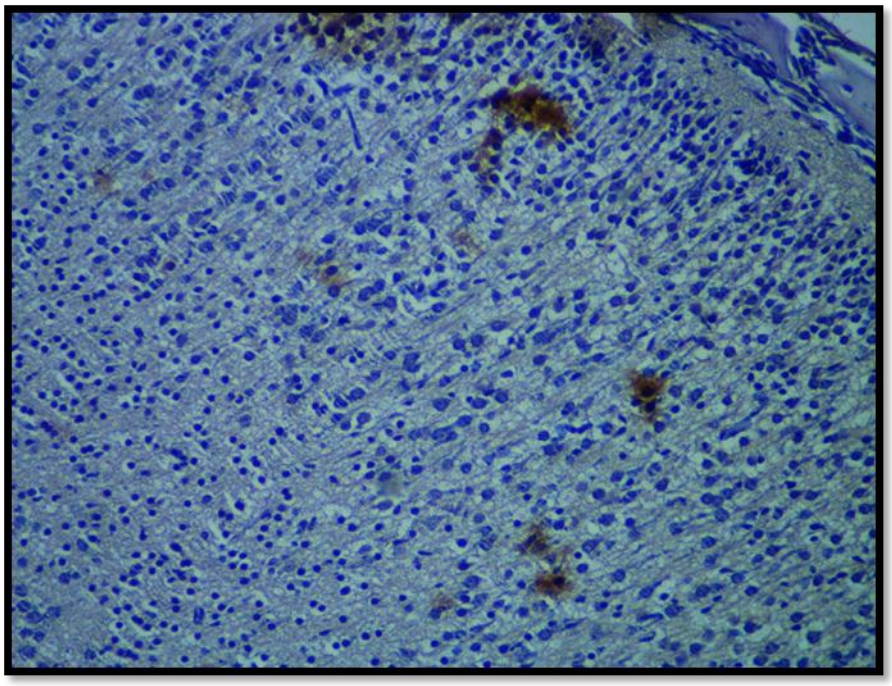


Figure 1. Sagittal paraffin section. Frontal cortex of the neonate mouse of the experimental group. The low magnification immunohistochemical labeling showed scattered clumps of brown staining distributed randomly in the frontal cortex, these brown staining was localized in the extracellular matrix and having different intensities and more intense accumulations of the immunohistochemical reactivity was seen in the superficial layers of the frontal cortex. Immunohistochemical Anti-APP staining (X200)

APP immunohistochemical reaction in the frontal and parietal cortices of newborn mice in control group

The evaluation of the counted mean values in the frontal cortex of neonate mice of control groups revealed statistically non-significant difference compared to parietal cortex of the same group (Figures 2 and 3).

The mean value of the number of strongly positive pixels was (1.6818±7.88) in frontal cortices; the mean value in parietal cortices of control group was (0.7273±3.19).

The mean value comparison between frontal and parietal cortices in the control group showed statistically non-significant difference and p values of the mean counted pixels was ≥ 0.602 of the mean counted pixels (Table 1). Both these cortices showed very weak immunohistochemical reactivity.

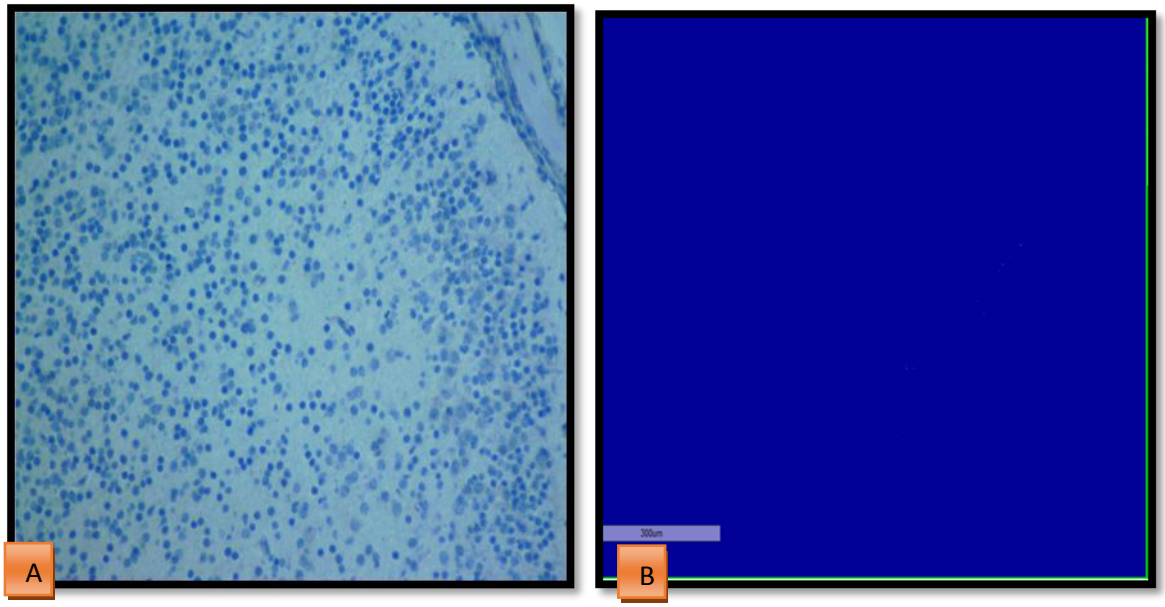
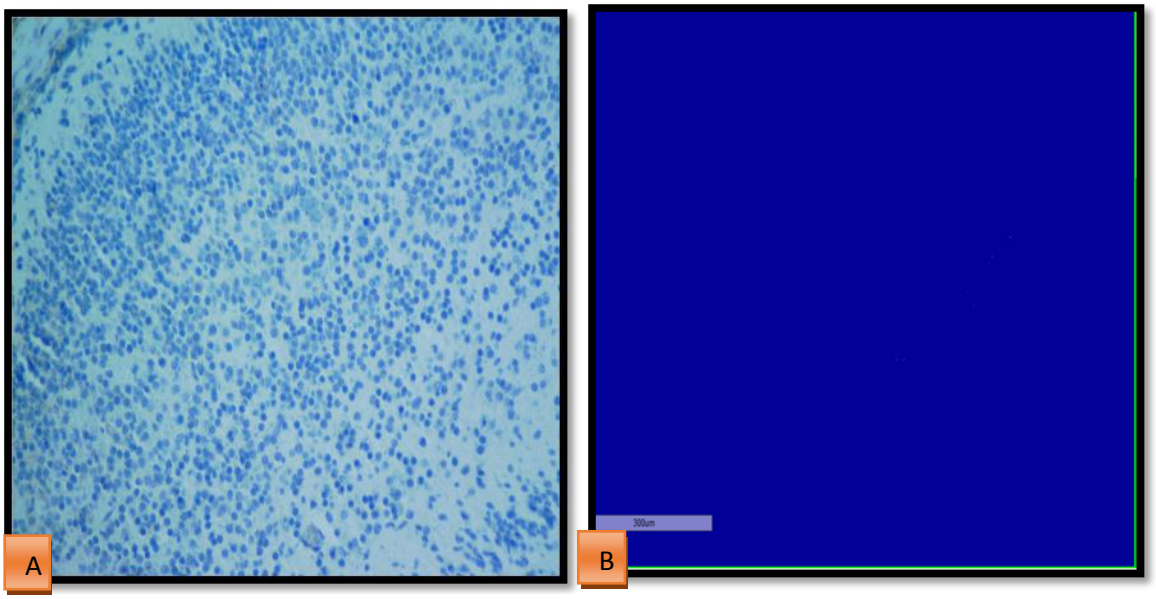


Figure 2. (A) APP reactivity in frontal cerebral cortex of neonate mice from control group. The APP negative stain is seen in all layer of cerebral frontal cortex. X 200 (B) The snap shoot as analyzed by Aperio positive pixel count algorithm



The APP immunohistochemical reaction in the frontal and parietal cortices of newborn mice in experimental group

The mean of strong positive pixel in the frontal cortex was higher than the mean of parietal cortex experimental in groups (Figure 4).

The estimation of mean values of staining intensity for the Amyloid precursor protein immunohistochemical reaction in the frontal and parietal cerebral cortices of the neonate mice of experimental group showed that the mean value from the neonate frontal cortices is 9734.05±1074.82, and the mean value of parietal cortices is 948.82±1168.22 (Figures 5, 6 and 7).

The mean of the strong positive pixel obtained from the frontal cortices showed statistically significant difference from those of the parietal cortices experimental groups (P ≤ 0.000) (Table 1).

Table 1. The mean strong positive pixel obtained by the Aperio Image Scope analysis of frontal and parietal cerebral cortices sections of newborn mice

Group		Mean±SD	P value
Frontal and parietal cortices of control group	Frontal cortex Parietal cortex	1.69±7.89 0.73±3.2	0.602 *(not-significant)
Frontal and parietal cortices of experimental group	Frontal cortex Parietal cortex	9734.05±1074.82 948.82±1168.22	0.000 * (significant)
Frontal cortices of experimental and control groups	Frontal cortex of experimental group Frontal cortex of control group	9734.05±1074.82 1.682±7.89	0.000 * (significant)
Parietal cortices of experimental and control groups	Parietal cortex of experimental group Parietal cortex control group	948.82±1168.22 0.73± 3.2	0.000 * (significant)

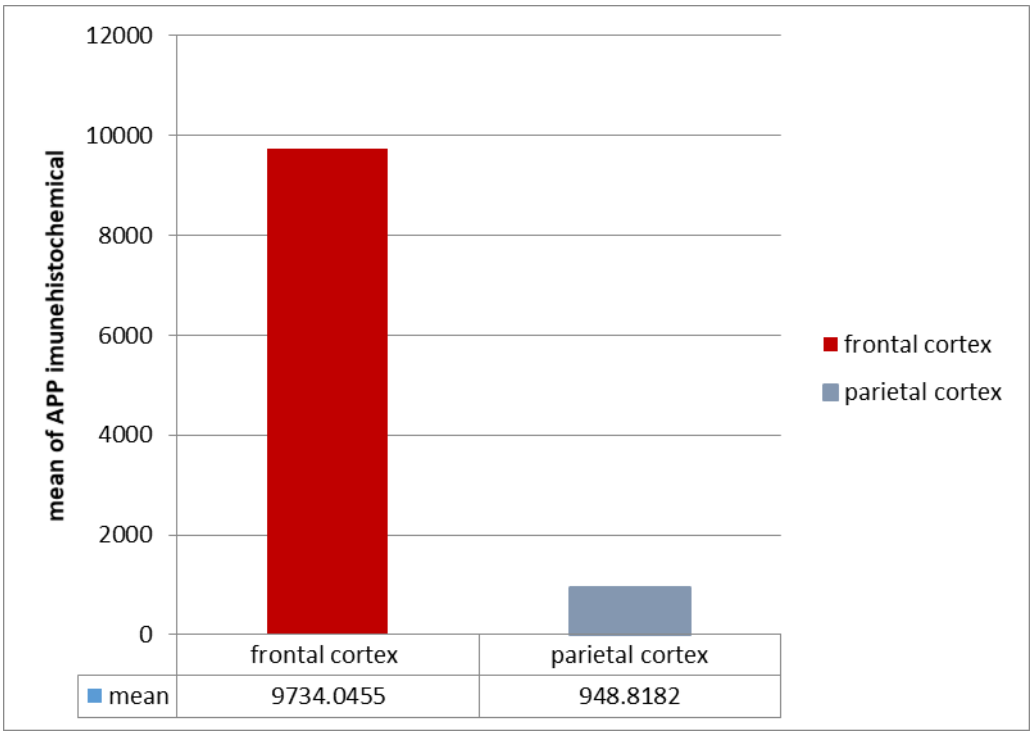


Figure 4. The values of APP immunohistochemical marker reactivity obtained from frontal and parietal cortical section in newborn mice of the experimental groups

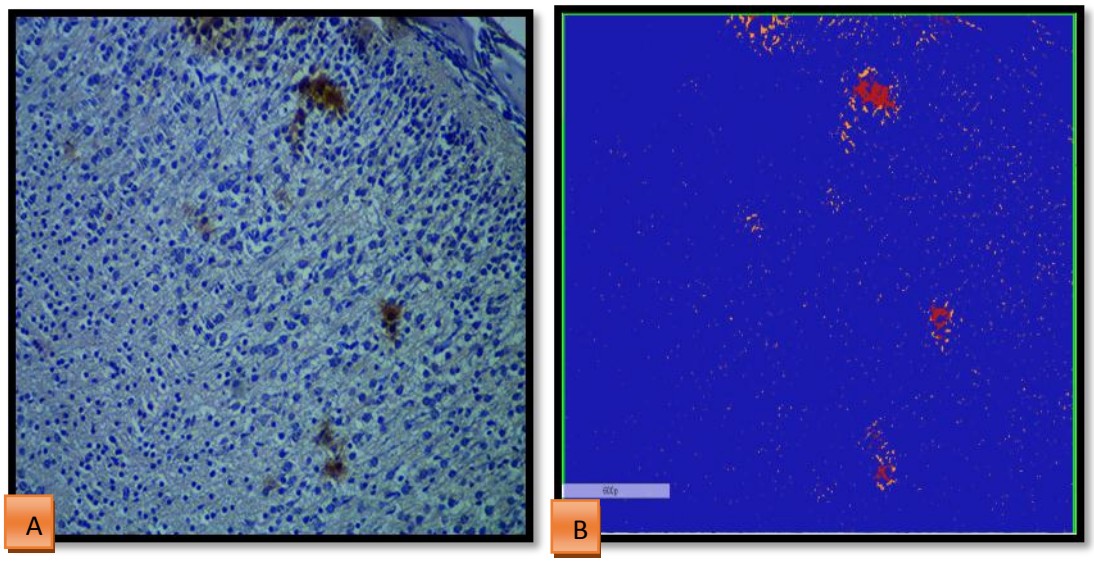


Figure 5. (A) sagittal section APP in immunohistochemical reactivity in frontal cerebral cortex of neonate mice from experimental group. APP positive stain is seen in all layer of cerebral frontal cortex. X 200 (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm

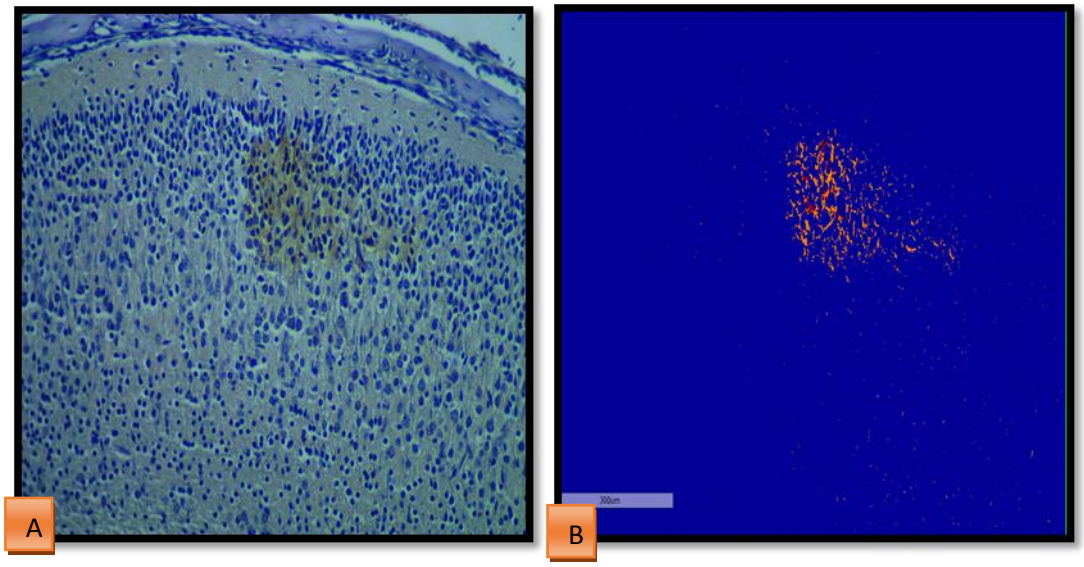


Figure 6. (A) sagittal section APP in immunohistochemical reactivity in frontal cerebral cortex of neonate mice from experimental group. APP positive stain is seen in all layer of cerebral frontal cortex. X 200 (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm

Statistical evaluations of APP immunohistochemical reaction between frontal cortices of experimental group and control group

The statistical analysis of the values of the strongly positive pixels obtained from evaluation of APP immunohistochemical expression of on neonate mice frontal cortices of experimental and control group showed that the mean of strong positive pixels in frontal cortices of experimental group is 9734.05±1074.82 and mean of strong positive pixels in control group is 1.69±7.89. This difference was statistically significant (P ≤ 0.000) (Table 1).

Statistical evaluations of APP immuno-histochemical reaction between parietal cortices of experimental group and control group

The mean values of strong positive pixel of parietal cortices of experimental group were the highest mean value when compared to parietal cortices of control groups. The mean number of the strongly positive pixels of immunohistochemical reaction of parietal cortex of mice that was injected with ketamine is 948.82±1168.22, while the mean of strong positive pixel of parietal cortex of mice inject with distal water is 0.73±3.2.

The differences between the treated and control group were statistically significant (p ≤ 0.000) (Table 1).

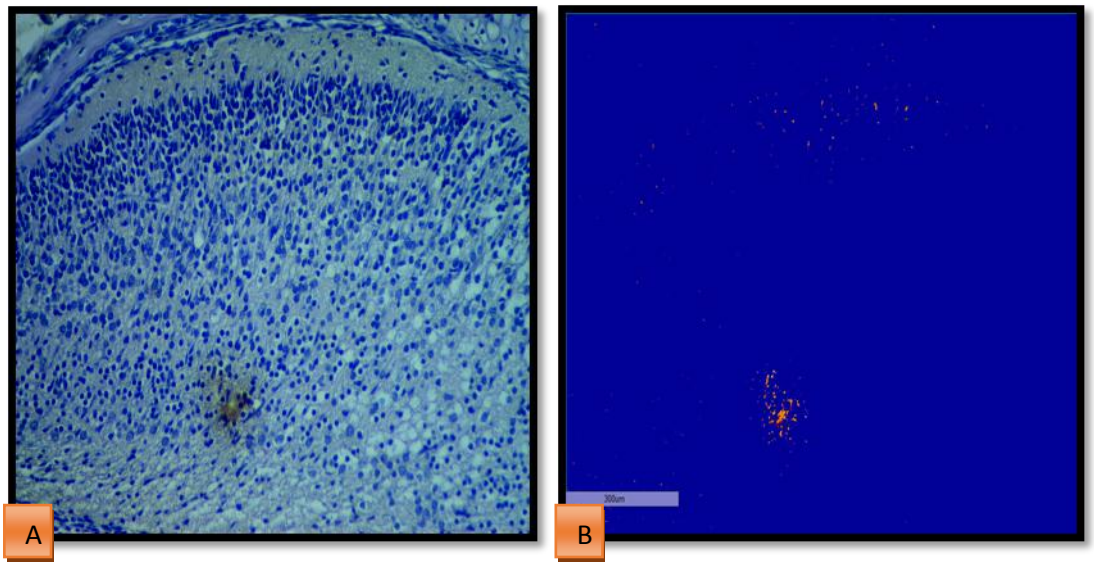


Figure 7. (A) sagittal section APP in immunohistochemical reactivity in parietal cerebral cortex of neonate mice from experimental group. APP positive stain is seen in all layer of cerebral frontal cortex. X 200 (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm

Discussion

The adverse effects of ketamine had been investigated extensively in many researches. Different aspects were considered in regard to neonatal and prenatal consequences of ketamine on the developing brain. Variables appraisals were done that demonstrated changes as neuronal apoptosis, synaptic transmission and plasticity, and deficits in learning-related behaviors [11].

The immunohistochemical APP reactivity showed different intensities in the frontal cortex of the animals involved in this study that were prominently noticeable compared to the parietal cortex. This conclusion has been confirmed by the statistical evaluations of the

mean values of strongly positive pixels algorithm found in this study.

This result simulates the histological changes occurring in the cerebral cortex of rat affected by prenatal ketamine exposure, massive histological changes seen in frontal cortex compared to parietal cortex [12,13]. Also, the regional variability of APP accumulation found in this study was highly significant in the frontal cortex, which indicates more susceptibility of frontal cortex to ketamine's compared to parietal cortex. The possible explanation of this agreement is the frequent exposure to ketamine though out pregnancy as a common contributing factor to frontal cortical pathology in this study and that was done during the other experimental procedures [13].

The cortical lamina variability of APP accumulation after prenatal exposure to ketamine in this study was not seen in experimental researches considering neuroapoptotic consequences of prenatal ketamine exposure. It was reported in previous literatures that Ketamine have more prominent damaging effects on the fetal than neonatal brain. This speculation was implicated in both clinical anesthesia and drug abuse precautions [13]. This assumption could also be adopted according to the results of our study as the cortex of fetuses was massively affected by ketamine exposure of pregnant mice. Further researches could be done in future to compare the significance of ketamine exposure of neonate but with fetus.

The accumulation of APP in this study was more marked in the superficial lamina at the frontal cortex. The APP is an integral membrane protein concentrated in the synapses of neurons. It had been implicated as a regulator of synapse formation, the most-substantiated role for APP is in synaptic formation and repair [14]. The study of Brambrink et al. (2012) [13] reported that none of the ketamine-exposed neonate brains showed a laminar pattern of cell death in any specific layers of the cortices. However, the laminar-specificity of ketamine-induced changes in the frontal cortex was reported by Jeevakumar et al. (2014) [15] when ketamine was administered during the first 2 postnatal weeks, these changes were attributed to the effect of ketamine on the maturation of NMDA dependent of interneurons [16]. The distribution of these interneurons across layers of the rodent cortex was found to be highest in layer 5 [17], thus layer 5 does not show significant functional impairment by NMDA dependent of interneurons cellular loss caused Ketamine exposure compared to the superficial layers 2/3 [15]. This interpretation is supported by the reports of previous studies that systemic injection of the NMDA receptor antagonists significantly increased the drug adverse outcome (including apoptosis) in rodent pups [18].

APP expression was suggested to be upregulated during neuronal differentiation and after neuronal injury [19]. The more AAP in superficial lamina explored in this study could be related to the requirement of this substance in repair and differentiation of the developing NMDA dependent interneuron in the superficial lamina that were impaired by prenatal ketamine exposure. The synapses formation associated with the increased dendritic branching in the frontal cortex affected by ketamine may be regulated by accumulations of AAP in the frontal cortex. The impaired maturation of these interneurons in the deeper lamina of the frontal cortex in mice does not have functional consequences due to higher percentage of these neurons in the deep lamina that balances the deficit in differentiation of these interneurons caused by prenatal ketamine exposure. This speculation supported the conclusions reported by Jeevakumar et al. (2014) [15].

The APP immunohistochemical accumulation in mice cerebral cortex after prenatal exposure to ketamine represented a developmental requirement of cortical tissue during development needed to provide the necessary adaptive functional maturation. These developmental requirements for APP aggregation could involve functional demands including synaptogenesis and neuronal network formation [20], neurite growth, neuronal adhesion and axonogenesis [21].

APP proteolysis generates beta amyloid (Aβ), which is the primary component of amyloid

plaques found in the brains of Alzheimer's disease patients [22]. The possibility of pathological sequel of the accumulated APP in the cerebral cortex after prenatal ketamine exposure in post-pubertal life could not be excluded.

This study concluded that the immunohistochemical APP reactivity showed different intensities and different morphology in the frontal and parietal cortices of the animals involved in this study that was prominently noticeable compared to the parietal cortex, these differences could be related to the requirement of this substance in repair and differentiation of the developing NMDA dependent interneuron impaired by prenatal ketamine exposure.

The impaired maturation of these interneurons in the deeper lamina of the frontal cortex in mice does not have functional consequences due to higher percentage of these neurons in the deep lamina that balances the deficit in differentiation of these interneurons caused by prenatal ketamine exposure.

Acknowledgments

Regard and gratefulness should be presented to the staff members Department of Human Anatomy at the College of Medicine, Al-Nahrain University for their assistance and cooperation.

Authors contribution

Najm: The MSc. candidate performing the laboratory research work. Dr. Mubarak: The advisor of the research performing the interpretation of the results. Mohammed: Assist for performing production of the statistical results.

Conflict of interest

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

Funding

The research funding was provided by the authors.

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Received Sep. 13th 2017
Accepted Dec. 26th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

The Level of 27-hydroxycholesterol and Oxysterol 7α-hydroxylase (CYP7B1) in Tissues of Women with Breast Tumors

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Abstract

Background In Iraq, breast cancer is the commonest type of malignancy in females. The disease is a genetically and clinically heterogeneous. The cholesterol metabolite 27-Hydroxycholesterol (27HC), a primary metabolite of cholesterol and an estrogen receptor (ER) and Liver X receptor (LXR) ligand, increases ER-positive breast cancer. 27-hydroxycholesterol (27HC) is metabolized by oxysterol 7α-hydroxylase (CYP7B1), CYP7B1 expression has been decreased in ER-positive tumors compared with normal breast tissues.

Objective To study and investigate the possible association of clinic-pathological parameters 27HC and CYP7B1 level in sera of women with benign and malignant breast tumors and in control group by ELISA technique and Investigate the possible relationships of 27HC with CYP7B1.

Methods This case control study was conducted on sixty patients with breast diseases were divided into three group, group I contained twenty patients with benign breast diseases, group II consisted of twenty premenopausal patients with breast cancer. Group III comprised twenty postmenopausal patients with breast cancer with the mean age and standard deviation (25.25±7.87, 38.65±6.28, 58.5±7.02 years). 27HC and CYP7B1 were measured in tissues by instrument ELISA technique.

Results The tissue homogenates of women with premenopausal and postmenopausal breast cancer groups showed a significant elevation of 27HC in comparison with benign ($p > 0.001$), whereas there is no significant difference observed between both breast cancer groups ($p = 0.542$). The tissue homogenates of women with premenopausal and postmenopausal breast cancer groups showed a significant decrease of CYP7B1 concentration in comparison with benign ($p = 0.003$) and ($p = 0.001$), whereas there is no significant difference observed between both breast cancer groups ($p = 0.868$).

Conclusion A higher incidence of 27HC and a lower incidence CYP7B1 were obtained in malignant than benign breast tumor tissues with positive estrogen receptors. These indicate that the levels of 27HC and CYP7B1 in breast tumor tissues may be used as new biochemical markers for breast tumor prognosis.

Keywords 27-Hydoxycholesterol, CYP7B1, breast cancer, estrogen receptor

Citation Mohammed ZK, AL-Saeed HH, Nile AK. The level of 27-hydroxycholesterol and oxysterol 7α-hydroxylase (CYP7B1) in tissues of women with breast tumors. Iraqi JMS. 2018; 16(2): 201-206. doi: 10.22578/IJMS.16.2.12

List of abbreviations: 27HC = 27-Hydoxycholesterol, CYP7B1 = oxysterol 7α-hydroxylase, CYP27 = Cytochrome P450 sterol 27-hydroxylase.

Introduction

The cholesterol metabolite 27-hydroxycholesterol (27HC) formed by the mitochondrial cytochrome P450 sterol 27-hydroxylase (CYP27), an enzyme particularly expressed in the vascular endothelium, macrophages and the liver [1,2]. Introduction of a hydroxyl group allows the otherwise hydrophobic cholesterol molecule to pass amphiphilic membranes more easily [2,3]. Because of these physicochemical properties,

27HC has been postulated to be secreted from cells independently of transporters and extracellular lipoprotein acceptors and thereby to facilitate an alternative route for apolipoprotein (apo) A-I/high density lipoproteins (HDL)-mediated transport of cholesterol from macrophages to the liver [2]. In the liver, 27HC is an important intermediary product of the so-called alternative bile acid synthesis pathway, which contributes ~10% to de novo bile acid biosynthesis [2]. In addition, 27HC is an important ligand of at least two types of nuclear hormone receptors. It activates liver-X-receptors (LXR) alpha and beta, which regulate the transcription of several genes involved in lipid and lipoprotein metabolism [4,5]. Most recently 27HC was identified as the first endogenous selective estrogen receptor modulator (SERM). Both in vitro and in vivo 27HC was found to modulate the transcriptional activity of estrogen receptors tissue-specifically either as an agonist or antagonist [6].

The cholesterol metabolite 27HC is the most abundant oxysterol in the circulation [4], the plasma levels of 27HC have been found to correlate with the cholesterol content in atherosclerotic lesions and the severity of coronary artery disease [7]. Patients with genetic CYP27 deficiency suffer from cerebrotendinous xanthomatosis and develop premature atherosclerosis despite having normal levels of plasma cholesterol [2]. In addition, there are greater 27HC levels in tumor samples compared with controls. Furthermore, survival of cancer patients is markedly poorer for patients with low versus high tumor CYP7b1 expression. In mouse models, 27HC promoted the tumor growth and metastasis by independent mechanism [8].

This study aimed to determine of 27HC levels and CYP7B1 in tissues of women with benign and malignant breast tumors. Also, to investigate the possible relationships of 27HC with CYP7B1.

Methods

The study was executed during the term from February 2017 to June 2017, it included 60 patients of woman with breast tumor that divided into 3 groups; Group I contained 20 patients with benign breast tumor, Group II consisted of 20 premenopausal patients with breast cancer, Group III comprised 20 postmenopausal patients with breast cancer with the mean age and standard deviation (25.25±7.87, 38.65±6.28, 58.5±7.02 years) respectively. All samples were collected from Al-Imamein Al-Kadimein Medical City, Medical City of Baghdad Teaching Hospital, Al-Kadimiyah Privet Hospital and Al-Numan Hospital. They were histologically proven, newly diagnosed and not underwent any type of therapy. Patients suffered from any disease that may interfere with this study were excluded.

Collection of specimens

The tumor tissues were surgically removed from breast tumor patients by either mastectomy or lumpectomy. The specimens were cut off and immediately immersed in ice-cold isotonic saline solution. They were collected individually in plastic receptacle and stored at -20 °C until homogenization. The level of 27HC and CYP7B1 was measured by monoclonal antibody Enzyme Linked Immuno Sorbent Assay (ELISA) technique using number of kit two for each 27HC and CYP7B1 from China.

Data were statistically analyzed using SPSS statistical software (version 23). Values were expressed as the mean ± standard deviation. A unpaired t test was used to compare mean levels of 27HC and CYP7B1 in women with breast tumors.

Results

In tissue homogenate samples, 27-HC was significantly elevated in postmenopausal breast cancer group compared with benign group ($p< 0.001$) and elevated in premenopausal breast cancer group compared with benign group ($p< 0.001$) as shown in table (1) and figure (1). In

tissue homogenate samples, CYP7B1 was significantly decreased in postmenopausal breast cancer group compared with benign group (p= 0.001) and CYP7B1 was significantly decreased in premenopausal breast cancer group compared with benign group (p= 0.003) as shown in table (2) and figure (2).

The possible relationships of 27-HC with CYP7B1 in tissue homogenates of women with breast tumors is shown in table (3).

Table 1. Comparison the concentration of 27-Hydroxy cholesterol (ng/l) in sera and tissues homogenate of women with breast tumors

Group	No.	Mean±SD in tissue	P value
Group I Benign	20	15.90±4.84	< 0.001
Group II Pre	20	26.95±5.68	
Group I Benign	20	15.90±4.84	< 0.001
Group III Post	20	28.25±7.55	

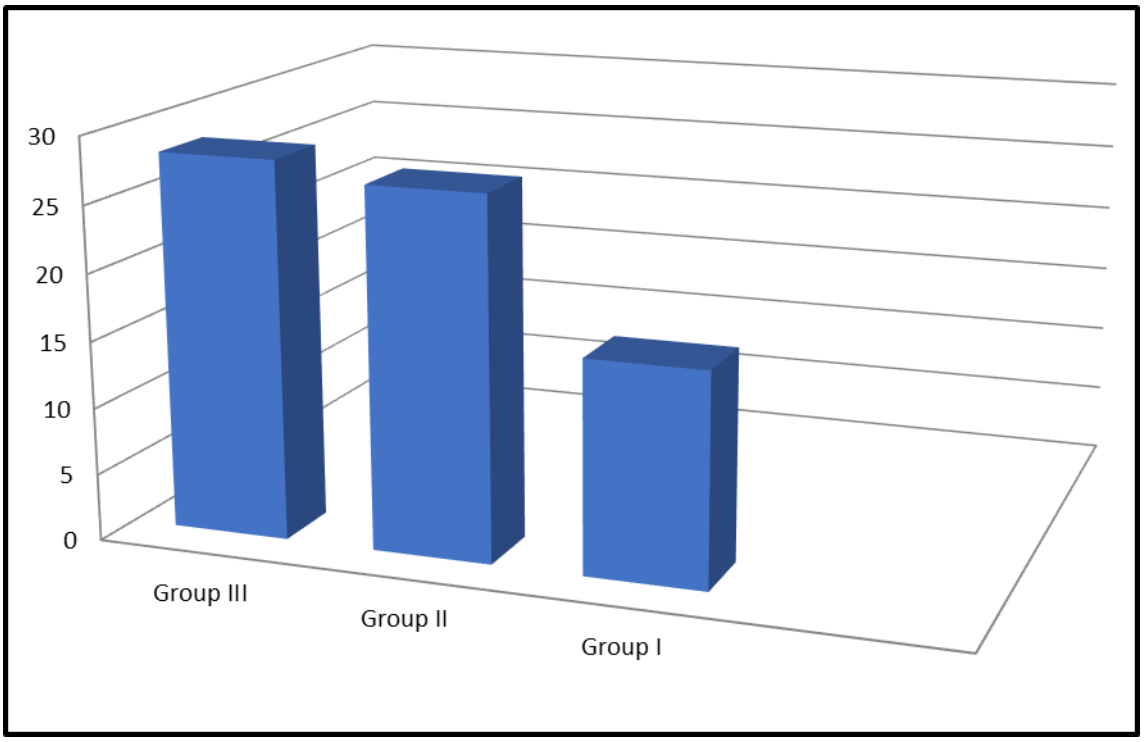

Figure 1. Comparison the concentration of 27-Hydroxy cholesterol (ng/l) in tissues of woman with breast tumor

Table 2. Comparison the concentration of CYP7B1 (U/ml) in tissues homogenate of women with breast tumor

Group	No.	Mean±SD in tissue	P value
Group I Benign	20	8.85±3.96	0.003
Group II Pre	20	5.30±2.36	
Group I Benign	20	8.85±3.96	0.001
Group III Post	20	5.19±1.75	

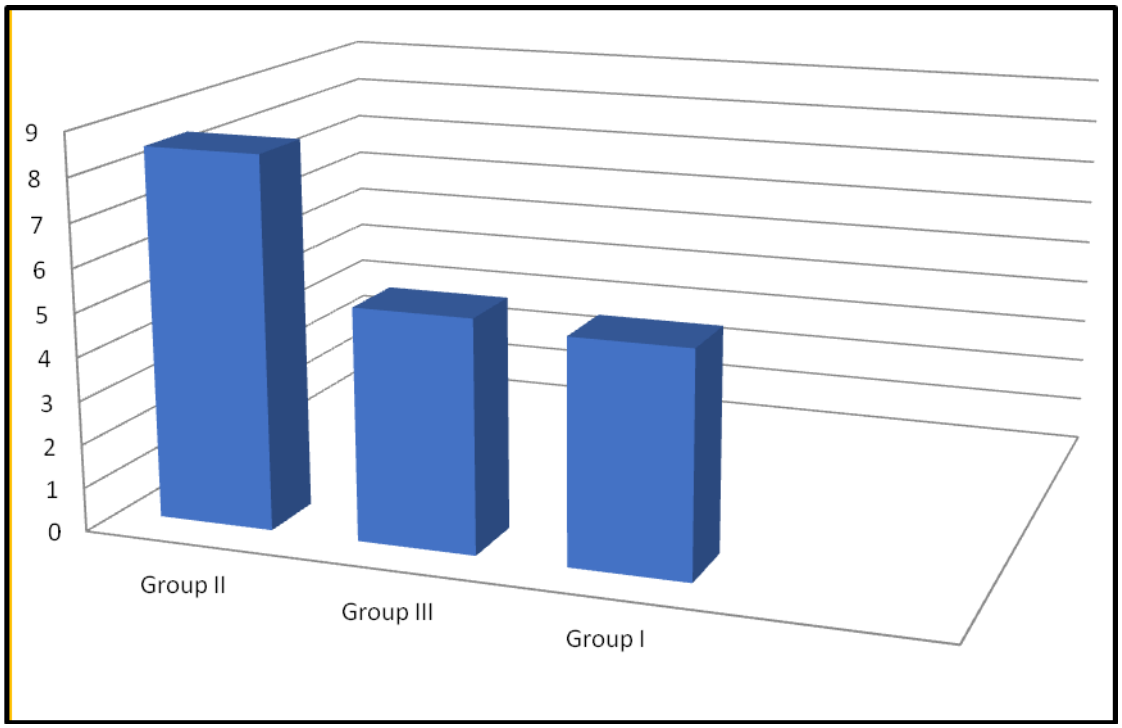

Figure 2. Comparison the concentration of CYP7B1 (U/ml) in tissues of women with breast tumor

Table 3. Correlation of 27-hydroxycholesterol with CYP7B1 in tissue homogenates of women with breast cancer

Value	Group I Benign N=20	Group II Pre N=20	Group III Post N=20
r	-0.135	-0.492	-0.314
P	0.570	0.028	0.178

Discussion

In tissue homogenate samples, in present study the mean and standard deviation of 27-HC in benign, pre and postmenpausal women with breast cancer in ng/l (15.90±4.84, 26.95±5.68, 28.25±7.55 ng/l) respectively. The result of the present study found that 27-HC was significantly elevated in pre and postmenopausal breast cancer group compared with benign and control group at ($p< 0.001$), and this agreed with studies by (Wu et al. [8] Schor [9] and Kimbung et al. [10].

27HC abundance is also predictably elevated in the setting of hypercholesterolemia and with obesity, which is frequently a comorbidity with hypercholesterolemia [11,12].

In women, both dyslipidemia and obesity raise breast cancer risk and severity, with obesity particularly having an adverse impact in postmenopausal women [13-16].

Study by Lee et al showed when applied on animal model enabled us to compare the impact of 27HC with or without hypercholesterolemia. Now found that elevations in 27HC via the deletion of CYP7b1 caused exaggerated atherosclerosis with-out altering lipid status in the setting of normo- and hypercholesterolemia; in addition,

estrogen-related atheroprotection is markedly attenuated [17].

The results of the present study revealed that the mean and standard deviation of CYP7B1 in tissues of benign, pre, postmenopausal women with breast cancer were (8.85±3.96, 5.30±2.36 and 5.19±1.75 U/ml) respectively. These results indicate that CYP7B1 was significantly decreased in postmenopausal breast cancer group compared with benign group (p= 0.001) and was significantly decreased in premenopausal breast cancer group compared with benign group (p= 0.003), and this agreed with studies accomplished by the following researchers Lee et al. [17] and Stiles et al. [18].

CYP7B1 is diminished in breast tumor compared with normal breast tissue. There is more than 7-fold poorer overall survival in women whose tumors display low CYP7B1, compared with women with high tumor CYP7B1 [19]. This may be related to estrogen deprivation because in mice E2 up regulates hepatic CYP7B1 expression in an ERα-dependent manner without impacting CYP27A1 [20].

The conclusion of this study revealed a higher incidence of serum 27-HC and a lower incidence of CYP7B1 found in malignant than benign breast tumors with positive estrogen receptors. These indicate that the presence of 27-HC and deficient of CYP7B1 in sera of patients with breast tumors may be used as a favorable prognostic indicators and diagnostic tools in breast tumors.

Acknowledgments

The authors are grateful to the staff of Chemistry and Biochemistry Department and Breast Examination Unit in the Al-Imamein Al-Kadhimein Medical City for their technical help.

Authors contribution

Dr. Al-Saeed suggests the study; Dr. Nile select the suitable patients and both of them co-writes the manuscript for study. Mohammed collected the blood samples, conducted the necessary analysis of the study, writes the paper and analyzed the results statistically.

Conflict of interest

There was no conflict of interest.

Funding

The research was funded by College of Medicine, Al-Nahrain University.

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doi: http://dx.doi.org/10.1016/S1470-2045(02)00849-5.

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Received Sep. 19th 2017
Accepted Nov. 15th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

Clinical Utility of Urinary Antigen Test and Molecular Method for Detection of Legionella Pneumophila

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Abstract

Background *Legionella pneumophila* (*L. pneumophila*) is gram-negative bacterium, which causes Legionnaires' disease as well as Pontiac fever.

Objective To determine the frequency of *Legionella pneumophila* in pneumonic patients, to determine the clinical utility of diagnosing *Legionella pneumonia* by urinary antigen testing (LPUAT) in terms of sensitivity and specificity, to compares the results obtained from patients by urinary antigen test with q Real Time PCR (RT PCR) using serum samples and to determine the frequency of serogroup 1 and other serogroups of *L. pneumophila*.

Methods A total of 100 pneumonic patients (community acquired pneumonia) were enrolled in this study during a period between October 2016 to April 2017; 92 samples were collected from patients attended and admitted to Al-Imamein Al-Kadhimein Medical City and 8 samples from those in the (Center of Kidney Diseases and Transplantation) in the Medical City of Baghdad. All patients were under therapy with antibiotics. Serum and urine specimens were obtained from all patients; urine samples were processed for urinary antigen test (rapid test). Serum samples were collected and submitted to DNA extraction for detection of *L. pneumophila* mip gene by q RT PCR assay.

Results The percentage of *L. pneumophila* in two hospitals in Bagdad was 30%. Of these 26% was serogroup 1 detected by urinary antigen testing (UAT). In the other hand, 23% of samples were positive by q RT PCR based mip gene, of these 19 % were serogroup 1 and 4% were another serogroup. The sensitivity of UAT is high (P value < 0.001), which means statistically highly significance than q RT PCR.

Conclusion LPUAT is a rapid tool for early diagnosis of Legionella infection, which highlights the need of using this test in hospitals and health institutions and there is a high prevalence of *L. pneumophila* in Iraq that refer to the necessity of considering this microorganism point of view in future studies for detection and treatment in pneumonic patients.

Keywords *L. pneumophila*, mip gene, quantitative real time PCR, urinary antigen.

Citation Gauad SA, Abdulrahman TR, Muhamad AK, Jawad AA, Hassan JS. Clinical utility of urinary antigen test and molecular method for detection of Legionella pneumophila. Iraqi JMS. 2018; 16(2): 207-215. doi: 10.22578/IJMS.16.2.13

List of abbreviations: DFA = direct fluorescent antibody, IHC= immunohistochemistry, LD = Legionnaires' disease, LPUAT = Legionella pneumonia by urinary antigen testing, mip = Macrophage infectivity pointier gene, q RT PCR = quantitative real time polymerase chain reaction, sg1 = serogroup one, TALD = Travel-associated legionnaire's disease, UAT = urinary antigen test

Introduction

Legionnaires' disease (LD) is an acute pneumonia caused by Legionella spp. Legionella is Gram-negative bacteria ubiquitous in both man-made and natural aquatic reservoirs, it is reported that up to 90% of cases of LD is caused by *Legionella pneumophila* [(1)]. Of the 50 species and 72 serogroups belonging to the genus Legionella, *L. pneumophila* serogroup one (sg1) is responsible for at least 80% of human infections [(2)].

Legionellosis has two distinct clinical presentations: Pontiac fever, a self-limited, febrile, flulike illness; and LD, which is an atypical pneumonia [3]. Peoples living in warm climate area are considered as at-risk population due to the direct and prolonged exposure to air-conditioning and air-circulating systems [4].

Risk factors for legionellosis include whirlpool spa exposure, recent overnight travel or plumbing repairs (two weeks prior to onset of symptoms), immunosuppression, alcoholism, diabetes, malignancy, hepatic or renal failure, chronic obstructive lung disease, smoking history, and patient age >50 years [5].

Conventional methods for diagnosis of LD consist of culture, antigen detection in urine (UAT), serological testing, and direct fluorescent antibody (DFA) staining or immunohistochemistry (IHC) [6].

L. pneumophila urinary antigen testing (LPUAT) is a rapid tool for early diagnosis of Legionella infection in urine sample [6]. Real time PCR (RT PCR) a rapid and sensitive method for the detection of LD based on the use of macrophage infectivity potentiator gene (mip gene), which encodes a 24-kDa protein virulence factor [7].

mip gene facilitates the entry of legionellae into amoebae and macrophages. It has sufficient sequence variability between the Legionella species to also afford the specific detection of L. pneumophila by PCR [7].

This study aimed to determine the frequency of *L. pneumophila* in pneumonic patients in addition to determine the clinical utility of diagnosing LPUAT in terms of sensitivity and specificity then compares the results of UAT with q real time PCR using serum samples. In Iran the seroprevalence rate of *L. pneumophila* in admitted patients with pneumonia was 17.3% while in Austria and Trinidad were 0.65% and 31.7% respectively [8].

Methods

Study setting and design

A total of 100 patients with (community acquired pneumonia) were confirmed by chest radiography (CXR) and enrolled in this study during a period between October 2016 to April 2017, 92 serum and urine specimens were obtained from patients admitted to admitted to Al-Imamein Al Kadhimein Medical city and 8 samples from the (Center of Kidney Diseases and Transplantation) in the Medical City of Baghdad.

Clinical inclusion criteria: Patients older than 20 years, Respiratory Care Unite patients and medical word, diabetic, smoking, chronic lung disease, renal transplant, immuno-compromised and cancer patients and those receiving corticosteroid treatment.

Exclusion criteria: patients with atypical pneumonia less than 20 years old.

Urine samples were processed for UAT (rapid test) and DNA was extracted from serum to detect the presence of mip gene of *L. pneumophila* by q RT PCR assay.

Urinary antigen detection test

The presence of *L. pneumophila* sg1 antigens in urine specimens was determined according to manufacture instructions using qualitative immunochromatographic (CerTest; Spain) assay.

DNA extraction

Bacterial DNA was extracted from serum samples using DNA-Sorb-B extraction kit components (Sacace™/Italy).

Real Time PCR Protocol

According to the company and factory instructions, controls were prepared for each quantitative test as follow: 10 µl of DNA-buffer was added to the tube labeled amplification negative control; 10 µl of QS1, QS2 and QS3 were added to the tubes labeled QS1, QS2, QS3 respectively. A required quantity of PCR-mix-1 reaction tubes were prepared for samples and controls, 7 µl of PCR-mix-2 was added on the wax surface, 10 µl of extracted DNA then added to appropriate tube. RT PCR was performed on Rotor-Gene Q, (Qiagen Germany) and DNA was amplified according in

the thermal cycler for thermal profile showed in table 1.
The analysis results are considered valid, only if the control samples of the quantitative test results comply the following, table 2.

Table 1. *Legionella pneumophila* q RT PCR amplification profile

Step	Rotor-type Instruments[1]			Plate type Instruments[2]		
	Temperature °C	Time	Repeats	Temperature °C	Time	Repeats
1	95	15 min	1	95	15 min	1
2	95 60 72	5 s 20 s 15 s	5	95 60 72	5 s 20 s 15 s	5
3	95 56 72	5 s 20 s fluorescent signal detection 15 s	40	95 56 72	5 s 30 s fluorescent signal detection 15 s	40

Table 2. *Legionella pneumophila* q RT PCR amplification profile

Control	Stage for control	Ct channel FAM/Green	Ct channel JOE/Yellow	Interpretation
C-	DNA isolation	Pos	Neg	OK
C+	DNA isolation	Pos	Pos	OK
DNA buffer	Amplification	Neg	Neg	OK
QS1	Amplification	Pos	Pos	OK
QS2	Amplification	Pos	Pos	OK
QS3	Amplification	Pos	Pos	OK

***Legionella pneumophila* DNA quantification**
The concentration of *L. pneumophila* DNA in control and samples were calculated according to the following equation:
C L.pn. DNA (cop/L) = K L.pn. DNA/KIC x CIC x 2

- C L.pn. DNA (cop/L) – quantity of *L. pneumophila* DNA copies in 1 L of sample
- K L.pn. DNA (cop/ml) – calculated quantity of *L. pneumophila* DNA copies in 1 ml of sample; KIC (cop/ml) - calculated quantity of IC DNA copies in 1 ml of sample
- CIC (cop/ml) - quantity of IC DNA copies in 1 ml of IC according to Data Card
- 2 – adjustment for sample filtration

1. The calibration curve correlation coefficient R2 must be more than 0.97
2. The Efficiency value must be in range: 0.85 – 1.15

Questionnaire
All participants were surveyed using a standard questionnaire, whereby the following information was collected: (age, gender, work); environmental risk factors (residence, travel and bad water supplies); epidemiological risk factors (smoking, diabetes, chronic obstructive pulmonary disease (COPD), asthma, hepatic or renal failure, kidney transplant, malignancy)

and serum sodium electrolyte as laboratory finding.

Statistical Analysis

The statistical analysis of this prospective study performed with the statistical package for social sciences (SPSS) 21.0 Software and Microsoft Excel 2013. Numerical data were described as mean, standard deviation and median with 95% confidence intervals. Chi-square test or Fisher exact test was used to describe the association between variables.

Results

In this study, the percentage of *L. pneumophila* in two hospitals in Baghdad was 30 (30%) by both UAT and q RT PCR. Of these, 26 (26%) was sg1 detected by UAT. In the other hand, 23 (23%) were positive by q RT- PCR based mip gene, 19 (82.6) out of 23 patients were positive for mip gene-based PCR and UAT, which considered to be true positive for sg1 while only 4% were another serogroup. The sensitivity (95%CI) of UAT is 82.61 % and Specificity (95%CI) 90.91 % with (P value < 0.001), which means statistically highly significance than q RT PCR, (table 3).

The mean of mip gene was 8.76 copies/ml for pneumonic patient with LD. Minimum copy was 0 and maximum 68.35 copies/ml. figure (1).

Table 3. Comparisons of q RT PCR in serum samples versus UAT in urine

		q RT PCR		Total
		Positive	**Negative**	
UAT	Positive	19 (82.6%)	7 (9.1%)	26 (26.0%)
	Negative	4 (17.4%)	70 (90.9%)	74 (74.0%)
Total		23	77	100
P value		<0.001**		
Sensitivity (95%CI)		82.61 (62.86 – 93.02)		
Specificity (95%CI)		90.91 (82.4 – 95.53)		
Positive Predictive Value (95%CI)		73.08 (53.92 – 86.3)		
Negative Predictive Value (95%CI)		94.59 (86.91 – 97.88)		

**: Highly statistical significance (p≤0.001)

LD according to the age group.

In the current study, the minimum age of enrolled was20 years while the maximum age was 90 years. Their mean age was 60.12 years and median was 63.

The age group was ranged between (< 30 - ≥ 80) years. The highest age group with positive results with UAT was (60-69) years and the higher incidence of LD is between 50 and 79 years. The distribution of LD according to the age group by urinary antigen test compared with q RT PCR showed in table 4.

Subjects demographic according to gender

This study enrolled 100 pneumonic patients with atypical pneumonia. Among these; 66 (66%) were females, and 34(34%) were males; male to female ratio was 1:1.9. The comparison between q RT PCR and UAT according to gender showed in table 5.

Residence, travel and water associations with LD

The current study has evaluated the frequency of LD according to the residence. The result showed that, higher frequency of LD in rural 10 (28.57%) than in urban 16 (24.62%). Statically non-significance (p>0.05) association between residence and infection with LD.

Both of travel history and bad water system were risk factors for infection. 10/30 (33.33%) of patients had travel history before 2 weeks

from onset of the symptoms of LD. Bad water system was highly statistical significance (p≤0.001) comparable with q RT PCR, which also showed statistical significance (p=0.012), 12/31, 38.71%).

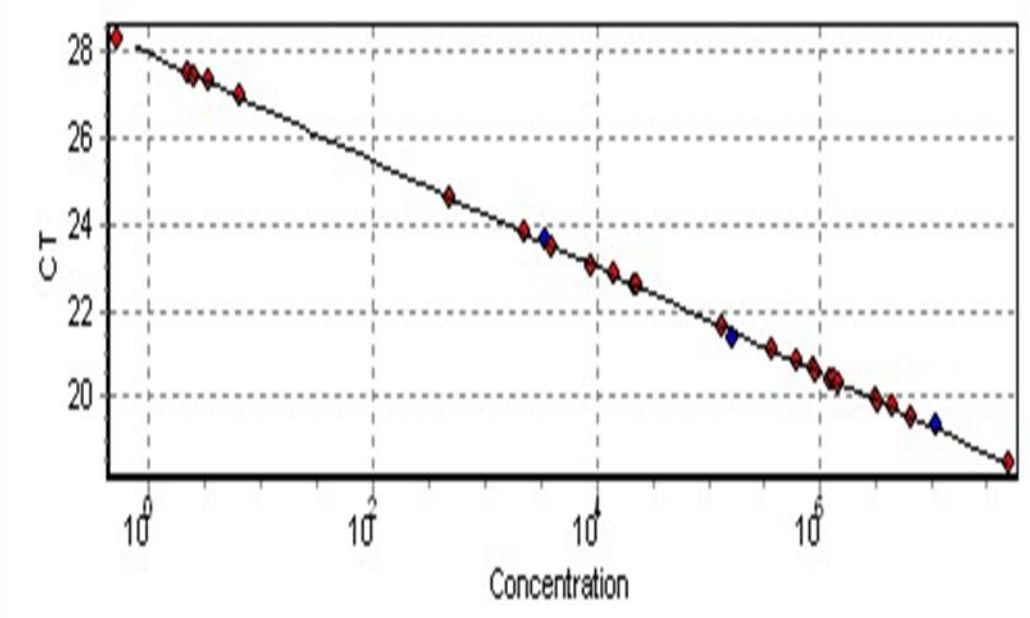

Figure 1. The standard curve of q RT-PCR *L. pneumophila* mip gene including standards represented by 3 blue squares and 23 positive cases represented by red squares

Table 4. Distribution of LD according to the age group in comparable between UAT and q RT PCR

Age group	UAT		q RT PCR	
	Positive No. (%)	Negative No. (%)	Positive No. (%)	Negative No. (%)
< 30 years	1 (16.67)	5 (83.33)	1 (16.67)	5 (83.33)
30-39 years	3 (50.0)	3 (50.0)	3 (50.0)	3 (50.0)
40-49 years	2 (22.22)	7 (77.78)	2 (22.22)	7 (77.78)
50-59 years	6 (40.0)	9 (60.0)	4 (26.67)	11 (73.33)
60-69 years	7 (20.59)	27 (79.41)	8 (23.53)	26 (76.47)
70-79 years	6 (28.57)	15 (71.43)	4 (19.05)	17 (80.95)
≥ 80 years	1 (11.11)	8 (88.89)	1 (11.11)	8 (88.89)
Total (%)	26	74	23	77
P value	0.507 NS		0.726 NS	

NS: none statistical significance (p>0.05)

Table 5. Comparison between UAT and q RT PCR according to the gender

Gender	U Ag		q PCR		Total (%)
	Positive No. (%)	Negative No. (%)	Positive No. (%)	Negative No. (%)	
Male	7 (20.59)	27 (79.41)	6 (17.65)	28 (82.35)	34 (34.0)
Female	19 (28.78)	47 (71.21)	17 (25.76)	49 (74.24)	66 (66.0)
Total (%)	26(26)	74(74)	23(23)	77(77)	100(100)
P value	0.376 NS		0.361 NS		

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Both of travel history and bad water system were risk factors for infection. 10/30 (33.33%) of patients had travel history before 2 weeks from onset of the symptoms of LD. Bad water system was highly statistical significance (p≤0.001) comparable with q RT PCR, which also showed statistical significance (p=0.012), 12/31, 38.71%).

Risk factors for legionnaire's disease

The most important risk factors for LD are diabetes mellitus (DM), smoking, old age, COPD, asthma, renal failure, alcoholism, kidney transplant and malignancy.

According to the risk factors, this study showed that 13 (25.49%) DM patients, 9 (25%) smokers, 5 (33.33%) COPD, 1 (12.5%) kidney transplant and 1 (10%) as cancer patients. All were developed LD according to UAT and q RT PCR.

Serum electrolyte in LD

Serum electrolyte especially sodium (Na) may considered as an important laboratory investigation in pneumonic patients suspected LD. Normal range of sodium from (135-145) mmol/l. In this study, the percentage of hyponatremia were the same as 9/28 (32.14%) in LD patients by each UAT and q RT PCR testes. Statistically, this study showed non-significant association (p>0.05) between hyponatremia and LD.

Discussion

In this study, by using UAT and q RT PCR based mip gene, the prevalence of LD in two hospitals in Baghdad were 30%. In Iran the seroprevelance of LD was 17.3% [8]. The spread of this disease in Iraq is due to bad water system. The percentage of sg1 of *L. pneumophila* detected by both UAT and q RT PCR was 82.6%, which is considered true positive and the predominant serogroup responsible for LD in Iraq. Because of those patients have antigen shedding in their urine and gene in their serum is considered to have severe pneumonia. Chen et al. (2015) demonstrated that the percentage of L. pneumophila detected by those tests were 32% [9]. Legionella DNA can be detected in serum within the first two weeks after the onset of symptoms [10]. In the other hand, 7 out of 77 (9.1%) patients tested negative in PCR but positive in UAT.

This result may be due to secretion of antigen is early within 2-3 days after infection with *Legionella spp*. and lasting for months and even for a year as well as the UAG assay may remain positive for up to a year after treatment. While because of low bacterial DNA load in patient's serum or loosing during extraction and storage, PCR assay become negative soon after initiation of therapy [11].

This study reported that four (17.4%) samples give positive by q RT PCR but negative by UAT, which considered as other serogroups or may be the concentration of antigen in urine is low that Certest determine the detection limit

value is 6.25 ng/ml. This result agreed with study showed that approximately 8% of patients with LD do not excrete antigen in their urine [12].

The UAT had higher sensitivity (P value< 0.001), which means statistically highly significance. Also, the present study showed that UAT highly specific than q RT PCR based mip gene. In France, Maurin et al. (2010) reported that Legionella UAT was positive in (100%) of patients and the q RT PCR was displayed a sensitivity 80.4% [13]. Avni et al. (2016) disagree with current results where they noticed that the using of PCR in respiratory samples was much better and higher significance (p= 0.001) than that of UAT [14]. These differences in result because of differences in sample type, which is include sputum in addition to serum.

In this study, the mean and standard deviation of copy/ml of mip gene for *L. pneumophila* DNA in serum of patients were 8.76 and 16.65 respectively. Bacterial DNA mip gene load were ranged from (0-68.35) copy/ml. This result nearly agreed with mean result but disagree with bacterial DNA load of Maurin et al (2010), they determined the mean were 6.48 ± 7.29 and q RT-PCR mip gene ranged from 1.9–8.11 log10 DNA copies/mL which considered high bacterial loads in LRT samples. According to the current result, such patients required prolonged hospitalization in an intensive care unit [13].

The mip gene much higher in serum sample so it is more suitable than sputum for identification of *L. pneumophila* because it has been shown that fewer than half of the Legionnaires' disease patients produce sputum [15] or scanty sputum in atypical pneumonia was release contrasted with large amount of blood obtained from patients. The age group in the current study ranged between (20-90 years) with mean 60.12 and median 63. Study published in Netherlands in 2008 showed that the mean age of man was 57 years and women were 60 years and the total mean for both were 58.5 while the median age of patients confirmed LD was 54 years [16]. This variation with the current study may be due to the differences in number of samples, in addition to different target gene used in RT PCR assay, which is 23S-5S rRNA intragenic spacer region in the comparative study [17].

All age groups were showed positive UAT. The higher incidence of LD is between 30 and 79 years. Touray et al. (2014) reported that there was a higher occurrence among middle-aged participants between 45 and 69 years compared to older and younger participants, which showed low or no infection [18]. The current result because of these ages are more associated with environmental, clinical risk factors and most common out door.

This study enrolled 100 pneumonic patients, (66%) were females, and (34%) were males. Female infected more than male. The female/male ratio was 1.9:1. A study fulfilled in the European working group on legionella infections criteria of LD demonstrated that (61%) were males and (39%) were female and the females / males ratio was 1: 1.4 [16]. While a study achieved in Bulgaria, 2015 showed that female 22/54 more than male 5/12 and the female/male ratio was 4.5:1 [19]. This limitation because of most of the male prefers to treat out of the hospital and the differences in the techniques used which is based neither antigen nor gene but antibody IgG, IgM in the comparative studies.

The result showed that, higher frequency of LD in rural (28.57%) than in urban (24.62%). This result disagreed with study conducted in Iran showed that LD occurs in city more than in village [8]. The explanation of the current result that most of Iraqi rural residence depends on rivers and bad tank water for bathing and house work occupancies.

In this study, 33.33% of cases were reported as travel-associated whereas 22.86% were non-travel which is statistically non-significant for LD in Iraq. Four countries (France, Italy, Netherlands and UK) reported that 72% of all Travel-associated legionnaire's disease (TALD) cases were a risk factor for LD because of tourism [20].

Water system is very important risk factor for infection because *L. pneumophila* is aquatic pathogen. The current study showed highly statistically significant of bad water system as a source for infection. In Iraq, a study conducted on water of Basrah city revealed that the

percentage of *L. pneumophila* in water were 71.2% [21]. Other study in Najaf city reported that *L. pneumophila* isolated from water of dental units, which has been confirmed as an etiological agent for hospital-acquired respiratory tract infection [22]. This may be due to variety of health technologies applied in medical and dental practice associated with formation of fine water droplets/aerosols. This presents the potential risk of both the patients and the personnel acquiring Legionnaires' disease [19].

The risk factors for LD include cardiopulmonary disease, cigarette smoking, age >50 years, diabetes, malignancy, and immunosuppressive state including glucocorticoid use [17]. Accurate existing study detected that DM (25.49%), smoking (25%), COPD (33.33%), kidney transplantation (12.5%) and cancer (10%) were non-significant. Predisposing factors for LD asthma (16.67%) and renal failure (0), were significantly not concedes as a risk factors associated with LD. Cargnelli et al. (2016) disagree with current results, they proved the high prevalence of cigarette smoking (71%) with LD, then diabetes (29%), chronic renal insufficiency (29%), chronic lung disease (14%), immunosuppression (14%), which have the same propensity [17].

This study includes (10) patients with hematological and solid organ malignancy. Results revealed that only one patient 1 (10%) was positive for UAT and q RT PCR mip gene in serum samples, this result disagree with a study done in Iran in 2014, which detected that percentage of mip gene of *L. pneumophila* was (47.5%) in serum samples, this difference may be due to the differences in sample size [23].

The most common laboratory abnormalities included hyponatremia <134 in LD. In this study, (32.14%) were hyponatremic patients infected with *L. pneumophila* and detected by UAT and q RT PCR respectively. Statistically, it was no significance (p >0.05) because of the result recorded at time of admission. Fiumefreddo et al. (2009) showed hyponatremia in (46%) was significantly association with LD patients [24]. Hyponatremia is a non-specific finding among patients with pneumonia, and it has not been consistently shown to have an association with L. pneumophila when compared to those who had pneumonia due to other causes [25].

This study concluded that high frequency of LD caused by *L. pneumophila* sg1 was observed in Iraqi patients, UAT is a rapid, highly specific and sensitive as a detection tool of LD in pneumonic patients and q RT PCR is a rapid and sensitive tool as a molecular detection of different serogroups of *L. pneumophila*.

Recommendations

This study recommended to prove the other serogroups of these bacteria in Iraqi patients, rapid UAT is reliable for the diagnosis of LD in Iraqi hospitals and serum samples should be used in the diagnosis of LD by q RT PCR.

Acknowledgments

Authors would like to acknowledge Al-Imamein Al-Kadhimein Medical city and Center of Kidney Diseases and Transplantation in the Medical City of Baghdad for their cooperation in accomplishing this study.

Authors contribution

Gauad: collection of specimens, DNA extraction, RT PCR and writing of the manuscript. Dr. Abdulrahman: Supervision and performance of DNA extraction and RT PCR run, writing of the manuscript. Dr. Muhamad: Consultant, collection of data and specimen. Jawad: DNA extraction, Gel electrophoresis, DNA purity and concentration measurements. Dr. Hassan: RT PCR interpretation.

Conflict of interest

Authors declare no conflict of interest.

Funding

There is no funding source for this research.

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Received Nov. 2nd 2017
Accepted Jan. 21st 2018

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

The Effect of Ginger Extracts on Bacterial Isolates from Patients with Suppurative Otitis Media and Externa: In Vitro Study

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Abstract

Background Otitis media is a group of complex inflammatory disorders affecting the middle ear, which can be acute or chronic. Otitis externa is an inflammation on the skin of the external auditory canal usually associated with secondary bacterial and/or fungal infection of macerated skin and subcutaneous cellular tissue.

Objective To determine the effect of ginger extracts on bacterial isolates from patients suffering from otitis.

Methods Two hundred patients suffering from suppurative otitis media and externa, who were attending to ENT Department, Al-Imamein Al-Kadhimein Medical City. The powder of ginger rhizomes was soaked with the solvent left in a shaking water bath at 40 °C for 24 hours and then filtered using Whatman No.1 filter paper. Each extract was concentrated using a rotary evaporator with vacuum to get the final crude extract after the procedure of ginger extract was done. The activity of this extract was tested against bacterial isolates from patients with otitis.

Results The results of this study revealed that otitis externa was less common infection than the other types of otitis 29 (14.5%), while acute otitis media comprised 96 (48.0%), and chronic suppurative otitis media consisted of 75 (37.5%). The most common bacterial isolates that caused otitis were *Pseudomonas spp*. followed by *Staphylococcus spp*. The results showed that, there are different effects among (chloroform, methanol, and aqueous) extracts of ginger against pathogenic bacteria.

Conclusion Generally, ginger extracts had a good effect on isolated bacteria. Chloroform extract was the most effective one, followed by methanol extract, while aqueous extract showed the least activity in this regard.

Keywords Otitis externa, otitis media, ginger extraction

Citation Mohammed MM, Al-Attraqchi AAF, Al-Hasseni JMK, Sahib HB. The effect of ginger extracts on bacterial isolates from patients with suppurative otitis media and externa: in vitro study. Iraqi JMS. 2018; 16(2): 216-222. doi: 10.22578/IJMS.16.2.14

List of abbreviations: AOM = Acute otitis media, CSOM = Chronic suppurative otitis media, DMSO = Dimethyl sulfoxide, OE = Otitis externa, OM = Otitis media

Introduction

Ear infection is one of the most common diseases occurring throughout the world. Different etiological agents are responsible for ear infections [(1)]. Ear infections are three types; the first type is otitis externa (OE) that intricate the outer ear and ear canal. In OE, the ear seems to be painful when touch and tugging, it is also called swimmers ear. Otitis media (OM) is the second type in ear infection; in which the ear is contaminated with fluid behind the ear drum, in the habitually air filled in space of middle ear. The infection of middle ear is very common in childhood. In some cases, this condition

requires a surgical procedure called myringotomy and tube insertion. The third type is otitis interna, which involves the inner ear including sensory organs [2].

Ginger the rhizome of Zingiber officinale (family Zingiberaceae) is globally one of the most common spices and has been used as a culinary agent for over 1,000 years in Asia. The Zingiberaceous plants are characterized by their tuberous or non-tuberous rhizomes, which have strong aromatic and medicinal properties [3]. Recently, in Iraq, ginger was experimentally cultivated during one year with the attempt to grow and distribute the plant [4]. The main active components in ginger from its phenolic substance are gingerol and shogaol, while zingiberene is obtained from ginger oil [5]. Ginger has a wide range of action on the human body and has been found to be effective in the treatment of cataract, heart disease, migraines, struck amenorrhea, athletes foot, bursitis, chronic fatigue, cold, flu, coughs, depression, dizziness, fever, kidney stones [6]. Powdered derived ginger root is made into capsules and sold in pharmacies for medical use [7].

This study was carried out to determine the effect of ginger extracts on bacterial isolates from patients suffering from otitis.

Methods

Samples collection

Two hundred patients suffering from suppurative otitis media and externa who were attending ENT department, Al-Imamein Al-Kadhimein Medical City were enrolled in this study from November 2016 to the end of April 2017. Ear swabs specimens were collected from each patient in sterile swabs. All specimens were transported to the laboratory for processing and investigations at the same day. Samples processing occurred according to standard operating procedures which included: Cultivation on culture plates (blood, chocolate, MacConkey) agar for the isolation of bacterial pathogens. Gram staining for bacteria, biochemical tests as diagnostic tools for bacterial pathogens.

Preparation of ginger extract

Five hundred grams of dried rhizomes of ginger (Zingiber officinale) was purchased from local market in Baghdad, Iraq and authentication was done in Department of Pharmacognosy/ College of Pharmacy, AL-Mustansyriah University. The dried rhizomes were grind into very fine powder using a heavy-duty grinder. The powder of ginger rhizomes was divided into 12 portions then each portion extracted sequentially with three solvents beginning with the non-polar solvent and ascending to the most polar solvent (chloroform, methanol and distilled water, respectively) with a ratio of 1:7 W/V (30 gm of powder/ 210 ml of solvent); the extraction repeated twice for each solvent and the process of extraction used was the cold method, i.e., maceration. The powder of ginger rhizomes was soaked with the solvent and left in a shaking water bath at 40 °C for 24 hours and then filtered using Whatman No.1 filter paper to take the clear extract. Each extract was concentrated using a rotary evaporator with vacuum to get the final crude extract. The extract powder was kept in sterile bottles, labeled accordingly and stored in the refrigerator [8]. One gm of the crude extracts of (chloroform, methanol and aqueous) ginger extracts were dissolved in 10 ml DMSO (Dimethyl sulfoxide) to became the concentration 100mg/ml as stock solutions.

Antibacterial test of extracts using agar well diffusion method

The antibacterial activity of different extracts against bacteria was evaluated by using agar well diffusion method [9]. Isolated colonies were selected from nutrient agar plates culture and transferred to 3 ml of 0.85% normal saline to a density equivalent to the turbidity of the (0.5) McFarland standards. A sterile cotton swab was dipped into the bacterial suspension; excess fluid inoculum from the swab was removed by pressing the swab firmly on the side wall of the tube above the fluid level. Streaking of the inoculum was done over the entire sterile agar surface. This procedure was repeated by streaking 2 more times, rotating the plate approximately 60º each time to

ensure an even distribution of inoculum as a final step, the rim of the agar was swabbed. The plates were left at room temperature for 15 minutes to allow for any excess surface moisture to be absorbed. Wells of 5 mm was punctured with the help of a sterilized cork-porer into the pre-solidified Mueller Hinton agar plates containing the test organism. Using the micropipette, 20 µl of each extract (chloroform, methanol, and aqueous) was poured into the different wells of the inoculated plates. DMSO well was used as a negative control and bacterial plates were incubated at 37 °C for 24 hrs. The diameters of inhibition zones were measured, later on [10].

Statistical analysis

Data of this study were entered using EPI INFO7 Windows Version and analyzed by using statistical package for social sciences (SPSS) version 20. Descriptive statistics were presented as frequencies, percentage (%), means and standard deviation (SD). Chi square test was used to estimate the association between two categorical variables. Level of significance of ≤ 0.05 was considered as significant. Analysis of variance (ANOVA) used for comparison among more than two groups. Paired samples T test used for comparison between two groups.

Results

Gender and age distribution of patients with ear infection

A total of 200 patients suffering from otitis were enrolled in this study. The mean age of patients was (30.04) with standard deviation (20.41), ranged from 7 days - 80 years old. It was found that a half of patients were males as 109 (54.5%) and 91 (45.5%) were females. the first group which is the largest group included 50 patients (25%) when the age factor was (≤10) years old, while the seventh which is a last group of (>60) years included 16 patients (8%) (Table 1).

Table 1. Classification of patients with otitis regarding age groups

Age group	NO.	Percentage %
<10 years	50	25.0
11-12 years	23	11.5
21-30 years	35	17.5
31-40 years	25	12.5
41-50 years	30	15.0
51-60 ears	21	10.5
>60 years	16	8.0

Bacterial isolates from patients with otitis

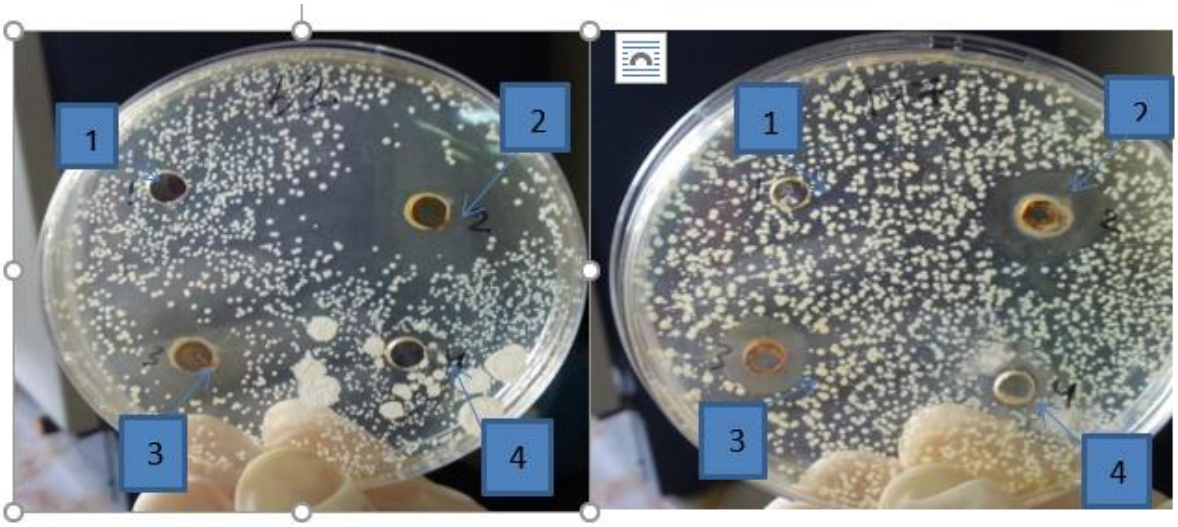
Table (2) shows the most bacteria isolates from patients suffering from ear discharge were *Pseudomonas spp.*, followed by *Staphylococcus aureus*. In this study also isolated *Ewingella americana* and *Pasturella pneumoniae* this first study isolate these bacteria.

Antibacterial activity of (chloroform, methanol, and aqueous) ginger extracts:

The antibacterial activity of ginger extract was prepared with different solvents (aqueous, methanol, and chloroform) tested against pathogenic bacteria species. Table (3) demonstrates the most effect of extract was chloroform ginger extract on the most bacterial isolate and less or no effect was aqueous ginger extract.

Table 2. Percentages of bacterial isolated from patients with otitis

Bacterial culture	Otitis media		Otitis Externa	Total
	AOM	CSOM		
Citrobacter spp.	1 (1.0%)	0 (0.0%)	0 (0.0%)	1 (0.5%)
E. coli	4 (4.2%)	5 (6.7%)	3 (10.3%)	12 (6.0%)
Enterobacter spp.	1 (1.0%)	1 (1.3%)	0 (0.0%)	2 (1.0%)
Ewingella americana	1 (1.0%)	0 (0.0%)	0 (0.0%)	1 (0.5%)
Klebsiella spp.	4 (4.2%)	3 (4.0%)	2 (6.9%)	9 (4.5%)
Morganella morganii	1 (1.0%)	0 (0.0%)	0 (0.0%)	1 (.5%)
Pasturella pneumoniae	4 (4.2%)	2 (2.7%)	2 (6.9%)	8 (4.0%)
Proteus spp.	5 (5.2%)	1 (1.3%)	0 (0.0%)	6 (3.0%)
Pseudomonas spp.	20 (20.8%)	29 (38.7%)	3 (10.3%)	52 (26.0%)
Serratia spp.	2 (2.1%)	1 (1.3%)	0 (0.0%)	3 (1.5%)
Staphylococcus aureus	17 (17.7%)	9 (12.0%)	3 (10.3%)	29 (14.5%)
Staphylococcus epidermidis	5 (5.2%)	2 (2.7%)	4 (13.8%)	11 (5.5%)
Staphylococcus capitis	5 (5.2%)	3 (4.0%)	2 (6.9%)	10 (5.0%)
Sterptococcus viridans	3 (3.1%)	1 (1.3%)	0 (0.0%)	4 (2.0%)
Streptococcus pneumoniae	2 (2.1%)	0 (0.0%)	1 (3.4%)	3 (1.5%)
Streptococcus pyogenes	1 (1.0%)	0 (0.0%)	0 (0.0%)	1 (0.5%)
No growth	20 (20.8%)	18 (24.0%)	9 (31.0%)	47 (23.5%)
Total	96 (100%)	75 (100%)	29 (100%)	200 (100%)



(A): Staphylococcus aureus. (B): Pseudomonas spp.

Figure 1. Antibacterial effect of aqueous extract of ginger, chloroform, and methanol extract. 1. Negative control (DMSO), 2. Chloroform extract, 3. Methanol extracts, 4. Aqueous extract

Table 3. The antibacterial activity of ginger extracts against pathogenic bacteria isolated from ear discharge

Bacterial isolate	Means of diameters of inhibition Zones (mm)			
	Chloroform	Methanol	Aqueous	Negative control
Citrobacter spp.	16	16	8	0
E. coli	15.92±4.5	11.25±4.79	6.25±3.05	0
Enterobacter spp.	9±1.41	8±1.41	0	0
Klebsiella spp.	12.56±4.16	12.56±4.56	5.22±3.96	0
Morganella morganii	12	13	0	0
Pasturella pneumoniae	11.88±5.96	8±6.99	7.38±4.81	0
Proteus spp.	13.33±2.42	7.17±3.6	8.33±1.37	0
Pseudomonas spp.	14.92±3.8	11.1±3.71	8.44±3.1	0
Serratia spp.	11.33±3.06	9.67±2.52	5.67±4.93	0
Staphylococcus aureus	14.45±5.88	11.66±3.88	3.07±4.07	0
Staphylococcus epidermidis	12.27±5.06	11±6.78	4.18±4.87	0
Staphylococcus capitis	15.8±2.94	13.8±3.71	5.4±4.99	0
Streptococcus viridans	13.75±2.63	11±3.37	4.5±5.26	0
Streptococcus pneumoniae	15.33±3.21	13.67±2.08	2.67±4.62	0

Discussion

In the present study, results indicated that males expose for OM more than females may be because males are more exposed to different conditions in work such as dust, humidity, and may be more actively involved in outdoor activities, hence to be more exposed to contaminated environment. On the other hand, females wearing of scarfs may be considered an important factor to decrease infection. This result agrees with other obtained by Almamory et al. (2014) [11] who mentioned that the rate of ear infection in males was higher than females, while disagree with that obtained by Khammas et al. (2010) [12] who mentioned that the rate of ear infection in females was higher than males. In the current study, all age groups could develop otitis with significant differences, the highest infection rate was so cases occurred in the age group (≤10) years were (50) cases, the plausible explanation of these result that children and infants may have low resistance to infection, and because of relative short and straight Eustachian tube [13], the lower immune system of children compared to adults, and the fact that bacteria adhere better to epithelial cells of children than adults [14]. This result agrees with other study done by Jayakar et al. (2014) [15] who proved that there were significant differences in the distribution of age in ear infection.

Bacterial isolates from patients with otitis

The current study revealed that the percentage of AOM was (48.0%), while CSOM was (37.5%), with lower rate of infection with OE, which was (14.5%). This study agrees with other done by Ayub et al. (2015) [2] who found that OM was the most frequent type of ear infection. The number of bacterial isolates was (153), it was found that the highest common bacterial isolates among patients with otitis was *Pseudomonas spp*. as 52 (26%), followed by *Staphylococcus aureus* as 29 (14.5%). A study done by Ibrahim (2013) [17] and AL-Ataar (2015) [18] who proved that *Pseudomonas spp.* and *Staphylococcus aureus* were the most commonly cause of CSOM and in another study done by Jaafar et al. (2014) [19] *Proteus spp.* in CSOM was more frequent than *Staphylococcus aureus. Streptococcus pneumoniae* and *Streptococcus pyogenes* isolated only from AOM cases in this study, which agrees with other study done by Almamory et al. (2014) [11]. Concerning *Morganella morganii* these

bacteria was isolated from AOM only, while in another study done by Chirwa et al. (2015) [20] these bacteria were isolated from CSOM.

Antibacterial activity of (chloroform, methanol, and aqueous) ginger extracts

The average diameter of inhibition zones by chloroform and methanol extracts against *Citrobacter spp*. (16 mm), while aqueous extract was (8 mm). These results disagree with Ogbonna et al. (2014) [21] who found that aqueous extract of ginger was found to have potent antimicrobial activity against *Citrobacter spp.* with mean diameter as (38 mm). This discrepancy of results might be due to the presence of different phyto-compounds, which may include terpenoides, alkaloids and phenolic compounds that may interfere with the results [22]. In *E. coli*, it was shown that chloroform extract was gave the largest mean of diameter of inhibition zones as (15.92±4.5 mm), this difference in responses might be due to the chemical compound found in chloroform that dissolved some compound that found in ginger. This study disagrees with Yassen and Ibrahim (2016) [23] who mentioned the mean of diameter of inhibition zones of methanol extracts were (15 mm). Methanol extract of ginger showed a stronger effect on *Morganella morganii* as (13 mm) than chloroform extract as (12 mm) with no effect of aqueous extract, there were no previous compatible studies to compare with these results. Chloroform extract gave the largest mean of diameter of inhibition zones in Pseudomonas spp. as (14.92±3.8 mm), ginger known to contain resins and volatile oils, which may be responsible for its potent antimicrobial activity against for all bacteria [26], this result disagrees with another by Abdulzahra and Mohammed (2014) [24] who found that chloroform extract has no effect at all against *Pseudomonas spp*. In case of *Staphylococcus aureus*, the diameters mean of chloroform extract gave the largest inhibition zone, followed by methanol extract, while aqueous extract of ginger gave the lesser inhibition zones chloroform and methanol ginger extract will have an effect on external membrane of Gram-negative, because bacteria renders then to be highly hydrophilic surfaces, whereas the negative charge of the surface of the Gram-positive wall may reduce their resistance to antibacterial compounds [25]. This study agrees with Ahmed et al. (2012) [25] who mentioned that chloroform and methanol extracts, were exhibited highly antimicrobial activity against Staphylococcus aureus. Ginger rhizome has several components which have antibacterial and antifungal effects, gingerol and shagol identified as more active agents of ginger [26].

From this study it is concluded that the most frequent of bacteria was isolated from patients with otitis was *Pseudomonas spp.* followed by *Staphylococcus aureus*. The percentage of AOM was the highest among the other types of infection and the lower one was OE. The age group ≤10 years old were the highest among other groups in developing otitis. Chloroform extract of ginger was the most effective as antibacterial followed by methanol extract, while aqueous extract was the weaker.

From this study authors recommended further study needed to investigate the bioactive materials by column chromatography and to identify the concentration of active constituent by HLPC. Also, further study needed to explain the mechanism of action.

Acknowledgments

The authors are grateful to all staff members of the Medical Microbiology Department, College of Medicine, Al-Nahrain University for their help and cooperation. Special thanks to the staff member of ENT Department in Al-Imamein Al-Kadhimein Medical city for their kind assistance in sample collection

Authors contribution

Mohammed: Msc student, Dr. Al-Attraqchi: supervision, Dr. Al-Hasseni: sample collection, Dr. Sahib: consultation of the pharmaceutical part of research.

Conflict of interest

The authors declare that they have no competing interests.

Funding

Self-funding

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E-mail: mahamicrobiology@yahoo.com
Received Dec. 3rd 2017
Accepted Jan. 21st 2018

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(2)

Identification of Common Aerobic Bacterial Isolates among Conjunctivitis in Sulaymaniyah Province / Iraq

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Abstract

Background Bacterial conjunctivitis is a microbial infection of the mucous membrane of the conjunctiva of eye that occurs both in adults and children. It is produced by an array of microorganisms that is frequently isolated from the conjunctiva of healthy subjects. The normal conjunctival flora represents both bacterial colonization and transient or recurring bacterial contamination. Coagulase-negative *Staphylococci* and *Corynebacteria* are frequently present on the healthy conjunctiva, but more traditionally pathogenic organisms, such as coagulase-positive *Staphylococci*, *Streptococci, Haemophilus species, Moraxellae* and Gram negative coliform rods occasionally isolated from normal, non-inflamed eyes.

Objective To determine etiological agents of aerobic bacterial conjunctivitis and the prevalence of each bacteria and its relation with the conjunctival discharge.

Methods This study conducted on 100 conjunctival samples from patients with conjunctivitis, and 50 samples from healthy normal persons. All samples were cultured on enriched media like blood agar and chocolate agar, anti-microbial susceptibility of all 100 samples from patients were analyzed.

Results The prevalence of aerobic bacterial conjunctivitis is 53 %with predominance age of 1-10 years. The most common bacterial isolates were *Staphylococcus epidermidis* followed by *Streptococcus pneumonia*. Most of the isolates were sensitive to cefotaxime and ofloxacillin, while different result was detected regarding resistance of each isolated bacteria.

Conclusion Aerobic bacterial isolates can be detected both in conjunctivitis and normal healthy individual with similarity regarding the type of bacteria that affect all age group.

Keywords Conjunctivitis, commensal flora, conjunctival discharge

Citation Anoar KA, HassanTM, Majid BT. Identification of common aerobic bacterial isolates among conjunctivitis in Sulaymaniyah Province / Iraq. Iraqi JMS. 2018; 16(2): 223-229. doi: 10.22578/IJMS.16.2.15

List of abbreviations: API = Analytic profile index, CLNI = Clinical and Laboratory Standard Institute of antimicrobial susceptibility testing

Introduction

Conjunctivitis is a nonspecific term used to describe inflammation of ocular surface from either infectious or none infectious causes [(1)]. Viruses and bacteria are the most common infectious causes, noninfectious conjunctivitis includes allergic, toxic, as well as inflammation secondary to immune mediated diseases and neoplastic processes [(2)]. The acute infective causes (viruses and bacteria) are the most frequently encountered ocular disorders in primary care, making up to 1-2% of all family medicine consultations [(3)].

The conjunctival flora is found on the ocular surface of healthy individuals and under normal conditions comprises noninfectious

microorganisms and these microorganisms have an important role in the maintenance of normal conjunctival functions and the prevention of ocular infections [4].

Infection can involve the eye itself and or the tissue surrounding the eye, may be unilateral or bilateral (one or both eyes), or it can spread from one eye to infect other one [5].

The most prominent symptoms of acute infective conjunctivitis are mild pruritus, foreign body sensation, and intolerance to light and redness while most prominent signs include crusted eyelids that are often matted together, especially after sleep, watery or purulent discharge from one or both eyes [6].

The prevalence of conjunctivitis varies according to the underlying cause, which may be influenced by the patient's age, as well as the season of the year. Viral conjunctivitis is the most common cause of infectious conjunctivitis both overall and in the adult population and is more prevalent in summer [7], while bacterial conjunctivitis is the second most common causes and is responsible for the majority of cases (50-75%) and observed more frequently from December through April [8]. While allergic conjunctivitis is the most frequent cause, affecting 15-40% of the population, and is observed more frequently in spring and summer [9].

The most common etiologic agents of conjunctivitis are Gram-positive organisms such as *Staphylococcus species*, *Streptococcus pneumoniae, Streptococcus viridians* [10]. Infections with *Streptococcus pneumoniae* and *Haemophilus Influenzae* are more common in children, while *Staphylococcus aureus* most frequently affects adults [11], while other organisms such as *Bacteroides, Corynebacterium diphtheriae* and *Moraxella catarrhalis* account for the lesser percentage of causes [12]. Gram negative organisms, such as *Escherichia coli, Klebsiella pneumoniae, Serratia marcescens, Proteus, Enterobacter,* and *Pseudomonas species* have been implicated also as lesser causes of conjunctivitis [13]. *Chlamydia trachomatis* and *Neisseria gonorrhea* are the two well described agents associated with ophthalmia neonatorum, and are known to be associated with systemic complication [14]. The majority of infectious neonatal conjunctivitis cases are due to bacterial etiology, though most of these cases are benign, some of them may progress to systemic complications or visual loss [15].

The aim of this study was to identify etiological agents of aerobic bacterial conjunctivitis in all age group and finding any relation with conjunctival discharge.

Methods

This study was conducted in Outpatient Ophthalmology Clinic of Shahid Aso Hospital in Sulaymaniyah, Iraq from a period of March - October 2017, after obtaining informed consent from all the participants: swabs from conjunctiva were collected from two groups; 100 from patients suspected to had acute conjunctivitis and 50 samples from healthy control persons. Age occupation, sex and eye discharge were asked from both groups and recorded.

All the samples were collected using a cotton tipped swab applicator pre-moistened with sterile saline. The swab was placed in the transport medium and transferred to the Microbiology Lab in College of Medicine, University of Sulaymaniyah. All the samples were processed through culturing on different culture media (blood, MacConkey agar, chocolate agar) under aerobic condition and using candle jar for incubation of streaked chocolate agar for 24-48 hours. After incubation final diagnosis were done by doing Gram stain on positive culture and using several biochemical tests such as coagulase, catalase, oxidase mannitol fermentation test and Analytic Profile Index (API) system for final diagnosis of bacterial species [16].

Antibiotic susceptibility was examined for all bacterial species according to Clinical and Laboratory Standard Institute of antimicrobial susceptibility testing (CLNI) 2015 by using 10 selected antimicrobial agents [17].

Results

This study included 100 patients with clinical history of conjunctivitis and 50 healthy control group. Female 45, male 55 among conjunctivitis and 31 female ,19 male in control group. Different age group were participated in the study from less than 1 year to 80 years with mean age 40.5, with predominance of 1-10 years as the most affected age group by infection. Table 1 demonstrates age and sex of participant among conjunctivitis group.

Table 1. Age and gender of patients with conjunctivitis

Age group	Male	Female	Total
< 1	0	3	3
1-10	22	14	36
11-20	4	3	7
21-30	4	3	7
31-40	4	8	12
41-50	11	6	17
51-60	4	2	6
61-70	3	3	6
71-80	3	3	6
Total	55	45	100

The prevalence of aerobic bacterial conjunctivitis is 53% with predominance of *Staphylococcus species* 34% followed by *Streptococcus pneumonia* 13% and 2% for each of *Diphtherioid, Neisseria gonorrhea* and Gram-negative bacteria, while bacterial isolates among control group account for 52% with predominance of *Staphylococcus epidermidis* (Figure 1).

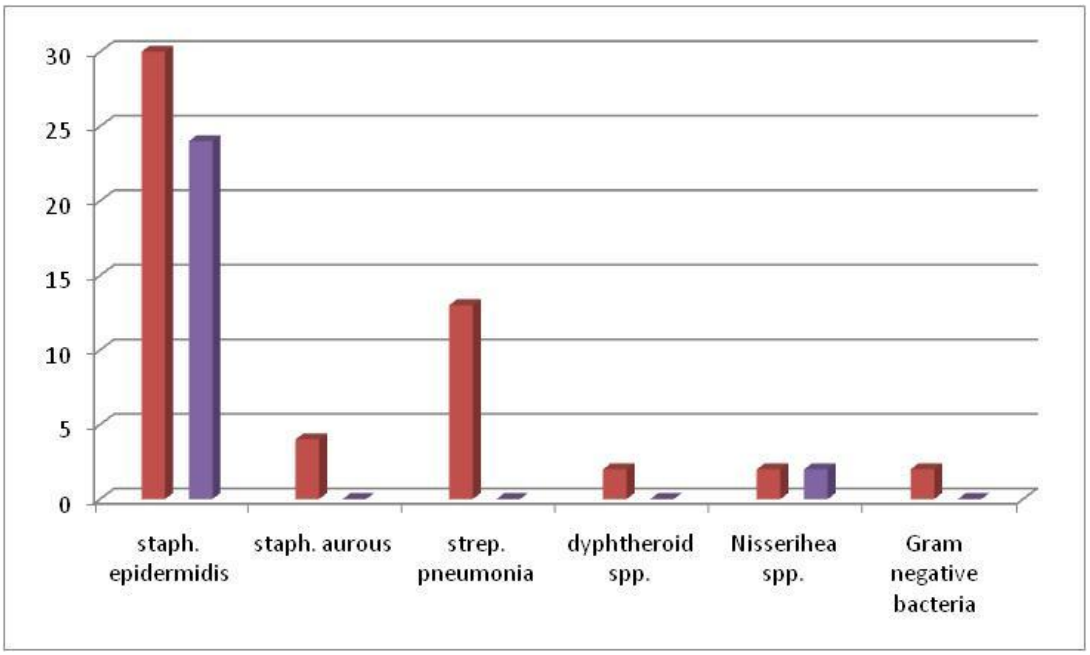

Figure 1. Distribution of aerobic microorganisms among conjunctivitis and control group

One of the symptoms of conjunctivitis is red eye which was observed among all patients with conjunctivitis followed by presence of eye discharge. Table 2 illustrates the presence of eye discharge among positive and negative culture (p> 0.05).

Table 2. Distribution of eye discharge in relation of culture result among conjunctivitis

Culture results	Eye Discharge		Total
	Positive	Negative	
Positive culture	41	12	53
Negative culture	37	10	47
Total	78	22	100

X2 = 0.270, DF = 1, P = 0.869

The type of discharge also analyzed in this study and it was differ according to the isolates and each type of bacteria producing different discharge but purulent discharge is the commonest one (Table 3) although statistically this variation is not significant p value > 0.05.

Table 3. Distribution of eye discharge in relation of culture result among conjunctivitis

Microorganisms isolated	Type of discharge				Total
	Mucoid	Watery	Purulent	None	
Staphylococcus species	5	8	9	12	34
Streptococcus species	2	4	7	0	13
Diphtheria species	0	1	1	0	2
Neisseria species	0	0	2	0	2
Gram negative	1	1	0	0	2
Total	8	14	19	12	53

P value = 0.838

Antimicrobial susceptibility was done for all isolated bacteria from conjunctivitis patients and the result was different according to each species of bacteria as shown in table 4.

The most resistant drugs against *Staphylococci* is tetracycline (76.66%) followed by erythromycin (70%), trimethoprim (56.66%), while gentamycin was the most resistant drug against *Streptococcus pneumonia* (69.23%).

The best drug to treat conjunctivitis is cefotaxime which has 76.66% efficiency against *Staphylococcus epidermidis* and 100% against *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Diphtheria species*. On the other hand, sensitivity to ofloxacillin was detected in 56.66% of *Streptococcus epidermidis*, 75% of *Streptococcus pneumoniae* and 84.61% of *Staphylococcus aureus.*

Discussion

Bacterial conjunctivitis can be contracted directly from infected individuals or can result from abnormal proliferation of the native conjunctival flora, contaminated fingers, ocular genital spread and contaminated fomites are common routes of transmission [17].

The prevalence of aerobic bacterial conjunctivitis is 53% with predominance of Gram positive bacteria such as *Staphylococcus epidermidis* as the major pathogens followed by *Streptococcus pneumonia*, which is compatible with other studies [18-21]. In spite of this result, there is study in which Gram-negative bacteria such as Pseudomonas aeruginosa is the predominance microorganisms followed by Staphylococcus aurous [5].

Negative culture was detected among 47% apart from symptoms and signs of conjunctivitis, in which other etiology may the

causes such as viral, allergic, anaerobic bacteria and *Chlamydia* that they require special technique for their isolation and they are not included in this study.

Table 4. Antimicrobial susceptibility results for all bacterial isolates from conjunctivitis

isolated microorganisms	antimicrobial antibiotics										
	No%	C	T:	E:	CN:	CIP:	OFX	CTX	MEM	TM	CRO
staphylococcus epidermidis (30)	R	10 (33.33)	23 (76.66)	21 (70)	12 (40)	13 (43.33)	13 (43.33)	7 (23.33)	11 (36.66)	17 (56.66)	11 (36.56)
	S	20 (66.66)	7 (23.33)	8 (26.66)	17 (56.66)	13 (43.33)	17 (56.66)	23 (76.66)	19 (63.33)	13 (43.33)	18 (60)
	I	0	0	1 (3.33)	1 (3.33)	4 (13.33)	0	0	0	0	1 (3.33)
Staphylocous aurous (4)	R	0	1 (25)	2 (50)	2 (50)	2 (50)	1 (25)	0	1 (25)	0	0
	S	4 (100)	3 (75)	2 (50)	2 (50)	2 (50)	3 (75)	4 (100)	3 (75)	4 (100)	4 (100)
	I	0	0	0	0	0	0	0	0	0	0
streptococcus pneumonia (13)	R	2 (15.38)	6 (46.15)	7 (53.84)	9 (69.23)	2 (15.38)	0	0	3 (23.07)	6 (46.15)	1 (7.69)
	S	11 (84.61)	7 (53.84)	6 (46.15)	4 (30.76)	11 (84.61)	11 (84.61)	13 (100)	10 (76.92)	7 (53.84)	12 (92.3)
	I	0	0	0	0		2 (15.38))	0	0	0	
Diphtheriod Species (2)	R	0	0	0	0	1	0	0	0	1 (50)	0
	S	2 (100)	2 (100)	2 (100)	2 (100)	1 (50)	2 (100)	2 (100)	2 (100)	1 (50)	2 (100)
	I	0	0	0	0	0	0	0	0	0	0
Gram negative bacteria (2)	R	0	0	0	0	2 (100)	1 (50)	0	2 (100)	0	0
	S	2 (100)	2 (100)	2 (100)	2 (100)	0	1 (50)	2 (100)	0	2 (100)	2 (100)
	I	0	0	0	0	0	0	0	0	0	0
Niserihea species (2)	R	0	1 (50)	2 (100)	1 (50)	0	0	1 (50)	1 (50)	2 (100)	1 (50)
	S	2 (100)	1 (50)	0	1 (50)	2 (100)	2 (100)	1 (50)	1 (50)	0	1 (50)
	I	0	0	0	0	0	0	0	0	0	0

C: Chloramphinicol(30µg) , T: Teteracycline(30µg) , E: Erythromycin(15µg) , CN: Gentamicine(10mcg) , CIP: ciprofloxacine(10mcg) , OFX: Ofloxacine(5mcg) , CTX: Cefotaxime(30mcg) , MEM: Meropenem(10mcg) , TM: Trimethoprime(5mcg) , CRO: Ceftriaxone(30µg)

Bacteria may frequently be isolated from the conjunctiva of healthy subjects and is frequently comprised of same microorganisms as the skin flora and upper respiratory tract [22]. The present study revealed the major causative pathogens both in conjunctivitis and healthy group are the same in spite of that conjunctival flora forms a defensive barrier against infection, it also includes major pathogens of ocular infections [23].

The most affected age group is between 1-10 years and 40-50 years of age, which is compatible with study done [24,25] as children more prone to infection due improper sanitary condition by their parents and adult above 40 are prone to infection due to contact with dirty subject in their work.

One of the hallmark of conjunctivitis is the presence of eye discharge and it was present in all positive culture and in 37% of negative culture, although different eye discharge was

observed but purulent discharge is the commonest specifically among Streptococcus pneumonia and both cases of Neisseria as other study done [26].

Although 60% of cases of suspected or culture-proven acute bacterial conjunctivitis are self-limiting within 1 to 2 weeks of presentation [7] the antimicrobial susceptibility profile done in this study and revealed that cefotaxime and ofloxacillin is the best drug to treat conjunctivitis as observed also by others [22] followed by gentamycin, ciprofloxacillin and meropenem but there are others research in which gentamycin is the more sensitive drug with ciprofloxacillin [27-29] and other conclude macrolide antibiotic eye drop containing 1% azithromycin that was approved in the USA in 2007 is the drug of choice [30]. The resistant pattern also differs according to bacterial species, in general tetracycline and erythromycin were the most drugs that bacteria exhibited resistance against, while *Staphylococcus epidermidis* showed resistance to chloramphenicol which gave better result against *Staphylococcus aureus* and *Streptococcus pneumonia* than tetracycline [31]. However, there is some researches, in which *Streptococcus pneumonia* is highly resistant type of bacteria [32,33].

This study concluded that bacterial conjunctivitis is common in all age group with predominance of Gram positive microorganisms similar to what was isolated in normal healthy subject. Cefotaxim and ofloxacillin regarded as the best drug of choice for treatment of conjunctivitis.

Acknowledgments

The authors would like to thanks Ophthalmology Unit in Shahid ASO Hospital in Sulaymaniyah for their cooperation regarding taking of samples from patients and great thanks to all the patients and control group that participated in the study for their cooperation and allowance for taking samples.

Authors contribution

Dr Hassan: taking patients history and examination, all the samples were analyzed and processed by Dr Anoar and Majid, and all of three authors participated in writing of paper.

Conflict of interest

no conflict of interest regarding all three authors that participated in this study to any other company or any organized sit with best regards.

Funding

The whole fund of the research was paid by all three authors themselves regarding all material that needed for bacteriological process.

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Received Oct. 18th 2017
Accepted Feb. 25th 2018

المجلد السادس عشر، العدد الثاني، 1439 هـ، 2018م
DOI: 10.22578/IJMS.16.2.

المجلة العراقية للعلوم الطبية

عنوان المراسلات إلى المجلة العراقية للعلوم الطبية، صندوق بريد 70044 بغداد، العراق. تلفون (7717516090 964+).

رقم الإيداع في دار الكتب والوثائق ببغداد 709 لسنة 2000

Iraqi Journal of Medical Sciences

IRAQI JMS

المجلة العراقية للعلوم الطبية

Volume 16, Number 2, 2018
April - June

P- ISSN 1681-6579
E- ISSN 2224-4719

Contents