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Iraqi Journal of Medical Sciences

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Cancer Stem Cells

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Three customs of cancer research are shared together to provide increasing precision to the causal mechanisms of tumor heterogeneity and recognition how these are linked to therapy resistance, tumor progression, and recurrence. Advanced genome sequencing has established that cancer within a single patient is a heterogeneous mixture of genetically distinctive subclones that arise through branching evolution [1,2] . The exclusive driver mutations within each subclone can impact the cancer hallmarks in a different way, thus contributing to functional heterogeneity. Correspondingly, strong confirmation is emerging that nongenetic determinants, principally connected to developmental pathways and epigenetic modifications (DNA methylation, histone modification, chromatin openness, microRNA [miRNA], and other noncoding RNA) contribute to functional heterogeneity [3-5] .These determinants are commonly attributed to the continuation of normal tissue stem cell hierarchies. Equally, nongenetic determinants create hierarchically ordered tumor tissues where a subpopulation of cancer stem cells (CSCs) sustains the long-term clonal maintenance of the neoplasm. Proof from both experimental models and clinical studies indicate that CSCs survive many generally employed cancer therapeutics. Furthermore, the properties and transcriptional signatures specific to CSC are highly predictive of overall patient survival pointing to their clinical importance [6].

CSC is a general term referring to the cancer cells talented of differentiation and self-renewal which is the role of CSCs chemotherapy resistance. The description of CSCs does not decide their origin and the term "Cancer Stem Cell" does not denote that cancer begins from stem cell. CSCs are more differentiated than stem cells together with a more restricted spectrum of the cells existing in a tissue [7]. In 1994, CSCs were isolated for the first time. In 1855, German pathologist stated that cancers arise from the activation of dormant, embryonic- like cells present in mature tissue and argued that cancer does not simply appear spontaneously [8].

The initial cell that develops cancer is not essentially a cancer stem cell, despite the fact that cancer-initiating cell and cancer stem cell are occasionally used interchangeably. The existence of CSCs was initially projected 40 years ago, though analysis of its details leftovers a mystery until the development of advanced investigation tools [9]. The most excellent evidence to support the existence of CSCs arose from the study of hematological malignancies [10]. Taking into consideration the task of embryonic stem cells and self-renewal in mature cells like blood cells, the definition of CSCs was discovered [11].

In addition to developing tumors, CSCs direct to the migration and propagation of tumors in new sites that take place in metastasis. Although the role of CSCs in the renewal and initiation of tumors has been exposed, the correlation between CSCs and metastasis is yet

to be found out [12]. Hermann *et al* [13] used pancreatic cancer as a model to investigate the relationship between CSCs and metastasis. They analyzed the initial tumors and established that a chief part of the tumor has the aptitude to form tumor after implantation. They comprise a sub-category of CD133+ cells which have the tumorigenesis and high resistance features of Gemcitabine. These tumorigenic CD133+ cells were channeled serially, signifying their capability for self-renewal, and that they were capable to create tumor heterogeneity by manufacturing differentiated, non-tumorigenic progeny [14].

Long term cell culture, FACS (fluorescence-activated cell sorting), and MACS (magnetic cell sorting) are the chief techniques used to isolate CSCs. CSCs enrichment can be completed by means of the FACS technique. Cells are as well isolated based on the expression of special proteins of cellular-level, cell culture, epigenetic changes and expression pattern of such cellular-level markers as CD 24, CD133, ALDH1 and CD44. CSC characteristics can be determined through mRNA and miRNA expression analysis, copy number variation, etc. Subsequently phenotypic and genotypic characteristics can be linked with in vitro and in vivo clinical data [15].

In spite of advances in radiation therapy and chemotherapy, the prognosis of patients with advanced malignant tumors remains poor. Unproductive targeting of CSCs has been suggested as one explanation for present treatment failure [16]. CSCs have been recognized to be resistant to a variety of chemotherapeutic agents and radiotherapy [17]. The resistance of CSCs to chemotherapy may engage augmented expression of drug efflux pumps, more efficient DNA repair [18], and interactions of CSCs with their microenvironment [19]. With reference to CSC resistance to conventional therapeutic agents, development of alternative/novel therapeutic strategies that can specifically and efficiently target CSCs is needed to improve the efficacy of other therapeutic agents. There are a number of hypothetical reasons which give a rationale for rising immune approaches to target CSCs. It is clear that CSCs and their more differentiated progeny exhibit distinct gene expression profiles and consequently express diverse antigens. Immunologic approaches directed against whole tumors are principally biased toward more differentiated tumor cells which form the bulk of the tumor and which express "differentiation" antigens. This suggests that effective immune targeting of CSC may necessitate the specific targeting of this cell population. Additionally, within a tumor, CSCs may themselves exhibit heterogeneity resultant from both genetic and epigenetic regulation related to tumor progression and metastasis. For case in point, breast CSCs uphold that flexibility to transition between mesenchymal (EMT) and epithelia (MET) states in a process regulated by the tumor microenvironment. The capability of immunotherapies to target multiple antigens makes these approaches right to the targeting of these heterogenous CSC populations [20].

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The Changes in PNA, WGA, and UEA I Lectin Binding Pattern in the Uterine Tube Epithelium by Effects of Estradiol and Progesterone Therapy in Rat

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Abstract

Background The carbohydrate histochemistry in the epithelium of the uterine tube was found to be related to the uterine tube physiology and morphology.

Objectives This study investigated the changing binding pattern of peanut agglutinin, wheat germ agglutinin, and ulexeuropaeus agglutinin I lectin to the epithelial cells of the rat oviduct ampullary region in response to the effect of combined estrogen and progesterone hormonal administration.

Methods This experimental study was based on injecting the animals with doses of estradiol and Progesterone hormonesduring the successive estrous cycles. Then, the ampulla of uterine tubes were identified and prepared for paraffin sections. The peanut agglutinin, wheat germ agglutinin, and ulexeuropaeus agglutinin I lectins were used to label the tube section; the sections were examined by fluorescent microscope.

Results The three lectins used in this study showed analogous pattern of binding behavior. The uterine tube epithelium of the controlled group showed bimodality of binding, while that of the treated group showed a third pattern of lectin binding in some of the epithelial cells.

Conclusion The ciliated cells that are transformed to display a secretory function are called the transitional cells, the formation of these cells is influenced by hormonal factors and the pattern of the lectins binding to these cells could be considered as a histochemical marking.

Key word Lectin, uterine tube, carbohydrate histochemistry, hormone.

List of abbreviation: PNA = peanut agglutinin, UEA I = ulexeuropaeus agglutinin I, WGA = wheat germ agglutinin, NBF = neutral buffer formalin, FITC = flouresceinisothiocyanate, PBS = phosphate buffered saline.

Introduction

The uterine tube is responsible for transport of gametes and embryos; in addition it is a physiologically suitable sit for both fertilization and early development [(1)].

The uterine tube wall is formed of external tunica serosa, intermediate tunica muscularis, composed of inner circular and outer longitudinal layers and an internal tunica mucosa; however, there is no submucosa [(2)].

The epithelial cells of the mucosa are of a simple columnar type resting on a basement membrane and a lamina propria. These mucosal epithelia are of four types; the ciliated columnar cells, the non-ciliated (secretory) cells, the intercalary (intercalated) cells, and the basal (reserve or indifferent) cells [(3)].

The uterine tube epithelium changes in height and thickness during the estrus cycle. In rodents, the secretory cells predominate the ampullary region during the estrous and metestrous phases, while the ciliated columnar cells predominate during the latter part of the diestrous and the proestrous phases [(4)].

Ciliogenesis and deciliation processes in the lining epithelium are influenced by the level of ovarian hormones [5].

The uterine tube showed much histochemical reactivity indicating the existence of many chemical compounds including; carbohydrates, proteins, lipids and others, these histochemical compounds were found to have certain role to maintain the uterine tube physiological functions. The histochemical carbohydrate constituents are found to be important structural cytoplasmic elements of the uterine tube, these carbohydrates were found mainly in the cytoplasm, glycocalyx, secretory granules and connective tissues and other parts of the epithelium [6].

Lectins are carbohydrate binding proteins of a non- immune origin that agglutinate cells and/or precipitate glycoconjugates. The lectins have no enzymatic activity, may be soluble or membrane bound, and are of bacterial or plant origin [7]. There are various biological roles of lectins in animals [8]. At the level of the tissues, lectins had been used in many directions, the lectins are considered as specific probes for various cell types[9], the lectins are used to define cells at various stages of differentiation or maturation [10], and are also used as probes to detect microenviroment [11] and detecting the functional changes [8].

The aim of this study was to investigate the binding pattern of PNA, WGA, and UEA I lectins to the epithelial cells of the rat oviduct ampullary region, and elaborate the alteration in binding configuration in these epithelial cells affected by a combined estrogen and progesterone hormonal administration.

Methods

Fourteen adult healthy female albino rats (*Rattus Norvegicus*) weighing between 200-280g, and having an estrous cycle of 4 days period used for this study were obtained from the animal house of the High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University.

The following drugs were used in this study:

- B-Estradiol 17-acetate (Sigma-Aldrich) 1g, in a solid form, was dissolved at a concentration of 20µg/ml sesame oil.
- Primolute Depot (Bayer Schering Pharma AG/Germany) in form of Hydroxyprogesteron ecaproate 250mg/1ml in oily solution.
- Purified Sesame oil used as a vehicle for dilution of both hormones. The total quantity of the oil used was adjusted to be fixed in every daily dose or doses to be a net of 2 ml.

The animals were divided into 2 main groups; a Control group (6 rats) and treated group (8 rats) treated with the 10 µg/day of estradiol for a period of two successive estrous cycles (i.e. 8 days).

The Progesterone hormone was given in a dose of 4 mg/kg body weight for the treated group on the third and fourth days of the two successive estrous cycles. The drugs were given daily in the morning, as a subcutaneous injection at the lower back region of the rats.

Vaginal cytology smears were collected from the experimental animals to check the length of the estrous cycle and to identify the estrous phases for the rats depending on the criteria of vaginal cytology.

In this study; 4 days estrous cycle rats have been selected. The vaginal smear done using Ayres Spatula that was placed deeply inside the vagina of the rat, then the spatula rotated through 360 degree maintaining contact with the vaginal wall.

After the swab was removed from the vagina, the cells were transferred to a clean glass slide by rolling the swab along the surface of the slide. Intact cells were obtained during transfer by rolling the swab. Once the cells have been transferred, the slides were fixed immediately using absolute alcohol for 2-6 hours.

The vaginal smear for the control group was done with the rat under anesthesia and just before termination, while for the treated groups it was done two times (for each animal), the first one done before starting treatment, and the second done after finishing the

treatment and just before sacrificing the animal.

The sacrificed animals were terminated 24 hours after the last dose of drugs by cervical dislocation. Then, the right and left uterine tubes of each animal were identified and the ampullary region (0.2-0.3 cm long) was removed on both sides, fixed in 10% Neutral Buffer Formalin (NBF) prepared for paraffin sections according to Bancroft and Stevens (1982) for lectin binding study [12].

Procedure of lectin histochemistry

Three types of Lectins (Sigma™ USA) were used in this study:

1. Peanut agglutinin (PNA) from Arachis-hypogaea (peanut) which has affinity to bind the D-Galactose sugar.
2. Ulexeuropaeus agglutinin I (UEA-I) from Ulexeuropaeus which is a Fucose binding lectin.
3. Wheat germ agglutinin (WGA) from Triticumvulgaris (wheat) which has the affinity for binding the N- acetylglucosamine and Neuraminic (sialic) acid.

All lectins used were flouresceinisothiocyanate (FITC) labeled.

The Procedure of lectin histochemical staining was as follow [13]:

- Dewaxing of the paraffin sections was done by use of xylene for 30 minutes.
- Hydration of the sections through graded concentrations of ethyl alcohol.
- Hydrated paraffin sections were washed in phosphate buffered saline (PBS).
- Keep the slides flooded by the lectin-PBS solution.
- Sections were flooded by lectin-PBS solution and kept for 1.5 hours.
- Sections were washed in PBS and mounted in non-fluorescent fractoilmountant.
- Sections were examined under the ultraviolet light of the fluorescent microscope.
- Digital camera (Sony cyber shot) was used for capturing pictures after microscopic evaluation of the fluorescent reactivity.

Results

The PNA binding

PNA binding to the controlled group

The PNA lectin binding to the mucosal epithelium of the ampullary region of the uterine tube in the control group (Fig. 1) showed predominate intracellular binding cells given positive fluorescence activity, these cells are low columnar with central round nucleus.

The epithelium showed also singly settled narrow columnar cells having weaker fluorescence activity (especially in the supranuclear cytoplasm), these cells show central elliptical nucleus that is arranged parallel to the long axis of these cells.

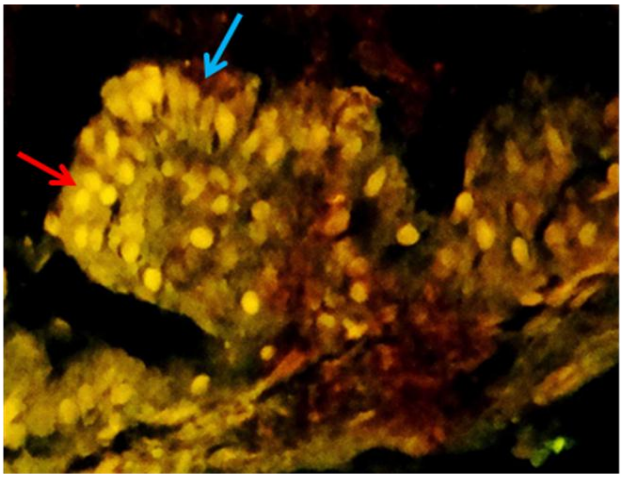

Fig. 1. PNA binding to the uterine tube mucosa of the control group showing the predominate intracellular binding cells (red arrow); singly settled narrow columnar cells having weaker fluorescence activity (blue arrow). X400.

PNA binding to the treated group:

The PNA binding to the mucosal epithelial cells showed 3 levels of fluorescence activity (fig. 2), the predominant cells showed strong intracellular fluorescence, others showed progressive diminishing fluorescence reaching to the least fluorescent cells that have very weak fluorescence activity. The latter showed the same criteria in the epithelium of the controlled group as these cells are singly settled with narrow columnar cell body and central elliptical nucleus arranged parallel to the long axis of these cells. A part from these

columnar cells, the predominant cells of the epithelium have rounded central nuclei, the cellular boundaries of the whole epithelial cells seemed obscure that make the fluorescence of these cells appearing cloudy with 3 different florescent activities, even the nuclei were masked compared to those nuclei of the cells in the epithelia of the controlled group. The predominant epithelial cells are lower columnar in shape compared to those seen in the controlled group and have intracellular fluorescence.

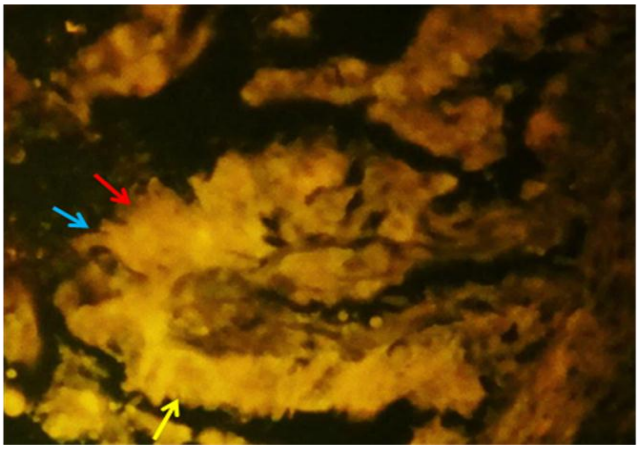

Fig. 2. PNA binding to the mucosal cells of the treated group showed 3 levels of activity, the least fluorescent cells showed singly settled with narrow columnar cell body (longer blue arrow). X400.

The WGA binding

WGA binding to the controlled group:

The WGA lectin binding to the mucosal epithelium of the ampullary region of the uterine tube in the control group (Fig. 3) showed predominate intracellular binding cells given positive fluorescence activity, these cells are low columnar with central round nucleus. The singly settled narrow columnar cells having central elliptical nucleus arranged parallel to the long axis of these showed equivalent fluorescence at the supranuclear region, but the perinuclear cytoplasm inferior to the nucleus showed weaker fluorescence activity.

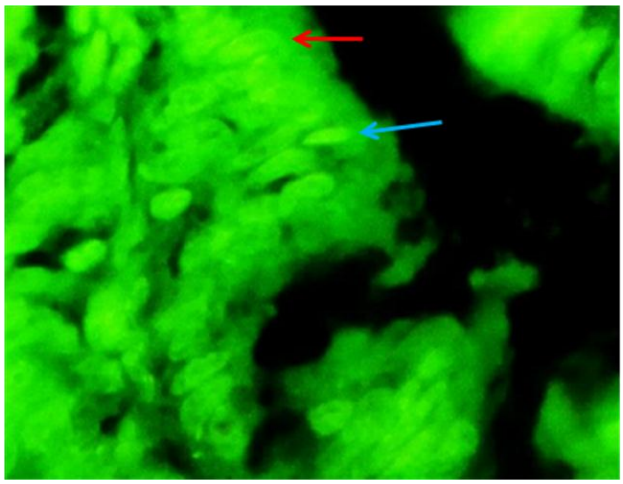

Fig. 3. WGA lectin binding to the uterine tube mucosal epithelium of the control group. The predominate intracellular binding cells (red arrow) and the singly settled narrow columnar cells (blue arrow) are seen. X400.

WGA binding to the treated group

The fluorescence activity of WGA binding to the epithelial cells showed equivalent pattern and intensity in both forms of cells described above (the predominant and the single settled cells), apart from few of the predominant cells have comparable weaker fluorescent activity (fig. 4). The epithelial cellular boundaries are also obscured.

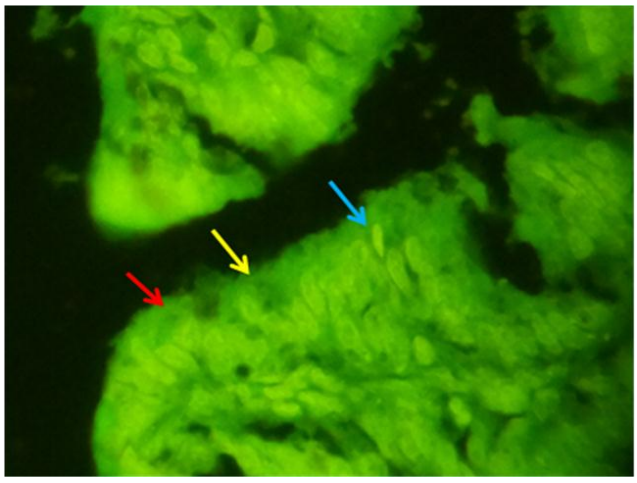

Fig. 4. WGA binding to the uterine tubal epithelium in the treated group showed predominant (red arrow); single settled cells (blue arrow) with few of predominant cells having comparable weaker (yellow arrow). X400.

The UEA I binding

UEAI binding to the controlled group

The predominant cells of the mucosal epithelium showed cell surface florescence of UEAI binding with variable intensities; this fluorescence is brighter on the luminal surface of these cells. The single settled narrow cells showed weak intracellular UEAI binding (fig. 5).

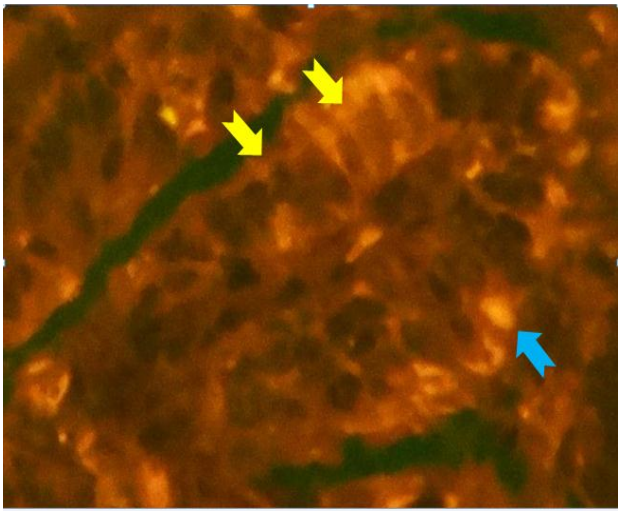

Fig. 5. UEA I binding in the uterine tube mucosal epithelium of the control group. The predominant cells showed cell surface florescence (yellow arrows), the single settled narrow cells showed weak intracellular binding (blue arrow). X400.

UEAI binding to the treated group

The UEAI binding to the mucosal epithelial cells showed similar pattern and intensities as that seen in the epithelial cells of the controlled group. In addition, some of the predominant cells showed intracellular reactivity (fig. 6). The cellular boundaries of the whole epithelial cells are obscured resulting in elusive pattern of negative rather than cell surface fluorescence. The nuclei were masked compared to those nuclei of the cells in the epithelia of the controlled group, especially in the intracellular fluorescent predominant.

Discussion

The three lectins used in this study showed analogous pattern of binding behavior. The uterine tube epithelium of the controlled group showed bimodality of binding, represented in the epithelial predominant cells (low columnar with central nucleus) and the single settled cells (narrow columnar cells).

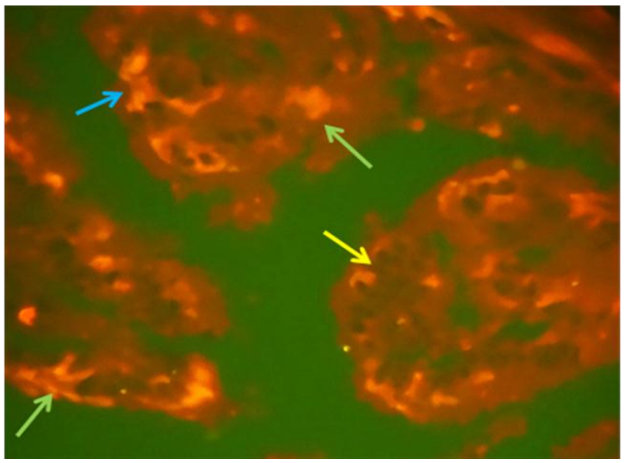

Fig. 6. UEAI binding to the mucosal epithelial cells of the treated group showed similar pattern and intensities as that seen in the epithelial cells of the controlled group. Some of the predominant cells showed intracellular reactivity (green arrows). X400.

The epithelium of the treated group showed a third pattern of lectin cellular binding only in some cells matching in their histological criteria to the predominant epithelial cells, and hence these cells may represent a deviation in the binding pattern of the predominant cells in response to hormonal treatment. These results may suggest that hormonal treatment did not have consequences on the bimodal pattern but it is associated with the appearance of a third mode of lectins binding pattern.

The predominant cellular binding pattern represents the marking pattern of ciliated epithelial cells, and the pattern of singled settled cells is the marking pattern of the secretory epithelial cells. This conclusion has been predicted depending on the illustrious histological descriptions verified previously that traditionally described the predominant ciliated cells and the scattered secretory cells in the tubal epithelium of rat [(14)].

It was reported that some of the ciliated cells have the ability to be transformed to display a

secretory function, these cells were describe to have the same light microscopic morphological criteria but with a paler cytoplasm. The ultrastructural criteria of these transformed cells differ from the predominant ciliated epithelial cells. These transformed cells were nominated as the transitional cells between ciliated and secretory cells, and the formation of these cells was suggested to be influenced by hormonal factors [15]. Hence, the third pattern of the lectins binding may be interpreted as the marking pattern of a transitional cells developing by the effect hormonal treatment applied in this study.

These analyses of the results of this study was congruently reported by Gheril et al (2001) that described no difference in sugar localization and distribution in a study on the lectins binding pattern in the uterine tube epithelium at variable physiological circumstances [13].

In this study, the PNA binding in the controlled group showed intracellular binding to the predominant cells (i.e., the ciliated cells), but weaker supranuclear binding in the singly settled cells (i.e., the secretory cells). This finding is supported by the histochemical elaboration of Schulte et al (1985) that demonstrated apical localization of galactose in the ciliated but not secretory cells. These authors suggested that there is no menstrual phase related variability of the carbohydrate distribution in the epithelial cells [16]. This definite analysis may indicate that the iatrogenic hormonal manipulation can cause an alteration that is not seen in normal hormonal variation.

The WGA binding to the controlled group showed intense apical cytoplasmic binding in the secretory cells, the ciliated cells showed diffuse intracellular WGA reactivity. The WGA binding in the treated group showed weaker reactivity in few of the epithelial cells having the histological criteria simulating the predominant ciliated cells. The pattern of WGA binding in ciliated epithelial cells in the treated and control group was supported by the results reported by Gehri et al (2001) [13], therefore, the lectins PNA and WGA could be considered as the marker of the ciliated epithelium during physiological condition as the hormonal treatment done in this study results in fading of fluorescence WGA reactivity in these cells.

Ingrid and Bavdek (1997) reported that WGA and UEAI showed binding reactivity in the supranuclear Golgi apparatus and in the secretory granules of the epithelial cells of the ampulla of the uterine tube [17]. This description supported the result found in this study demonstrating supra-nuclear WGA binding in the single settle secretory cells. Accordingly, this supra-nuclear WGA pattern maybe interpreted as an WGA binding to the Golgi apparatus at the supra-nuclear cytoplasm position between the nucleus and the secretory surface [18].

The UEAI binding to the secretory single settle cells showed intracellular binding in the controlled group, this pattern sustained the interpretation of Ingrid and Bavdeck (1997) considering the UEAI binding to the Golgi apparatus and the secretory granules.

The lectin binding specificity to the carbohydrate has been discussed in consideration to the intracellular machinery and in relation to the intercellular milieu in many literatures [19]. The binding pattern of the lectins used in this study could be related to the physiological and pharmacological hormonal adjustments of the uterine mucosal epithelium.

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Resistant Hypertension in Chronic Renal Failure

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Abstract

Background Several factors involved in the pathogenesis of hypertension among chronic renal failure patients including sodium and water retention, increased activity of vasoconstrictive systems, decrease activity of vasodilatory systems, increased intracellular calcium, increased arterial stiffness, sleep apnea, hyperparathyroidism, erythropoietin and renovascular disease and dialysate composition and prescription.

Objective To assess the prevalence of resistant hypertension in chronic renal failure patients and to study the relation between primary causes of renal failure, smoking, non-steroidal anti-inflammatory drugs, body mass index and hepatitis C positive with resistant hypertension.

Methods A case-control study of five hundred patients with hypertension and on antihypertensive drugs was conducted during the period from August 2013 to December 2014. Three hundred of them are complaining chronic renal failure. All patients underwent a history and physical examination at baseline. Blood pressure measurement was done for them and blood urea, serum creatinine, serum electrolytes, hemoglobin level and random blood sugar were estimated.

Results Patients with office blood pressure 140/90 mmHg or those treated with antihypertensive drugs are considered as hypertensive, which made up 87.9% of patients with chronic renal failure. The prevalence of resistant hypertension is 31%; all of them are receiving more than three drugs. Resistance hypertension in chronic renal failure associated with high body mass index, left ventricular hypertrophy, and symptom complaint and non-steroid anti-inflammatory drugs but not related to urea level.

Conclusion Resistant hypertension in chronic renal failure is almost always multifactorial in etiology, may be more sensitive to sodium than the general hypertensive population. Resistance to diuretic in chronic renal failure contributes mainly to resistant hypertension.

Keywords Hemodialysis, resistant hypertension, chronic renal failure

List of Abbreviation: RH = resistant hypertension, BP = blood pressure, ABPM = ambulatory blood pressure measurement, CRF = chronic renal failure, OFB = office blood pressure, GFR = glomerular filtration rate, NSAID = non-steroidal anti-inflammatory drugs.

Introduction

Resistant hypertension (RH) is a common clinical problem faced by both primary care clinicians and specialists. While the exact prevalence of resistant hypertension is unknown, clinical trials suggest that it is not rare, involving perhaps 20% to 30% of study participants. As aging and obesity are two strongest risk factors for uncontrolled hypertension, the incidence of resistant hypertension will likely increase as the population becomes more elderly and heavier. The prognosis of RH is unknown but cardiovascular risk is undoubtedly increased as patients often have a history of long-standing severe hypertension complicated by multiple other cardiovascular risk factors such as obesity, sleep apnea, diabetes, and chronic kidney disease [(1)].

RH is defined as blood pressure (BP) above a goal despite adherence to at least 3 optimally dosed antihypertensive medications of different classes, one of which is a diuretic. Evaluation of possible RH begins with an assessment of adherence to medications [(2)].

Relevant factors involved in the pathogenesis of hypertension in dialysis patients include sodium water retention, dialysate composition and prescription, increased activity of vasoconstrictive systems (sympathetic nervous system, renin-angiotensin system, endothelin and vasopressin), decreased activity of vasodilatory systems (nitric oxide, kinins), increased intracellular calcium, increased arterial stiffness, sleep apnea, hyperparathyroidism, erythropoietin and renovascular disease [(3)].

Clinic BP measurements may not indicate the 'real' BP load in the fluctuating BP profile of hemodialysis patients. Indeed, interdialytic ambulatory BP monitoring is agreed by most as the best method to estimate BP in haemodialysis patients, mostly due to its better reproducibility [(4)].

Furthermore, ambulatory BP measurement (ABPM) provides PB during sleep, where most dialysis patients fail to experience a drop in BP (non-dipping) [(5)].

RH is almost always multifactorial in etiology. Treatment is predicated on identification and reversal of lifestyle factors contributing to treatment resistance; accurate diagnosis and appropriate treatment of secondary causes of hypertension; and use of effective multi-drug regimens. Lifestyle changes, including weight loss; regular exercise; ingestion of a high-fiber, low-fat, low-salt diet; and moderation of alcohol intake should be encouraged where appropriate. Potentially interfering substances should be withdrawn or down-titrated as clinically allowable. Obstructive sleep apnea should be treated if present [(6)].

Inaccurate measurement of BP can result in the appearance of treatment resistance. Two of the most common mistakes—measuring the BP before letting the patient sit quietly and use of too small cuff—will result in falsely high BP readings [(7)].

In recent years, there has been growing interest in nonpharmacologic interventions to treat RH. Electrical stimulation of the carotid sinus baroreceptor has been shown to decrease BP. A few small studies have demonstrated that an implantable baroreflex stimulator is feasible and may be quite effective [(8)].

Catheter-based radio-frequency renal denervation is another promising approach that currently is being studied [(9)]. Renal sympathetic activity contributes to hypertension in part through simulation of renin release, increased sodium reabsorption, and neurogenic mechanisms. Selective denervation of the renal nerves responsible for these effects has been shown to reduce BP. In the Symplicity HTN-2 Trial, resistant hypertension patients (with mean baseline BP of 178/96 mm Hg) randomized to catheter-based radio-frequency denervation had a 6-month mean reduction of office BP that was 31/12 mm Hg greater than controls [(10)].

An expansion in extracellular volume, which can be either relative or absolute, frequently contributes to RH. Volume overload may be related to a high-sodium diet, chronic kidney disease (leading to sodium retention), or both. Volume overload may not manifest as peripheral edema detectable on physical examination, yet it should be considered in the patient with persistently elevated BP despite multiple medications, even when one of the medications is a low-dose thiazide diuretic.

Patients with RH may be more sensitive to sodium than the general hypertensive population. In one study in which patients with RH were randomized to low-salt versus high-salt diets, mean office BP was reduced to 23/9 mm Hg more in the low-salt diet group [(10)].

The objectives of this study was to assess the prevalence of resistant hypertension in chronic renal failure patients and to study the relation between primary causes of renal failure, smoking, non-steroidal anti-inflammatory

drugs, body mass index and hepatitis C positive with resistant hypertension.

Methods

This case-control study was performed in Al-Imamain Al-Kadhimain Medical City during the period from August 2013 to December 2014. Five hundred patients (250 males and 250 females) complaining of hypertension were involved in this study with different age groups ranging from (15 to 70) years (mean of age 47.6 year). Three hundred patients are known cases of chronic renal failure (CRF) with glomerular filtration rate (GFR) ≤ 60; one hundred and fifty of them were on regular hemodialysis.

Each patient in end stage renal disease was subjected to hemodialysis for period of 4 hours in two or three sessions per week. All patients underwent a history and physical examination at baseline. The investigations include blood urea, serum creatinine, serum calcium, phosphorus, sodium, potassium, hemoglobin level and random blood sugar. BP was measured before, during and after dialysis in the dialysis unit as well as its measurement at home depending on patient's readings.

The BP has been measured accurately using an appropriately sized cuff, with the patient correctly positioned and after at least a 5-minute rest. Hypertension was determined according to European Society of Hypertension criteria: office BP (OBP) = BP 140/90 mmHg, ABPM 125/80 mmHg [(10)].

The patients were instructed on how to perform home BP measurements and were observed to make sure that they performed it correctly. Lastly, a systematic approach to collecting of measurements should be used. Those who missed three hemodialysis sessions or more, bleeding complication, infectious disease, secondary hypertension and chronic atrial fibrillation, white coated hypertension and pseudo resistance hypertension (including lack of BP control secondary to poor medication adherence) are excluded from the study. Statistical analysis was performed using chi-square test. At level of significance p≤o.o5 regarded as statistically significant.

Results

The number of patients who completed BP measurement in the home with a percentage of valid measurements > 80%. Two hundred patients are excluded from this study due to normal renal function, three hundreds were complaining of CRF. The patients on regular hemodialysis were 150 (64 women and 86 men). They were 55.8 ± 16.2 years old, on hemodialysis for 28±12 months; and spKt/V was 1.2±0.34. Patients with OBP 140/90 mmHg or those treated with antihypertensive drugs are considered as hypertensive, which made up of 87.9% in CRF of the study population as shown in table 1.

The prevalence of RH in this study as mentioned in table 2 is 31%; all of them are receiving more than three drugs including diuretic, angiotensin converting enzyme inhibitor, calcium channel blocker, beta blocker and angiotensin receptor blocker.

There are many factors associated with RH in CRF include body mass index, hepatitis C virus infection, staging of renal impairment, smoking, non-steroidal anti-inflammatory drugs (NSAID), symptom complaint such as (headache and dizziness) and complication such as cardiovascular disease and stroke (Table 3).

Discussion

Most of the patients with chronic kidney disease have hypertension especially elderly patients. In this research, 264 patients (87.9%) have hypertension either as a cause of renal failure or as manifestation of CRF. Among them, 30% were patients taking only one drug, mostly angiotensin receptor blockers or angiotensin converting enzyme inhibitors and 27% patients taking two drugs mostly a combination of calcium channel blocker (CCB) and diuretics, and 33% patients taking three or more drugs, one of which is a diuretic, among them 31% patients are RH.

Table 1. Baseline data of patients with chronic renal failure

Characteristics		number	percentage
Number	Total	500	100
	Hypertension + Chronic renal failure	300	87.9
	On regular hemodialysis	150	50
Age range (yrs)	17-76		
Sex	Males	166	
	Females	134	
Diabetes mellitus		190	38
Body mass index	< 18.9	48	16
	19-24.9	117	39
	25-29.9	94	31.4
	≥ 30	41	13.6
Smoking		125	28
Alcoholics		20	4
On NSAID		65	8
Family history of hypertension		58	58
Left ventricular hypertrophy		290	97
Hepatitis positive in hemodialysis	C	59	38
	B	14	9.5

Table 2. Relation between resistance hypertension and causes of chronic renal failure

Causes of CRF	patients with CRF No. (%)	Patients with RH No. (%)
Diabetes mellitus	105 (35)	24 (8)
Hypertension	96 (32)	36 (12)
Obstructive uropathy	33 (11)	6 (2)
Glomerulonephritis	30 (10)	15 (5)
Polycystic kidney	18 (6)	3 (1)
Vasculitis	12 (4)	6 (2)
Hereditary	6 (2)	3 (1)
Total	300 (100)	93 (31)

CRF = chronic renal failure, RH = resistant hypertension

RH is more in CRF in comparison to general population such as in a recent study in Spain which had found a rate 12 % [12]. This result is due to many mechanisms of hypertension in CRF such as resistance to diuretics and …etc. as mentioned previously.

Examination survey data suggests that among hypertensive adults treated with medication, approximately 13% have RH [13].

RH in CRF patients of the current study is present in approximately 31%. The high prevalence of RH in CRF is due to secondary causes and resistance to diuretic and other mechanism as mentioned previously.

In this study, RH is more common in patients who are already hypertensive before development of CRF in comparison with renal failure due to other causes such as vasculitis and obstructive uropathy (12% versus 2%). This could be multifactorial including long duration of hypertension which affects multi systems in the body such as increase stiffness of the

arterial wall, left ventricular hypertrophy, more glomerular sclerosis, lower GFR and increase activity of the renin angiotensin system.

Table 3. Relationship between resistance hypertension and many factors

Factors		Number of patients with			*P* Value
		CRF + hypertension	Controlled hypertension	Resistant hyoertension	
Hepatitis	C	65	42	23	0.881
	C and B	14	12	2	
	negative	221	177	54	
Body mass index	< 18.9	48	46	2	< 0.001
	19-24.9	117	105	12	
	25-29.9	94	85	9	
	≥ 30	41	13	28	
Renal impairment	< 100	42	39	3	< 0.305
	100-150	84	79	5	
	150-200	108	102	6	
	≥ 250	66	52	14	
Smoking		28	20	8	0.156
NSAD		24	17	7	0.8396
Complain symptom		132	110	22	≤ 0.001
Complications (CVS, stroke)		243	187	56	< 0.001

CRF = chronic renal failure, CVS= cardiovascular system, NSAID=non-steroid anti-inflammatory drugs

In an analysis of Framingham study data, the strongest predictor of lack of BP control was older age, with participants > 75 years being less than one fourth as likely to have systolic BP controlled compared with participants <60 years of age [(14)].

RH has significant effect on cardiovascular complication such as left ventricular hypertrophy and stroke.

The next strongest predictors of RH which are significant were the presence of diabetes mellitus and obesity (body mass index more than 30 Kg/m^2) (due to disregulation of endothelial factors regulating contractility of the smooth muscle of blood vessels, hyperlipidemia and other endocrine abnormalities such as secondary hyper-parathyroidism) which is similar to other studies such as in ALLHAT [(15)].

Most of the patients with RH complain from headache, dizziness, cardiovascular complication (heart failure, ischemic heart disease or stroke). Blood urea level does not correlate with RH which could be due to effect on contractility of the heart and impairment of left ventricular dysfunction in CRF. Hepatitis C and B infection and smoking in CRF have no effect on RH.

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Conflict of interest

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The Value of Mixed Somatosensory Evoked Potential in the Diagnosis of Lumbosacral Spinal Canal Stenosis

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Abstract

Background Lumbosacral spinal canal stenosis is a common cause for chronic low back pain. The diagnosis is mostly radiological, yet, the extent of neural impairment cannot be expressed by radiological means. It is hypothesized that somatosensory evoked potential indicate a nerve root involvement complementary to the neurological examination.

Objectives To evaluate the usefulness of different parameters of mixed somatosensory evoked potential in the diagnosis of lumbosacral stenosis.

Methods Thirty five patients with Lumbosacral stenosis, clinically and radiologically confirmed by MRI examination and 20 normal individuals were enrolled in the study. Mixed-somatosensory evoked potentials of tibial nerve was done using subdermal monopolar needle electrodes at 4 channels; cortical, lower thoracic, lumbar and popliteal. From these channels negative waves (N45, N25, N20 and N10) were studied for both latency and amplitude, besides the central sensory conduction time which represents inter-peak latency between N25 and N45.

Results The cutoff values of N25, N45 and N20 wave latencies presented highly significant differences between affected sides and controls; with the highest difference given by N25 wave ($P< 0.0001$). There was no significant difference regarding N10 and N25-N45 latencies. The mixed somatosensory wave amplitude cutoff values showed equivocal results about the sensitivity and specificity percentages.

Conclusions Mixed somatosensory evoked potential study can be used as a supplementary test in the diagnosis of Lumbosacral stenosis. N25 wave has the highest diagnostic yield due to having the highest sensitivity and specificity. Equivocal results of the evoked potential amplitudes and their lower sensitivities and specificities compared to evoked potential latencies, lower their validity in the diagnosis of Lumbosacral spinal stenosis.

Keyword Cutoff, sensitivity, specificity, referred.

List of abbreviation: L3S = Third lumber spinous process, LSS = Lumbosacral spinal canal stenosis, MRI = Magnetic resonance image, TN = tibial nerve, ROC = receiver operating characteristic, SEP = Somatosensory evoked potential, $T_{12}S$ = Twelfth thoracic spinous process.

Introduction

Lumbosacral spinal canal stenosis (LSS) is defined as narrowing of the lumbosacral spinal canal, its lateral recesses, and neural foramina which can cause compression of the lumbosacral nerve roots. The stenosis can be symptomatic or asymptomatic. It can be the result of congenital or acquired causes. Frequently, they are combined [(1)]. The extent of narrowing of the spinal canal correlates poorly with symptom severity and radiologically significant lumbar stenosis can be found in asymptomatic individuals [(2,3)].

The electrophysiological techniques may help in the definitive diagnosis of LSS particularly when the patient's clinical and MRI findings are

incompatible; there are root compressions at more than one level; the patient's history and clinical findings suggest radiculopathy but the MRI examination is normal, or the patient's history and clinical findings do not allow a distinction among plexopathy, mononeuritis, and radiculopathy [4]. It is hypothesized that electrophysiological recordings, especially somatosensory evoked potentials (SEPs), indicate a nerve root involvement complementary to the neurological examination. They provide confirmatory information in less obvious clinical conditions and help in the exclusion of other abnormalities [5].

The existing literature on the use of dermatomal somatosensory evoked potentials in lumbosacral spinal stenosis is limited. The aim of this study is to evaluate the value of different parameters ofmixed somatosensory evoked potential of the tibial nerve in the diagnosis of LSS.

Methods

Thirty five patients with clinically suspected and radiologically confirmed LSS were randomly collected among those attending the neurosurgery clinic in Al-Imamain Al-Kadhimain Medical City- Baghdad/ Iraq.

Electrophysiological assessment was done and patient were excluded if clinical, radiological and/or electrophysiological evaluations revealed signs of lumbosacral plexopathy, neuromuscular disorder, peripheral neuropathy associated with any systemic disease, spinal tumors, post traumatic or surgical stenosis or any previous disk-related operation.

A control group of 20 healthy subjects free from any musculoskeletal or neurological deficits confirmed clinically and radiologically were also included for the determination of the normal electrophysiological values.

All subjects were subjected to thorough history taking and full neurological examination of both lower limbs by the neurosurgeon, MRI examination and conventional electrophysiological studies by a neurophysiologist to exclude lumbosacral plexopathy, neuromuscular disorders or peripheral neuropathy. Bilateral mixed-SEP of the tibial nerve (TN) study was done for all subjects.

Mixed tibial SEP study was done with individuals lying in prone position in a quiet environment and was instructed to lie comfortable on the coach and the limbs were kept extended and relaxed and advised not to move or blink in order to decrease muscle contraction artifacts which obscure the waves of SEPs.

TN was stimulated just behind the medial malleolus at both sides, with an intensity enough to create a slight twitch in the toes, with the cathode placed at mid-point between medial malleolus and Achilles tendon and the anode about 3 cm distal to the cathode [6].

The study was performed using Micromed computerized EMG/EP device and the responses were recorded at 2µV/Division gain, 100 ms time base, and 14 Hz–2.5 kHz filtration range. The average of 150-200 cortical responses was taken and each measurement was carried out at least twice to confirm the reproducibility of the SEP.

Recordings were made by using subdermal monopolar needle electrodes that were put in the following positions:-

a) Active electrode was placed at Cz´ (2 cm posterior to Cz) and referred to Fz according to the international 10–20 system (for channel 1).

b) Active electrode was placed at twelfth thoracic spinous process ($T_{12}S$) and referred 4 cm rostrally (for channel 2). $T_{12}S$ is the first blade-like spinous process, felt by tracing upwards and inwards on the floating 12th rib to find it.

c) Active electrode was placed at third lumber spinous process (L_3S) and referred 4 cm rostrally (for channel 3). L_3S felt midway in line between right and left iliac crest with the spinous process above.

d) Active electrode was placed at the popliteal fossa (4-6 cm above popliteal crease) and

referred to medial knee (for channel 4). This site is between the tendons of the semitendinosus and semimembranosus muscles.

From these channels negative waves (i.e. pointing upward from isoelectric line) were recorded. From channel-1, N45 was recorded. N25 was recorded from channel-2. N20 and N10 were recorded from channel-3 and channel-4; respectively. For each waveform, both latency and amplitude were recorded as well as central sensory conduction time (CSCT) which represents the inter-peak latency between N45 and N25.

The results of the demographic characteristics of the studied groups were presented as mean ± SD for the age and as numbers and percentages for the gender. Unpaired Student's t-test was used to compare ages between patients and controls. Chi-square test, on the other hand, was used to express differences in gender ratio between patients and controls. Cutoff value, sensitivity and specificity percentages of latencies and amplitudes of SEP waves were estimated using receiver operating characteristic (ROC) test. Chi-square and Fisher Exact tests were used to evaluate the differences of the studied parameters between two groups (affected and control groups); where the number and percentage of abnormal values in any of the studied parameters were calculated from the determined cutoff values.

Results

Thirty five patients (42.86% males and 57.14% females) were enrolled in this study. Their mean age was 46.43 years (ranged from 25 to 63 years). The control group consisted of 20 individuals, ten males (50%) and 10 females (50%). Their mean age was 38.95 years (ranged from 23 to 56 years). There was a significant difference regarding the mean ages between patients and control groups (*P*= 0.0092); whereas there was no significant difference in sex between the two studied groups (*P*=0.779) (Table 1).

Table 1. Demographic Characteristics of the Studied Patients and Control Groups

Parameters		Patients No (%)	Control No (%)	*P* value
Male Female		15 (42.86) 20 (57.14)	10 (50) 10 (50)	0.779 *
Age (years)	Mean±SD Range	46.43±10.11 (25-63)	38.95±9.5 (23-56)	0.0092 **

* = using chi-square test, ** = using unpaired t- test.

Mixed SEP-TN study included 4 main negative evoked potential waves; which were N10, N20, N25 and N45 with two studied parameters for each wave (peak latency and amplitude) as seen in (Fig. 1).

Since the pathophysiological abnormalities in LSS affect different nerve roots independently with no commitment to the sides affected; therefore, results of the mixed-SEPs were presented as affected sides compared to control sides, i.e. results of the right and left sides were added together as one group. Therefore, the study included 70 affected sides (35 patients on each side) and 40 control sides (20 controls on each side).

According to table 2, the best cutoff value with the highest sensitivity and specificity % was that of N25 latency (24.02ms, 70% and 72.5%; respectively), followed by N45 and then N20 that have lower sensitivity and specificity percentages; respectively, while N10 latency cutoff value (9.61ms) shows the lowest sensitivity and specificity%. Likewise, the cutoff value of the central sensory conduction time

(N25-N45) showed a very low sensitivity and specificity (Table 2).

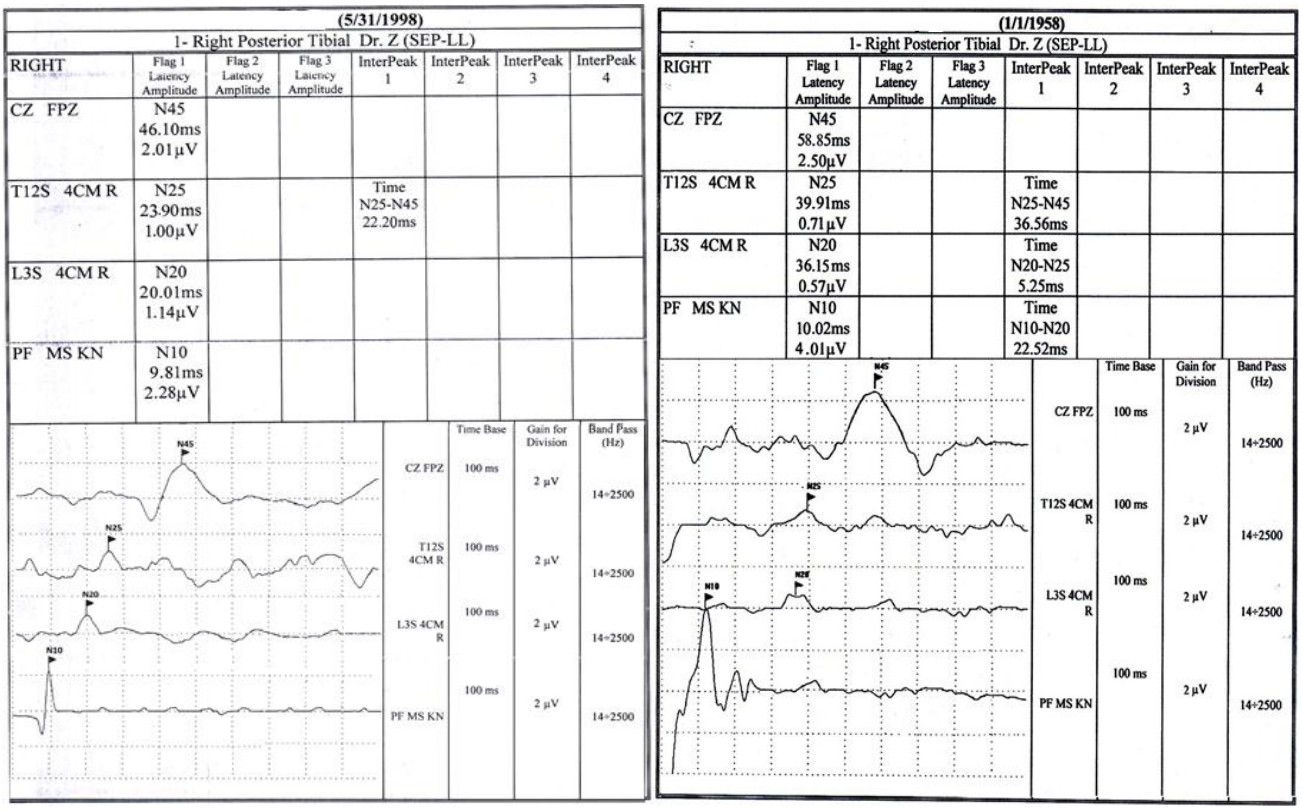

Fig. 1. Mixed SEP-TN (Left) of normal values, (Right) of abnormal values.

Table 2. Cutoff Value, Sensitivity and Specificity Percentages of Peak Latencies of Mixed SEP-TN Using ROC Test

Mixed SEP-TN Latencies	Cutoff value (ms)	Sensitivity (%)	Specificity (%)
N10	9.61	48.6	52.5
N20	20.705	67.1	67.5
N25	24.02	70.0	72.5
N45	46.22	67.1	70.0
N25 - N45	22.28	48.6	50.0

After dividing the studied patients and controls into 2 groups: prolonged and normal latency groups according to the chosen best cutoff values; results showed that cutoff values of the three N25, N45 and N20 SEP wave latencies presented highly significant differences between affected sides and controls; with the highest difference given by N25 (P< 0.0001, P=0.0003 and P=0.0007; respectively). However, there was no significant difference between affected sides and controls regarding N10 latency cutoff value (P= 1.0). As well, the N25-N45 cutoff value showed no significant difference between affected sides and controls (P = 1.0) (Table 3).

Regarding the amplitude of the measured SEP-TN waves, cutoff values showed equivocal results about the sensitivity and specificity %. The cutoff value of N25 amplitude (0.94µv) presented the best harmonized sensitivity and specificity %. Although N20 and N45 amplitudes have higher specificity % than N25 amplitude, their sensitivities were lower. On the other hand, the cutoff value of N10

amplitude has lower sensitivity and specificity (55% and 57%, respectively) compared to N25.

Table 3. Comparison of Mixed SEP Latencies between Affected Sides and Control Group by Chi Square and Fisher Exact Test

Parameters	Status	Affected sides N=70		Control Group N=40		*P* value
		No.	%	No.	%	
N10	Prolonged	34	48.57	19	47.50	1.000
	Normal	36	51.43	21	52.50	
N20	Prolonged	47	67.14	13	32.50	0.0007
	Normal	23	32.86	27	67.50	
N25	Prolonged	49	70.00	11	27.50	< 0.0001
	Normal	21	30.00	29	72.50	
N45	Prolonged	47	67.14	12	30.00	0.0003
	Normal	23	32.86	28	70.00	
N25 - N45	Prolonged	34	48.57	20	50.00	1.000
	Normal	36	51.43	20	50.00	

Table 4. Cutoff Value, Sensitivity and Specificity Percentages of Amplitudes of Mixed SEP-TN Using ROC Test

Mixed SEP-TN Amplitudes	Cutoff value (µV)	Sensitivity (%)	Specificity (%)
N10	2.77	55.0	57.1
N20	0.94	55.0	75.7
N25	0.94	60.0	64.3
N45	2.08	57.5	68.6

Amplitude cutoff values of the studied TN-SEP waves differentiate the studied patients and controls into 2 groups: low and normal amplitudes. N20, N25 as well as N45 amplitude cutoff values presented significant differences between affected sides and controls (*P*= 0.0018, 0.0171 and 0.0092; respectively); despite that the differences are more significant in cases of N20 and N45. On the other hand, no significant difference was obtained between affected sides and controls using the N10 amplitude cutoff value (*P*= 0.2395) (Table 5).

Discussion

Neurophysiological testing such as SEPs are helpful in determining the function of the nerve roots in LSS patients. They can be very helpful by providing objective information about the existence of, extent and severity of, and prognosis of neurologic deficits [1].

In this study, the presence of significant difference regarding the mean ages between patients and controls can be explained by the fact that LSS increases in occurrence with advancing age [1] explaining that most of included patients were of older ages; as compared to the younger ages of most of the control group.

In the current study, the lesser effect played by N20 latency cutoff value can be explained by the fact that the difference in conduction velocity between fast and slow fibers within a family of nerve axons would be intensified by increased distance of recording, which can be further exaggerated by the presence of compression neuropathy [7]. On the other hand, N45 measures a different entity of

somatosensory pathway of neurons (third order neurons) which may explain the lower sensitivity and specificity of its peak latency cutoff value compared to that of N25.

Table 5. Comparison of Mixed SEP Amplitudes between Affected Sides and Control Group by Chi Square and Fisher Exact Test

Mixed SEP-TN Amplitudes	Status	Affected sides N=70		Control N=40		*P* value
		No.	%	No.	%	
N10	Low	40	57.14	18	45.00	0.2395
	Normal	30	42.86	22	55.00	
N20	Low	53	75.71	18	45.00	0.0018
	Normal	17	24.29	22	55.00	
N25	Low	45	64.29	16	40.00	0.0171
	Normal	25	35.71	24	60.00	
N45	Low	48	68.57	17	42.50	0.0092
	Normal	22	31.43	23	57.50	

The least sensitivity and specificity percentages obtained by N10 latency cutoff value were expected because it measures the latency of peripheral part of the pathway (before the site of the pathology of the studied disease).

N25-N45 latency cutoff value showed a very low sensitivity and specificity and this proves that conduction time in the segment proximal to the lumbosacral spine has poor effect in the diagnosis of LSS.

Applying the selected cutoff values demonstrates that there were no significant differences between affected sides and controls concerning N10 peak latency (representing the conduction time in the peripheral segment of the somatosensory pathway) and N25-N45 latency (representing the central sensory conduction time) and these results are expected from the lowest sensitivities and specificities of these cutoff values and the fact that they represent the conduction time in the proximal and distal segments to the presumed site of compression, and hence logically they should not be affected by the pathology of the disease.

Significant differences were witnessed between affected sides and controls when applying the N20, N25 and N45 latency cutoff values, mostly by N25 latency. These rational results are expected, even more for N25 latency, due to their high sensitivity and specificity percentages and as these SEP parameters represent conduction times of different distances across the supposed site of compression. Therefore, these parameters are useful in the diagnosis of compression in the somatosensory pathway at the level of lumbosacral spine.

Results of the current study are in agreement with Eltantawi and his group [5], who found that there was a significant difference in SEP latency between patients and controls with a *P*=0.001 compared to (0.0003) *P* value in this study.

The results in this study disagree with that of Bingöl and co-workers [4] who stated that cortical SEP latency and the spinal SEP latency showed no significant differences between patients and control groups. This can be explained by using different statistical procedure, having low number of patients or studying patients with one level root compression. As peripheral mixed nerves such as TN contains fibers from multiple roots, results of SEP-TN can be normal despite the existence of a single root compression due to the diluting effect from the remaining unaffected roots.

Again, N25 amplitude cutoff value showed the highest harmonized sensitivity and specificity percentages, in the same way as the results of peak latency. However, other EP amplitudes showed higher specificities but lower sensitivities in some or lower both specificities and sensitivities in others than N25 amplitude. These equivocal results can be explained by the complex relationship between neuronal pathways in the central nervous system, with the presence of different order neurons and lots of convergence and divergence, which probably lower the validity of the amplitude measurements of the different evoked potentials in the somatosensory pathway.

Results of the chosen amplitude cutoff values showed significant differences between affected sides and control in all the SEP wave amplitudes, apart from N10 being a measure of peripheral conduction. These results demonstrate a diagnostic value of mixed SEP-TN amplitude in LSS; despite having lower sensitivities and specificities compared to latency. These results are in accordance with Eltantawi *et al* 2012 [5] who found that the cortical SEP amplitude had high significant difference between patient and control ($p < 0.05$) but disagree with Bingöl and his group 2010 [4] who found no significant difference ($p = 0.09$) between symptomatic and asymptomatic sides which may be explained by the same reasons mentioned earlier.

In this study abnormal mixed SEP-TN found in 70% of patients according to results of N25 wave; similar findings were detected by Egli *et al* 2007 [8] who found mixed SEP-TN abnormalities in 78% of the studied 54 patients.

In conclusion, mixed SEP study can be used as an add-on test in the diagnosis of LSS. N25 of the mixed SEP has the highest diagnostic yield in LSS because of having the highest sensitivity and specificity. Equivocal results of the SEP wave amplitudes and their lower sensitivities and specificities compared to SEP latencies, lower their validity in the diagnosis of LSS.

We recommended a combination of clinical, radiological and electrodiagnostic test like SEP to be included in the evaluation of patients with suspected LSS. Data on MRI findings of the lumbar spine of asymptomatic subjects should be supported by SEP studies as radiologic findings may not represent physiologically important LSS. Further studies correlating imaging and electrophysiological procedures with operative findings need to be done to further document the role of mixed SEP study in accurate evaluation of LSS patients.

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Author Contribution

Dr. Essa performed the SEP study while Dr. Al-Hashimi did the conventional NCS and EMG for exclusion criteria and both share the writing of the paper. Dr. Nema was responsible for collection of patients who participate in the work and their clinical examination.

Conflict of Interest

The authors declare no conflict of interest concerning this work.

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Antibacterial activity of Fenugreek essential Oil against *Pseudomonas aeroginosa*: *In vitro* and *in vivo* Studies

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Abstract

Background Multiple drugs resistance has increased due to the random use of available antimicrobial drugs in treatment of infectious diseases.

Objective To investigate probable antibacterial effects of fenugreek essential oil extract against *Pseudomonas aeruginosa*

Methods Twenty eight isolates of *P. aeruginosa* were collected from skin infected patients in Al- Yarmook teaching hospital in Baghdad. Antimicrobial susceptibility tests of 14 antibiotics were performed using Vitek2 compact system. The antibacterial activity of essential oil was evaluated using agar well diffusion method with minor modifications**.** The broth micro dilution method was used to determine minimum inhibitory concentration. Then animal experiment was performed in five groups of mice (n=7, for each) as following: control, induction, treated with fenugreek alone, treated with Gentamycin alone, treated with combination of fenugreek and Gentamycin. Then histopathological examination was done after seven days of the treatment.

Results *P. aeruginosa* isolates are highly resistance to trimethoprime/ sulfamethaxazole, while sensitive to amikacin. Minimum inhibitory concentration of fenugreek essential oil for highly resistance *P. aeruginosa* isolates (n=10) as followed: 6 isolates with minimum inhibitory concentration = 1.2gm/100µl, and 4 isolates with MIC= 0.6gm/100µl**.** Minimum inhibitory concentration of gentamycin was equal to>=16.

Conclusion Fenugreek essential oil has higher antibacterial effect alone and in combination with gentamycin than gentamycin alone.

Keywords Antibacterial activity, Fenugreek, *Pseudomonas aeruginosa.*

List of abbreviation: FICs = Fractional inhibitory concentration values, MDR = multidrug resistance, FLC = Fast Liquid Chromatography, MIC = minimum inhibitory concentration.

Introduction

Multiple drugs resistance has increased due to the random use of available antimicrobial drugs in treatment of infectious diseases. Bacteria are more common on normal skin than other microorganisms [(1,2)]. A number of antimicrobial agents, including a number of Beta–lactams are active against *P. aeruginosa.* Extended-spectrum penicillins often used to treat infections caused by this bacterium. Although most cephalosporins are not active against *P. aeruginosa* [(3)]. Of the carbapenems, meropenem has slightly better activity against *P. aeruginosa* than imipenem-cilastatin. The fluoroquinolones have the advantage of being exists in both oral and intravenous formulations and are thus attractive options for treating *P. aeruginosa* infections in the outpatient setting. Of the fluoroquinolones agents, Ciprofloxacin is the most active against *P. aeruginosa*. In

conclusion, the aminoglycosides have been mainstays in the treatment of these infections [4].

The aim of the study is to investigate probable antibacterial effects of fenugreek essential oil extract against *P. aeruginosa* in sample from skin infections and detection of active component behind these antibacterial effects, and to study the combination effect of fenugreek essential oil with gentamycin against *P. aeruginosa*.

Methods

Isolation and detection of *P. aeruginosa*

All specimens were diagnosis microscopically (Gram stain), morphorgically and biochemically according to standard methods [5,6], and some biochemical tests were achieved bycommercial kits (GN VITEK2 gram negative colorimetric identification kit) for *P. aeruginosa* bacteria (BioMerieux, France).

Antibiotics susceptibility

Antibiotics susceptibility tests by using the Biomérieux VITEK2 compact system (BioMerieux, France) against the following antibiotics: pipracillin, pipracillin- tazobzctum, ticarcillin, ticarcillin/ clavulonic acid, ceftazidim, cefipim, imepenem, meropenem, amikacin, tobramycin, gentamycin, trimethoprim / sulfamethaxazole, and ciprofloxacin.

Extraction of fenugreek essential oil

Fenugreekessential oil is extracted from seeds of plant (supply from local markets in Baghdad). The oil is extracted by steam distillation methods from dried plant and yield 1.2gm for fenugreek for each 100gm of plant materials [7].

Assessment of antibacterial activity of fenugreek essential oil

It was evaluated using agar well diffusion method with minor modifications [8]. The broth micro dilution method was used to determine minimum inhibitory concentration (MIC). All tests were performed in muellerhinton broth Salucea (Netherlands) supplemented with Tween 80 (BDH (England) at a final concentration of 0.5% (v/v) [9]. Fractional inhibitory concentration values (FICs) for antimicrobials combinationswas used to determine the effect of antimicrobials combinations on multidrug resistance (MDR) isolates of bacteria. FIC values used to assess the synergism between gentamycin with fenugreek essential oil for *P. aeruginosa* [10].

Separation active ingredient of fenugreek essential oil

Separation of active ingredients of fenugreek essential oil was done by Fast Liquid Chromatography (FSL) (Shimadzu, North America) equipped with binary delivery pump model 2010, using 3µ particle size column (50 × 4.6 mm H.D) C-18 (Injection 10 µl of essential oil in column), Mobile phase: 0.01M ammonium phosphate buffer (BDH, England) A: acetoitrile B (BDH, England). Eluted by linear gradient from 0-100% B in 10 min. Detection of eluted peak were monitored by UV-Vis spectrophotometer (Cecil, France) set at 254nm, flow rate 1.0 ml/min, temperature 30˚C [11] (Fig. 1).

Animals experiment (*In vivo* method)

Thirty five healthy, domestic male mice, weighing 23-25 gram were used in this study; they were obtained from animal house in High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, in the period between Aug. 2014 and Oct. 2015. These mice were kept in separated cages; the room temperature was maintained at 20 -25^{o}C.

Animals grouping

The mice randomly divided into five groups (n=7, each) according to following:

Group 1 (control): control group infected just by phosphate buffer saline (China).

Group 2 (induction group): infected by bacteria without treatment

Group3: treated with fenugreek essential oil alone for 7 days.

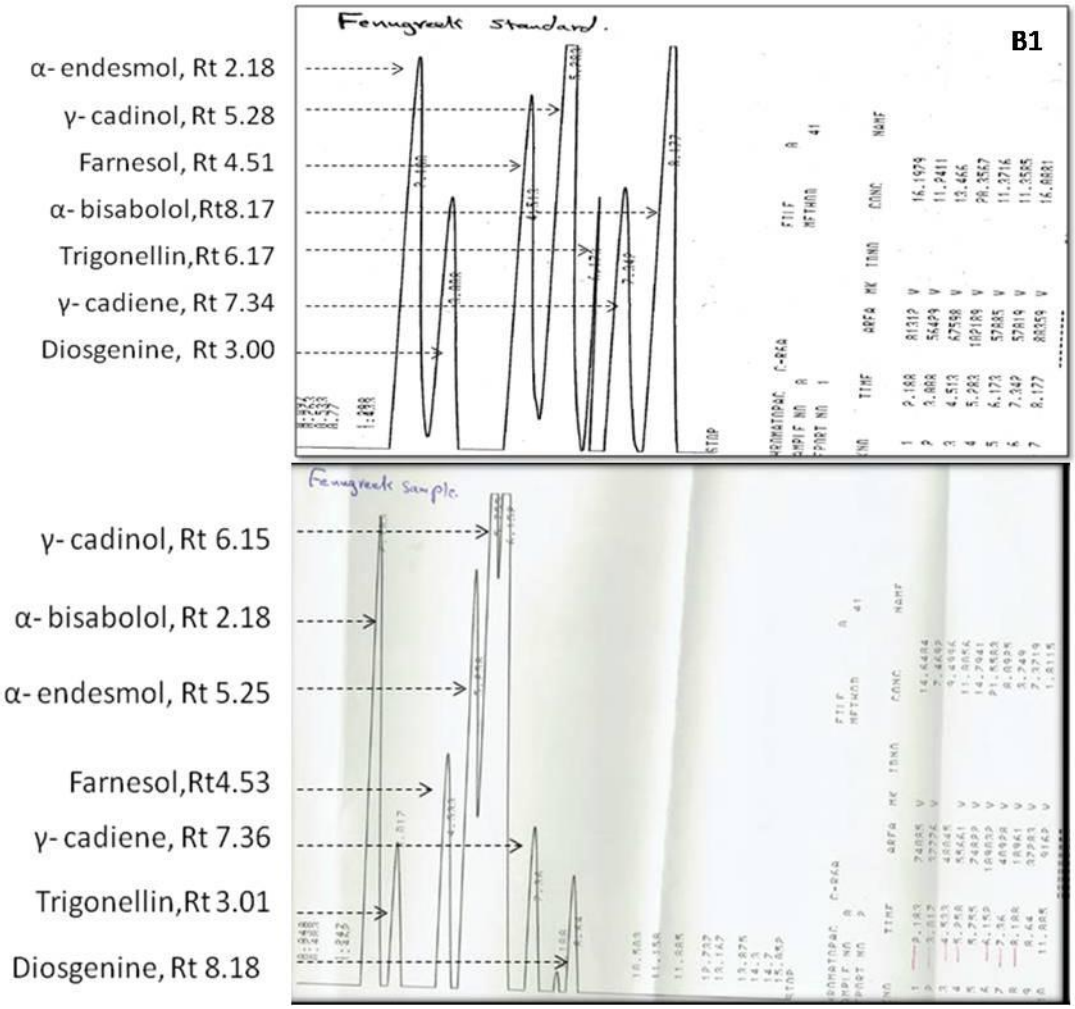

Fig. 1. HPLC chromatography of fenugreek standard and sample, B1: fenugreek standard, B2: fenugreek (Rt = retention time)

Group 4: treated with gentamycin cream (SDI) alone for 7 days.

Group 5: treated with combination of Gentamycin cream and fenugreek essential oil for 7 days.

For preparation of inoculate, the bacteria were sub cultured onto brain heart agar (BHA) (Oxoid, England) and incubated at 37^{o}C overnight. Then one colony was inoculated into brain heart broth (BHB) (Oxoid, England) and incubated overnight at 37^{o}C, the overnight culture was diluted 1:100 in fresh BHB and grown until the mid-exponential phase (approximately 3 hours). The bacteria washed twice and resuspended in sterile phosphate buffered saline (PBS) [12].

Before inoculation the mouse models of bacterial skin infection were sedated with ether. The flanks of the sedated mice were shaved with clippers when necessary and cleansed with an ethanol solution (BDH (England), and then make wound by scalpel cuts. The wounds were subsequently inoculated by 50 µl of the bacterial suspension. Then the mice were returned to their cages and observed. All mice had free access to food and water throughout the duration of the experiments. Animals were observed daily and skin lesion size, swelling, redness, amount of buss were noticed. The treatments with antimicrobial used in this study begin after 4 hrs of bacterial inoculation and continued at the regimens of 7 days [12,13].

Statistical Analysis: Data were analyzed using SPSS version 16 and Microsoft Office Word and Excel 2007. Nominal data were expressed as

number and percent. Independent sample T-test was used for comparison of mean. *P*-value less than 0.05 were considered significant.

Results

Out of 300 specimens obtained from different skin infection, 28 isolates (9.3%) were *P. aeruginosa* which are highly resistance to most of antimicrobial agents while show moderate resistance to ciprofloxacin, imepenem, meropenem and amikacin. The percentage of resistant isolates to each antibiotic is shown in fig. 2.

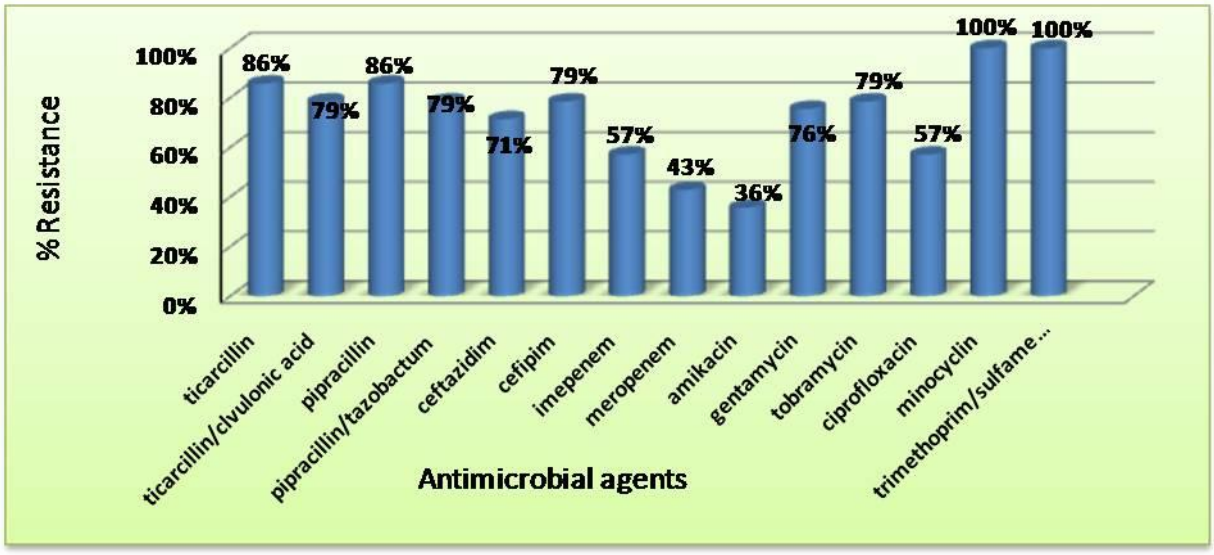

Fig. 2. Resistant *Pseudomonas aeruginosa* isolates to antimicrobial agents (n=28)

MIC of fenugreek essential oil for highly resistance *P. aeruginosa* isolates (n=10) as followed: 6 isolates with MIC= 1.2gm/100µl, and 4 isolates with MIC= 0.6gm/100µl (Table 1). MIC of gentamycin for isolates was determined using VITEK 2 AST method which represent >=16.

The effects of fenugreek essential oil alone and in combination against *P. aeruginosa* are shown in table 1.

Table 1. Effect of fenugreek essential oil alone and in combination with gentamycin on multidrug resistance *P. aeruginosa* isolates

Isolates no.	Fenugreek MICgm/100µl	Fenugreek 1/4 MIC	Gentamycin 1/4 MIC	1/4+1/4 MIC	1/2+1/2 MIC	FIC Values FIC/Interpretation	
1	1.2	+	+	+	-	1	Indifference
2	1.2	-	+	-	-	0.5	synergism
3	0.6	+	-	+	-	1	Indifference
4	1.2	-	+	-	-	0.5	synergism
5	0.6	+	+	-	-	0.5	synergism
6	0.6	+	+	-	-	0.5	synergism
7	1.2	-	+	-	-	0.5	synergism
8	1.2	+	-	-	-	0.5	synergism
9	1.2	+	+	-	-	0.5	synergism
10	0.6	+	+	+	-	1	Indifference
240 mg		240 mg	0.00325 mg	2.4 mg			
0.77		0.77		0.77			

– (no growth), + (growth), fractional inhibitory concentration (FIC) was determine as follow: ≤0.5 = synergism, 0.5-<1 = additive, 1-<4 = indifference, ≥4 = antagonism, *P*-value less than 0.05 were considered significant.

The treatments with antimicrobial used in this study for *in vivo* study begin after 4 hrs of bacterial inoculation and continued at the regimens of 7 days. Then after seven day part of lesion area was tested for histopathological examination.

Group 1: the mice infected just by phosphate buffer saline, no lesion, no redness, no swelling, no death, and histopathological section showed normal skin without inflammatory cell infiltration, also no vascular congestion, no edema and no necrosis as showed in fig. 3.

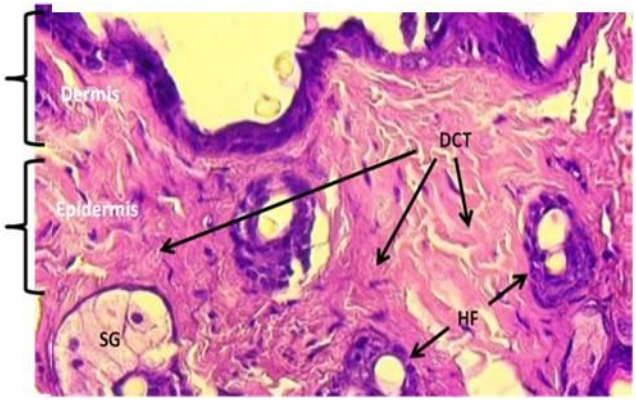

Fig. 3. Section of mouse infected by phosphate buffer saline showing DCT = dermatomal connective tissue, HF = hair follicle, SG = Sebaceous gland (H&E) x40

Group 2: this group infected by *P. aeruginosa* isolate without treatment. The skin swallowed, red, heavy pus, lesion size=0.8 cm, four mice died, and under histopathological examination the section showed market inflammatory cell infiltration, market edema and market vascular congestion of dermis and subcutaneous tissue as shown in fig. 4.

Group 3: Redness, swallowed, lesion (0.6 cm), and pus continued from first day until four day. Few pus and redness were remaining, no death was occurred, the histopathological examination showed moderate edema, moderate inflammatory cell infiltration and moderate vascular congestion as showed in fig. 5.

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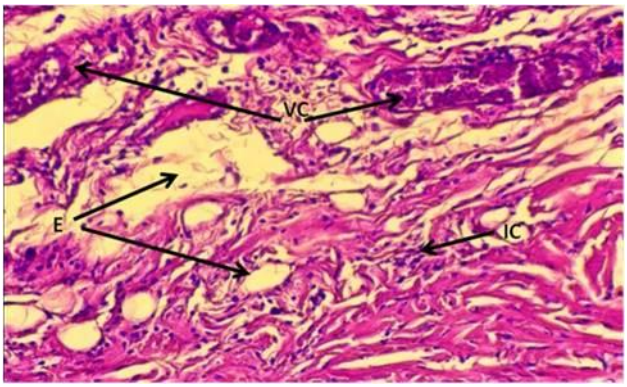

Fig. 4. Section of mouse infected by *P. aeruginosa* without treatment showing IC = inflammatory cells, E = edema, VC = vascular congestion (H&E) x40

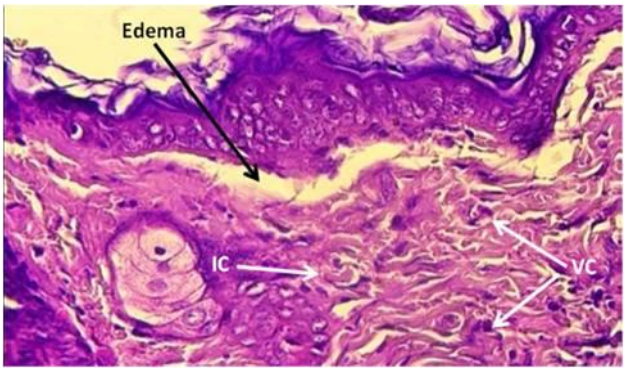

Fig. 5. Section of mouse infected by *P. aeruginosa* treated with fenugreek showing VC = vascular congestion, IC = inflammatory cells (H&E) x40

Group 4: the degree of redness, lesion (1cm), swelling and pus were decreased until third day, healing begun in day six and only one mouse was died in third day. Histopathological examination showed moderate edema, and moderate inflammatory cell infiltration as showed in fig. 6.

Group 5: in this group swallowed, lesion (0.8cm) and pus continue until day five and only one mouse was died in third day.

Histopathological examination showed just mild edema, no vascular congestion and scanty inflammatory cell infiltration as shown in fig. 7.

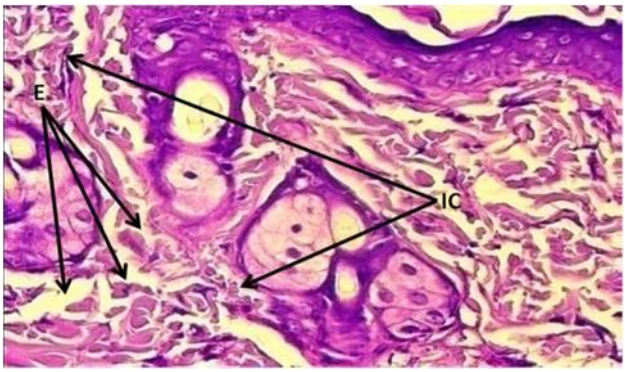

Fig. 6. Section of mouse infected by *P. aeruginosa* treated with gentamycin showing E = edema, IC = inflammatory cells (H&E) x40

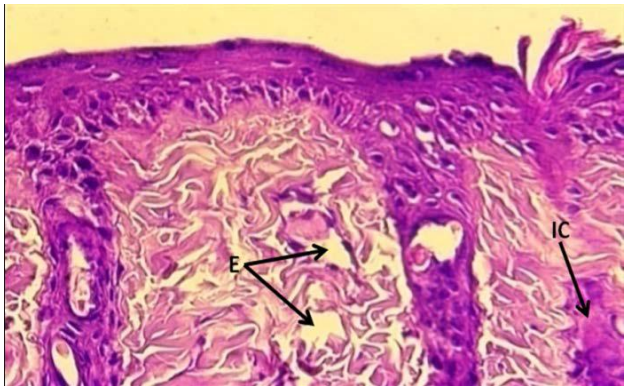

Fig. 7. Section of mouse infected by *P. aeruginosa* treated with combination of fenugreek and gentamycin showing E = edema, IC = inflammatory cells (H&E) x40

Discussion

In the present study, correct identification rate of *P. aeruginosa* was 100 % (28 isolate/28 total isolates). Ines *et al* [14] found that correct identification rates of *P. aeruginosa* were 90.1%.The result of current study demonstrate that *P. aeruginosa* represent 9.3% among patients with skin and soft tissue infection, the results of present study coincides with previous studies in Iraq [15] in which 9.7% of *P. aeruginosa* isolates obtained from burn wound swabs in patients admitted to Al-Karama teaching hospital in Baghdad.

Zone of inhibition of fenugreek essential oil after 48 hrs against *P. aeruginosa* range from 12 to 22 mm of concentrated essential oil (100%), and some essential oil did not have any antibacterial effect against two isolates. Mayssaa [16] revealed that zone of inhibition of fenugreek against *P. aeruginosa* was 16mm and some isolates showed resistant to fenugreek extract. In this study it was found that MIC of fenugreek essential oil range from 0.6-1.2 gm/100ml. Other investigations revealed that MIC values of fenugreek essential oil were ranging from 0.8-6.4 gm/100ml against both gram positive and gram negative bacteria [17].

The result of the present study was in agreement with the finding of Al-Derzi [18] that has found the resistance of *P. aeruginosa* to gentamicin was 79.1%, while in agreement with him that the resistance rate to amikacin was 89.7% and this result is higher than the result of the current study.

The aminoglycosides inhibit protein synthesis in bacterial cell by binding to 30S subunit of the ribosome and the aminoglycoside- resistance in *Pseudomonas sp.* is primarily due to modification in target enzymes and in activation of the antibiotics [19,20].

Aminoglycosides have numerous groups of resistance mechanisms: enzyme modification, low outer membrane permeability, active efflux and, not often, target modification [21,22].

Synergistic effect was seen from combination of gentamycin with fenugreek essential oil against most isolates of *P. aeruginosa* as shown in table 1. The important point of Abascal and Yarnell [23] study is on the combining of herbs with antibiotics to decrease drug resistance acting synergistically with drugs to kill microbes. Generally, their action is the result of the combined effect of together active and inactive compounds [24,25].

The growth of the organism was clearly observed in all inoculated mice. Lesions cultures was confirmed the infections by bacteria. After usage of the plants as topical treatment for one week, the lesions and

wounds were healed dramatically. Control groups were used to prove that healing was not spontaneously.

In recent years, different reports from different countries were indicated that there were antimicrobial activities of medicinal plants, for many years, the effect of herbal medicine on burn wound has been noted. Herbal products seem to possess moderate efficacy and are less expensive as compared with synthetic drugs. Many plants and plants-derived products have been shown to possess potent wound-healing activity [26].

In-vivo-sensitivity of the plants studied on the infected mice proved to be very active. All the infected mice were cured by local application of the plants on the lesions. No spontaneous improvement was detected on the infected control mice. The result of histopathological examination in the present studies show that antibacterial activity of fenugreek essential oil alone and in combination with Gentamycin is greater than antibacterial activity of gentamycin alone, this effect may belong to the active compound in the plants which have bactericidal effect against *P. aeruginosa*. This indicates that thecure of the tested mice was due to the action of theseplants studied. The use of that plant in the form of topical therapy in infected mice was proved the affectivity of fenugreek plants as medicinal purpose [27].

Most of the medicines are mixture of many plants, but none of these traditional ointments were scientifically studied.In our study, fenugreek extract was compared with gentamycin as the standard treatment for burn wounds in mice. The actual mechanism of improved healing is still unclear, the probable mechanism are providing necessary material for healing, increasing blood flow to burn area, decreased inflammatory response, and decreasing rate of infection. A new skin medication can be introduced by usage of herbal medicines with fewer adverse effects and shorten the period of healing thus decrease the rate of hypertrophic scar. The result findings denotes of fenugreek in healing of burn injuries as an inexpensive and available herbal medicine [28].

In conclusion, fenugreek essential oil has antibacterial effect against skin infection with *Pseudomonas aeruginosa* and combination of fenugreek with gentamycin shows synergistic effect and is more effective than gentamycin alone.

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Author contribution

Dr. Abdul Kahaleq conducted the study; Dr. Abu-Raghif organizes the idea, finalizes the protocol, and selects the herbs; and Dr. Kadhim contributes through technical support of the research.

Conflict of interest

The authors declare no conflict of interest.

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Ultrasound Guided Aspiration versus Drainage under General Anesthesia in Breast Abscesses

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Abstract

Background Despite the fact that breast abscess is becoming less common in developed countries, it has remained one of the leading causes of morbidity in women in developing countries. Ultrasound has been shown to be useful in diagnosis of breast abscesses, guiding needle placement during aspiration and also enables visualization of multiple abscess loculation and thus useful in needle aspiration of breast abscesses and is associated with less recurrence, excellent cosmetic result and has less cost.

Objective To establish whether ultrasound guided needle aspiration is a feasible alternative treatment option for breast abscesses.

Methods A prospective interventional study conducted on 144 female patients with and age range from 15 to 55 years. One hundred and twenty four are lactating and the other 20 are non-lactating. They were divided into two groups; the first group comprised 72 patients treated as outpatients by ultrasound assisted aspiration of pus while the second group serves as the control comprised 72 patients who are treated by drainage under general anesthesia. For both groups, data regarding early complications, hospital stay, return to daily activity and late complications were recorded. Follow up for up to 3 months was done for both groups.

Results Healing rate of the two groups had no statistically significant difference both overall and at each visit. There was only 1.4% recurrence rate observed in the ultrasound guided needle aspiration group while there was 6.9% recurrence rate observed in the incision and drainage group.

Conclusion Sonographically guided percutaneous aspiration of breast abscesses represents a less invasive and very promising alternative to surgical incision, showing the following advantages: no general anesthesia required a superior cosmetic result and shorter hospitalization.

Key words Breast abscess, Ultrasound guided aspiration, Incision and drainage

List of abbreviation: BA = breast abscess, PPW = primiparous women, GA = general anesthesia, U/S = ultrasound, UGNA = ultrasound guided needle aspiration.

Introduction

Breast abscess (BA) is a common cause of morbidity in women. Mastitis is a potential complication of breast-feeding that occurs more commonly in primiparous women (PPW). The reported incidence varies widely, from 1% to 24% [1,2]. BA can result as a complication of mastitis, especially if treatment is delayed or inadequate [3,4]. Treating mastitis with a combination of breast-emptying procedures and a course of antibiotics usually prevents abscess formation [4]. On occasion, an abscess may occur after medically induced weaning [5]. The reported incidence of abscess in lactation-related mastitis is 4.8% to 11% [1]. PPW, mothers over 30 years of age and those giving birth post-maturely may be more likely to develop BA during lactation than other groups [5].

It has been demonstrated earlier that stress to the mother and fetus during labor and delivery

are risk factors for delayed lactogenesis. It is possible that the release of oxytocin as a result of suckling may be somewhat slower in mothers, whose labor may have been induced with artificial oxytocin, predisposing them to engorged breasts, subsequent inflammation and abscess formation. The delaying of pregnancy may play a part in lactation difficulties [6].

While they are less common in developed countries as a result of improved maternal hygiene, nutrition, standard of living and early administration of antibiotics, BA remains a problem among women in developing countries [7].

Traditionally, management of BA involves incision and drainage; however this is associated with need for general anesthesia (GA), prolonged healing time, regular dressing, difficulty in breast feeding, and possible unsatisfactory cosmetic outcome [8].

The treatment of BAs sometimes represents a difficult clinical problem. Even with the aggressive approach of incision and drainage combined with use of antibiotics, BAs recurrence rate is reported to be between 10 and 38% [9].

The breast is one of female sex organs, in case of breast disease care should be taken to insure that its beauty is minimally compromised in order to preserve its value and function.

BAs can be treated by repeated needle aspiration with or without ultrasound (U/S) guidance [10-12]. U/S has been shown to be useful in diagnosis of BAs, guiding needle placement during aspiration and also enables visualization of multiple abscess loculation and thus useful in needle aspiration of BAs [13].

This procedure has been used successfully and is associated with less recurrence, excellent cosmetic result and has less coasty [14].

The aim of our study is to establish whether ultrasound guided needle aspiration (UGNA) is a feasible alternative treatment option for BAs.

Methods

A prospective interventional study conducted for 144 female patients with an age range between 15 and 55 years (mean 36 years) who attend the Accident and Emergency Department and Breast Outpatient Clinic in Al-Kindy Teaching Hospital, Baghdad, Iraq for the period between March 2013 and October 2014.

One hundred and twenty two out of the total patients are lactating and the rest 22 were not. Patients with recurrent or chronic BA and those with necrotic skin overlying the abscess or abscess already draining were excluded from the study.

The clinical diagnosis based on the presence of breast pain, swelling, fever and presence of a fluctuant tender breast swelling. The patients diagnosed clinically were subjected to U/S scan equipped with high frequency linear transducer of 7.5 MHZ (HD11 XE, Philips) in the radiology department. The diagnosis was confirmed by the presence of a thick walled echo complex mass, predominantly cystic with internal echoes and septations. The radiologist studied the site, size, number of abscess cavities as well as presence or absence of any other concomitant breast pathology, so we met the inclusion criteria which included the well-formed pus cavities, superficial or peripherally located pus cavity, absence of concomitant breast lump and no suspicion of malignancy.

The patients were divided into two groups; the first group comprised 72 patients who were treated as outpatients by U/S assisted aspiration of pus, for those a short history was taken regarding demographic data and lactation state. This group was subjected to U/S assisted aspiration of pus by 16G needles and a 20 ml syringe after scrubbing the field by suitable antiseptic solution and infiltration of 1 - 4 ml xylocaine 2% at the site of maximum tenderness. Those patients were followed up by U/S in the next day; if no pus residual seen, no further action will be required other than oral antibiotics for few days. If there was still residual collection, another session of

aspiration was performed until improvement in general conditions and U/S showing no residual collection. The time interval between each session of aspiration and the next was 24 hours and the median number of these sessions was 3.

The other group which was treated by surgical drainage also comprised 72 patients who either refused the aspiration or those with large cavity collection (more than 10 cm) when the pus is too superficial with skin changes. For those patients, preparation was done including investigations (HB, blood sugar, blood urea, chest x-ray and ECG when indicated). Drainage under GA was done, sample of pus was sent for bacteriological study, injectable or oral antibiotics were given for all patients postoperatively**.**

For both groups, data regarding early complications, hospital stay, return to daily activity, and late complications were recorded. Follow up for up to 3 months was done for both groups.

In this study, healing was defined as achieving BA resolution and the latter defined as clinically no breast tenderness, swelling or wound at the previous site of the abscess and via U/S complete absence of fluid collection, normal breast glandular and fibro-fatty tissue with no edema**.**

Statistical analysis

Statistical analysis was done using Statistical Package of Social Sciences (SPSS) computer software version 17. Categorical data was summarized into proportions, percentages and rates. Continuous data was summarized into mean, median and range. Tables were used to present data. Statistical significance was defined as a P value of less than 0.05.

Results

Out of the total number, 72 (50%) patients underwent U/S assisted aspiration under local anesthesia with age range from 15 to 47 year (mean 34), those referred to as group A. The remaining 72 patients (50%), those treated by classical incision and drainage under GA with age range from 20 to 55 years (mean 36) and referred to as group B. Sixty 60 patients (83 %) of group A were lactating and 12 patients (17%) were non-lactating, whereas 62 patients (86%) of group B were lactating and 10 patients (14%) non-lactating**.** Median size of pus cavity was 3 cm (range 2 - 5 cm) for group A while for group B, the median size was 9 cm (range from 5-15) as shown in table 1.

Table 1. Number of patients, their age and abscess size of each group

Character		Group A	Group B
No. of patients		72	72
Lactation	Yes	60	62
	No	12	10
Age range (mean)		15-47 (34)	20- 55 (36)
Abscess size		2-5cm (3)	5-15 (9)

Of the 72 patients of the group A, 67 patients (93%) had peripherally located abscesses and 5 patients (7%) with centrally (subareolar) located abscesses. In group B there were 70 patients (97%) with peripherally located and 2 patients (3%) with subareolar location.

Regarding the number of U/S assisted aspiration of group A, there were 55 patients (76.3%) treated successfully with single aspiration, 10 patients (13.8%) were re-aspirated on the first visit due to persistence of the abscess and 5 patients (6.9 %) treated successfully and efficiently by third session. Two patients underwent surgical drainage under GA, one for residual mass (which was excised and proved to be a benign inflammatory mass) and other for persistent fistula because of ectatic duct.

The median number follow up U/S examinations was 3 (range from 2 to 5). In group B, the median number of post operative visit for dressing change was 7 visits (5-10).

Regarding the hospital stay, for group A, 65 patients (90.3%) were discharged within 1 or 2 hours after aspiration to be attend at the next

day for re-examination clinically and ultrasonically, 7 patients (9.7%) were admitted for one day to be discharged on the next day after second aspiration. They return to normal daily activity on the same day and for lactation (for lactating women) on the next day. In group B, 20 patients (27.78%) were discharged in the same day, 43 patients (59.72%) were admitted overnight, 7 patients (9.72%) were admitted for 48 hours and 2 patients (2.78%) were admitted for 5 days because of mammary fistulas. They return to daily work after 48 hours in 55 patients (76.4%) and 72 hours in 15 patients (20.8%) and 2 patients (2.78%) after 10 days (Table 2).

Table 2. Duration of hospital stay in both groups

Duration of hospital staying	Group A	Group B
Few hrs (<1 day)	65 (90.3%)	20 (27.78%)
One day	7 (9.7%)	43 (59.72%)
48 hrs	0	7 (9.7%)
>2days	0	2 (2.78%)

Lactation was resumed after 24 hours in 32 patients, after 48 hours in 18 patients, on third day in 6 patients, 4 days in two patients and after 10 days in 2 patients (Table 3).

Table 3. Resuming of lactation in both group

Resume lactation	Group A	Group B
Within 24 hrs	62	---
1^{st}-2^{nd} day	---	32
2^{nd}-3^{rd} day	---	18
3^{rd}-4^{th} day	---	6
4^{th}-5^{th} day	---	2
>5 days	---	2

Regarding wound complication, there was only one patient belong to group A with hematoma formation, one with recurrent abscess formation within 3 months. No patient with residual scar formation. In group B, a large or hypertrophic scars in 2 patients, mammary fistula in 2 patients (one in an ectatic ducts and other in uncontrolled diabetic patient), and both fistulas were treated conservatively and closed within 10 and 14 days respectively (Table 4).

Table 4. Complication rate at both groups

Complication	Group A No (%)	Group B No (%)
Hematoma	1 (1.39)	0
Fistula	0	2 (2.78)
Hypertrophic scar	0	2 (2.78)
Recurrence after 3 months	1 (1.39)	5 (6.9)

In group B, wick drains were used in 55 patients while corrugated plastic drains were used in 17 patients. The drains removed after 24 hours in 44 patients, after 48 hours in 10 patients, after 3 days in 8 patients, after 5 days in 8 patients and in 2 patients removed after 10 and 14 days. Dressing and follow up were for 5 - 20 days.

During the study period, one BA recurrence was observed in the U/S aspiration group; while 5 (6.9%) recurrence were recorded in the group B. Almost all the patient (97%) treated by U/S aspiration highly accepted the procedure.

Seventy (65.5%) patients belongs to group A exhibited complete healing by the third visit and 68 (58.1%) in group B. There was no difference in healing rate between the two study arms at all the three visits (P = 0.55) as observed figure 1.

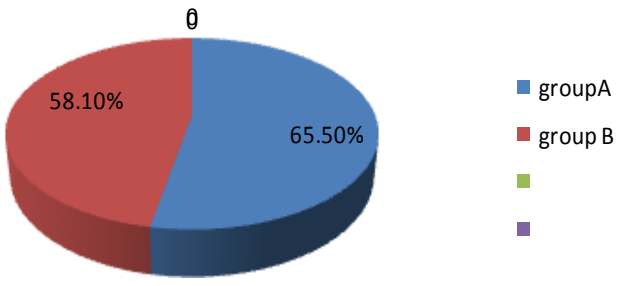

Fig. 1. Pie chart showing healing rate after 3 month (P = 0.63)

Discussion

Healing rate of the two groups had no statistically significant difference both overall and at each visit and this was similar with what was found by other study [10]. This similarity in the healing rate between the two treatment option could be explained by the fact that regardless of the way pus is removed from the cavity (that is incision and drainage, needle aspiration or spontaneous rupture onto the skin surface) the healing process is the same which is by collapse of the cavity wall and adherence to one another by fibrin, later by granulation tissue. The remaining bacteria destroyed by polymorphs [15].

There was only one recurrence (1.4%) of breast abscess observed in the ultrasound guided needle aspiration group during the study period and this may be explained by small pus cavity selection for group A. There was 6.9% (5 /72) recurrence rate observed in the incision and drainage group; however this recurrence rate was less than 31% recurrence in the incision and drainage group which has been reported in another study [14]. This small recurrence rate observed may have been resulted from a short follow up period and it was not possible to compare the recurrence rate of the two study groups.

Almost all the patients treated with ultrasound guided needle aspiration highly accepted this modality (97%). This was consistent with other studied [14,16,18]. This high acceptance rate may have been resulted from the convenience of the procedure which was an outpatient one, having no wound to nurse and absence of scar after healing.

The total cost of ultrasound guided aspiration was found to be much less than that of incision and drainage, thus indicating that ultrasound guided aspiration provides savings to the hospital and the patient, hence more cost effective than incision and drainage. This was consistence with what was found elsewhere [17,19]. Since ultrasound guided aspiration is an outpatient procedure as opposed to the incision and drainage which is inpatient procedure. Studies done to compare outpatient versus inpatient surgical procedures showed that outpatient procedures were cost effective [20,21].

In conclusion, there is no difference in terms of healing rate of breast abscess between ultrasound guided aspiration and surgical incision and drainage. Ultrasound guided needle aspiration is highly accepted by women with breast abscesses. Ultrasound guided aspiration is more cost effective than incision and drainage in management of breast abscess, therefore ultrasound guided needle aspiration was an effective treatment option for both lactating and non-lactating breast abscess. Sonographically guided percutaneous aspiration of breast abscesses represents a less invasive and very promising alternative to surgical incision, showing the following advantages: no general anesthesia required, a superior cosmetic result and shorter hospitalization

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Author contribution

Dr. Al-Marzooq did the study conception, design and acquisition of data; Dr. Mehsen analyze and interpret the data and Dr. Al-Timimy made the drafting of manuscript and critical revision.

Conflict o Interest

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Immunohistochemical MDA Changes of the Newborn Rat Frontal Cortex Affected by Prenatal Ketamine Exposure

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Abstract

Background Ketamine has an excellent analgesic property and is widely used currently to provide "sedation" for minor procedures. Ketamine has been proved to potentiate deletion of large numbers of neurons from the developing brain.

Objectives To investigate the neurotoxic effect of prenatal ketamine exposure on newborn rat frontal cortex using malondialdehyde antibodies as immunohistochemical marker.

Methods Seventy two pregnant rats were divided into three groups I, II and III (24 rat for each group) and exposed to ketamine at different gestational periods (7th day, 11th day, and 18th day, respectively). Each group was subdivided into four subgroups including the control subgroup A injected intraperitoneally with normal saline, and the experimental subgroups B, C and D rats injected intraperitoneally with different doses of ketamine (5mg/kg, 10mg/kg, and 20mg/kg respectively). Paraffin sections of the frontal cortex of the newborn rats were immunohistochemically stained with Expose Mouse and Rabbit Specific HRP/DAB Detection Kit. The Aprio Image Scope v.9 software was used to evaluate the anti-MDA antibodies immunohistochemical reaction.

Results Non-significant variability between the subgroups B and C of group I, while significant variability was found between these two subgroups in groups II and III. The values obtained from subgroup B in all the groups I, II, and III had no significant variability compared to the control subgroup (A). The values obtained from subgroups (C) and (D) showed statistically significant changes compared to the control subgroup (A) in all the groups (I, II, and III). The results showed significant variability by comparing the results of subgroup (D) with both subgroup (B) and subgroup (C) in all the groups.

Conclusion The anti-MDA immunohistochemical reactivity shown in this study suggested that lipid peroxidation is an event occurring during ketamine induced neurotoxicity, this event leads to apoptosis.

Keywords Cortex, prenatal, ketamine, neurotoxicity, immnuohistochemistry.

List of abbreviation: MDA = malondialdehyde.

Introduction

Neurotoxicity is defined as the abnormalities of the nervous system following exposure to a chemical, biological, or physical agent. The susceptibility to neurotoxicity is more during development as the blood brain barrier is not completely developed and neurogenesis and synaptogenesis are taking place at high rates [(1)].

The frontal lobes in human include several functionally different regions that are grouped into three regions; motor, premotor, and prefrontal. The frontal lobes connect to all cortical regions through association fibers. It receives mainly strong input from limbic cortex, amygdala and septal nuclei, and areas

involved in emotional responses [2]. There are wide varieties of symptoms that are associated with frontal lobe lesions; these include disorders of motor functions, failure of divergent thinking, impaired response inhibition and inflexible behavior, reduced memory, and impaired social and sexual behavior imaging [3].

Ketamine has been used in the surgical emergencies requiring anesthesia, it has been suggested that ketamine can be used safely for anesthesia in infants and children [4]. Ketamine is a nonbarbiturate, dissociative anesthesia used for short diagnostic and surgical procedures and to supplement low-potency anesthetics such as nitrous oxide. The adverse reactions associated with ketamine including visual hallucinations, nightmares or illusion, and post-anesthesia delirium [5].

Considering the effect of ketamine on developmental tissue, ketamine was placed in the class of other competitive and noncompetitive *N*-methyl-d-aspartate antagonists. Ketamine affects neuronal functioning in the developing brain of the rat, and significant decreases were found in neural cell adhesion molecules and postsynaptic densities after single exposure to ketamine during neuronal development [6].

The teratogenic potency of ketamine hydrochloride in mice has been proved experimentally [7]. It was suggested that ketamine has the potential to delete large numbers of neurons from the developing brain by a mechanism involving interference with the action of the neurotransmitters glutamate and gamma-amino butyric acid at *N*-methyl-d-aspartate and gamma-amino butyric acid receptors during the synaptogenesis period. This transient interference during the synaptogenesis period (the last trimester of pregnancy and the first several years after birth in humans) causes millions of developing neurons to commit suicide (die by apoptosis) [8].

The current study was formulated to investigate the prenatal ketamine neurotoxicity in rat. This aim was proposed as the neuronal number, location, differentiation, and type are affected by the events occurring in embryonic life before neuronal differentiation. It was reported that there are 2 distinct periods of cell proliferation in the nervous system, the first occurs embryonically and correlates with neurogenesis and the second occurs postnatally and correlates with gliogenesis [9].

Immunohistochemical antibodies to malon-dialdehyde (MDA) have been used in this study to investigate the prenatal ketamine neurotoxicity. MDA is a natural product formed in all mammalian cells as a product of lipid peroxidation. It is a highly reactive byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism. MDA can combine with many functional groups on proteins, lipoproteins, and DNA. MDA is toxic and has been implicated in many biological events [10].

Methods

In this study, female Wistar rats (*Rattus Norvegius*) aged 4-6 weeks and weighted between 150-250g were brought from the laboratory animal house, College of Medicine, Baghdad University. The study was performed during the period from November 2013 to may 2014. These female rats were mated, and pregnancy was confirmed by the observation of vaginal plug.

All animals were treated according to National Institute of Health Guidelines for the Care and Use of Laboratory Animals [11].

The total number of pregnant rats used in this study was (72), these animals were divided into three groups I, II, III (24 rat for each group). Animals of these groups were exposed to ketamine at different gestational periods (at the 7^{th} day, 11^{th} day, and 18^{th} day, respectively).

The pregnant rats of each group were subdivided into four subgroups (six animals for each subgroup) including the control subgroup A, received intraperitoneal injections of normal saline, and the experimental subgroups B, C

and D, received intraperitoneal injections of ketamine in different doses (5mg/kg, 10mg/kg, and 20mg/kg, respectively). Female rats which were found to have no signs of pregnancy were excluded.

Pregnant rats of the experimental subgroups received intraperitoneal ketamine hydro-chloride injections (Kanox, ketamine 50 mg/ml preservative; chlorobutanol 5%, batch number 122228E, Duopharma), the control subgroup A received intraperitoneal normal saline injections.

The injections were done at 6 consecutive doses every 1.5 hours (for a total of 9 hours of therapy) at each of the 7^{th}, 11^{th} and 18^{th} days of pregnancy.

Each female rat delivered (8-16) neonates, from which 6 neonates were selected randomly for this study.

Newborn animals were sacrificed by decapitation during the first hour on the first day of delivery, their brains were removed from the cranium, and coronal paraffin sections of 5µm thickness of the frontal cortex were prepared after fixation in 10% formalin [12].

Digital camera (Sony cyber shot) was used for documenting tissue staining and histology.

Anti-MDA antibodies

These antibodies were provided from Abcam (code no. ab6463). They are rabbit polyclonal antibodies containing small molecules of synthetic malondialdehyde conjugated to bovine serum albumin.

The immune-histochemistry detection kit is called Expose Mouse and Rabbit Specific HRP/DAB Detection IHC Kit from Abcam (code no. ab80436).

Six sections were randomly selected from the sections of the frontal cortex of neonates delivered by the rats of each subgroup (A, B, C, and D) in each of the groups (I, II, and III).

Aperio Image Scope version 9 software was used for the evaluation of MDA antibodies immunohistochemical reaction. This image analysis software involves counting the number of strong positive pixels to evaluate the immunohistochemical stain.

The list of positive pixel count algorithm includes parameters obtained from the application of this software to quantify the amount of a specific stain present in a scanned slide image.

These parameters when first selected have been pre-configured for brown color quantification. Pixels which are stained, but do not fall into the positive-color specification, are considered negative stained pixels.

Results

Analysis of variance (ANOVA) statistical evaluation of the mean values of MDA immuno-histochemical reactivity in the frontal cortex of neonates delivered by rats of subgroup A (the control subgroup) showed non-significant differences among all the groups (I, II, and III).

Anti-MDA reaction in the frontal cortex of neonates delivered by rats of group (I) (Fig. 1-3):

The evaluation of the counted mean values obtained by the application of the Aperio Image Scope software in the cortex of neonates delivered by rats of subgroups (B, C, and D) revealed statistically significant variability compared to those of the control subgroup A.

This counting of the mean value of the number of strong positive pixels was highest in subgroup D (11562.6 ± 764.6). The mean values in subgroup B and C were 4400.1 ± 234.1 and 4925.6 ± 149.4 respectively. The *P* values were ≤ 0.001 for the subgroups B, C, and D.

The multiple comparison statistical tests done for the mean differences between the experimental subgroups B and C of group I showed none significant variability ($P \geq 0.072$), while high significant variability was shown between the subgroups B and C compared with subgroup D of group I ($P \leq 0.001$).

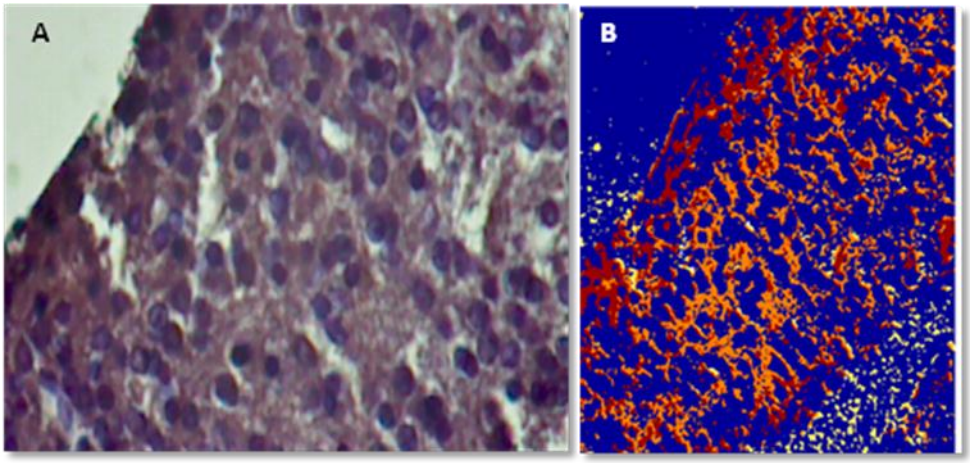

Fig. 1. (A) Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup B of group I. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.

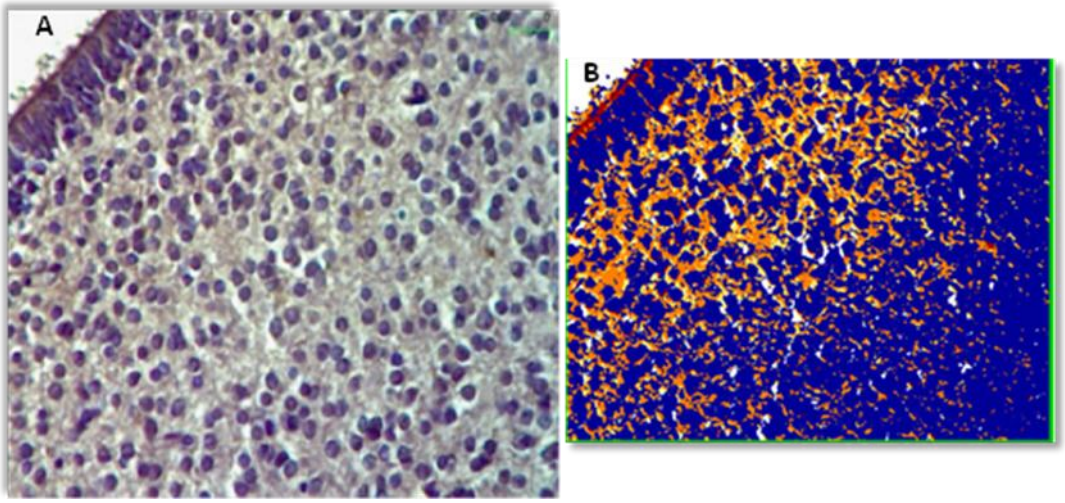

Fig. 2. (A) Anti MDA reactivity in frontal cerebral cortex of neonate rat from subgroup C of group I. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.

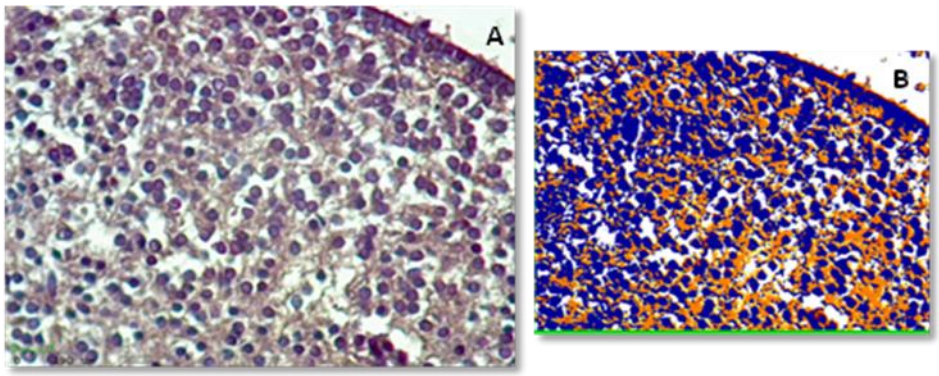

Fig. 3. (A) Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup D of group I. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm (400X).

Anti-MDA reaction in the frontal cortex of neonates delivered by rats of group (II) (Fig. 4-6):

The counting for the mean values of the number of strong positive pixels obtained from the cortex of the neonates delivered by female rat of the experimental subgroups (B, C, and D) and those of the control subgroup A of group II showed equivalent analysis compared to that of group I.

The highest mean value was in subgroup D (16791.3 ± 1325.9), the mean values of subgroup B and C were 5571.3 ± 348.3 and 9965.1 ± 436.6, respectively. The *P* value was ≤0.001 for subgroups B, C, and D.

The multiple comparison statistical tests done for the mean differences between the frontal cortices of neonates in experimental subgroups B, C, and D of group II showed high significant variability ($p \leq 0.001$).

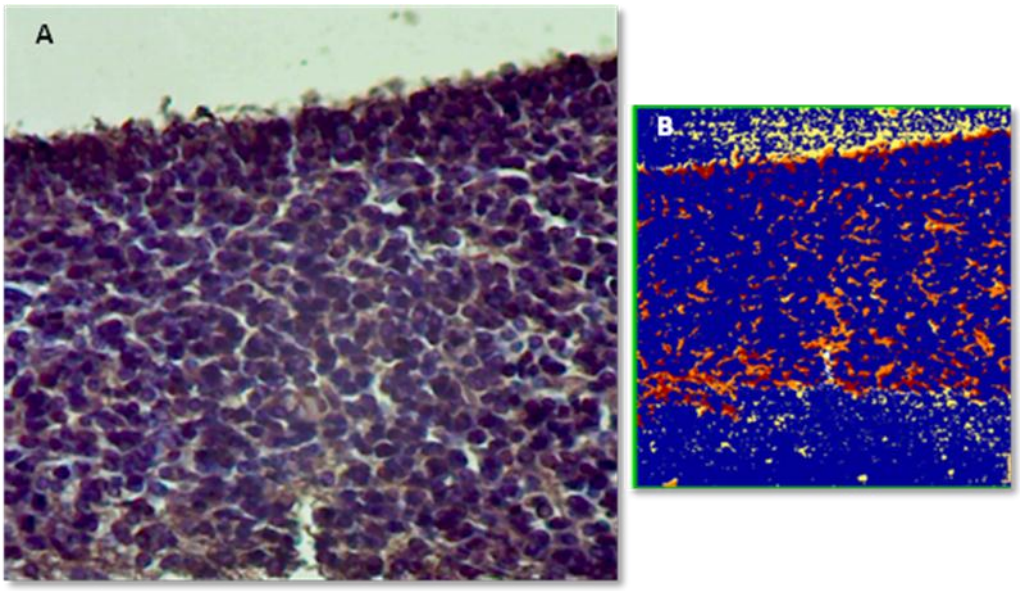

Fig. 4. (A) Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup B of group II. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.

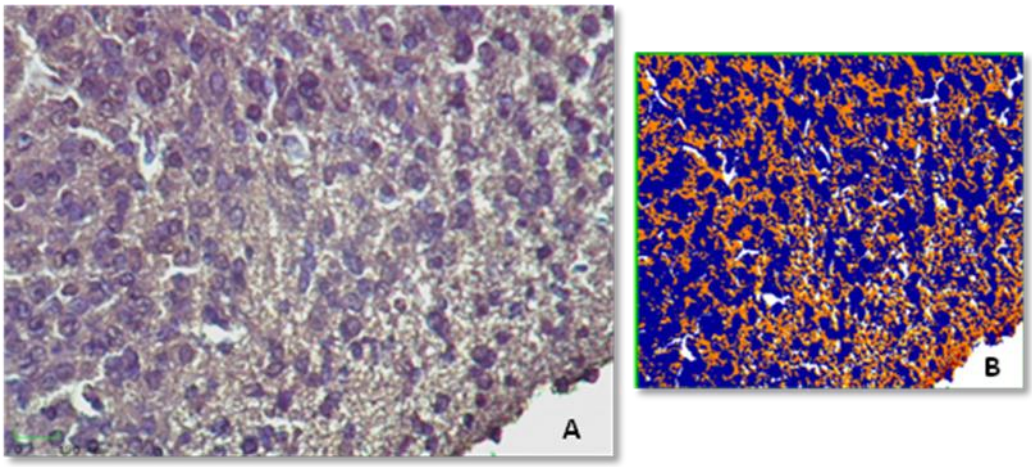

Fig. 5. (A) Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup C of group II. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.

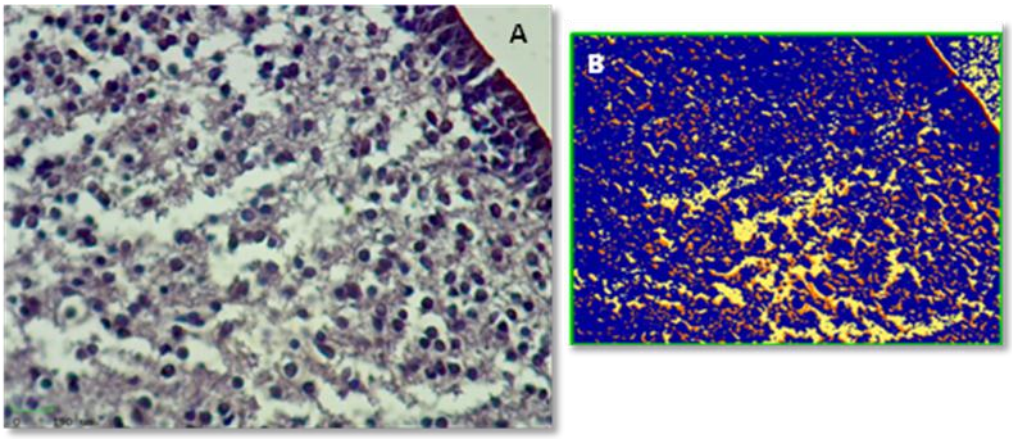

Fig. 6. (A) Anti- MDA reactivity in frontal cortex of neonate rat from subgroup D of group II. Anti-MDA positive stain is seen in all layer of frontal cortex (400X) (B) The snap shoot of Aperio Positive Pixel Count Algorithm.

Anti-MDA reaction in the frontal cortex of neonates delivered by rats of group (III) (Fig. 7-9):

The neonate frontal cortex of group III showed marked increase in the mean value of subgroup D (42147.1 ±1058.0) compared to that of the subgroups B and C (5544 ±447 and 13676.5 ±419.6, respectively), these results were parallel to the results obtained from the analysis of group I and II. A significant variation between the experimental subgroups (B, C, and D) was recorded at $p \leq 0.05$ compared to the control subgroup A.

Equivalent statistical results were obtained for the mean differences between the treated subgroups B, C, and D of group III. Highly significant variability ($P \leq 0.001$) was found between these subgroups B, C, and D.

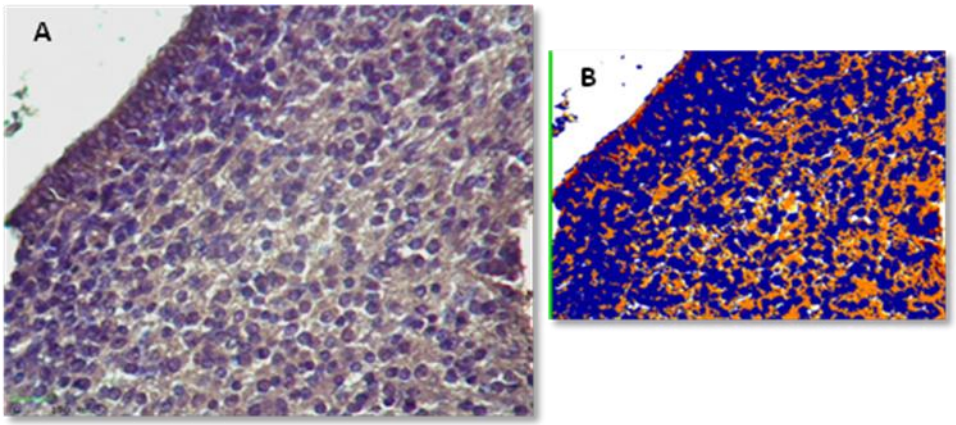

Fig. 7. (A) Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup B of group III. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.

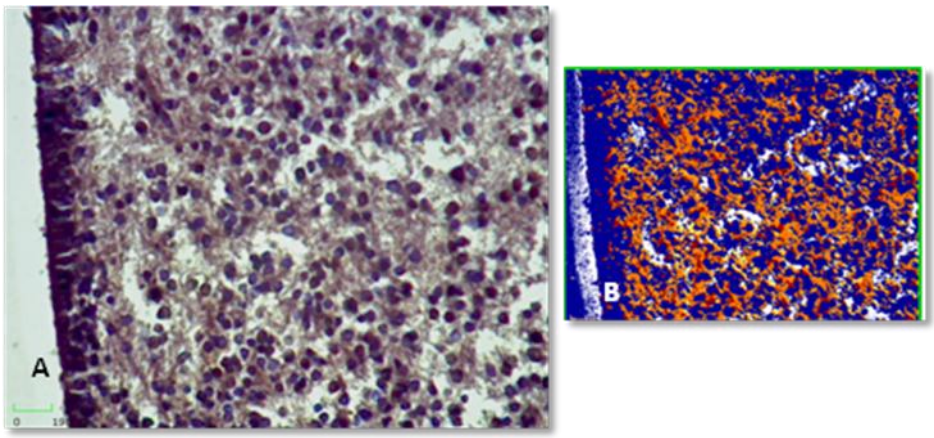

Fig. 8. (A): Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup C of group III. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.

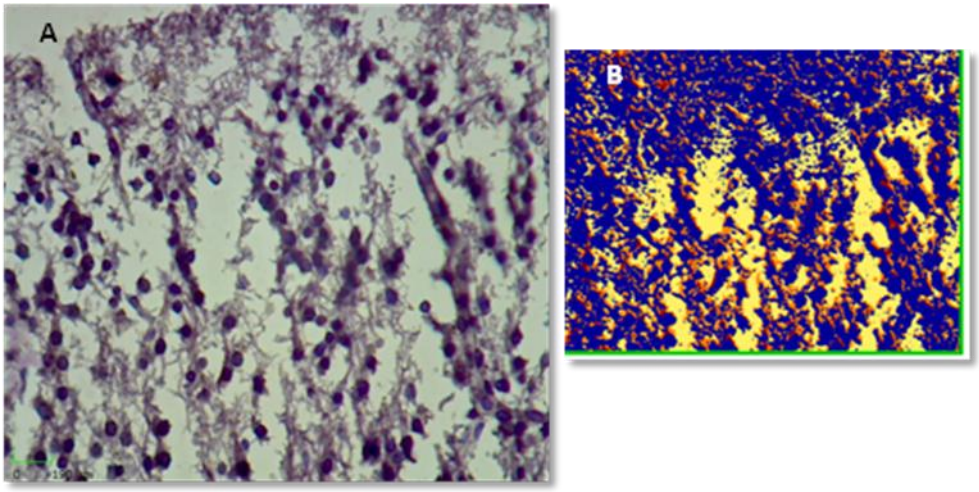

Fig. 9. (A) Anti- MDA reactivity in frontal cerebral cortex of neonate rat from subgroup D of group III. Anti-MDA positive stain is seen in all layer of cerebral frontal cortex (400X) (B) The snap shoot as analyzed by Aperio Positive Pixel Count Algorithm.

Appraisal of anti- MDA reaction of each subgroup in all the groups:

ANOVA statistical analysis of the counted mean values obtained for subgroup B in all groups (I, II, and III) compared to that of the subgroup A (the control subgroup) showed non-significant variability ($P \geq 0.253$). While significant variability was found from the values of comparing the subgroup C with control subgroup A ($P \leq 0.024$), and a highly significant variability was found for subgroup D in all groups ($P \leq 0.001$).

Appraisal of anti- MDA reaction of all the subgroups in each of the groups:

ANOVA statistical analysis of the counted mean values for all the subgroups in group I showed non-significant variability compared to those of group II ($P \geq 0.179$), while the statistical analysis of these values in group I compared to group III showed highly significant variability ($P \leq 0.001$). Also, the analysis of the values obtained from group II compared to group III showed significant variability ($P \leq 0.01$).

Discussion

Evaluation of the anti-MDA immune-histochemical changes caused by 5mg/kg of ketamine exposure (subgroup - B):

The anti-MDA immunohistochemical reactivity shown in the results of this study suggested that ketamine-induced lipid peroxidation is an event occurring during the process of ketamine neurotoxicity. Lipid peroxidation after cellular injury leads to apoptosis and autophagy; cellular membranes, because of their high lipid content, are especially susceptible to damage because lipid peroxidation reactions can alter the structure and function of critical membrane lipids leading to cell injury and cell death [13].

Therefore, the statistical analysis of the results in this study, showing the non-significant variability between subgroups B and C of group I, suggested that the cortical cellular membrane damage by lipid peroxidation reactions is of the same severity in the newborns of these subgroups. This explanation indicates that the doses of 5mg/kg and 10mg/kg of ketamine produce equivalent neurotoxic effect when used during the 7th day of gestation.

Statistical evaluation showed the least values from the subgroups (B) compared to the other subgroups in each of the groups; therefore, it could be assumed that the least neurotoxic effect of ketamine is seen when using the drug in dose of 5mg/kg.

The analysis of the results in subgroup B of all the groups I, II, and III have no significant variability compared to the control subgroup (A). This is a supportive evidence for considering 5mg/kg as a dose of least neurotoxicity.

Evaluation of the anti-MDA immune-histochemical changes caused by 10mg/kg and 20mg/Kg of ketamine exposure (subgroups B and D):

The newborn frontal cortex of these subgroups (C) and (D) showed statistically significant changes compared to the control subgroup (A) in each of the groups (I, II, and III).

The anti-MDA staining analysis showed significant variability by comparing the results of subgroup (D) with both subgroup (B) and (C) in each of the groups; this is considered as a supportive evidence that the injection of 20mg/kg is the highest toxic dose. The results of this study showed that the mean of the number of strong positive pixels counted to evaluate the histochemical reactivity in the cortex of the subgroup (C) have an intermediate values between that for subgroup (B) and (D) in each of the groups. The mean for the subgroup (D) was the highest in each of the groups.

Also the results of the counted values obtained from the subgroups (C) and (D) in all the groups showed significant variability from those of the control subgroup (A).

The neurotoxic effect of ketamine injection on spatiotemporal development of the cerebral cortex:

The important question whether anesthetic drugs can trigger neuroapoptosis in the developing non-human primate brain was first addressed by Slikker and colleagues [14] who reported that intravenous infusion of ketamine triggered neuroapoptosis in the (5) day old rhesus macaque brain. These reports are supportive to the results of this study, suggesting that lipid peroxidation detected by the anti-MDA immunohistochemical technique indicates neuroapoptosis in the frontal cortex by the neurotoxic effect of ketamine.

In agreement with the results of this study, it was reported that the pattern of augmented neuroapoptosis in the ketamine-exposed fetal brains was widespread [15]. Therefore, the results of this study supported the fact that ketamine exposure during development is responsible for inducing neuroapoptosis in the developing brain.

All of the recent human epidemiological studies pertaining to developmental anesthesia neurotoxicity [16] have focused on full-term infants and children; the focus of future human research should be expanded to include third

trimester fetuses and prematurely born infants.

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Author contribution

Dr. Gaeb performs the laboratory research work; Dr. Mobarak and Jaffar interpret the results.

Conflict of interest

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

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Prevalence of Coronary Artery Disease in Symptomatic Patients with Zero Calcium Score Undergoing Coronary CT Angiography

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Abstract

Background Non-invasive coronary angiography is being increasingly performed by computed tomography angiography to assess obstructive coronary artery disease. There is increasing interest in the absence of coronary artery calcification, as a "negative" cardiovascular risk factor. The frequency and clinical relevance of coronary artery disease in patients without coronary artery calcification are unclear.

Objective To assess the presence and the severity of coronary artery disease in symptomatic patients without coronary artery calcification (Calcium score of zero).

Methods One hundred and ten cases (62 females and 48 males) with mean age of 50.4 years with no detection of calcified plaques in the coronary arteries (coronary artery calcification score=zero) were studied. Known or suspected cases of coronary artery disease underwent a coronary computed tomography angiography examination. Calcium score examination was conducted immediately before coronary computed tomography angiography. Degree of stenosis was found by comparing the luminal diameter of the narrowest segment of the artery with that of a more proximal or distal normal segment of the same artery.

Results Stenosis was found in 23/110 patients, giving a prevalence of (20.9%), among the 23 cases with stenosis: the right coronary artery stenosis was found in 19/23 (82.6%), left anterior descending in 11/23 (47.8%) and left circumflex artery in 5/23 (21.7%). Mean percentage of stenosis was highest in right coronary artery (59.9%). In 52.2%, only one vessel was involved, in (43.5%) two vessels were involved and in (4.3%) three vessels were involved.

Conclusion Coronary computed tomography angiography can clearly demonstrate noncalcified atherosclerotic coronary plaques in a large group of patients with suspected coronary artery disease. The absence of coronary artery calcification does not exclude the presence of significant stenosis in symptomatic patients with no coronary Calcium.

Keywords Coronary artery disease, Zero Calcium score, CT coronary angiography

List of abbreviation: CAD = coronary artery disease, CT: computed tomography, CACS = coronary artery calcium scoring, CCTA = coronary computed tomography angiography, ECG = electrocardiogram, RCA = right coronary artery, LAD = left anterior descending, LCXA = left circumflex artery.

Introduction

Coronary artery disease (CAD) is the leading cause of death in the world. Identifying new risk factors and improving the screening methods for CAD are continuously evolving processes. The presence of calcium in coronary arteries is pathogenomonic of atherosclerosis, as confirmed by histopathology and intravascular ultrasound studies [1-3]. The implementation of multidetector computed tomography (CT) at the end of the 1990s resulted in the widespread use of Coronary artery calcium scoring (CACS) [4]. The latter represents a

reliable linear anatomic estimate of total plaque burden [5]. Nearly all prospective studies have found moderate-to-high CAC to be an independent and incremental predictor of future cardiovascular events over conventional risk factors and the Framingham Risk Score [6-15]. Therefore current guidelines recommend measurement of CAC for further risk stratification of intermediate risk individuals, in whom treatment with long-term aspirin and statin therapy is most uncertain [6, 16].

Calcified plaques represent older lesions, and newer plaques are more likely to be lipid rich and poor in calcium [17]. There is increasing interest in the absence of CAC (a calcium score of 0) as a "negative" cardiovascular risk factor [18,19]. Absence of CAC might reliably exclude obstructive coronary disease in asymptomatic and selected symptomatic individuals and seems to be associated with a low cardiovascular event rate, suggesting that less aggressive pharmacotherapy might be indicated in this population [20].

However, published event rates for individuals with 0 CAC vary, likely owing to differences in baseline risk, follow-up period, as well as outcome ascertainment and verification [19]. The characteristics of the few individuals with 0 CAC who subsequently develop cardiovascular events have not been well- described. Less is known about the prognosis of a low positive CAC score (CAC 1 to 10), because most studies are underpowered to report this as a distinct group. Some studies have reported increased and variable non-calcified soft coronary plaque in patients with low CAC [21]. In patients with a CAC score <10, coronary computed tomography angiography (CCTA) provides excellent diagnostic performance with a very high specificity [22].

One concern is the presence of isolated lipid-laden (soft) plaque in the setting of a negative study (zero calcium score). The ability to detect lipid-laden coronary plaques with cardiac CT angiography would possibly improve risk stratification of these patients [23]. Recent studies found varying degrees of noncalcified plaques using coronary CT angiography in patients with a CAC score of zero [21,24,25].

CCTA has become a noninvasive diagnostic option for detecting critical coronary artery stenosis in patients with low or moderate risk [26]. and can be employed as a screening tool for selected populations in the identification of patients at higher risk for ischemic events. Those people would benefit from further testing and more aggressive risk factor modification [27]. The determination of significant stenotic disease in persons with some level of calcification will undoubtedly be useful to the clinician and patient [28].

The aim of the study is to assess the presence and the severity of CAD in symptomatic patients without coronary artery calcification (Calcuium score of zero).

Methods

A prospective cross-sectional study was employed at AL-Sader Medical City in Al- Najaf health directorate, performed from April to December 2014. A total of 110 patients (62 females, 48 males; mean age, 50.4 years [range, 21-75 years] were enrolled in the study. All the patients included in the study had no detectable calcified plaques in the coronary arteries (CAC score=zero). A known or suspected cases of CAD (patients complaining from chest pain and atypical angina) underwent a coronary CT angiography examination; Ca score examination was conducted immediately before coronary CT angiography.

The exclusion criteria were: patients with Ca score above zero, the coronary CT angiography examination was suboptimal, and the coronary arteries could not be sufficiently evaluated, history of coronary artery bypass graft and/or prior stent placement, as well as lack of sinus rhythm or history of any allergic reaction to contrast agent, renal function impairment, patient with myeloma or any patient in whom administration of contrast will be risky. Other patient-related factors that can interfere with the diagnostic quality of CTCA are irregular

heart rhythm (atrial fibrillation or frequent extra systoles) and inability to sustain a breath hold for at least 15 to 20 seconds.

The CT examination was performed in a calm and comfortable atmosphere (e.g., lights were dimmed, and the staff speaks quietly), avoiding anything that might affect the patient's heart rate, because a constant rate is crucial for image quality and diagnostic accuracy in CCTA. Patients asked to avoid anything that can increase their heart rate, such as talking during the scan or moving too much, also avoid caffeine, smoking and advice B blocker (metoprolol 50 mg) for one day before exam. CT coronary angiography was performed with a 64-slice scanner (Aquillon 64, V4.51 ER 010, Toshiba Medical Systems, Tochigi; Japan) with retrospective electrocardiogram (ECG) gating.

As soon as the patient has been placed on the table in the supine position with the arms above the head he or she should not move, in order to ensure that the planned scan region matches the region actually scanned and that the entire coronary tree is imaged. The patient should be shifted slightly to the right side of the table, so that the heart is as close to the center as possible (since spatial resolution is highest in the center of the scan field). The ECG electrodes should be placed so that they do not disturb the patient, while ensuring optimal identification of R-wave signals by the ECG monitor.

In the CCTA examination, about 80-100 ml of iodinated contrast agent (Omnipaque, 350 mg/mL iodine) was administered by dual head injector through an 18–20 G cannula, which was placed in the right antecubital vein. Then 40 ml saline was administered. The optimal scan time was determined using the automatic bolus tracking method.

Before Multi-Slice CTA, a non-contrast CT was acquired to measure calcium score according to the Agatston and volumetric methods for the whole heart (total heart calcium) as well as the individual coronary arteries [left main stem (LM), left anterior descending artery (LAD), left circumflex artery (LCX) and right coronary artery (RCA)] using sequence scan with slice thickness of 3 mm. When the patient heart rate was more than 65 bpm, a β-blocker (metoprolol 50 mg orally was administered before the scan. Breath holding exercise were done for all patient A bolus of 80 -100ml contrast medium (Omnipaque, 350 mg/mL iodine) was injected intravenously at a rate 5 ml/s, followed by 40 ml of normal saline. The scan was obtained from the aortic arch to the level of the diaphragm during a single breath hold.

With ECG triggered scanning protocol was performed, the following parameters were used: Collimation width 32.5×32.5 cm, slice thickness 0.5 mm, rotation time 0.35 s, tube voltage 120 kV, maximum effective tube current 890 mA, table feed 0.3 mm/rotation AT 75% of R-R cardiac cycle. The examination time about 10 seconds.

CT images were reconstructed using a smooth kernel (B25f) with a slice thickness of 0.5 mm (increment 0.3mm). CT data sets were transferred to dedicated workstation (VITREA 2 WORKSTATION vital image Plymouth, Minnesota, USA) for image analysis. The total calcium score of coronary arteries were calculated by Agatston and volumetric methods. To avoid observer variability, two radiologists had measured and read the calcium scoring.

The composition (calcified, noncalcified/soft, or mixed) of the plaques was established. Only the cases that had Agatston score=zero were included in the study and the patients were divided into 2 groups: with and without plaques (stenosis and without stenosis) as observed in coronary CT angiography. The degree of stenosis was assessed by comparing the lumen diameter of the narrowest segment with that of a more proximal or distal normal segment. Stenoses were classified as mild (<39% stenosis), moderate (40-69% stenosis) and severe (70-99% stenosis).

Statistical analysis

Data are presented as mean ± standard deviation or as numbers and percentages. Categorical data are expressed as frequencies and were compared with Pearson's Chi-square test. Continuous variables are presented as the mean ± standard deviation and were compared using ANOVA (analysis of variance). A probability (P) value of less than 0.05 was considered statistically significant. Data were analyzed with SPSS software version 20.

Results

There were 110 patients enrolled in this prospective study. The mean age of the studied group was 50.4 ± 9.1 (range: 21-75) years, furthermore, 17 patients (15.5%) aged ≤ 40 years, 46 patients (41.8%) aged 41-50 years, 34 patients (30.9%) aged 51-60 years and 13 patients (11.8%) aged > 60 years. Regarding the gender distribution, males were 48/110 represented (43.6%) of the studied group and females were 62/110 and represented (56.4%), with a female to male ratio of (1.3:1).

According to the CCTA findings of the studied group, stenosis of different degree was found in 23 patients (20.9%) while the remaining 87 patients (79.1%) had no stenosis.

Among the 23 patients with stenosis, right coronary Artery (RCA) stenosis was the most prevalent and found in 19 patients (82.6%), left anterior descending artery (LADA) stenosis in 11 (47.8%) and left circumflex artery (LCXA) in 5 patients (21.7%), this was statistically significantly (P = 0.015).

Further analysis revealed that mean percentage of stenosis was 59.9 ± 14.7 in RCA, 47.7 ± 12.8 in LADA and 56 ± 19.5 in LCXA, There is statistically significant difference when comparing stenosis in RCA and LADA, P value is 0.046 as shown in table 1.

Table 1. Mean stenosis percentage of the involved arteries among positive group

Artery	No.	Mean ± SD	Range	P value
RCA	19	59.9 ± 14.7	26-80	RCA vs. LADA = 0.046
LADA	11	47.7 ± 12.8	27-75	RCA vs. LCXA = 0.43
LCXA	5	56.0 ± 19.5	30-80	LADA vs. LCXA = 0.28

RCA = right coronary artery, LAD = left anterior descending, LCXA = left circumflex artery

In 12 patients (52.2%) only one vessel was involved, in 10 patients (43.5%) two vessels were involved and in one patient only (4.3%) three vessels were involved, indicated that one vessel involvement was the more frequent finding, followed by two vessels and the least frequent was the three vessels involvement, this findings was statistically significant (P = 0.011).

Mild RCA stenosis was found in 4 patients (21.1%), moderate in 9 patients (47.3%), and severe RCA stenosis was found in 6 patients (31.6%). Regarding the LADA, each of mild and moderate stenosis was found in 5 patients (45.5%), and severe stenosis was found in only one patient (9.1%). The mild LCXA stenosis was found in one patient (20%), moderate stenosis in two patients (40%), and severe in 2 patients (40%). By comparing the severity of stenosis between the three arteries, no significant differences had been found in the degrees of severity (mild, moderate and severe) between these arteries, P = > 0.05, as shown in table 2.

The prevalence of stenosis was significantly increased with advanced age, this findings was statistically significant (P = 0.015), furthermore, the mean age was significantly higher in patients with stenosis than those without; 55.5 ± 7.8 years vs. 49.1 ± 8.8 years, respectively. Regarding the association between stenosis and gender, no statistically significant difference had been found in the prevalence of stenosis between males and females, 18.8% vs. 22.6%, respectively (Table 3 and Fig. 1-3).

Table 2. Distribution of severity according to the involved vessel

Severity of stenosis	Artery						P value
	RCA (n=19)		LADA (n=11)		LCXA (n=5)		
	No.	%	No.	%	No.	%	
Mild (25 - 49%)	4	21.1	5	45.5	1	20.0	0.32
Moderate (50 - 69%)	9	47.3	5	45.5	2	40.0	0.95
Severe (70 - 99%)	6	31.6	1	9.1	2	40.0	0.29
Total	19	54.3	11	31.4	5	14.3	0.48

RCA = right coronary artery, LAD = left anterior descending, LCXA = left circumflex artery

Table 3. The age and gender distribution of the studied group with positive and negative stenosis

Parameter		Positive (N=23)		Negative (N=87)		Total	P value
		No.	%	No.	%		
Age (Years)	≤ 40	0	0.0	17	100.0	17	0.015
	41 - 50	8	17.4	38	82.6	46	
	51 - 60	9	26.5	25	73.5	34	
	> 60	6	46.2	7	53.8	13	
	mean ± SD	55.5 ± 7.8	-	49.1 ± 8.8	-	-	0.002
Gender	Male	9	18.8	39	81.2	48	0.62
	Female	14	22.6	48	77.4	62	
Total		23	20.9	87	79.1	110	

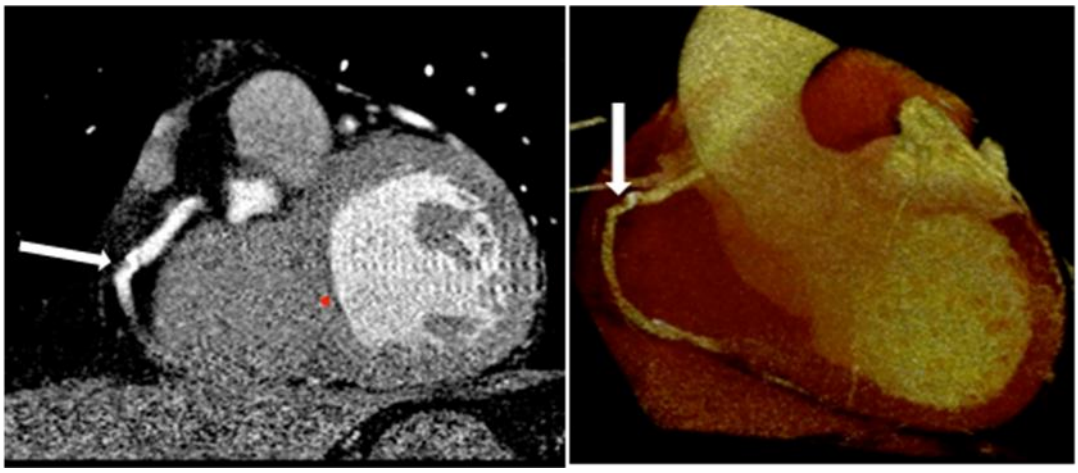

Fig. 1. 60 year-old symptomatic man. Curved planar reformatted (Left) and volume-rendered (Right) CT angiography images show a noncalcified soft plaque (*white arrows*), which is causing a significant (65%) stenosis in the RCA

Discussion

Because non calcified plaques can be found in cases of CAD in addition to calcified plaques, the CAC score examination has numerous limitations in detecting coronary atherosclerosis. Recent studies found varying degrees of noncalcified plaques using coronary CT angiography in patients with a CAC score of

zero. Acute coronary syndromes frequently result from the rupture of these noncalcified small plaques, which are generally not flow-limiting and do not cause stenosis. Calcification is a marker of plaque stability, whereas an unstable plaque is characterized by a large lipid core, a thin fibrous cap, and inflammation. An unstable plaque has been termed the "vulnerable plaque". The early detection of these plaques is important because they have a tendency to rupture but respond to medical treatment [26].

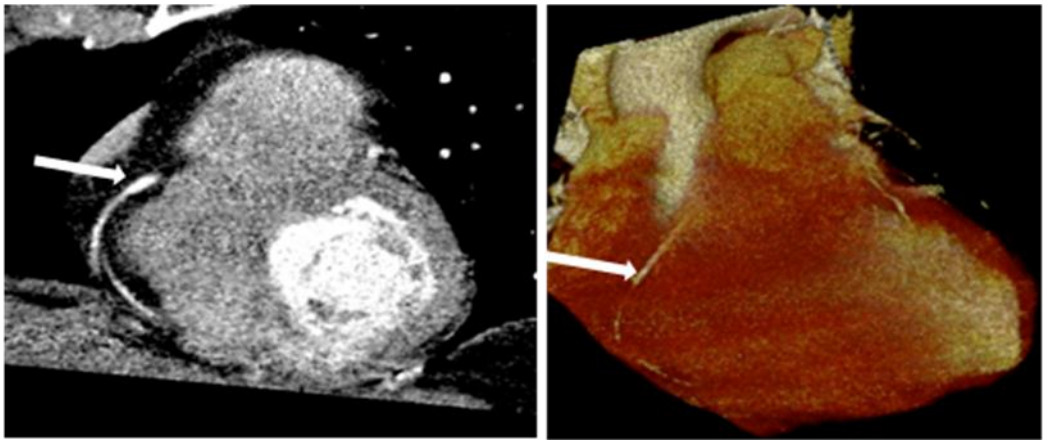

Fig. 2. A 65-year-old symptomatic woman. Curved planar reformatted (Left) and volume-rendered (Right) CT angiography images show a noncalcified soft plaque (*white arrows*), which is causing a significant (70%) stenosis in the RCA

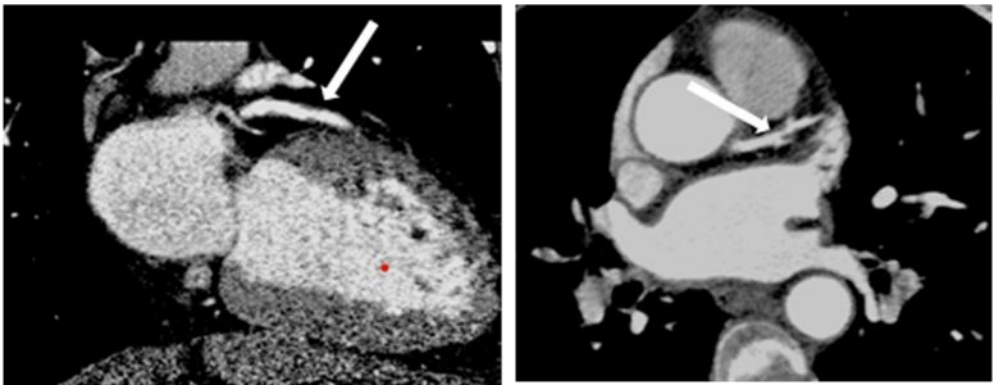

Fig. 3. A 54-year-old symptomatic woman. Curved planar reformatted (Left) and axial (Right) CT angiography images show a noncalcified soft plaque (*white arrows*), which is causing a significant (60%) stenosis in the LAD

The most important result of this study was the presence of coronary atherosclerosis at a high prevalence of 20.9% in patients with a CAC score of zero. The presence of noncalcified plaques in cases with a CAC score of zero has been reported at varying frequencies in the literature [21,25,29-31]. These rates were reported to be 6.5% by Cheng et al [21], 10% by Choi et al [29], 12% by Sosnowski et al [25], and 20% by Ergün et al [30]; however, Kelly et al. [31] reported a rate as high as 51%. These different rates may have resulted from the differences in the characteristics of the patient populations that were included in the studies.

Regarding the gender distribution, in our study, we observed that there is no significant difference between male and female gender (P value 0.62), this agreed to Kitagawa et al [32]

and Iwasaki et al [33]. On the other hand Büyükterzi et al [26] in a study Of 238 patients without plaques according to coronary CT angiography, they found that 126 (53%) were males and 112 (47%) were females, although the frequency of plaques was higher in males, this increase was not statistically significant (*P* = 0.153).

Regarding the site of stenosis we observed that among the 23 patients with stenosis, the RCA stenosis was the more prevalent it was found in 19 patients (82.6%) , LADA stenosis in 11 (47.8%) and LCXA in 5 patients (21.7%) , according to this findings, the RCA stenosis was significantly the more prevalent among the positive stenosis group, P = 0.015. Tse-Min Lu et al [34] found significant RCA disease was much more common than other arteries in their population. We also found that the mean percentage of stenosis was 59.9 ± 14.7 in RCA, 47.7 ± 12.8 in LADA and 56 ± 19.5 in LCXA with statistical significance between RCA and LAD (P value= 0.046). In Tse-Min Lu et al [34] study, they found that in a total of 164 patients included in the study 95 patients (57.9%) had significant RCA stenosis and 69 (42.1%) patients without stenosis. In patients with RCA disease, the majority had more than 70% stenosis (80/95, 84%), and 9 chronic total occlusion of RCA).

Regarding the number of vessels involved we found that In 12 patients (52.2%) only one vessel was involved, in 10 patients (43.5%) two vessels were involved and in one patient only (4.3%) three vessels were involved, indicated that one vessel involvement was the more frequent, followed by two vessels and the least frequent was the three vessels involvement, P = 0.011. This is agreed with Villines et al [35] who found that the majority of patients with a CAC score of 0 and obstructive CAD had single-vessel disease (82%), with a lower prevalence of 2-vessel (12%) and 3-vessel (6%). Gulin et al [36] found that in patients with diabetes mellitus single-vessel CAD was observed in 26%, two-vessel in 41% and three-vessel in 32%, whereas in patients without DM, 52% single-vessel CAD, 30% two-vessel and 18 % three-vessel CAD

By comparing the severity of stenosis between the three arteries, no significant differences had been found in the three degrees of severity (mild, moderate and severe) between these arteries, in all comparison. In the reported literature no study is available for comparison with our results; further study is recommended for more evaluation of this finding.

In our study, we observed that the mean age of the patients with noncalcified atherosclerotic plaque (55.5years), was higher than the mean age of the cases without plaques (49.1years). Similarly, Ergün et al [30] and Kelly et al [33] found that the mean age of the cases with atherosclerotic plaque, as detected by coronary CT angiography were 53 and 54.4 years, respectively) and it was higher than the mean age of the cases without plaques (49 and 50.4 years, respectively). The results of our study demonstrated that the rate of plaque detection by coronary CT angiography in the patient population with a CAC score of zero was higher in patients over 40 years of age; however, it is difficult to determine a threshold value for the age limit because studies have reported that the risk for CAD is higher in patients 45–50 years of age [37].

In conclusion, CCTA can clearly demonstrate noncalcified atherosclerotic coronary plaques in a large group of patients with suspected CAD. The absence of coronary artery calcification does not exclude the presence of significant stenosis in symptomatic patients with no coronary Calcium.

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Author Contribution

Dr. Kadhim did the study design, supervise data collection, writing part of the discussion and revising the manuscript; Dr. Al-Saadi participates in result interpretation, statistical analysis and writing part of the introduction and Dr. Hadi collect the data, writing the draft of the manuscript, interprets the results and made the statistical analysis.

Conflict of Interest

There is no conflict of interest for the authors of this manuscript and its potential publication.

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Antimicrobial Resistance Patterns of *Escherichia Coli* O157:H7 Isolated from Stool Sample of Children

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Abstract

Background Enterohemorrhagic *Escherichia coli* is a subset of Shiga toxin-producing *E. coli* that can cause diarrhea or hemorrhagic colitis in humans. Hemorrhagic colitis occasionally progresses to hemolytic uremic syndrome, is a major cause ofacute renal failure in children and morbidity and mortality in adults.

Objective To determine the susceptibility and resistance of the most effective antibiotic to *E.coli* O157:H7 associated with bloody diarrhea.

Methods Two hundred patients with bloody diarrhea were enrolled in this study. *Escherichia coli* were isolated on Sorbitol MacConkey agar with Cefixime and Tellurite and tested by latex agglutination test. The susceptibility and resistance for all bacterial isolates were identified by standard procedures resistance patterns such as disk diffusion test and minimum inhibitory concentration.

Results *E.coli* O157:H7 found in 37 (18.5%) out of two hundred stool samples. The highest rate was found in 18 cases (48.64%) out of 37 infants aged 3-12 months, 12 cases (32.43%) in infants aged 13-24 months, and the lower rates was in children over two years old (18.9%). *E.coli* O157: H7 was completely resistant to gentamicin, ampicillin, nalidixic acid and co-trimoxazole; high rate of resistance to cefotaxime and ceftazidime, moderate-to-low rate of resistance to ciprofloxacin, amikacine, ceftriaxone and Imipenem and no resistances rate to levofloxacin. Out of 37 isolates 29 (78.3%) were β-lactamase producer and 8 (21.6 %) isolates resistant to β-lactamase antibiotic patterns but not produce β-lactamase enzyme.

Conclusion The high-incidence rate of *E. coli* O157:H7 infection in children associated with limited number of drugs effective against *E. coli* O157:H7 with high prevalence of resistance to more than three antibiotics.

Keywords *E.coli* O157:H7, Enterohemorrhagic *Escherichia coli*, disk diffusion test, minimum inhibitory concentration.

List of abbreviation: EHEC = Enterohemorrhagic *Escherichia coli*, Stx = Shiga toxin, HUS = hemolytic uremic syndrome, SMAC-CT = Sorbitol MacConkey Agar with Cefixime and Tellurite, DDT = disk diffusion test, MIC = minimum inhibitory concentration, VTEC = Verotoxin-producing *E. coli*, CLSI = clinical laboratory institute.

Introductions

Enterohemorrhagic *Escherichia coli* (EHEC) is a subset of Shiga toxin (Stx)-producing *E. coli* that can cause diarrhea or hemorrhagic colitis in humans. Hemorrhagic colitis occasionally progresses to hemolytic uremic syndrome (HUS), which is a major cause of acute renal failure in children and morbidity and mortality in adults [(1)]. Humans acquire EHEC O157:H7 by direct contact with animal carriers, their feces, and contaminated soil or water, or via the ingestion of undercooked beef, or other animal products, and contaminated vegetables and fruit. The infectious dose is very low, which increases the risk of disease [(2)]. Verotoxin-producing *E. coli*

(VTEC), including O157:H7 was identified in 1982 as an important human pathogen [3]. Antimicrobial resistance in *Enterobacteriaceae* poses a critical public health threat, especially in the developing countries [4,5] much of the problem has been shown to be due to the presence of transferable plasmids encoding multidrug resistance and their dissemination among different enterobacterial species [6,7]. The usefulness of antimicrobial therapy for *Shiga Toxin E. coli* (STEC) infections is unresolved. Because antimicrobials may lyse bacterial cell walls, thereby liberating Shiga toxins [8] and/ or cause increased expression of Shiga toxin genes in vivo [9,10].

Methods

Over six months from September 2014 to February 2015 stool samples obtained from children with bloody diarrhea. Two hundred patients whom admitted or outpatients to Central Child hospital, Abu-Ghraib hospital or from private clinic in Baghdad were enrolled in this study; patient's selections were restricted to those who had bloody diarrhea because *E.coli* O157 was mostly associated with this clinical feature. Stool samples were divided into two portions. One portion for the direct stool examinations and the second portion was inoculated in tetrathionate broth for 24 hr at 37^{o}C, and then inoculated in MacConkey agar for isolation and identification of lactose fermenter *E. coli* bacteria. *E. coli* was isolated by sub-cultured on Sorbitol MacConkey Agar with Cefixime and Tellurite (SMAC-CT). This media considered as selective and differential medium for the detection of *Escherichia Coli* serotype O157:H7 which prepared as follows: peptone (20gm), Bile salts (1.5gm), Sodium chloride (5gm), Neutral red (0.03 gm), Crystal violet (0.001 gm), D-Sorbitol (10gm), Cefixime (0.05 mg), Potassium Tellurite (2.5 mg) and Agar (15 gm). These ingredients were suspended in one litter distilled water; PH was adjusted to (7.1) sterilized at 121 ^{o}C for 15 minutes, left to cool before pouring into plates [11,20].

SMAC-CT is modified MacConkey Agar using sorbitol instead of lactose with cefixime and tellurite added. Cefixime inhibits *Proteus* spp. and tellurite inhibits non-O157 *E. coli* and other organisms, thus improving the selectivity of SMAC-CT for *E. coli* O157:H7. Differentiation of enteric microorganisms is achieved by the combination of sorbitol and the neutral red indicator. Colorless or pink to red colonies are produced depending upon the ability of the isolate to ferment the carbohydrate sorbitol, *E. coli* O157:H7 considered as sorbitol negative [11]. For confirmatory identification of *E.coli* O157:H7 Latex agglutinations test for *E.coli* O157:H7 (OXOID-England) were also used in current study. The stranded isolates from central public health laboratory *E.coli* ATCC25922 used as anegative control.

Acidimetric test for β -lactamase production by tube method

β-lactam ring Hydrolysis lead to carboxyl group formations, acidity resulting can be tested in tubes or on filter papers. For the tube method, 2 mL of 0.5% (w/v) aqueous phenol red solution is diluted with 16.6 mL distilled water and 1.2 g of benzyl penicillin is added. The pH is adjusted to 8.5 with 1 M NaOH. The resulting solution, which should be violet in color, can be stored at -20^{o}C. Before use, 100 µl portions are distributed into tubes or microtitre wells and inoculated with bacteria from culture plates (not broth) to produce dense suspensions. A yellow color within 5 min indicates β-lactamase activity. Positive and negative controls must be run in parallel [12].

Antimicrobial susceptibility tests

Resistance patterns of *E.coli*O157:H7 to various selected antibiotics were determined by disk diffusion test (DDT) and minimum inhibitory concentration (MIC). The following antimicrobial discs were tested: cephalexin (30µg), cefotaxime (30µg), ceftriaxone (30µg), amikacine (30µg), gentamycin (10µg), imipenem (10µg), ampicillin (10µg), co-trimoxazole (25µg), ciprofloxacin (5µg),

levofloxacin (10µg), nalidixic acid (30µg), when the incubation was complete, the diameter of the inhibition zone around the disks was measured and compared with the break points of clinical laboratory institute (CLSI) [13]. The MIC was performed by a standard agar dilution method and has been applied for determination the lowest antibiotics concentration that inhibits growth of *E.coli* O157:H7. Stock solutions of each antibiotic at concentrations of 10 mg/ml, 1mg/ml, and 0.1 mg/ml; then two fold dilutions from 512-0.5 µg/ml for all antibiotics were prepared. Muller Hinton agar medium was prepared, sterilized by autoclaving, after cooling, 25 ml were added to each antibiotic container; the content mixed well and poured into petridishes. The inoculum density was adjusted by using 0.5 McFarland standard tubes and then 20 microliters of each inoculum were spotted on the agar surface of Muller Hinton agar medium and incubated at 37°C for 24 hr [12]. After incubation ensure that all of the organisms have grown on the antibiotic-free control plate and observed the growth of bacteria on Muller Hinton agar plate for each antibiotic dilution [12].

Results

Two hundred patients with bloody diarrhea were enrolled in this study, *E. coli* O157:H7 was found in 37 (18.5%) patients, the highest rate were in infants aged (3-12) months, which were 18 cases out of 70 (25.7%) and in (13-24) months, which were 7 cases out of 42(16.7%) and the lowest rates was in children over two years old (13.6%) out of 88 (Table 1).

Table 1. Age distribution among patients with E. coli O157:H7 isolates

Age group (months)	Escherichia coli		
	Positive No. (%)	Negative No. (%)	Total
3-12	18 (25.7)	52 (74.3)	70
12-24	7 (16.7)	35 (83.3)	42
>24	12 (13.6)	76 (86.4)	88
Total	37 (18.5)	163 (81.5)	200

In this study all isolates of *E. coli* O157H7in (SMAC-CT) media gave positive reactions with *E. coli* O157:H7 latex agglutination test.

Antibiotics resistance of *E. coli* O157 H7

Regarding antibiotics resistance by disc-diffusion method, results in current study revealed that *E. coli* O157: H7completely resistance to gentamicin, ampicillin, nalidixic acid and co-trimoxazole, high resistance to cefotaxime and ceftazidime, moderate-to-low resistance to ciprofloxacin, amikacine, ceftriaxone and imipenem. No resistances rate to levofloxacin was observed in this study (Table 2).

Table 2. Number and percentage of resistance *E. coli* O157: H^7 isolates against different antibiotics

Antibiotics	RI	SI	R %
Gentamicin	37	0	100
Ampicillin	37	0	100
Nalidixic acid	37	0	100
Cefotaxime	20	17	54
Amikacine	25	12	67.5
Ceftriaxone	15	22	40.5
Ceftazidime	18	19	48.6
Ciprofloxacin	17	20	45.9
Leafofloxacin	0	37	0
Imipenem	5	32	13.5
Co-trimoxazole	37	0	100

RI = Resistance isolates, SI = Sensitive isolates, R = Resistance

Minimum inhibitory concentration of *E. coli* O157: H7 isolates

The MIC of all antibiotic used in this study were determined by an agar dilution method to measures more exactly the concentration of an antibiotic necessary to inhibit growth of a standardized inoculums under defined conditions [13]. *E. coli* O157: H7was characterized as resistant if the MIC was greater than the breakpoint MIC defined by CLSI while it will be susceptible if it is less than the MIC [13].

MIC results revealed that the resistance pattern of 37 isolates to cefotaxime were as follows: 17 isolates were highly resistant with MIC (512 µg/ml), nine isolates with MIC (256 µg/ml), six isolates with MIC (128 µg/ml), five isolates with MIC (64 µg/ml). MIC of ceftriaxone shows eleven isolates had MIC (256 µg/ml), seven isolates with MIC (128 µg/ml), ten isolates with MIC (64 µg/ml), nine isolates exhibiting high level resistance with MIC (512 µg/ml). MIC of ceftazidime shows three isolates had MIC (256 µg/ml), six isolates (128µg/ml), and twenty isolates had MIC (32 µg/ml).

Imipenem MIC determination shows that two isolates exhibiting highly resistance (512 µg/ml), one isolates had (64 µg/ml), and two isolates (32 µg/ml). MIC respectively determination of MIC to ciprofloxacin reveals that five isolates with MIC (512 µg/ml), seven isolates with MIC (256 µg/ml), two isolates with (128 µg/ml), and three isolates with MIC (16 µg/ml). The results of MIC against Levofloxacin revealed that only one isolates of *E. coli* O157: H7 exhibited less susceptibility with MIC (2 µg/ml), MIC resistant isolates to gentamycin, ampicillin, nalidixic acid and co-trimoxazole reveals completely resistance to this drug. MIC to amikacine showed 25 isolates were highly resistance rate with MIC (512 µg/ml).

Regarding β-lactamase production, current study found that 29 out of 37(78.3%) were β-lactamase producer, and there was 8 (21.6 %) isolates resistance to β-lactamase antibiotic patterns but not produce β-lactamase enzyme which it gave negative results in this test.

Discussion

E. coli O157:H7 recognized as major etiologic agents of two life threatening complication in humans, hemorrhagic colitis and HUS makes *E. coli* O157:H7 infections a public health problem [(14)]. The present study showed that the risk developing of bloody diarrhea caused by *E. coli* O157:H7 was in the first two years of life and the lowest rate was in children over two years of life. Results in the present study are in agreement with previous studies by Baqir *et al* [(15)] and it was in disagreement with finding of Trung *et al* [(16)] who found that *E. coli* O157:H7 was not found in any of the samples of children under two years old. The new medium (TC-SMAC) gave substantial suppression of non-O157 strains and also inhibited non sorbitol fermenting (NSF) bacteria such as proteus species [(17,18)].

Results in current study showed that all isolates on SMAC-CT gave positive results with latex agglutination test, there for the sensitivity of the SMAC-CT media was estimated to be 100% compare with latex agglutination test. This result corresponded with results mentioned by Aseel *et al* [(19)]; Zadik *et al* [(20)]. Result in the current study revealed that out of37 isolates 29 (78.3%) were β-lactamase producer isolates were resistant to β-lactam drugs, high incidence of β-lactamase- producing *E. coli* O157:H7 was previously reported by Israa *et al* [(21)] and Panus *et al* [(22)]. The reminder isolates 8 (21.6 %) resistance to β-lactamase antibiotic patterns but not produce β-lactamase enzyme. The possible explanation for such results is the presence of other mechanisms that make *E. coli* O157:H7 resistant to β-lactamase antibiotic patterns such as efflux pump, alteration of an antibiotic target protein, penicillin-binding protein 2 and reduced antibiotic penetration is also a resistance mechanism for several classes of antibiotics [(23)].

The antimicrobial sensitivity tests showed that *E. coli* O157:H7 isolates were fully resistant to gentamicin, ampicillin, nalidixic acid and co-trimoxazole, a result which quite accord with studies done by Israa *et al* [(21)] and Daniel *et al* [(24)]. The resistance patterns of *E. coli* O157:H7 to several antibiotics were as follows: cefotaxime (54%), amikacine (67.5%), ceftriaxone (40.5%), ceftazidime (48.6%), ciprofloxacin (45.9%). The possible explanation to high level of resistance to this drug may be as a result of it being the most commonly available antibiotic used as a routine therapy among gastrointestinal infections and people readily purchase it across the counter for self-

medication in last years. This could be a reflection of use and misuse of these antibiotics in the society. This finding as a result to fact that outside the hospital environment the general population have easy access to various antibiotics from any pharmacy without prescription from a doctor.

In this study, the majority of *E. coli* O157:H7 isolates showed multidrug resistance to the antibiotics at various percentages. This result is in agreement with the findings by other researchers, who reported multidrug resistance among *E. coli* O157:H7 isolates [25,26].

The development of antimicrobial resistance by the *E. coli* O157:H7 isolates to these drugs poses a major challenge in both human and animal medicine because these drugs are commonly used in the treatment of human patients and in veterinary practice [27].

Results from this study indicate that imipenem and leafofloxacin had high sensitivity since few of the isolates was resistant to them and that might be attributed to the recent introduction of this agent for the treatment of infection in both human and animals in Iraq.

In conclusion, our study made it evident that high incidence rate of *E. coli* O157:H7 infection in children associated with limited number of drugs effective against *E. coli* O157:H7 with high prevalence of resistance to more than three antibiotics, this may provide an evidence for antimicrobial abuse and should be attract more attention about control strategies and used of antibiotic in human and veterinary medicine.

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Conflict of interest

The author declares no competing interests.

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Expandable Metal Esophageal Stent Deployment in Patients with Malignant Dysphagia

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Abstract

Background Palliative therapy is the only treatment for inoperable malignant dysphagia. Therapeutic option of self expandable metallic stent has been proven more effective and safest than other conventional palliative therapy like esophageal dilation, plastic stent and laser therapy in malignant dysphagia.

Objectives To study the outcome, efficacy and complications of self expandable metallic stent deployment in patient with malignant dysphagia.

Methods Fifty patients with malignant dysphagia comprised of 38 male and 12 female with malignant dysphagia treated palliativelly with self expandable metallic stent were studied. All the patients were submitted to upper endoscopy and forceps biopsy before self expandable metallic stent deployment. Analysis of stent expansion, mean dysphagia score, complications and mean survival period were carried for the study group.

Results Technical success rate of self expandable metallic stent placement was 86% in first trial and reached to 100% in the second trial. Early technical or patient's complications were negligible. Most important late complication was tumor overgrowth obstructing the stent. The mean dysphagia score point 1.9 and the mean survival period were 3.1 months.

Conclusions Self expandable metallic stent deployment is successful and rewarding procedure for immediate relief of disabling malignant dysphagia with negligible morbidity and zero mortality.

Keyword Self expandable metallic stent, Malignant dysphagia, Esophagus, Iraq

List of Abbreviations: MD = Malignant dysphagia, SEMS = self expandable metal stent, TSR = technical success rate, MDSP = mean dysphagia score points, BICAP = multipolar electrocoagulation, ELT = endoscopic laser therapy, PDT = photodynamic therapy, TTS = through the scope, FNA = fine needle aspiration, EUS = endoscopic ultrasonography, TOF = tracheo-esophageal fistula, PPI = proton pump inhibitor, GERD = gastroesophageal reflux disease, ND = not determine, , MSP = mean survival period,.

Introduction

Malignant dysphagia (MD) in term is given to any difficulty in swallowing due to malignant tumors that arise from the esophagus such as squamous cell carcinoma and adenocarcinoma, or malignant tumors arising from adjacent structures and infiltrating the esophagus. Squamous cell carcinoma represents 90% of primary esophageal cancer. There are many risk factors for this tumor; smoking, excess alcohol intake is most important acquired and avoidable risk factors [1-4]. The product of tobacco which is nitrosamine [5] is found to be cancerous to the esophagus.

Adenocarcinoma is less than 10% which is usually in the lower third of esophagus, since more than 90% for the patients with adenocarcinoma occurs as sequel to the Barrett's disease which is considered the most important risk factor for development of this type of tumor. The other rare non epithelial primary malignant tumors represent about 1%

arise from muscles of the esophagus, i.e., liomomyosarcoma [4,5].

Secondary esophageal tumors infiltrating the esophagus lead to MD. The commonest adjacent organs that the malignant tumors arise from are the lung, stomach especially the cardia, mediastinum and breast. Carcinoma of cardia cause sever dysphagia *per se* or due to infiltration to lower third of esophagus but in most of the time it is impossible to differentiate the primary site of adenocarcinoma whether arise from the esophagus or cardia even by endoscopic and histopathological examination [1,4,5].

Dysphagia grade was described by Stoller et al in 1977 and Atkinson et al in 1979 as follow: [1]

0 = no dysphagia, 1 = able to swallow most foods, 2 = able to swallow a soft diet, 3 = able to swallow liquids only and 4 = unable to swallow saliva.

The aim of stent placement is to improve dysphagia score points. The prognosis is dismal, at the time of diagnosis and the overall 5-year survival is 5%. More than 60% of patients are inoperable at the time of diagnosis, and because of debilitation or advanced age patients with comorbid diseases and metastasis at the time of presentation, so the palliation is the only realistic therapeutic option for these patients [4].

Self expandable metallic stent (SEMS) invented in 1980 by Boston scientific/microvasive in USA, but the placement of the SEMS for the first time by Doctor Frimberger in 1983 by per oral method [5] (Fig. 1 and 2). SEMS is promising and rapidly developing medical branch. Complications may include migration and perforation, occlusion, recurrent dysphasia, chest pain and bleeding [1,5].

The intention of this study is to study the outcome, efficacy and complications of SEMS deployment in patient with MD.

Methods

Eighty five patients with MD referred during Jan. 2004 to Jan. 2006 to the Gastroenterology and Hepatology Teaching Hospital in Baghdad for palliative therapy and were candidates for stent. They comprised 64 males and 21 females with an age range between 38 and 82 years (mean 62 years).

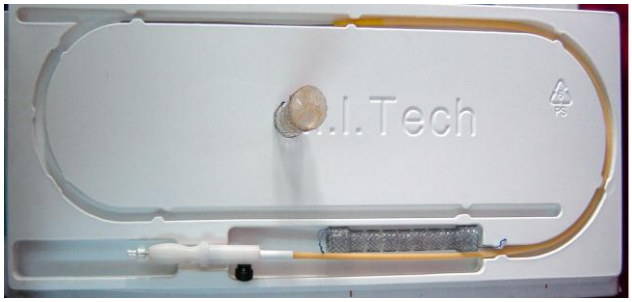

Fig. 1. Choostent Covered Esophageal; Diameter 18mm Total Length 140mm and its Delivery Device; Diameter is 6mm and usable length is 70 cm.

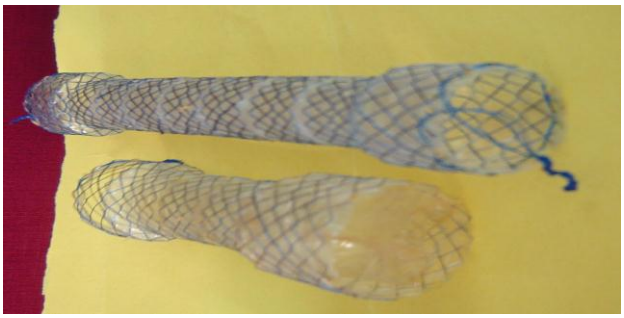

Fig. 2. Choostent SEMS with full expansion without anti reflux valve (upper), with tricuspid anti reflux valve (lower).

Fifty one males were smokers versus thirteen females and fourteen were alcoholics versus none of the females. Out of the total number, 35 patients were not fit for stent because of upper esophageal tumor (16 patients), tumor extent to fundus and body of stomach (7 patients) and complete obstruction to esophageal lumen (12 patients). We failed to identify the distal edge of the tumor and thus referred for another palliative treatment.

The rest fifty patients were inoperable (38 male and 12 female); were reevaluated thoroughly with biochemical, serological, hematological assessment with special concerns to viral screening, thrombin and partial prothrombin times. In addition, imaging studies including abdominal ultrasonography,

chest radiology, computed tomography scanning, and barium swallow when feasible. All the patients underwent endoscopy and biopsy was taken from the esophageal tumor.

Trial of SEMS was done to all patients using the following materials:

a. Olympus scope (GIFQ 160) or (GIF XP 120) used as upper endoscopy of the patients.
b. Choostent (cover, uncover), M.I. Tech, Seoul, Korea with (delivery device; guide wire 0.038 inch, diameter 6mm, usable length 70 cm).
c. Fluoroscope machine model 9800 C-arm.
d. Piano guide wire (metallic stainless steel floppy)
e. Contrast used (Omnipaque 240 mg/ml) equal to lohexol 518 mg + tromtamol 1.2 mg + sodium. Calcium editate 0.1mg/ml.
f. Savary-Guilliard polyvinyl dilator (wilsoncock)

We used the per oral rout method under complete aseptic technique in the theater. Covered stent was selected for those patients with tracheoesophageal fistula or with risk of perforation of esophageal wall by tumor. Uncovered stent was used when the tumor was in the cardia or when there was invasion to the mucosa and submucosa. All the patients sedated with intravenous 50-100 mg of pethidine.

The patients were kept in the hospital for two days and they were allowed to take clear liquid diet within 6 hours. Dysphagia score was recorded after 24 hours and the endoscopy repeated in the next day for the assurance of proper placement and expansion of the stent. The patients then discharged when there was no complication. If the patients tolerated the liquid diet well they were instructed to take soft food in the second week with dysphagia score (Fig. 3).

Result

The technical success rate (TSR) was 86% (43 out of 50) as 1 patient with migration and 6 patients there with high suspicion of perforation and the procedure postponed to the next day after the perforation had been excluded.

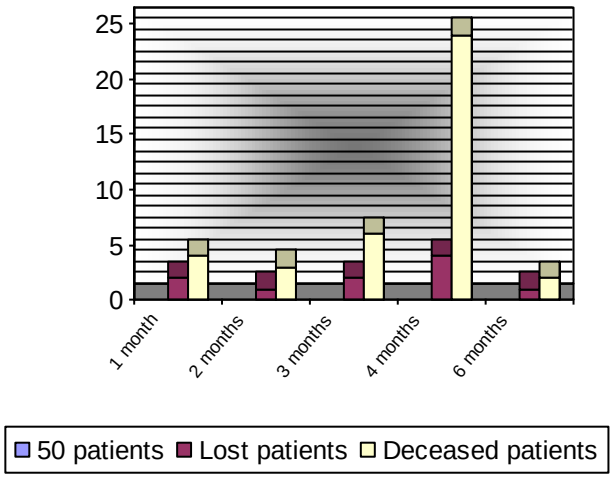

Fig. 3. Follow up period of 50 patients with malignant dysphagia after stent deployment

The criteria of technical success were expansion of the distal flange of stent 2-3 cm from distal edge of tumor and proximal flange of the stent 2-3 cm from proximal edge crossing the tumor with endoscopic and radiologic follow (Fig. 4-6).

Chest pain occurred in 24 out of 50 patients (48%) after stent placement, 12 patients with uncovered SEMS and 12 patients with covered SEMS. This was the commonest immediate complication after stent deployment in patients with malignant dysphagia (Fig. 4-6).

Recurrent dysphagia occurred in 6 patients in the first 2 weeks and the cause in all the case was food impaction and they were treated by excessive wash through the endoscope.

Migration occurred in 2 patients in the first two weeks of placement both with covered stent and another stent was re-inserted.

Perforation, sudden death, and sepsis were not recognized in any patients. Two weeks later, chest pain was seen in 6 patients (gave history of retrosternal discomfort) out of the 24 patients.

Late complications started to appear after first month includeing recurrent dysphagia by tumor obstruction and were increasing proportionally with duration of stent

deployment (Table 4). The mean dysphagia score points (MDSP) had been measured twice (24 hours 1 week later). The MDSP score was 3.3 before the stent and became 1.4 after stent deployment.

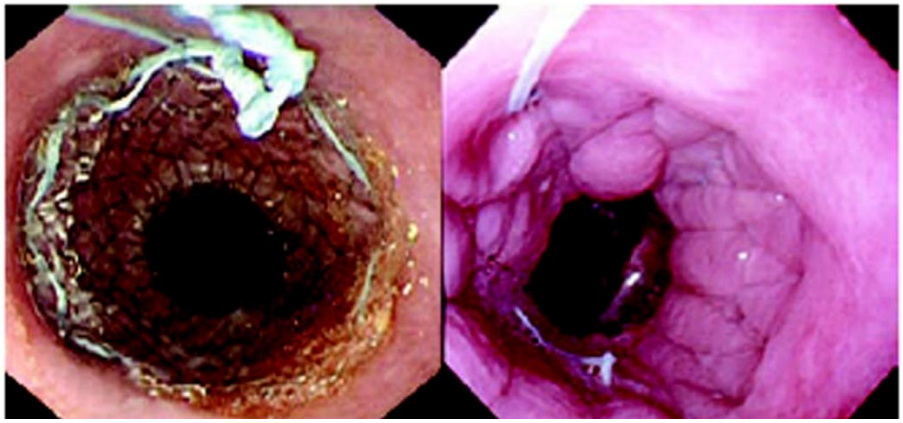

Fig. 4. Endoscopy shows fully expanded flange of covered stent after deployment (Left); shows firmly fixed flange of stent into the esophageal mucosa after insertion of uncover stent (Right)[7]

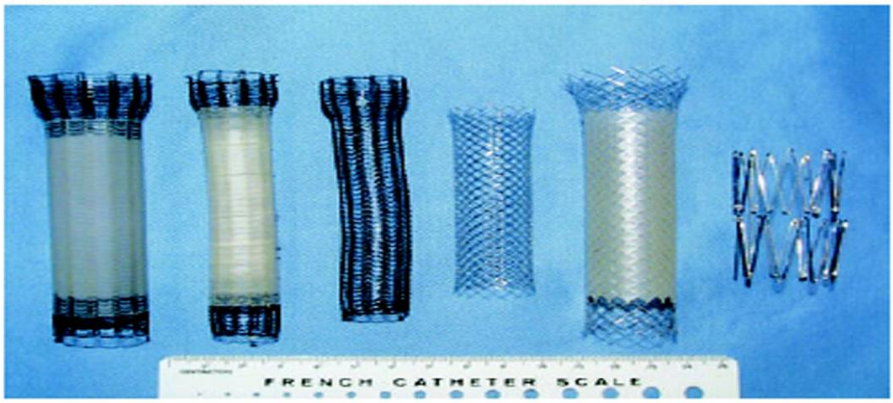

Fig. 5. Esophageal SEMS (Left to right): Ultraflex, large diameter, covered; Ultraflex, covered; Ultraflex uncovered; wallstent, uncovered; wallstent, large diameter, covered, Z-stent [15,16]

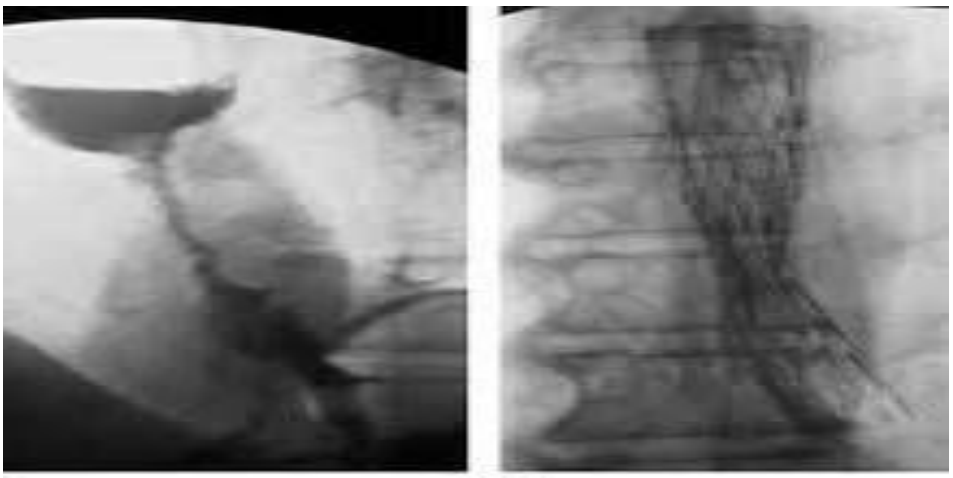

Fig. 6. Radiological view for SEMS before and after deployment

Discussion

The range of age (36-82 years) in this study and mean age of 56 years is lower compared to U.S.A, S. Korea mean age 62 and 65 years respectively [6,7]. Male:female ratio was 3:1, which is similar to other centers in USA and South Korea (3:1 and 4.1:1; respectively) [6,7].

Duration of dysphagia was 3-6 months, Ginsberg and Fleischer [4], mentioned in their review that dysphagia is rarely present for more than one year.

The TSR was 86 % in the first trial and reached 100% in the second trial next day of the study. This was similar to study conducted by Gukovsky-Reicher et al [5] where ultraflex stent was used in 44 patients and the TSR was 95 %. Similar TSR is reported by Chan Sup Shim in 61 patients with covered Choostent SEMS. The results of TSR in most centers with variant stents are nearly equal or slightly different (Table 1). Analysis of success evaluated as relieve of dysphagia was assessed by calculating the MDSP. The score of our study was in close proximity to that of many different centers and variants stents (Table 1).

Table 1. TSR, MDSP and MSP Comparative results in different centers and variant stents

Worker	Country	Stent deployed	No. of patients	TSR	MDSP	MSP
Our study	Iraq	Choostent	50	86%	1.9	3.1
Gukovsky-Reicher et al [5]	USA	Ultraflex	44	95%	1.5	3
Shim and Jung [6]	Korea	Choostent	61	95.5%	1.8	4.2
Raijman et al [10]	USA	Wallstent	101	96%	2.2	5.2
Doo et al [12]	Korea	Choostent	17	100%	2.0	4.8
Cwikiel et al [15]	USA	Ultraflex	106	97%	1.8	6

TSR = technical success rate, MDSP = mean dysphagia score points, MSP = mean survival

Kozarek and colleagues [8] analyzed data from 75 SEMSs deployment and found no correlation between complication rate and patient's age, sex or stent location it was very close to this study.

In this study 12 patients had complete obstruction of the esophageal lumen who were excluded from study which is similar to the work of Barr et al on 80 patients in which 16 of them had complete obstruction of the lumen and were treated by four sessions of laser therapy before stent deployment [2].

Early technical complications in this study were rather low. Migrations were seen in 2%, failure of insertion in first trial was 12%. No failure in expansion or perforation. These results are nearly similar to Gukovsky-Reicher *et al* [5], Cwikiel and colleagues [15] and Shim and Jung [6] in which migration were reported as 2%, 1% and 1% respectively.

Early complications it is rather higher. Chest pains were reported as 36%. In half of those patients uncovered SEMS were deployed. This is not comparable with Gukovsky-Reicher *et al* [5], Cwikiel *et al* [15] or Raijman *et al* [10] where chest pain occurred in 4%, 8% and 13% respectively. Recurrent dysphagia due to food impaction was 12% which is higher than the compared centers. This is explained by failure of compliance of the patients to special fluid diet. Bleeding occurred in 4% which is similar to USA, South Korea's centers (Table 2).

Delayed technical complications increased proportionally with duration of stent deployment. In this study, obstruction of stent is reported in 36% by tumor overgrowth mainly, with in the follow up period, while obstruction by food impaction is seen in 6% which is higher than other center. In three American centers the results were 8%, 19%, and 5 % [6], while in South Korea it was 17% [7] (Table 3).

Table 2. Early complications in different centers and variant stent

Complications	Our study	Gukovsky-Reicher et al [5]	Cwikeil et al [15]	Raijman et al [10]	Doo et al [12]	Shim and Jung [6]
Food impaction	12%	0%	0%	0%	0%	0%
Migration	2%	0%	0%	0%	1%	0%
Failure of expansion	0%	0%	6%	1%	2%	4.8%
Stent misplacement	0%	0%	1%	0%	0%	1.5%
Fracture	0%	0%	0%	0%	0%	0%
Chest pain	46%	5%	8%	13%	10%	8%
Recurrent dysphagia	12%	0%	0%	0%	3%	0%
Bleeding	4%	0%	0%	5%	4%	0%
Perforation	0%	2%	1%	0%	0%	0%
Sudden death	0%	0%	0%	0%	0%	ND

Table 3. Late complications in different centers and variant stents

Complications	Our study	Gukovsky-Reicher et al [5]	Cwikeil et al [15]	Raijman et al [10]	Doo et al [12]	Shim and Jung [6]
Food impaction	6%	5%	5%	0%	2%	0%
Tumor obstruction	36%	13%	19%	6%	17%	1%
Migration	4%	8%	3%	3%	4%	0%
Stent kinking	2%	2%	0%	0%	2%	0%
Chest pain	12%	0%	0%	ND		0%
Recurrent dysphagia	48%	38%	23%	15%	16%	3.3%
GERD	30%	8%	ND	ND	6%	8%
Bleeding	6%	0%	3%	2%	2%	0%
Perforation	ND	0%	5%	0%	0%	0%
Sepsis	ND	0%	0%	0%	0%	ND

This could be explained on the basis that patients received proper chemotherapy. Migration of stent occurred in 4% and the stent kinking in 2%. All these results nearly similar to the USA and South Korea centers [6-8].

Recurrent dysphagia was reported in 48%. This is slightly higher than three American centers in which the results were 38%, 23%, and 15% [6] and in the South Korea was 16 % [7-9].

GERD was recognized in 24% which represent all patients with uncovered non anti-reflux stents and in 6% with covered anti-reflux. The clinical features were used for diagnosis of GERD and not 24 hours PH monitoring as it is not available in the center of this study.

Doo et al, in 17 patients treated with anti-reflux Choostent had only one patient complicated by GERD, this proved by 24 hours monitoring of PH [10].

The mean survival period in this study is 3.1 months which is comparable to four American centers 3, 6, 5.2, 4, 4 months [6,12-15], however in the South Korea experience the mean survival period is 4.6 months.

Dormann *et al* [16] and Costamagna *et al* [17] used the poly flex plastic stent in state of metallic stent and found there is less complications.

In conclusion, most of inoperable patients with MD have rather prolonged period of dysphagia range from 3-6 months, SEMS deployment resulted in immediate relief of dysphagia, because of high technical success rate of stent deployment, low morbidity related to this

procedure and no mortality, all these results are encouraging causes for using this type of palliative therapy, most of late recurrent dysphagia results from distal tumors overgrowth which obstruct the distal part of stent, and decrease mean dysphagia score point from 3.3 to 1.4 lead to improve in dysphagia and quality of life.

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Author contribution

Dr. Saadoon selects the cases, follows up them and participates in stent deployment in some cases and Dr. Shubbar arranges the study and gives the instructions throughout the study.

Conflict of Interest

None.

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None.

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Effect of CYP19 Gene on Polycystic Ovary Syndrome Phenotype in Iraqi Women

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Abstract

Background Ovarian androgen overproduction is the key physiopathologic feature of polycystic ovary syndrome and the bulk of evidence points to the ovary being the source of excess androgens, which appears to result from an abnormal regulation (dysregulation) of steroidogenesis. Aromatase is an enzyme complex responsible for a key step in the biosynthesis of estrogen. It is encoded by CYP19.

Objective To examine whether the rs2414096 of *CYP19* gene contributes to genetic susceptibility to the polycystic ovary syndrome hyperandrogenism in Iraqi women.

Methods A Case control study was conducted in the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University and Al-Nahrain Forensic DNA Unit, Baghdad, Iraq for the period from February 2012 to February 2013. Sixty-five healthy women serves as the control group and eighty-four infertile women with polycystic ovary syndrome, divided into two subgroups depending on the body mass index (< and ≥30 kg/m2) were studied. Restriction fragment length polymorphism analysis was performed to determine the genotypes of rs2414096 of *CYP19*. Clinical, anthropometric, hormonal and biochemical parameters were also estimated.

Results Genotypic distribution of rs2414096 of *CYP19* was significantly different in polycystic ovary syndrome patients from that of control women. The frequency of GG genotype was higher in the patients, while AA genotype was higher in control women. Those with GG genotype have lower estradiol, estradiol/testosterone and higher testosterone, luteinizing hormone, follicular stimulating hormone than those with AA genotype.

Conclusion The present data suggests that single-nucleotide polymorphisms rs2414096 in the *CYP19* gene is associated with susceptibility to polycystic ovary syndrome hyperandrogenism in Iraqi women.

Keywords Polycystic ovary syndrome, *CYP19* gene, reproductive hormones, Iraqi women.

List of Abbreviation: PCOS = polycystic ovary syndrome, FSH = follicle stimulating hormone, Kb = kilobase, SNP = single nucleotide polymorphism, BMI = body mass index, LH = lutinizing hormone, E_2 = estradiol, FBS = fasting blood sugar, LDL = low density lipoprotein, HDL = high density lipoprotein, VLDL = very low density lipoprotein, GnRH = Gonadotropin releasing hormone.

Introduction

The etiology of polycystic ovary syndrome (PCOS) is still unknown; there is increasing evidence to support a major genetic basis, since the syndrome is strongly familial [(1)]. It is clear, however, that more than one gene (and probably several) contributes to the heterogeneous phenotype [(2,3)].

Ovarian androgen overproduction is the key physiopathologic feature of PCOS and the bulk of evidence points to the ovary being the source of excess androgens, which appears to result from dysregulation of steroidogenesis [(4)]. Genetic variation at androgen receptor [(5)], suggesting that hyperandrogenism in PCO may be partly genetically determined.

Aromatase is an enzyme complex responsible for a key step in the biosynthesis of estrogens. This enzyme complex is composed of the cytochrome P450 aromatase and the nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P450 reductase. It is a member of the cytochrome P450 superfamily, which are monooxygenases that catalyze many reactions involved in steroidogenesis [6] and it catalyzes the conversion of C19 androgens to aromatic C18 estrogens. It is induced by follicular stimulating hormone (FSH) and is present in a number of different tissues including adrenals, muscle, placenta, skin, adipose and nervous tissue. Reduced aromatase activity may lead to the development of PCOS [7].

CYP19 gene encodes P450arom which is located on the long arm of chromosome 15 at position 15p21.1 and 130 kilobase (kb) long. Its 10 exons (the final nine of which are coding) are located within 30 kb of each other, and the 93 kb 50-flanking region is thought to have a regulatory role [8].

Several studies have reported the association of the single nucleotide polymorphism (SNP) rs2414096 in the CYP19 gene with hyperandrogenism [9,10].

The aim of this study is to examine whether the rs2414096 of *CYP19* gene contributes to genetic susceptibility to the PCOS hyperandrogenism in Iraqi women.

Methods

A Case control study conducted in the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University and Al-Nahrain Forensic DNA Unit, Baghdad, Iraq from February 2012 to February 2013. The study was approved by the Institute review Board of the College of Medicine, Al-Nahrain University, and written ethical consent was obtained from patients to participate in the study.

Sixty five healthy control group and 84 women who were diagnosed as PCOS patients according to the 2006 Rotterdam criteria were studied. All patients had a history of oligomenorrhea and evidence of hyperandrogenism (on clinical examination or documented by elevated testosterone levels). Women with any other cause of oligomenorrhea and hyperandrogenism were excluded. Only women who had PCO on ultrasonography were enrolled to ensure that the phenotype was definitely PCOS. Clinical and biochemical characteristics of women with PCOS patients and control women are given in Table 1. According to body mass index (BMI), each groups were subdivided in two subgroups (obese ≥30 kg/m^2 and non-obese <30 kg/m^2)

Whole blood samples were obtained from PCOS patients and control women to measure plasma FSH, luteinizing hormone (LH), estradiol (E_2), and testosterone hormones. Fasting blood sugar (FBS), blood glucose level after half, one and two hours, and lipid profile levels: cholesterol, triglyceride, low density lipoprotein (LDL), high density lipoprotein (HDL) and very low density lipoprotein (VLDL) were measured.

Genetic Analysis

Blood samples for molecular genetic studies were collected in tubes containing ethylenediaminetetraacetate as an anticoagulant. The genomic DNA was extracted from the blood of patients with PCOS and control group by using gene extraction kit supplied by Geneaid Company (Thailand). The restriction fragment length polymorphism analysis was performed to determine genotypes of rs2414096 of CYP19. Using Forward primer: 5'-TCT GGA AAC TTT TGG TTT GAG TG-3' Reverse primer: 5'-GAT TTA GCT TAA GAG CCT TTT CTT ACA-3'. A total volume of 25 µl containing genomic DNA 50 ng was used as template in the reaction mixture In addition to 6.25 pmol of each primer and 12.5 µl of Green Master Mix (Promega, USA). Cycling parameters were denaturation at 94^{o}C for 5 minutes, 30 cycles with 94^{o}C for 1 minute, 60^{o}C for 1 minute, 72^{o}C for 1 minute, and 72^{o}C for 10 [13]. Polymerase chain reaction (PCR)

products (189-bp) were digested with HSP92 II (promega, USA) for 4 hours at 37°C. Digested deoxyribonucleic acid (DNA) fragments were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized by UV trans-illuminator spectroline (USA). Hence, a single 189-bp band indicates homozygosity for the GG genotype. The presence of two fragments, 161-bp and 28-bp bands, indicates homozygosity for the AA genotype. The presence of three fragments, 189-, 161-, and 28-bp bands, indicates heterozygosity for the AG genotype (Fig. 1).

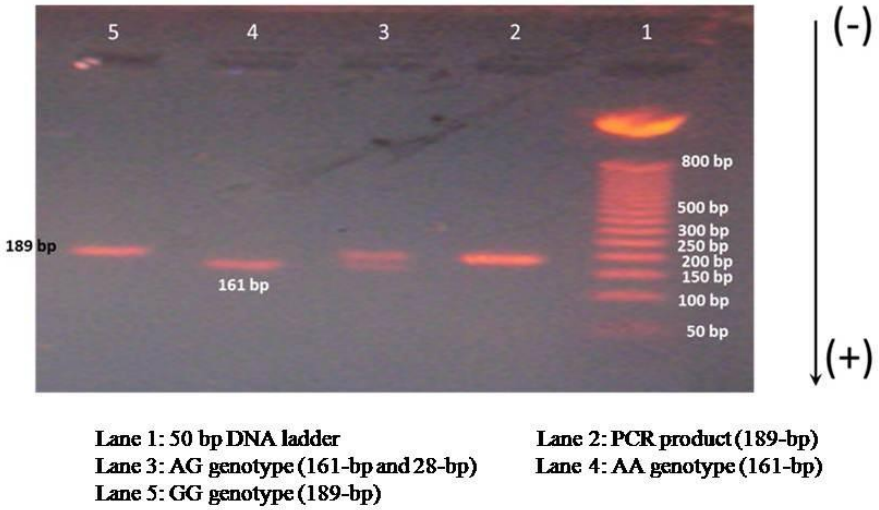

Fig. 1. Restriction fragment length polymorphism analysis of the A/G polymorphism of rs2414096 CYP19 genes. Agarose gel (2%) electrophoresis after HSP92 II digestion of the PCR.

Statistical analysis was performed by using statistical package of Science (SPPS); Version 17.0 and, Microsoft Excel Worksheet 2010. Numerical data analysis was done by using paired sample t-test for tables with mean and standards deviation of mean to compare PCOS and control subjects. Chi-square was used to examine the significance of gene and genotypes distribution in the two major groups and the subgroups. Analysis of variance (ANOVA) test was used to test the anthropometric parameters and its relation to genes. The differences between values were considered statistically significant at the level of ($p < 0.05$).

Results

The demographic data of the PCOS patients and control women are illustrated in table 1. Genotyping distributions of rs2414096 of *CYP19* gene (AA, AG and GG) in PCOS women as a whole or those who were obese were significantly different from that of the control group as a whole or those who were obese, respectively ($p < 0.05$).

On the reverse, the genotypic distribution of the non-obese PCOS patients was not significantly different from non-obese control women (Table 2). Furthermore, the genotypic distribution shows no significant difference between obese and non-obese PCOS patients and between obese and non-obese control women.

The frequency of rs2414096 *CYP19* genotype demonstrated that PCOS patients present low frequency of AA genotype ($p < 0.01$) but not with AG and GG genotypes as compared to the control women (Table 3).

Furthermore, lower A allele ($p < 0.05$), higher G allele ($p < 0.05$) but no difference in the frequency of A/G alleles was noticed in the obese PCOS patients versus obese control women. The alleles were not different in their frequencies in the non-obese PCOS patients

versus non-obese control women, obese PCOS versus non-obese PCOS patients and obese versus non-obese control women (Table 4).

Table 1. Comparison of demographic parameters between polycystic ovary syndrome patients and control women (using unpaired T-test)

Parameters	PCOS patients N = 84 (mean ± SD)	Control women N = 65 (mean ± SD)	p value
Age (yrs)	29.02 ± 4.50	30.31 ± 3.71	0.0584
BMI (kg/m^2)	29.9 ± 5.13	28.10 ± 4.51	0.0270
Waist/ Hip Ratio	0.82 ± 0.07	0.80 ± 0.06	0.0382
Waist/thigh ratio	1.42 ± 0.18	1.33 ± 0.11	0.0002
FSH (IU/ml)	5.35 ± 1.55	7.22 ± 2.32	0.0000
LH (IU/ml)	6.24 ± 3.00	3.97 ± 1.55	0.0000
LH/FSH	1.20 ± 0.52	0.56 ± 0.15	0.0000
E_2 (pg/ml)	60.27 ± 19.68	50.28 ± 18.90	0.0181
Testosterone (ng/ml)	0.79 ± 0.44	0.26 ± 0.15	0.0000
E_2/Testosterone	103.22 ± 58.34	230.62 ± 132.57	0.0000
FBS (mg/dl)	94.81 ± 15.30	87.07 ± 12.78	0.0132
BS (after 1/2 hr.) (mg/dl)	148.53 ± 36.84	133.08 ± 18.77	0.0092
BS (after 1 hr) (mg/dl)	142.79 ± 33.16	124.63 ± 17.39	0.0007
BS (after 2 hr) (mg/dl)	112.71 ± 24.32	99.12 ± 11.97	0.0005
Cholesterol (mg/dl)	163.70 ± 30.72	140.92 ± 17.87	0.0000
Triglyceride (mg/dl)	124.68 ± 33.51	111.25 ± 23.09	0.0109
VLDL (mg/dl)	24.85 ± 6.76	22.25 ± 4.62	0.0140
LDL (mg/dl)	98.90 ± 29.36	77.98 ± 17.36	0.0000
HDL (mg/dl)	39.11 ± 2.49	40.73 ± 2.30	0.0004

* = $P < 0.05$, ** = $P < 0.01$, PCOS = polycystic ovary syndrome, FSH = follicular stimulating hormone, LH = luteinizing hormone, E_2 = estradiol, FBS = fasting blood sugar, BS = blood sugar, VLDL = very low density lipoprotein, LDL = low density lipoprotein, HDL = high density lipoprotein

Table 2. Distribution of A/G Alleles of CYP19 gene in classified polycystic ovary syndrome patients and control women (using Chi square test)

Group	CYP 19 genotype			p value
	AA	AG	GG	
PCOS patients Control women	17 (20.24%) 26 (40%)	34 (40.48%) 23 (35.38%)	33 (39. 28%) 16 (24.62%)	< 0.022
Obese PCOS patients Obese Control women	5 (14.71%) 9 (40.91%)	12 (35.29%) 8 (36.36%)	17 (50%) 5 (22.73%)	< 0.045
Non-obese PCOS patients Non-obese Control women	12 (24%) 17 (39.54%)	22 (44%) 15 (34.88%)	16 (32%) 11 (25.58%)	0.273
obese PCOS patients Non-obese PCOS patients	5 (14.71%) 12 (24%)	12 (35.29%) 22 (44%)	17 (50%) 16 (32%)	0.233
Obese Control women Non-obese Control women	9 (40.91%) 17 (39.54%)	8 (36.36%) 15 (34.88%)	5 (22.73%) 11 (25.58%)	0.968

PCOS = polycystic ovary syndrome

Table 3. Allele frequencies of C/T polymorphism of rs2414096 CYP19 genes in polycystic ovary syndrome patients and control women (using Chi square test)

Genotype		PCOS N = 84	Control Group N = 65	p value
CYP19	AA genotype	17 (20.24%)	26 (40%)*	0.008
	AG genotype	34 (40.48%)	23 (35.38%)	0.526
	GG genotype	33 (39. 28%)	16 (24.62%)	0.059

* = p < 0.01, PCOS = polycystic ovary syndrome.

Table 4. Allele frequencies of A/G polymorphism of rs2414096 CYP19 genes in polycystic ovary syndrome patients versus control women (obese and non-obese) by BMI (using Chi square test)

Group	CYP19 gene					
	A allele	p value	AG allele	p value	G allele	p value
Obese PCOS patients Obese control women	5 (14.71%)* 9 (40.91%)	0.027	12 (35.29%) 8 (36.36%)	0.935	17 (50%)* 5 (22.73%)	0.041
Non-obese PCOS patients Non-obese control women	12 (24%) 17 (39.54%)	0.107	22 (44%) 15 (34.88%)	0.371	16 (32%) 11 (25.58%)	0.497
Obese PCOS patients Non-obese PCOS patients	5 (14.71%) 12 (24%)	0.298	12 (35.29%) 22 (44%)	0.425	17 (50%) 16 (32%)	0.097
Obese control women Non-obese control women	9 (40.91%) 17 (39.54)	0.915	8 (36.36%) 15 (34.88%)	0.906	5 (22.73%) 11 (25.58%)	0.800

* = p < 0.05, PCOS = polycystic ovary syndrome.

With respect to different CYP19 genotypic (AA, AG and GG) of PCOS patients, FSH (4.61 ± 1.01, 5.03 ± 1.15 and 6.07 ± 1.85; respectively) and LH (4.50 ± 2.21, 6.2 ± 2.75 and 7.22 ± 3.24 respectively) levels were significantly different (p = 0.002, p = 0.009; respectively) while no difference (p = 0.088) was observed in LH/FSH ratio (0.95 ± 0.42, 1.25 ± 0.51 and 1.29 ± 0.55; respectively). Likewise, in the control women, FSH (6.15 ± 1.66, 7.22 ± 2.12 and 8.71 ± 2.79; respectively) and LH (3.09 ± 0.95, 4.04 ± 1.41 and 5.07 ± 1.80; respectively) levels were significantly different (p = 0.024; p = 0.005; respectively) but not significant (p = 0.674) in the LH/FSH ratio (0.53 ± 0.16, 0.56 ± 0.17 and 0.59 ± 0.11; respectively).

With regard to the different genotype (AA, AG and GG), E_2 (72.78 ± 16.54, 61.66 ± 14.24 and 54.93 ± 22.61; respectively) and testosterone (0.54 ± 0.24, 0.63 ± 0.35 and 1.07 ± 0.45; respectively) levels and E_2/testosterone ratio (147.22 ± 44.90, 138.32 ± 49.27 and 61.17 ± 34.69; respectively) in PCOS patients were significantly different (p = 0.045; p = 0.000; p = 0.000; respectively). Similarly, in the control women, E_2 (60.62 ± 20.05, 48.87 ± 19.40 and 38.34 ± 10.42; respectively) and testosterone (0.18 ± 0.09, 0.21 ± 0.08 and 0.39 ± 0.16; respectively) levels and E_2/testosterone ratio (337.52 ± 148.13, 246.37 ± 81.07 and 116.88 ± 44.62; respectively) were significantly differ among the three genotypes (p = 0.011; p = 0.000; p = 0.000 respectively).

Discussion

Hyperandrogenism is a key diagnostic physiopathologic feature of PCOS [4]. Sisters of women with PCOS have increased androgen levels suggesting that hyperandrogenism may be partly genetically determined [11]. The *CYP19*

gene encodes aromatase enzyme (P450arom), is a key steroidogenic enzyme that catalyzes the final step of estrogen biosynthesis by converting testosterone and androstenedione to estradiol and estrone separately [12]. It is reported that several SNPs of the *CYP19* gene were associated with variation in serum androgen concentrations among women, both within and between racial/ethnic groups. Several studies have reported the association of the SNP rs2414096 in the *CYP19* gene with hyperandrogenism [9,10,13].

In the present study, there were significant differences in genotypic frequencies for the SNP rs2414096 in *CYP19* gene between PCOS patients and control women. The frequency of the AA genotype in the PCOS patients was significantly lower than that in the control group while the GG genotype was higher in PCOS patients (Table 3) which was comparable to that found by Jin *et al* [14].

According to BMI, GG genotype was presented more frequently in PCOS patients than control women (significantly in the obese and insignificantly in the non-obese). Likewise, the frequency of AA genotype was higher in the control women than PCOS patients also in both subgroups. On the other hand, the frequency of these genotypes did not differ significantly between both obese and non-obese PCOS and control women. The significant difference in genotype distribution probably indicates that the SNP of rs2414096 in *CYP19* gene is associated with the aromatase activity variation in PCOS women [14].

The estradiol/testosterone ratio provides important information about aromatase activity because conversion of androgens to estrogens is mediated by *CYP19*, which suggests that E_2/ testosterone ratio may be a direct marker of aromatase activity. The present study demonstrates that the rs2414096 AA genotype may be associated with activity of the aromatase and further affect the conversion of androgens to estrogens.

The E_2/ testosterone ratio of the AA genotype in PCOS was significantly higher than that of the other two genotypes and this agreed by Jin *et al* [14], who suggested that aromatase activity was augmented in the AA genotype. Reduced aromatase activity may lead to ovarian hyperandrogenism and the development of PCOS [9]. This is what was found in the present data where the testosterone level was higher in group of GG genotype both in PCOS patients and control women.

Also there was significant higher estradiol level in group of AA genotype which can be deduced from the facts that a higher frequency of PCOS is observed in people with augmented aromatase activity caused by mutant functional loss [7,15] and antral follicles taken from PCOS women exhibits no aromatase activity [16]. The augmented activity of the aromatase in the AA genotype may protect the ovary from the development of hyperandrogenism in PCOS patients.

FSH can induce aromatase activity, and this activity is positively correlated to the E_2 level. Thus, a reduced E_2 level can stimulate the production of FSH by negative feedback. This may account for the present observation that the concentration of FSH in the GG genotype, which demonstrated lower aromatase activity, was higher compared with the other two genotypes.

Furthermore, the data of this study shed the light on the effect of testosterone on the regulation of LH secretion, in which the LH level was higher in group of GG genotype, that have higher testosterone level, than other two genotypes. Testosterone augment pituitary sensitivity to gonadotrophin releasing hormone (GnRH) both by direct action on gonadotrophin synthesis and by enhancing GnRH-induced GnRH receptors [17], this is associated with an estradiol-related sensitization of pituitary LH release and hence an increase in LH secretion [18].

In conclusion, this study suggests that the SNP of rs2414096 in *CYP19* gene is positively

associated with PCOS hyperandrogenism in Iraqi PCOS women.

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Author contribution

Dr Muteb collects the data and analyzes it; Dr Hamdan and Al-Salihi interpret the data and revise the manuscript.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Diagnostic value of Somatosensory Evoked Potentials in Cervical Myelopathy

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Abstract

Background Cervical myelopathy is a condition caused by narrowing of the spinal canal leading to cord dysfunction. The most common causes are congenital stenosis and degenerative stenosis caused by spondylosis.

Objectives To confirm the diagnosis of cervical myelopathies using somatosensory evoked potentials and possibly to localize the level of the lesion.

Methods An electrophysiological study had been carried on 61 patients with cervical myelopathy (41 female and 20 male) aged 48.66±11.72 years and 41 healthy volunteers aged 44.8±10.53 years. Sensory and motor nerve conduction study and somatosensory evoked potential for all were done to evaluate the peripheral nerves and sensory central pathways.

Results No significant difference was demonstrated in the sensory and motor nerve conduction studies from the healthy subjects. Somatosensory evoked potentials showed statistically highly significant changes in the N13, N20 latencies, amplitudes and N13-N9 and N20-N13 central sensory conduction times of median nerve on both sides. N13 latency has the highest specificity and sensitivity among the somatosensory evoked potentials parameters. Those patients who had prolonged central sensory conduction time between N20-N13 suggests an upper cervical lesion while those having prolonged central sensory conduction time between N13-N9 suggest lower cervical cord and/or cervical root affection.

Conclusion Motor and sensory conduction studies are usually normal in CM. Among SSEPs parameters, N13 latency was prolonged bilaterally, CSCT abnormal bilaterally, N13-N9 and N20-N13 latencies unilaterally (Right side). Mononeuropathies, polyneuropathies, radiculopathies and plexopathies should be excluded before diagnosis of CM was made.

Key words Cervical myelopathy, electroneuromyograhpy, somatosensory evoked potentials.

List of abbreviation: CM = Cervical myelopathy, NCS = nerve conduction studies, CMAP = compound muscle action potential, SSEP = Somatosensory evoked potentials, CNS = central nervous system, EMG = Electromyography, EPs = Evoked potentials, SL = sensory latency, DML, distal motor latency, SNCV = sensory nerve conduction velocity, MNCV = motor nerve conduction velocity, CSCT = central sensory conduction time.

Introduction

Cervical myelopathy (CM) is a condition caused by narrowing of the spinal canal leading to cord dysfunction [(1)]. The most common causes are congenital stenosis and degenerative stenosis caused by spondylosis. [(2)]. The pathophysiology of CM involves static factors, which results in acquired or developmental stenosis of the cervical canal, and dynamic factors, which involve repetitive injury to the cervical cord [(3,4)]. The clinical findings of CM patient depending on the levels affected, involvements of the neural foramina and long tract. A variety of neurological signs and symptoms may be present, including sensory changes, reflex abnormalities, decreased dexterity, weakness, gait instability, bowel and bladder dysfunction, spasticity, presence of Hoffman's and/or Babinski's sign,

axial neck pain, radiculopathy, and even acute spinal cord injury [5-7].

Neurophysiology assessment of CM

Clinical neurophysiology is an area of medical practice focused primarily on measuring function in the central nervous system, peripheral nervous system and muscles as an extension of the neurologic evaluation; employs the same anatomic principles of localization as clinical examination [8,9], while nerve conduction studies (NCS) were used to evaluate the propagation of nerve action potentials along motor or sensory nerve fibers [10]. They provide means of confirming the presence and extent of peripheral nerve damage [11].

F-wave is a late compound muscle action potential (CMAP) [12]. Assessment of F-wave latency is very useful in clinical neurophysiology especially the proximal segment of the nerve [9,10]. While the somatosensory evoked potentials are presynaptic and postsynaptic responses recorded over the limbs, spine, and scalp following the stimulation of peripheral mixed motor and sensory nerves or cutaneous sensory nerves. The electrical potentials generated by various portions of the ascending sensory pathways can be easily elicited and recorded and can be used to examine the functional integrity of somatosensory pathways [8,11].

Median nerve responses are most commonly used [11]. It receives contributions from both the medial and lateral cords of the brachial plexus and contains fibers spanning from the C5 to T1 roots [8]. Following stimulation of the median nerve at the wrist, activity can be recorded at the Erb's point, cervical spine, and scalp. Several different peaks are identified with standard recording montages: N9, N11, N13, N14, and N20.

Abnormal somatosensory evoked potentials (SSEPs) can result from dysfunction at the level of the peripheral nerve, plexus, spinal root, spinal cord, brain stem, thalamocortical projections, or primary somatosensory cortex [13,14]. SSEP primary use is to determine compromised central nervous system (CNS) conduction. It confirms symptoms when few physical findings are noted. SSEP may confirm or reject the presence of a suspected conduction block and able to establish an anatomic region where the conduction disturbance or block occurs [15].

The intention of this study is to confirm the diagnosis of cervical myelopathies using SSEPs and conventional electromyography (EMG) study and possibly localize the level of the lesion.

Methods

This study was conducted at the Neurophysiology Unit at Al-Imamein Al-Kadhimein Medical City for the period extended from Apr. 2013 to Apr. 2014. An ethical consent was taken from each participant to be enrolled in the study.

Sixty one patients (20 males and 41 females) aged 48.66±11.72 years with documented CM diagnosed by a senior neurosurgeon were studied. The disease duration was 1 to more than 5 years.

Forty one healthy volunteers aged 44.8±10.53 years serve as the control group; they were clinically examined by the same neurosurgeon to be included in the study. The patients and controls with history of diabetes mellitus, alcoholism, uremia and other metabolic diseases were excluded from the study.

All studied subjects underwent the following neurophysiologic tests:

- Sensory nerve conduction (SNC) of the median and ulnar nerves (both sides)
- Motor nerve conduction (MNC) and F-wave studies of the median and ulnar nerves (both sides).
- SSEPs of both median nerves.

Routine computerized EMG /EP machine (Micromed, 8-channel elecromyograph) supplemented with different types of electrodes including grounding electrode used to protect the subject against electrical hazard

and to reduce stimulus artifacts and interference, stimulating surface electrodes was used to stimulate the nerves through the skin, surface recording electrodes and disposable subdermal monopolar electrodes.

Sensory nerve conduction study

An antidromic method was used for SNC determination in which the nerve was proximally stimulated from the trunk and the evoked activity was distally recorded from a finger. The parameters studied were the sensory latency (SL) measured in milliseconds (msec), Sensory amplitude measured in microvolt (µV) from peak to peak and SNCV measured by dividing the conduction distance in millimeter (d) by the SL in msec.

Motor Nerve Conduction Study and F- wave

The motor nerve was simulated at two points along its course, by applying stimuli at the distal and the proximal sites of the nerve and recording from the muscle innervated by that nerve. The parameters studied were distal motor latency (DML). Motor nerve conduction velocity (MNCV) measured by dividing the distance between the two stimulation points over the difference between the latencies of the recorded responses ensuring both CMAP configurations must be similar in addition to F wave latency measured from the stimulus artifact to the beginning of the evoked potential.

Somatosensory evoked potentials

The median nerve was stimulated by bipolar stimulating electrode placed over the median nerve at the wrist. The electrical stimuli were square-wave pulses given at rate of 2-3 /sec at high pass filter 4 Hz, low pass filter 500 Hz with time base 50 ms duration and gain 5 µv/Div. The stimulus intensity was adjusted to produce a visible twitch in the APB muscle without causing any discomfort. To confirm the reproducibility of the SSEP, each measurement was carried out at least three times.

The recording disposable subdermal monopolar needle electrode was placed at the following locations: Erb's point on each side (EPi) and (Epc), over the second and fifth cervical spine process (C2S, C5S), scalp over the contralateral cortex (CPc) and cephalic Fz electrode (Reference). The parameters studied in SSEPs study of median nerve include the latency, amplitude and central sensory conduction time (CSCT).

Statistical Analysis

The statistical analysis was obtained using statistical package of social sciences (SPSS) version 19 software and Microsoft Office Excel 2007. All data of were expressed as mean ± SD. Data from each patient and control group were compared using independent sample t-test to calculate differences between groups. Paired t-test was used to compare the right and left side within the same group. P-value of 0.05 or less was considered significant.

Cutoff values of the prolonged latencies, CSCT, accordingly the sensitivity and specificity were evaluated by using receiver operating curve (ROC). The percentage of abnormal values in SSEP tests is calculated according to cutoff value of the normal values for the control group.

Results

Nerve conduction study

The parameters of median and ulnar sensory and motor nerve conduction studies for the control subjects were presented in table 1. Paired t test was done and demonstrate no significant difference between the two sides.

Somatosensory evoked potentials

The latency, amplitude and CSCT of different SSEP components in the right and left upper limbs were presented in table 2. No significant difference was noticed between the two sides using paired t test.

Table 1. Nerve conduction parameters of the right and left median and ulnar nerves in the controls

Parameters	Nerve	Right side N= 41	Left side N = 41	P value
SL (msec)	Median	2.37±0.38	2.22±0.34	0.0864
	Ulnar	2.22±0.33	2.23±0.33	0.8973
SNAP (µV)	Median	26.93±5.3	28.95±6.56	0.1425
	Ulnar	26.63±5.06	28.5±7.17	0.1223
SNCV (m/sec)	Median	54.72±7.14	56.62±6.52	0.1301
	Ulnar	57.85±6.29	57.52±7.24	0.8277
DML (msec)	Median	2.79±039	2.91±0.32	0.1301
	Ulnar	2.56±0.37	2.59±0.41	0.0737
Distal CMAP (mV)	Median	7.16±1.49	7.85±2.21	0.064
	Ulnar	8.74±3.86	7.45±2.37	0.1034
Proximal CMAP (mV)	Median	7.33±1.78	6.59±2.75	0.2569
	Ulnar	7.94±2.96	7.03±1.92	0.1520
MNCV (m/sec)	Median	56.23±9.84	57.17±5.74	0.5391
	Ulnar	57.75±9.95	57.11±4.38	0.67747
F-wave latency (msec)	Median	26.52±1.69	26.54±1.26	0.9628
	Ulnar	24.92±2.06	24.57±2.0	0.5501

The data presented as mean±SD, SL = sensory latency, SNAP = sensory nerve action potential, SNCV = sensory nerve conduction velocity, DML, distal motor latency, CMAP = compound muscle action potential, MNCV = motor nerve conduction velocity.

Table 2. Somatosensory evoked potentials parameters recorded from right and left median nerves of the controls

SSEPs Parameters		Right side N=41	Left side N=41	P value
Latency (msec)	N9	9.27±0.23	9.22±0.41	0.9254
	N13	13.06±0.71	12.88±0.65	0.0866
	N20	20.59±1.23	20.83±1.1	0.2239
Amplitude (µV)	N9	4.70±1.43	4.46±1.32	0.1873
	N13	4.04±1.23	4.02±1.16	0.4764
	N20	4.74±1.67	5.64±1.71	0.0887
CSCT (msec)	N13-N9	3.84±0.87	3.66±0.69	0.1629
	N20-N13	7.53±1.43	7.96±1.34	0.0713
	N20-N9	11.37±1.3	11.56±1.19	0.2849

The data presented as mean±SD, SSEPs = somatosensory evoked potentials, CSCT = central sensory conduction time

Because there was no difference between the left and right side data; thus, they were pooled together and regarded as one group for further comparison with the patient data.

CM versus control subjects

Nerve conduction study

No significant difference was observed between the CM patients and control subjects

concerning the sensory and motor data of the median and ulnar nerves (Tables 3 and 4).

Table 3: Illustrate the data of median, ulnar sensory nerves in cervical myelopathy patient and control subjects (Unpaired t test).

Parameters	Nerve	CM Patients N =61	Control subjects N =82	P value
Sensory latency (msec)	Rt. Median	2.34±0.32	2.31±0.35	0.5564
	Lt. Median	2.26±0.34		0.4235
	Rt. Ulnar	2.32±0.29	2.23±0.33	0.0659
	Lt. Ulnar	2.23±0.32		0.9328
Sensory amplitude (µV)	Rt. Median	28.19±6.28	27.94±6.01	0.8077
	Lt. Median	30.05±6.83		0.0572
	Rt. Ulnar	28.86±7.91	27.57±6.24	0.2923
	Lt. Ulnar	26.69±8.25		0.4888
Sensory nerve conduction velocity (m/sec)	Rt. Median	55.66±5.6	56.39±6.42	0.4724
	Lt. Median	57.25±5.81		0.3992
	Rt. Ulnar	55.97±5.53	57.69±6.75	0.0964
	Lt. Ulnar	57.56±5.19		0.8962

The data presented as mean±SD, CM = cervical myelopathy

Table 4. Illustrate the data of median, ulnar nerves in cervical myelopathy patient and control subjects

Parameters	Nerve	CM Patients N =61	Control subjects N =82	P value
Distal latency (msec)	Rt. Median	2.96±0.56	2.91±0.32	0.1633
	Lt. Median	3.05±0.5		0.1029
	Rt. Ulnar	2.47±0.36	2.57±0.39	0.1115
	Lt. Ulnar	2.52±0.38		0.4207
Distal CMAP amplitude (µV)	Rt. Median	7.83±2.42	7.85±2.21	0.3905
	Lt. Median	8.15±1.78		0.4513
	Rt. Ulnar	8.07±3.2	8.09±3.25	0.9658
	Lt. Ulnar	8.25±3.21		0.7785
Proximal CMAP amplitude (µV)	Rt. Median	8.16±2.62	7.89±2.53	0.2163
	Lt. Median	8.63±1.93		0.0966
	Rt. Ulnar	7.49±2.59	7.48±2.52	0.998
	Lt. Ulnar	7.56±2.72		0.8729
Motor nerve conduction velocity (m/sec)	Rt. Median	56.36±9.23	56.75±7.95	0.7924
	Lt. Median	58.39±4.21		0.1146
	Rt. Ulnar	57.78±8.66	57.43±7.65	0.8022
	Lt. Ulnar	56.49±8.55		0.497
F wave latency (msec)	Rt. Median	27.67±5.48	26.53±1.48	0.1179
	Lt. Median	27.67±5.43		0.116
	Rt. Ulnar	24.94±1.94	24.75±2.0	0.5639
	Lt. Ulnar	25.25±1.77		0.1137

The data presented as mean±SD, CM = cervical myelopathy, CMAP = compound muscle action potential

Somatosensory evoked potentials

Apart from N9 latency and its amplitude, all other SSEPs components were significantly different between the studied groups (Table 5).

Table 5. Somatosensory evoked potentials data of median nerves in the cervical myelopathy patients and controls

Parameters			CM Patients N =61	Control subjects N =82	P value
Latency (msec)	Right	N9	9.35±0.28	9.26±0.22	0.0501
		N13	14.36±1.76	12.97±0.68	<0.0001
		N20	23.34±4.4	20.71±1.17	<0.0001
	Left	N9	9.36±0.33	9.26±0.22	0.0544
		N13	14.06±1.41	12.97±0.68	<0.0001
		N20	23.75±4.37	20.71±1.17	<0.0001
Amplitude (µV)	Right	N9	4.16±1.66	4.58±1.37	0.1091
		N13	3.1±1.41	4.11±1.14	<0.0001
		N20	3.63±2.02	5.25±1.41	<0.0001
	Left	N9	4.25±1.69	4.58±1.37	0.2119
		N13	3.49±1.79	4.11±1.14	0.0194
		N20	4.1±2.52	5.25±1.41	0.0019
CSCT (msec)	Right	N13-N9	5.01±1.84	3.7±0.7	<0.0001
		N20-N13	8.96±3.48	7.75±1.39	0.0117
		N20-N9	13.92±4.52	11.43±1.2	0.0001
	Left	N13-N9	4.7±1.37	3.7±0.7	<0.0001
		N20-N13	9.7±3.52	7.75±1.39	0.0001
		N20-N9	14.4±4.28	11.43±1.2	<0.0001

The data presented as mean±SD, CM = cervical myelopathy, CSCT = central sensory conduction time

Sensitivity and specificity of SSEP parameters

Median nerve

Cutoff values of the prolonged latencies and CSCT and lower amplitudes of the median nerves were estimated and accordingly the sensitivity and specificity were evaluated. N13 latency shows the highest specificity and sensitivity (Table 6).

Table 6. Cutoff value, sensitivity and specificity of the median somatosensory evoked potentials

Parameters		Cutoff	Specificity	Sensitivity
Latency (msec)	N9	9.35	52.0	50.0
	N13	13.25	70.7	69.7
	N20	21.15	62.2	59.8
Amplitude (µV)	N9	4.25	53.3	52.4
	N13	3.85	67.2	67.1
	N20	4.5	62.3	57.3
CSCT (msec)	N13-N9	4.05	68.3	62.3
	N20-N13	7.75	57.3	56.6
	N20-N9	11.75	62.2	60.7

CSCT = central sensory conduction time

The percentage of abnormal median nerves SSEP data according to cutoff value were presented in table 7. The N13 latency of right and left median nerves shows the higher percentage of abnormality.

Table 7. Percentage of abnormal median somatosensory evoked potentials data according to the cutoff value

Parameters		Cutoff	Right median		Left median	
			No.	%	No.	%
Latency (msec)	N9	9.35	29	47.5	28	45.9
	N13	13.25	41	67.21	42	68.85
	N20	21.15	37	60.6	34	55.7
Amplitude (µV)	N9	4.25	33	54	27	44.2
	N13	3.85	40	65.57	34	55.7
	N20	4.5	39	63.9	33	54.1
CSCT (msec)	N13-N9	4.05	39	63.9	37	60.6
	N20-N13	7.75	29	47.5	40	65.57
	N20-N9	11.75	35	57.37	38	62.3

CSCT = central sensory conduction time

Possibility of localization

The possibility of uni- and bilateral localization of lesion level through recording CSCT between N20-N13 and N13-N9 was assumed by cutoff values of the abnormal data (Table 8).

Table 8. Localization of lesion level by the cutoff value of abnormal central sensory conduction time of somatosensory evoked potentials

Level	N20-N13			N13-N9		
	Right	Left	Bilateral	Right	Left	Bilateral
C5-C1-cortex	29 (47.5)	40 (65.57)	25 (41)	-	-	-
C6-T1	-	-	-	39 (63.9)	37 (60.6)	37 (60.6)
Normal	32 (50.8)	21 (34.4)	17 (27.8)	22 (36.6)	24 (39.3)	19 (31.14)

Discussion

In the diagnosis of CM, conventional diagnostic methods such as neurologic findings, image study such as MRI and myelograms are usually performed, but conclusive diagnosis is sometimes difficult because many symptoms tend to be separate from the existing disease. The MRI demonstrates morphologic abnormalities of the cord but not the functional impairment, and not all cord compression shown by MRI is associated with cord dysfunction [16].

Control group

No side to side difference was observed in the control group regarding different SNC, MNC and SSEP. The current data were comparable with those reported by other researchers [9,11,17-22].

Patient versus control group

Conventional sensory and motor nerve conduction study

Conventional NCS are commonly used in lower motor neuron evaluation and they can provide an objective measure for nerve damage. They can confirm the clinical impression of nerve root compression and document or exclude other illnesses of nerves or muscles that could contribute to the patient's symptoms and signs [9,23].

Although the motor and sensory conduction studies are usually normal in CM, they still an essential part of their diagnostic evaluation. mononeuropathies, polyneuropathies, radiculopathies and plexopathies may all need to be excluded before an electrodiagnostic diagnoses of CM can be made; these all require relevant motor and sensory conduction studies.

In CM patients, motor and sensory conduction studies were within the normal limits of the control group and there was no side to side difference, a finding that was in close approximation to the data obtained by other researchers [24, 25].

The F-wave responses that provide information about the conduction rate in alpha motor fibers, especially when the pathological involvement is greater proximally or is located in anterior horn cells were normal in this study. This study documents that NCS remain complementary modalities in the evaluation of CM.

SSEPs study

In CM patients, SSEPs changed significantly from those of the control group (prolonged N13 and N20 latencies, low amplitudes and prolonged N13-N9 and N20-N13 CSCT). These findings were in harmony with the findings of other researchers [26, 27].

Patients who had abnormal Erb's point N9 latency and amplitude with normal median nerve sensory and MCS suggesting root affection and normal N9 potential in others indicates normal afferent volley reached the brachial plexus. In such condition, no slowing of impulse velocity exists in the afferent pathways.

The current study showed that N13 latency prolongation shows the highest specificity and sensitivity among other SSEPs parameters. Since studies presumed that N13 component is generated post-synaptically in the posterior horns of C2-C7 [28], more rostrally, possibly in the cuneate nucleus [29]. The timing of N13 with respect to N9 would, therefore, reflect the conduction velocity in the dorsal column fibers. Restuccia [30] found that SSEP segmental N13 medullary response was shown to be a sensitive indicator of medullary involvement in SCM and is believed to be a hallmark of potentially reversible segmental dorsal horn cervical cord dysfunction due to ischemia with a great potential for clinical improvement. Also focal demyelination of the cervical dorsal roots without blocking of the impulse transmission would obviously resulted in delayed N13 and increased N13-N9 conduction time. The N20 potential follows and is delayed in total time because of the conduction delays already demonstrated in the roots and dorsal columns.The current study presented prolonged N20-N13 interpeak latency (CSCT) suggesting an upper cervical lesion. Furthermore, N13-N9 interpeak latency was also prolonged which could suggest a lower cervical cord and/or cervical root affection. This cervical involvement may be secondary to vasculitis, degenerative disc changes or joint affection (preodentoid pannus and odentoid erosion) [25].

Moreover, the SSEPs study disclosed bilateral abnormalities in some of the patients. The increased N13-N9 conduction time may reflect a delay in impulse propagation either in the plexus, dorsal roots or the dorsal column [31]. Since lesions of brachial plexus invariably resulted in pathological N9 responses [29], SSEPs test results with normal N9 but increased N13-N9 conduction time points to a lesion proximal to the plexus, either in the cervical roots or dorsal columns.

The variability in SSEPs data of CM patients where some patients have normal results could invariably affect the sensitivity of SSEPs as a test in this group. It was observed that N13 and N20 components in some patients with CM can be entirely normal while prolonged in others.

The presence of normal SSEPs data may indicate that their symptoms either caused by an altered impulse pattern in otherwise normal large afferent fibers or by a lesion of thin, slowly conducting afferent fibers not tested by the present technique. Some patients who had evidences of radiculopathy with or without myelopathy have subjective symptoms as well as objective neurological signs of radiculopathy. The existence of N9 component and/or its normal latency indicates that a normal peripheral impulse will reach the cervical roots [32,33].

Normal SSEPs results therefore do not exclude pathological lesions outside the dorsal column/medial lemniscal system. When this system is affected, either the N13-N9 or the N20-N13 conduction times can be increased. The latter presumably reflects the conduction time between the dorsal column nuclei and the cortex, a pathway entirely located intracranially.

In conclusion the present study revealed that motor and sensory conduction studies are usually normal in CM, among SSEPs parameters, N13 latency was prolonged bilaterally, CSCT abnormal bilaterally, N13-N9 and N20-N13 latencies unilaterally (right side) and mononeuropathies, polyneuropathies, radiculopathies and plexopathies should be excluded before diagnosis of CM was made.

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Author contribution

Dr. Kaddori collectes and analyses the data; Dr. Hamdan interprets the data and approves the final version; and Dr. Mohammed examines the patients and referred them to the neurophysiology unit.

Conflict of interest

The authors declare no conflict of interest.

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Cardiac Electrophysiological Evaluation in Stroke Patients without Cardiac Abnormalities

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Abstract

Background Physicians have known for centuries that primary cardiac disorders can lead to stroke, but the realization that strokes may result in the cardiac abnormalities is much more recent. Cardiac disturbances are common following stroke such as cardiac arrhythmias or repolarization abnormalities. To provide optimal care, it is essential to distinguish whether cardiac abnormalities are caused by acute stroke or unrelated to it.

Objectives To evaluate the cardiac electrophysiological changes in patients with stroke and study the correlation of stroke type, electrocardiographic and echocardiographic findings with the holter findings.

Methods A case control study was conducted on 52 persons of either sex; involving 32 patients suffering from stroke for the first time without underlying cardiac disease, and 20 healthy persons. Electrocardiographic examination to record cardiac electrical changes, holter record for one hour to detect changes in heart rate variability parameters and echocardiogram to asses heart function have been done for each patient and subject control.

Results No significant difference was found between patient and control groups regarding sex and age, but there was a significant difference between the two groups regarding the SDNN, SDANN, rMSSD parameters, QT dispersion, ST segment shape and T wave shape.

Conclusion The Electrocardiographic changes can occur in stroke patients without cardiac problems. The non-invasive parameters (heart rate variability, QT dispersion) are useful tests in patient with stroke to assess cardiac abnormalities.

Key words Electrocardiographic changes, stroke, cardiac problem.

List of abbreviation: CNS = central nervous system, ECG = electrocardiography, SAH = subarachnoid hemorrhage, VC = ventricular contraction, NSVT = non-sustained ventricular tachycardia, SVT = supraventricular tachycardia, PVC = premature ventricular contraction, PSVC = paroxysmal supra ventricular contraction.

Introduction

Cardiac electrophysiological changes suggestive of cardiac pathology can be associated with intracranial pathology, most notably cerebral hemorrhage. Delay of operative therapy may have catastrophic results. Experimental data indicates massive sympathetic outflow results from stimulation of the lateral and posterior hypothalamic regions. Large amounts of norepinephrine are released into the systemic circulation, resulting in hypertension, tachycardia, dysrhythmias and electrocardio-graphic (ECG) changes.

Myocardial ischemia and injury can occur from the effects of this excessive sympathetic stimulation [(1)].

The association between central nervous system (CNS) disease and ECG changes was first described in 1938 [(2)]. These ECG changes were further detailed and categorized, and their frequent association noted in the presence of spontaneous subarachnoid hemorrhage (SAH) [(3)]. It is estimated that approximately 50% of patients with aneurismal SAH will have ECG

abnormalities [2]. One case report describes a patient with a known aneurismal SAH who had a preoperative ECG consistent with an anterior wall myocardial infarction. For this reason the operation was canceled, and the patient died soon afterward of a second hemorrhage. The autopsy revealed a normal heart with no evidence of recent infarction [3]. Cardiac abnormalities are present in more than 50% of patients with subarachnoid hemorrhage [4].

Several studies investigated the effect of brain injury on the heart but very few studies have addressed the prognostic significance of these changes. These abnormalities can lead to diagnostic and therapeutic difficulties for cardiologists and neurologists [3]. Khechinashvili *et al* in a systematic review compiled all information found in the literature on the prevalence of the ECG changes and QT prolongation during the acute phase of stroke and their coexistence with other abnormal cardiac findings [3]. Abnormalities, such as ischemic-like ECG changes and/or QT prolongation were present in more than 90% of unselected patients with ischemic stroke and intracerebral hemorrhage, but the prevalence was much lower after exclusion of patients with preexisting heart disease [5]. In patients with ischemic stroke and intracerebral hemorrhage, these ECG abnormalities (and QT prolongation) most often represent preexisting coronary artery disease. They found that the specificity of ECG changes to diagnose acute myocardial infarction is low in the acute phase of stroke [4]. In some cases ECG changes have been mistaken for those of myocardial infarction with resulting inappropriate treatment, especially the use of anticoagulants or delay of surgery [2]. Therefore, it is important to determine whether or not there is primary heart disease and subarachnoid hemorrhage or reversible cardiac abnormalities secondary to subarachnoid hemorrhage [6].

An understanding of the role the CNS plays in the genesis of ECG abnormalities has been obtained through numerous animal studies [7]. Apparently, the stimulation of certain central autonomic centers results in a massive sympathetic outflow, with a consequent release of large amounts of norepinephrine from the adrenal medulla. These centers in humans have been identified as lying in the lateral and posterior hypothalamic regions. These regions are stimulated by increased intracranial pressure, the irritant effect of blood, ischemia or a combination of these factors. Once stimulated, central efferent pathways produce norepinephrine release into the general circulation, with resultant acute hypertension and cardiac manifestations [8]. This study was conducted to evaluate the cardiac electrophysiological changes in patients with stroke and study the correlation of stroke type, electrocardiographic and echocardiographic findings with those of holter study.

Methods

A case control study was conducted on 52 persons with acute stroke attack who were attending Al Hussein Teaching hospital in Karbala Province for management, from December 2013 to June 2014. The study population consisted of two groups: thirty two patients with acute stroke attack for the first time without cardiac abnormalities and 20 healthy age and sex matched subjects were included in the study as control group.

Diagnosis of acute ischemic attack was established in all the patients by computed tomography (CT scan). Detailed history of hypertension, diabetes mellitus and neurological findings were recorded in all the patients. All the patients were evaluated for cardiac disease by detailed history and clinical examination. They were submitted to ECG recording and Holter monitoring for one hour, and also chest X-ray. Patients with history or clinically proved acute myocardial infarction, unstable angina or cardiac diseases were excluded from the study.

ECG findings done on the day of admission were considered for the analysis. Consultant expert cardiologists analyzed the electrocardiographic findings and cardiac

evaluation using echocardiography for all cases. ECG abnormalities were grouped into: invert T-wave, ST segment elevation/ depression.
The Holtor records were estimated by specialist physician and the following parameters were measured (SDNN, SDANN, rMSSD and Q-T dipersion)
To detect underlying cardiac disease, clinical and paraclinical evaluations were done by consultant cardiologists.

Statistical analysis

The data was analyzed using SPSS program version 17.0 and Excel program. The results were expressed as (mean ±SD). Student t- test was used to compare parametric values. While Chi- square test was used to compare non parametric values. For all these statistical comparisons, the threshold of significance was chosen as p=<0.05. The statistical significance was determined using the chi-square test and a p value of < 0.05 was considered significant

Results

The study included 32 patient group (range 32 - 80 yrs), 20 females and 12 males and the mean age was (61.31 ± 11.43). The control group consisted of 20 healthy persons, 16 females and 4 males and the mean age was (56.55 ± 9.93), (range 38 - 80 yrs) there is no significant differences in sex and age between two groups (Table 1).

Table 1. Demographic data of control group and patients group

Parameters	Control N=20		Patients N=32		*P* value
	Frequency	%	Frequency	%	
Male	4	20	12	37.5	**0.184**
Female	16	80	20	62.5	
Age (Years)	56.55 ± 9.93		61.31 ± 11.43		**0.131**

In stroke group 15 (46%) patients were hypertensive and 12 (37%) were hypertensive and diabetic, 4 (12%) of them were no hypertensive and no diabetic, 1 (3%) had only diabetes. In control group, history of no hypertension nor diabetes was found in 9 (45%), both hypertension and diabetes was found in 2 (10%) subjects and hypertension was found in 7(35%) subjects and diabetes mellitus in 2 (10%) subjects. The frequency of the abnormal ECG changes observed in 24 (75%) of patients with acute cerebral infarct (p = 0.0054) and 2 (10%) of the control group (p = 0.0103) (Table 2).

Table 2. Comparison between patients group and control group regarding ST segment and T wave shapes in patients group by Chi square test

Parameters		Patients N=32		Control N=20		*P* value
		No.	%	No.	%	
ST segment Shape	**Normal**	22	68.75	20	100	**0.0054**
	Abnormal	10	31.25	0	0.00	
T wave Shape	**Normal**	18	56.25	18	90.00	**0.0103**
	Abnormal	14	43.75	2	10.00	

ECG changes that were observed in stroke patients were inverted T-wave in 14 patients (43.75%), ST segment elevation/depression in 10 patients (31.25%).

Inversion of T wave and ST segment changes were the most common findings. The observed abnormalities were mostly related to myocardial repolarization abnormalities. In most of these patients ECG changes were transient according to follow- up period recordings.

All the HRV parameters showed a significant lower mean value in patients group with a significant differences (p = <0.05).

Values of SDNN, SDANN, rMSSD parameters in patients group were significantly lower than those of the control group (p = <0.0001, p = <0.0001, p = <0.0001, respectively).

Table 3. Comparison of holter finding between control group and patients group by t test

Parameters	Control (n=20) Mean ± SD	Patients (n=32) Mean ±SD	P value
Mean NN (ms)	725.45 ± 118.06	774.97 ± 129.44	0.1715
SDNN (ms)	127.15 ± 16.08	92.69 ± 13.58	<0.0001
SDANN (ms)	111.7 ± 14.44	58.19 ± 9.84	<0.0001
r MSSD (ms)	29.65 ± 7.3	17.13 ± 3.99	<0.0001

Discussion

The CNS regulates the heart rate, blood pressure, vasomotor tone, and cardiac output and plays an important role in myocardial metabolism and cardiac contraction. Further, the CNS can affect the cardiovascular system by altering fluid and electrolytes balance. Cardiac systolic function also is affected after an acute CNS [(9)].

Coronary artery disease and ischemic cerebrovascular disease are leading causes of morbidity and mortality. Coronary artery disease often coexists with asymptomatic carotid artery atherosclerosis, transient ischemic attacks, or ischemic stroke. Numerous studies have shown that mortality from all forms of ischemic cerebrovascular disease is primarily due to coronary artery disease. Thus, there is increasing interest in identifying coronary artery disease in patients with cerebrovascular disease, including those without clinical manifestations of heart disease. ECG changes are common in patients with ischemic stroke. ECG changes suggestive of ischemic heart disease were the common findings in this study [(10,11)].

In the current study there were cardiac electrical changes rather than structural cardiac disease. This is in agreement with most of the studies that prove the association between neural injury and the cardiac electrical defects; yet, the study groups were less selected than the aforementioned studies regarding the utility of ECG in the setting of acute stroke [(12,13)].

The study of relationship between HRV and acute cerebral stroke allows for the description of the arrhythmic profile of the acute phase of ischemic stroke in patients without heart disease. In particular previous studies it was noted in right-sided brain infarctions were found to be associated with more frequent arrhythmias than left-sided lesions [(14-16)]. Furthermore, right insular damage was associated with more complex dysrhythmias, namely ventricular contraction (VC), non sustaintained ventricular tachycardia (NSVT), and supraventricular tachycardia (SVT), than any other localization. These findings once more suggest a major role of the right insula in the pathogenesis of cerebrogenic cardiac disturbances [(17)].

Another interesting aspect of this study is the analysis of the possible interplay between HRV

abnormalities with acute brain infarction. To the best of our knowledge, this is the first study reporting the existence of a significant negative correlation between a specific HRV parameter, namely SDNN in the acute phase of ischemic stroke. In fact, lower values of 24-hour SDNN were associated with a higher number of premature ventricular contraction (PVC) and paroxysmal supra ventricular contraction (PSVC) and also predicted the presence of more complex arrhythmias, such as VC, NSVT, and SVT. Actually, SDNN, which is an estimate of the overall 24-hour HRV behavior, is the best known, best validated, and easiest HRV index to use [18-20].

A decrease of SDNN has been considered to reflect a diminished vagal activity directed to the heart, which may lead to a relative prevalence of sympathetic modulation and to a cardiac electrical instability [19-21]. This interpretation is in agreement with the clinical evidence that SDNN reduction is an independent predictor of an increased arrhythmic mortality in several conditions characterized by autonomic imbalance, such as heart failure, diabetes, and coronary artery disease [10,13,14]. Actually, HRV abnormalities associated with cardiac damage may be determined by derangement of neural activity of cardiac origin.

In particular, changes in the geometry of the beating heart, caused by the presence of diseased non contracting ventricular segments, may abnormally increase the firing of sympathetic afferent fibers [10,15]. This overflow of sympathetic nerve traffic may in turn attenuate vagal activity. However, in the acute stroke setting, cardiac autonomic abnormalities should have a central origin, despite being similar in terms of HRV behavior. Accordingly, acute brain and acute heart damage show the same final expression, with HRV abnormalities and arrhythmias representing a final common effect, possibly determined by a relative sympathetic prevalence on the sinus node and on the myocardium. A follow-up study clearly would be useful in order to assess the prognostic value of Troponin T and ECG changes in the longer term, not only regarding neurological sequels but also regarding cardiologic complications such as recurrent ischemia, myocardial infarction and congestive heart failure amongst these patients [21].

In conclusion, ECG changes can occur in stroke patients without cardiac problems; the non invasive parameters (HRV, QT dispersion) are useful tests to in patients with stroke to assess cardiac abnormalities and finally stroke may be associated with cardiac abnormalities.

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Author contributions

Dr. Hussein and Al-Hashimi analyze, interpret, writ and revise the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Effects of Metformin on Hormonal Profile and Seminal Fluid Analysis in Obese Infertile Male

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Abstract

Background Overweight and obese men have an up to 50% higher rate of sub-fertility when compared with normal weight men. Possible management options include weight reduction by dieting or surgery and medical treatment to correct specific endocrine abnormalities, but as yet none has been proven to be effective.

Objective To verify the impact of decreasing body mass index by giving metformin on hormonal profile and seminal fluid analysis in obese infertile male.

Methods Eighteen obese patients whose body mass index was 30-40 kg/m2 and with mean age of 29 years (range: 22-42 years) with idiopathic asthenozoospermia were enrolled in the study. Standard semen analysis according to WHO and hormones assay which include: follicular stimulating hormone, luteinizing hormone, prolactin, testosterone, and estradiole were performed at baseline and after 12 weeks of therapy. The enrolled patients were asked to take metformin 850 mg twice daily orally for 12 weeks.

Results A significant decrease ($p<0.001$) in sperm count and sperm activity after 12 weeks of treatment with metformin. While there is no significant differences with respect to other spermiological parameters. A statistically significant decrease in the level of serum prolactin after 12 weeks of treatment with metformin whereas no significant differences with respect to the level of other hormones.

Conclusions Although metformin has the capacity to decrease the level of prolactin, it decreases the number and activity of sperms. Further studies are recommended to investigate whether there is any association between infertility in human males and chronic metformin use

Keywords Metformin, infertility, male, prolactin, overweight, obese.

List of abbreviation: AMK = activated protein kinase, BMI = body mass index, FSH = follicular stimulating hormone, LH = luteinizing hormone, E_2 = estradiole.

Introduction

Metformin is one of antidiabetic drugs which belong to the biguanide class of oral antihyperglycemic agents. It was first synthesized in 1929 and was shown to be a potent hypoglycemic agent [(1)].

Metformin acts in the presence of insulin to increase glucose utilization and reduce glucose production, thereby countering insulin resistance. The effects of metformin include increased glucose uptake, oxidation and muscle glycogenesis, increased glucose metabolism to lactate by the intestine, reduced hepatic gluconeogenesis and possibly a reduced rate of intestinal glucose absorption [(2)].

The molecular mechanisms of metformin action are not fully known. Activation of the enzyme AMP-activated protein kinase (AMK) appears to be the mechanism by which metformin lowers serum lipid and blood glucose concentrations [(3)]. Metformin works through the Peutz-Jeghers protein, LKB1, to

regulate AMPK. LKB1 is a tumor suppressor and activation of AMPK through LKB1 may play a role in inhibiting cell growth [4].

The incidence of obesity is rapidly rising in almost every region of the world. Although obesity affects women more than men, male obesity is an issue of serious concern. In Europe, the International Obesity Task Force (2005) has indicated that obesity rates in adult men range from 10 to 27%, with this prevalence rising significantly in the last 10 years [5].

The adverse influence of obesity on various aspects of female reproduction and fertility has been realized for sometime [6] and management guidelines are now available [7]. More recently, data regarding male obesity and infertility have been accumulating [8,9]. There are now several population-based studies showing that overweight and obese men have an up to 50% higher rate of sub-fertility when compared with normal weight men [10,11]. One could argue that this could be related to confounding factors such as male age, smoking and alcohol use, and female partner obesity. However, once these factors have been excluded it was shown that for every three-point increase in a man's BMI, couples were 10% more likely to be infertile [12].

Kort *et al*. (2007) showed significant negative relationship between high body mass index (BMI) and sperm motility in 528 Danish men. In addition, men with BMI > 25 had fewer chromatin-intact normal-motile sperm cells per ejaculate [13]. Jensen *et al*. (2004) in accordance with other studies showed that overweight and obese men (BMI >25 kg/m2) had significantly lower sperm concentrations than those of normal-weight men (BMI 20–25 kg/m2) [14]. The prevalence of oligozoospermia was higher in overweight and obese men compared with normal-weight men. A substantial decrease in serum testosterone, sex hormone binding globulin and Inhibin B were also found with increasing BMI [14]. There are several etiological theories including endocrine abnormalities, genetic, sexual dysfunction and testicular hyperthermia. Of these, endocrine abnormalities are likely to be the most important, involving increased estrogen and increased insulin resistance, reduced androgens and reduced inhibin B levels. Possible management options include weight reduction by dieting or surgery and medical treatment to correct specific endocrine abnormalities, but as yet none has been proven to be effective [15]. The aim of current study was to assess the impact of decreasing BMI by giving metformin on hormonal profile and seminal fluid analysis in obese infertile male.

Methods

Patient Selection

Eighteen obese patients (BMI = 30-40 kg/m2) (mean age, 29 years; range, 22–42 years) with idiopathic asthenozoospermia were enrolled in the study. The patients were selected at the Department of Pharmacology, Faculty of Medicine, Al-Nahrain University and Urology Clinic of Al-Imamain Al-Kadhimiyian Medical City, Baghdad, Iraq, at period extended from Apr. 2012 to Sep. 2012. All subjects underwent medical screening, including history and clinical examination, and presented with a clinical history of primary infertility of at least 3 years.

Eligibility Criteria

The exclusion criteria were: [1] Infertile men with azoospermia [2] diabetes mellitus. [3] infectious genital diseases, anatomical abnormalities of the genital tract including varicocele, and anti-spermatozoa antibodies [4] systemic diseases or treatment with other drugs in the 3 months before enrollment in the present study [5] smoking, alcohol, drug addiction, or occupational chemical exposure.

Safety Assessment

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

Laboratory investigations

Hormones assay include: follicular stimulating hormone (FSH), luteinizing hormone (LH),

prolactin, testosterone, and estradiole (E_2) (by miniVIDAS 12 model)

Standard semen analysis**:** Freshly ejaculated semen samples were obtained by masturbation into sterile petri-dish containers under clean condition after (3-5) days of sexual abstinence. The specimens were placed in an incubator at 37^{o}C for (30) minutes to allow liquefaction, after liquefaction, semen samples were evaluated for semen volume, appearance, pH and viscosity, then specimens were analyzed for sperm concentration, progressive motility and normal morphology according to WHO criteria [16].

Study Design and Treatments

The enrolled patients were asked to take metformin (Merck, France), 850 mg twice daily orally for 12 weeks. Clinical examination, semen analysis, and hormonal assay were performed at baseline and after 12 weeks of therapy. All patients provided their written informed consent and completed the entire trial.

Statistical analysis

The data were analyzed using SPSS program. Results were reported as mean ± S.D. The total variations were analyzed by performing the statistical design T-test. Probability levels of less than 0.05 were considered significant [17].

Results

Metformin treatment was well-tolerated by all subjects. None of the subjects suspended the therapy due to side effects, although some experienced transient diarrhea and flatulence during the first month of treatment.

Effect on BMI

For the mean duration of the study (12 weeks), Metformin was given to 18 patients. The mean BMI decreased significantly during the treatment time, from 35.93 ± 5.7 to 34.85 ± 5.2 ($p < 0.001$).

Effect on seminal fluid analysis

The results of current study shows no significant differences with respect to semen volume, liquefaction time, pH, and normal morphology at baseline and after 3 months of treatment with metformin 850 mg two times / day. The (mean±SD) was (3.04 ± 1.12 vs. 3.08 ± 0.93) (39.17 ± 23.82 vs. 28.75 ± 2.26) (8.21 ± 0.50 vs. 8.21 ± 0.33) (62.08 ± 10.97 vs. 64.58 ± 4.98) before and after 3 months of treatment with metformin for semen volume, liquefaction time, pH, and normal morphology respectively.

In this study, the (mean±SD) before versus after 3 months of treatment with metformin 850 mg two times / day for sperm count and sperm activity was (19.00 ± 14.53 vs. 16.13 ± 13.76) (8.33 ± 5.77 vs. 3.83 ± 1.95) respectively. The results shows significant difference ($p < 0.05$) with respect to sperm count and sperm activity at the base line (before treatment) and after 3 months of treatment with metformin (Table 1 and Fig. 1).

Effect on hormonal analysis

The (mean±SD) before versus after 3 months of treatment with metformin of serum LH, FSH, E2, and testosterone was as follows respectively: (4.04 ± 4.21 vs. 3.63 ± 2.76) (7.19 ± 8.84 vs. 6.55 ± 7.77) (5.30 ± 5.54 vs. 4.21 ± 2.69) (4.33 ± 1.51 vs. 3.94 ± 1.18) and it shows no statistically significant differences between baseline and after 3 months of treatment with metformin.

The results of current study reveal statistically significant decrease in the level of serum prolactin before and after 3 months of treatment with metformin with mean±SD of 8.01 ± 5.73 vs. 6.93 ± 5.09.

Discussion:

Obesity has been shown to adversely affect male fertility, by reducing spermatogenesis. There are several etiological theories including endocrine abnormalities, genetic, sexual dysfunction and testicular hyperthermia. Of these, endocrine abnormalities are likely to be the most important, involving increased estrogen and increased insulin resistance, reduced androgens and reduced inhibin B levels [15].

Table 1. Semen analysis parameters before and after 3 months of metformin treatment

Semen parameters	Metformin Treatment		p value
	Before (mean± SD)	After (mean± SD)	
Semen volume (ml)	3.04 ± 1.12	3.08 ± 0.93	0.754
liquefaction time (min)	39.17 ± 23.82	28.75 ± 2.26	0.148
pH	8.21 ± 0.50	8.21 ± 0.33	1.000
Sperm count (10^6/ml)	19.00 ± 14.53	16.13 ± 13.76	0.018
Sperm activity %	8.33 ± 5.77	3.83 ± 1.95	0.009
Normal morphology (%)	62.08 ± 10.97	64.58 ± 4.98	0.352

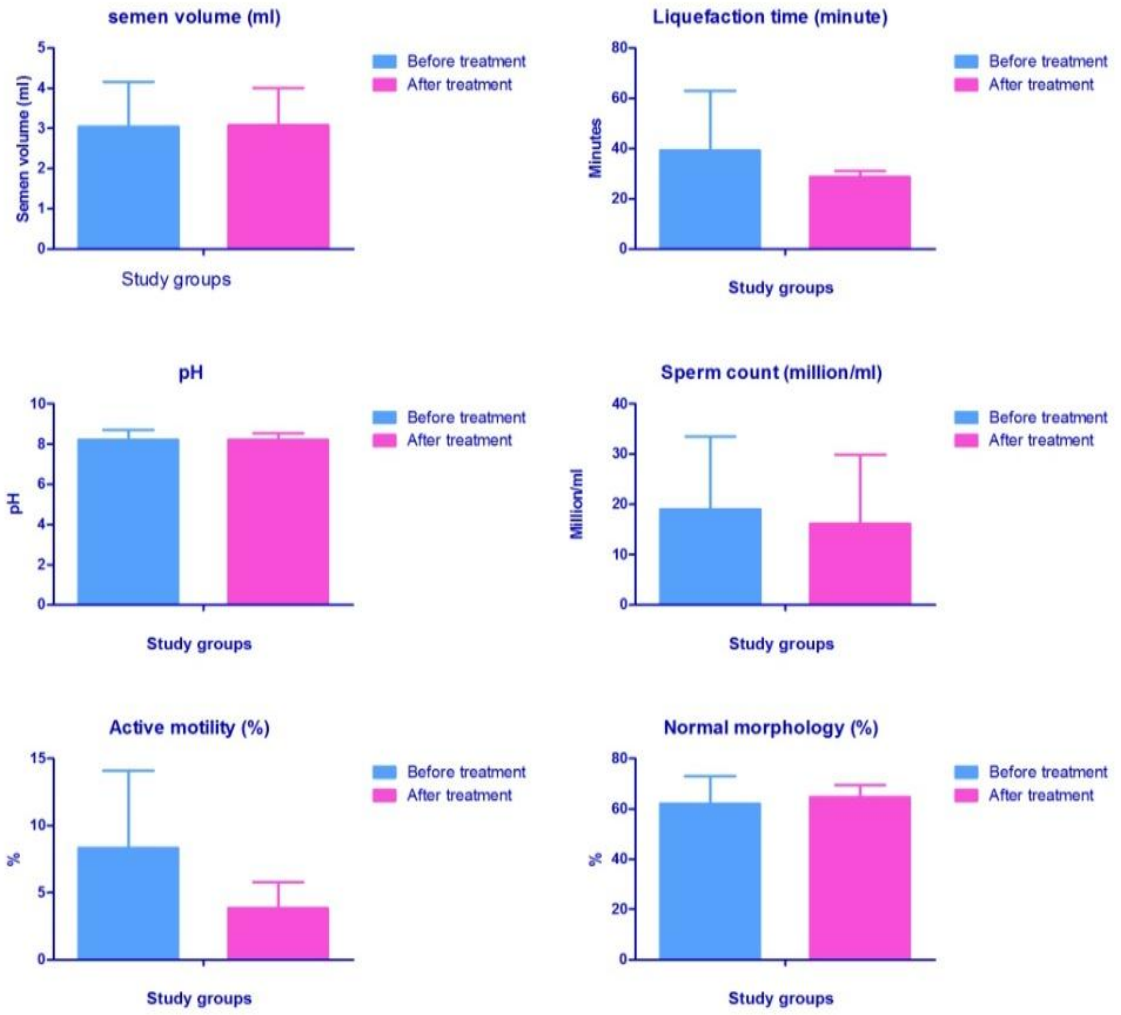

Fig. 1. Semen analysis parameters before and after 3 months of treatment with metformin

Insulin resistance could be the underlying pathogenesis of chronic hypospermatogenesis leading to oligospermia and azoospermia associated with other metabolic abnormalities in men. Metformin has proven as an effective medication for not only IR but several other aspects of the polycystic ovarian disease including reproductive abnormalities [15]. Therefore, insulin sensitizers, particularly metformin could probably have beneficial effects on overweight and obese patients with asthenozoospermia.

In our study, metformin was effective in reducing BMI significantly. These results are in accordance with recent observations made by several authors, such as Hosokawa *et al* [18] Garber *et al* [19] and De Fronzo *et al* [20] among others. The MOCA trial is the largest double-blind, randomized, placebo-controlled trial of metformin in obese non diabetic children and young people. The MOCA trial provides evidence that a short treatment course of metformin is clinically useful, safe, and well tolerated to halt further gain in adiposity and improve fasting glucose [21].

Table 2. Hormonal analysis before and after 3 months of metformin treatment

Hormones	Metformin treatment		P value
	Before (mean± SD)	After(mean± SD)	
Luteinizing hormone	4.04 ± 4.21	3.63 ± 2.76	0.403
Follicular stimulating hormone	7.19 ± 8.84	6.55 ± 7.77	0.074
Prolactin	8.01 ± 5.73	6.93 ± 5.09	0.019
Estradiole	5.30 ± 5.54	4.21 ± 2.69	0.235
Testosterone	4.33 ± 1.51	3.94 ± 1.18	0.196

In the current study metformin was given to 18 overweight and obese patients with asthenozoospermia in a dose of 850 mg twice a day for 12 weeks The results of present study demonstrate that administration of metformin decreases significantly sperm count and sperm activity while there is no statistically significant changes regarding semen volume, liquefaction time, pH, and normal morphology respectively.

A study by Naglaa *et al* showed that oral administration of metformin to both diabetic and non-diabetic rabbits resulted in a significant decrease in testicular weight, sperm count, sperm motility and serum testosterone with a significant increase in sperm anomalies and dead sperm percentage [22]. Naglaa *et al* has suggested that vitamin B_{12} deficiency may cause decreased sperm count and motility as it is well established that chronic use of metformin is associated with 20-30 % lower blood levels of vitamin B_{12}. This hypothesis is further strengthened by the finding that vitamin B12 supplements improve fertility in animals with abnormal sperm production. In this way, Naglaa *et al* have questioned the justification for the use of metformin in a frame of a therapeutic strategy for diabetes due to its resulting impact on male fertility and also put forward probable reasons behind this [22]. Moreover, vitamin B_{12} deficiency during pregnancy may induce irreversible damage in the germ cells of embryos and affect the maturation of spermatozoa. Chronic exposure of metformin induces DNA damage in mammalian cells [23] and also impairs the mitochondrial complex-1 activity which plays the vital role to maintain the normalcy of sperm motility [24].

However, another study by Morgante *et al* has shown that the use of metformin is associated with a statistically significant reduction in insulin resistance and sex hormone-binding globulin levels, a statistically significant increase in serum androgen levels, and a consequent improvement in semen characteristics [25].

The results of present study demonstrate that the (mean±SE) before versus after 3 months of treatment with metformin of serum LH, FSH, E_2, and testosterone shows no statistically significant differences while the level of serum prolactin reveal statistically significant decrease.

Metformin may change the affinity and/or the number of dopamine receptors or of receptors for other compounds regulating production, secretion and metabolism of prolactin, may enhance gastrointestinal absorption and/or

metabolism of bromocriptine, as well as may directly affect prolactin pharmacokinetics. Interestingly, animal studies carried out evidenced that metformin penetrates the blood–brain barrier, and its content in the pituitary is higher than in any other brain structure [(26)]. In the light of these results, it seems that the pituitary is an important target for metformin action and that the prolactin-lowering effect of this agent results, at least in part, from its action at the level of pituitary lactotropes. Taking into account that this drug was found to reduce plasma levels of other pituitary hormones [(27-28)].

In conclusion, results of the current study demonstrate that although metformin has the capacity to decrease the BMI as well as level of prolactin, it decreases the number and activity of sperms. Further studies are recommended to investigate whether there is any association between infertility in human males and chronic metformin use and *in vitro* effects of metformin on human spermatozoa to observe cytomorphometrical changes, biochemical alterations

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Conflict of interest

The author declares no conflict of interest**.**

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