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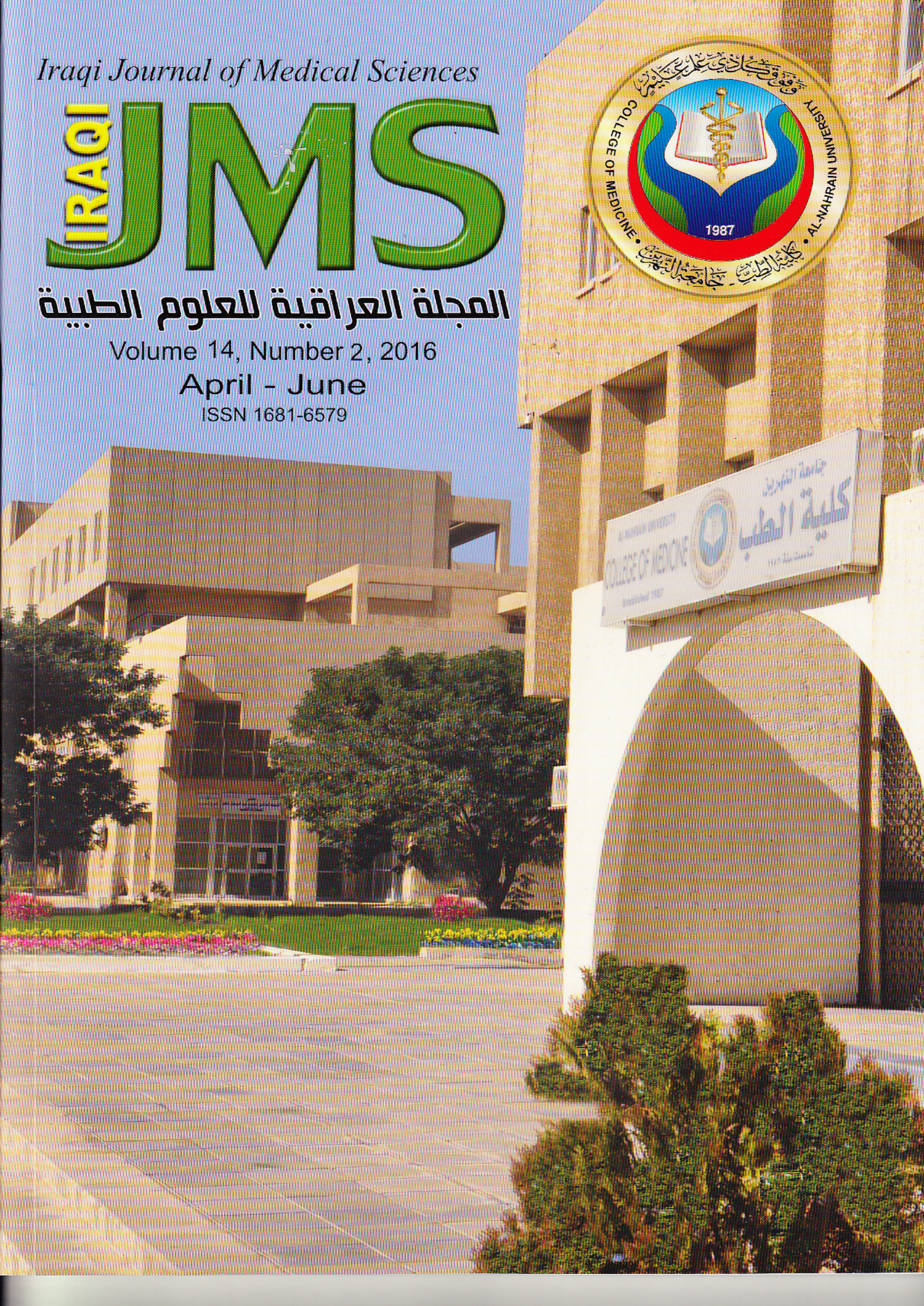**Al Nahrain University, College of Medicine 9ᵗʰ Scientific Conference.**

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3. Chapter in book: Phillips SJ, and Whisnant JP. Hypertension and strock. In: Laragh JH, and Brenner BM. editors. Hypertension: Pathophysiology, diagnosis, and management. 2nd ed. NewYork: Raven Press; 1995. p. 465-78.

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Medical Ethics in the Glorious Qur'an and Holy Shari`ah

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Abstract

It is believed that good ethical values flow out from one source, and follow a clear path that extends to every part of the world. These values honor the dignity of man as a human being honored by God almighty, and maintain man's essential rights, including life, freedom, preservation of property, health and sufficiency, throughout man's life.
The Glorious Qura'n and Holy Shari`ah had contribute actively in regulation these ethical aspects in medicine. Although, these principles have been adopted in practice since the early days of Islamic culture and cited by many Muslims scientists.
Nowadays, physicians are under prepared to deal with the new progress in medical technology like organ transplants, genetic engineering, and respirators that keep a person alive, only as a vegetable, for several years.

Keywords: Ethics, Medical Ethics, Relgion, Islam, The Glorious Qura'n, Holy Shari`ah

Medicine is a science as well as an art. The science is essential for competency in detection of illness and attempting to restore a good health. The art of medicine is helpful in realizing the differences between individual patient regarding the culture, religion, freedom, rights & responsibilities [1]. The most pressing needs of people while seeking medical advice , is to get relief from health disturbances, and therefore; they allow physician to touch, see, and manipulate every part in their bodies (including the most intimate) as they trust that this physician will act the best to help them [1].

Physicians themselves accountable only by themselves and to God in order to provide the core values of medicine which are the compassion, competence and autonomy [1,2].

Ethics is derived from the Greek word "ethike" that means habit, action or character [3]. Medical Ethics is a subdivision that is concerned with moral principles in medical practice whether clinical or investigational aspects [2]. It may include different values like right, responsibilities & virtues and it is regulated by law [1,2]. Concern for medical ethics has been expressed in the form of laws, assumptions and "oaths" prepared for or by physicians since the beginning of human history. Among the oldest of these are: the Code of Hammurabi in Babylon, Egyptian papyri, Indian and Chinese writings, in addition to early Greek" founder of medicine", Hippocrates recommendations [1,3].

Religion is highly appreciated framework that helps in admirable application of these interrelated issues of morality, law and ethics on a background of science [4,5].

A physician should trust in God when treating a patient and expect the cure to come from him .

No physician should count on his own power and work, nor depend wholly on that in any of his actions [1,4,5]. Good ethical values flow out from one source, and follow a clear path that extends to every part of the world. These values are clearly emphasized by the Glorious Qur'an as a human being honored by God almighty, and maintaining man's essential rights, including:

life, freedom, preservation of property, health and sufficiency, throughout life [2,4,6].

The sacred law of Muslims has dedicated the following principles in dealing with all aspects of human existence: [5,6]

1. The first principle that regulates medical ethics when dealing with patient is that human being **honored** by God

{وَلَقَدْ كَرَّمْنَا بَنِي آدَمَ} (الاسراء 70) [7]

"We have honored the children of Adam" (17:70) [7]

2. The second principle is that every human being has the **right to live**; his life is respected and protected

{وَمَنْ أَحْيَاهَا فَكَأَنَّمَا أَحْيَا النَّاسَ جَمِيعًا} (المائدة 32) [8]

"and if he saves [a life], it is as if he saved the lives of all people" (5:32) [8]

3. The third principle is **equity**, which is one of the purposes of messenger missions:

{لَقَدْ أَرْسَلْنَا رُسُلَنَا بِالْبَيِّنَاتِ وَأَنزَلْنَا مَعَهُمُ الْكِتَابَ وَالْمِيزَانَ لِيَقُومَ النَّاسُ بِالْقِسْطِ} (الحديد 25) [9]

"We have sent our messengers with clear signs and sent down with them the Book and the scale, so that men may stand in equity" (57:25) [9]

{إِنَّ اللَّهَ يَأْمُرُ بِالْعَدْلِ وَالإِحْسَانِ} (النحل 90) [10]

"God enjoins equity and charity" (16:90) [10]

On the other hand, there is a warn against all things that may upset a just situation:

{لاَ تَتَّبِعُواْ الْهَوَى أَن تَعْدِلُواْ} (النساء 135) [11]

"Do not, in following your desires, fail to be just" (4:135) [11]

The word used for "equity" in the Quran also implies equality.

Even the Sheet (the constitution of Medina), had emphasized for equal treatment and equity for all people who live within the Muslim community, whether they themselves are Muslim or non-Muslims:

" The Jews who follow us will enjoy support and equality, suffer no injustice, and have no united fronts formed against them" [6]. This means the greatest possible degree of equality in the distribution of health resources among society members and in providing them with preventive and therapeutic care, without the slightest discrimination on the basis of gender, race, belief, political affiliation, any social or judicial consideration, or any other factor [4,6].

4. The fourth principle is **doing well**. The Arabic word ihsaan, translated as doing well. It means the gentle, compassionate touch while "ihsaan" denotes a living conscience and mindfulness of God [1,6]. These issues are almost missing in modern medical practice.

5. The fifth principle is no harm and no causing harm. It is unacceptable to bring harm on one's self, or to cause harm to others or to society in any shape or form [1, 4-6].

{أَنَّهُ مَن قَتَلَ نَفْسًا بِغَيْرِ نَفْسٍ أَوْ فَسَادٍ فِي الأَرْضِ فَكَأَنَّمَا قَتَلَ النَّاسَ جَمِيعًا} (المائدة 32) [8]

"When a person who kills a soul – unless it is [in punishment] for a [murdered] soul or for corruption on earth – it is as if he killed all people" (5:32) [8]

Even WHO announcement emphasizes on these aspects as the main goals of medicine, which are applications of all the above principles:

1. "health for all": To offer the highest attainable standards of health to all people, both individuals and communities, in the place they live and at a cost they can afford [6].

2. "As physicians should be gentle with people, refrain from talking ill about them in their absence, and keep their secrets".

3. "If the physician treats one of a man's women folk, girls, or boys, he should cast down his eyes and not look beyond the afflicted part of the body" [2,5,6].

Historically, these principles have been discussed and well adopted in practice since the early days of this culture as cited by these Muslims scientists:

1) Al-Blatheri, in Futooh Al-buldaan (The conquest of countries).
2) Ibn Sa'd's, in Tabaqaat (Classes)
3) Al-Razi, in Akhlaaq Al-tabeeb (Ethics of the physician).
4) Al-Ruhawi, in Adab al-Tabib (Practical Ethics of the Physician) by a ninth-century physician residing under the Islamic caliphate in Iraq [2,4,6].

Nowadays, because of great progress in medical technology, there are much new different accountability (like patients, hospitals, licensing and authorities like in case of abortion, assisted reproduction, cloning, etc...); where physicians are under prepared to deal with such situations efficiently [1]. In addition to other new issues that required clarification from religion point of view like: organ transplants, genetic engineering, and respirators that keep a person alive, only as a vegetable, for several years.

The questions here; Do we have the right to:

- remove an organ from a living person? Or purchase it?
- take all the organs we want from a dead person?
- artificial insemination? test-tube babies? womb hiring?

The three rules of the "Goals of the Islamic Shari`ah" are the protection and preservation of life, intellect, and progeny [2,5]. Concerning termination of pregnancy it is known by sacred Shari`ah that Ensoulment occurred at 40 or 120 days after fertilization, according to different schools of thought. The right of the human fetus in Islam is similar to the rights of a mature [2,4,5]. Chorionic villous biopsy must be performed before ensoulment in first trimester while termination for any other reason is strongly and consistently discouraged, particularly after ensoulment has occurred [5]. It is not permissible for a doctor to switch off the medical apparatus

providing a Muslim patient with oxygen, even if he is brain dead, i.e. in vegetative state [12], or even the doctor should not give in to the demand of the patient or his family-members for stopping medical aid. If the doctor pulls out the plug and the Muslim patient dies because of it, he will be considered a killer .

It is permissible to transplant an organ from an animal (including dogs and pigs) to a human being; the transplanted organ will be considered as an organ of the recipient; all rules will apply to it [12].

It is not obligatory on a Muslim to investigate whether or not the medicine consists of forbidden ingredients, even if the process of reading a result is easy for him.

Examples of questions and answers concerning some medical ethics (Istifftaat)[12]

Question: Some experts of genetic engineering claim that they can improve the human race by altering the genes in the following ways: Removing the ugliness of the face; Replacing it with beautiful characteristics; By both of the above. Is it permissible for the scientists to engage in these kinds of activities? Is it permissible for a Muslim to allow the doctors to alter his genes?

Answer: If there are no side effects, then, in principle, there is no problem in it.

Question: Principles of donation.

Answer: As far as donating an organ by a living person to another person is concerned, there is no problem in it if it does not entail a serious harm to the donor. (For example, donating one kidney by a person who has another healthy kidney.)

As far as removing an organ from a deceased (as directed in his will) for the purpose of transplanting it into a living person is concerned, there is no problem in it so long as: The deceased was not a Muslim or someone who is considered a Muslim. Or the life of a Muslim depended on such transplantation. Apart from these two cases, there is a problem in enforcing the will [of the deceased] and in allowing the

removal of the organ. However, if the will had been made [by the deceased], there will be no indemnity on the person removing the organ from the dead body.

Question: Is the process of test-tube babies allowed? In the sense that the wife's ovum and the husband's sperm are extracted to be fertilized outside the body, and then placed in the womb [of the wife].

Answer: In principle, it is allowed.

Question: There are certain hereditary diseases that are transferred from parents to children and pose a danger to their lives in the future. Modern science has acquired the means of preventing some of such diseases by fertilizing the woman's ovum in a test tube and examining the genes to eliminate the problematic ones. Then it is returned the woman's womb. The remaining genes [i.e., ova] are destroyed. Is this religiously permissible?

Answer: In principle, there is no problem in it.

Question: In the process of fertilization in a lab, more than one ovum is fertilized at a time. Secured in the knowledge that implanting all fertilized ova in the mother's womb will endanger her life. Is it permissible for us to use only one fertilized ovum and destroy the remaining ones?

Answer: It is not obligatory to implant all the fertilized ova in a test tube into the womb. Therefore, it is permissible to use one ovum and destroy the remaining ones.

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Immunohistochemical Expression of p53, bcl2 and CD34 in Cervical Intraepithelial Neoplasias and Carcinomas

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Abstract

Background — Cervical cancer is the fourth most common cancer affecting women worldwide. Immunohistochemical expression of several biomarkers; including those regulating apoptosis and angiogenesis; may help to distinguish reactive conditions from precancerous and cancerous lesions of the uterine cervix.

Objective — To assess the IHC expressions of p53, bcl2 and CD34 in cervical intraepithelial neoplasias and carcinomas.

Methods — A cross sectional study included a total of 127 formalin-fixed paraffin-embedded cervical tissue blocks; of which 22 cases were chronic cervicitis, 24 cases were low grade squamous intraepithelial lesion (LSIL), 28 cases were high grade squamous intraepithelial lesion (HSIL) and 53) cases were invasive cervical carcinomas. Sections from each block were immunohistochemically stained for p53, bcl2 and CD34.

Results — p53 was not expressed in chronic cervicitis, with significant increase in its expression from LSIL through HSIL to carcinomas had been identified. A significantly higher IHC expression of p53 was observed in adenocarcinomas and adenosquamous carcinomas compared to squamous cell carcinomas. Bcl2 was expressed in all cases with non-significant differences. Regarding CD34 IHC expression; there was a significant increase in microvessel density (MVD) from chronic cervicitis through LSIL and HSIL to carcinomas. A significantly higher MVD was detected in adenosquamous carcinomas and adenocarcinomas, in poorly differentiated carcinomas and was significantly increasing with stage.

Conclusions — p53 plays an important role in the progression of the severity of intraepithelial cervical lesions. MVD can be utilized as ancillary marker for the risk of malignant transformation of cervical intraepithelial lesion.

Key words — LSIL, HSIL cervical carcinoma, p53, bcl2, CD34, MVD

List of abbreviation: CIN = cervical intraepithelial neoplasia, LSIL = low grade squamous intraepithelial lesion, HSIL = high grade squamous intraepithelial lesion, IHC = Immunohistochemical, TAH = total abdominal hysterectomies, SI = staining index, MVD = microvessel density

Introduction

Cervical cancer is the fourth mostly common cancer affecting women worldwide, after breast, colorectal, and lung cancers; it is most found in the lower resource countries of sub-Saharan Africa. It is also the fourth most common cause of cancer death (266,000 deaths in 2012) in women worldwide [1]. In Iraq, and according to the latest Iraqi cancer registry 2011, cervical cancer is out of the commonest ten cancers in Iraqi females [2].

Cervical intraepithelial neoplasia (CIN) is a premalignant (dysplastic) lesion that is characterized by abnormal cell proliferation, maturation and nuclear atypia. CIN may return to normal or progress to invasive cancer if left

untreated. Approximately one-third to one-half of cases of CIN-I and CIN-II regress without treatment, even cases of CIN-III have been noted to regress spontaneously. The more severe the abnormality of the lesion, the less likely it is to regress [3]. The average time for progression of CIN to invasive cancer has been expected to be 10 to 15 years, allowing long time windows for early detection and possible preventive therapy of cancer or precancerous lesions [4]. Because the decision with regard to patient management is two-tiered (observation versus surgical treatment), the three-tier classification system has been recently made easy to a two-tiered system, with CIN-I renamed low grade squamous intraepithelial lesion (LSIL) and CIN-II and CIN-III combined into one class referred to as high grade squamous intraepithelial lesion (HSIL) [5].

P53 is a tumor suppressor gene, which inhibit cellular proliferation by blocking entry into the S phase of the cell cycle and is also a principal regulator of apoptosis. Immunohistochemical (IHC) expression of p53 may be a useful marker which can provide information complementary to morphology, prognosis and survival outcome of the patients [6].

bcl2 is a protooncogene, which defends the cell from apoptosis. Inappropriate expression of bcl2 may prolong survival of defective and harmful cells, including those involved in human papilloma virus (HPV) infection, thus increasing the probability of malignant change [7].

Angiogenesis plays an important role in tumerogenesis and metastasis in most human solid tumors [8]. Study of angiogenesis can offer good information about the role of angiogenesis in pre-invasive and invasive cervical tumor progression and can assess the relationship with epidemiological and prognostic pathological parameters, which may develop evaluation models that constitute the basis of investigative trials for possible treatment targets [9].

The aim of the present study is to assess the IHC expression p53, bcl2 and CD34 in chronic cervicitis, LSIL, HSIL and cervical carcinomas and to study the relation of these expressions to certain clinicopathological parameters including patient's age, grade of cervical intraepithelial neoplasia, grade and stage of cervical carcinoma.

Methods

This cross sectional study was approved by Institute Review Board of the Collage of Medicine, Al-Nahrain University. During the period from March 2014 to December 2014; a total of one hundred twenty seven formalin fixed paraffin embedded cervical biopsies were collected (punch, cone and total abdominal hysterectomy). The histological diagnosis of the specimens included chronic cervicitis in 22 cases (17.3%), LSIL in 24 (18.8%) case, HSIL in 28 (22%) and invasive cervical carcinoma in 53 (41.7%) case. The carcinoma cases included 38 (29.9%) cases of squamous cell carcinoma, 12(9.4%) cases of adenocarcinoma and 3 cases (2.4%) of adenosquamous carcinoma types. Cases were retrieved from the archival materials of Teaching Laboratories and Oncology Teaching Hospital in Medical City, and Al-Imamain Al-Kadhimain Medical City for the period from January 2012 to October 2014. All the cases of chronic cervicitis were punch biopsies; whereas 20 cases of LSIL were punch biopsies and 4 cases were cone biopsies. Regarding HSIL, 20 cases were punch biopsies, 6 cases were cone biopsies and 2 cases were total abdominal hysterectomies (TAH); for invasive cervical carcinomas 45 cases were TAH, 6 cases were punch biopsies and 2 cases were cone biopsies.

All the clinicopathological parameters such as (age; grade of cervical neoplasia; histopathological type, grade and FIGO (International Federation of Gynecology and Obstetrics) pathological stage of cervical carcinomas) were obtained from patients' admission case sheets and pathology reports.

Any sample lacking the clinicopathological information was excluded from this study.

For each case, one representative (4 μ) section was stained with Hematoxylin and Eosin and the histopathological diagnosis was revised. Three (4 μ) sections were placed on positively charged slides and stained immune-histochemically using three steps- indirect streptavidin method for monoclonal mouse antibodies including anti-p53 antibody, clone (BP53-12), anti-bcl2 antibody, clone (Bcl2/100) and anti-CD34 antibody, clone (QBEND-10); all manufactured by Abcam (United States).

Interpretation of the results of IHC staining

IHC reaction is considered positive when brown staining is nuclear for p53 protein, cytoplasmic bcl2 and cytoplasmic (of endothelial cells) for CD34 protein. The positive control for both p53 and bcl2 IHC reaction was taken from the lymphoid tissue in non-Hodgkin lymphoma. The positive control for CD34 was obtained from normal lymph node tissue. Technical negative control for all was obtained by omission of the primary antibody.

The results of IHC expressions of the above molecular markers were analyzed in a semi-quantitative fashion as follow:

p53

The IHC expression of p53 was scored semi-quantitatively by assessing both staining intensity (0 = no staining, 1 = weak, 2 = moderate and 3 = strong) and percentage of stained cells (staining ratio), (1 = 1-5%, 2 = 6-25%, 3 = 26-50%, 4 = 51-75% and 5 = 76-100%). The staining index (SI) was calculated for each case as the product of staining intensity and staining ratio (SI = staining intensity + staining ratio), with final SI range from 0, 2-8 [10, 11].

bcl2

The IHC expression of bcl2 was scored semi-quantitatively by assessing both staining intensity (0 = no staining, 1 = weak, 2 = moderate and 3 = strong) and percentage of stained cells (staining ratio), (1 = 1-5%, 2 = 6-25%, 3 = 26-50%, 4 = 51-75% and 5 = 76-100%). The SI was calculated for each case as the product of staining intensity and staining ratio (SI = staining intensity + staining ratio), with final SI range from 0, 2-8 [10].

CD34

For CD34 IHC expression in carcinoma cases, intratumoral microvessel density (MVD) was calculated based on Weidner method. According to this method, sections were scanned at the low power (x10) for the blood vessels stained with CD34, and three hot spots area were chosen. The hot spot area is an area with the most dense vessel growth. Only hot spots in tumor cell cluster in viable (non-necrotic and non-sclerotic areas) have been taken into account in this study [12]. Regarding SILs cases, the microvessels were counted in the stroma along the basement membrane subtending dysplastic epithelium [13].

Once the region of interest (the vascular hot spot) was defined, a higher magnification was selected in order to be able to count the individual stained blood microvessels. The count of microvessels was done at power (x20) which represent a field size of 0.74 mm^2 which provide microvessel count. Three hot spots were selected for counting MVD. The mean of the examined fields was divided on the high power field area which is 0.74 mm^2 and this represented MVD [12].

Any single brown stained endothelial cell or endothelial cell clusters clearly separated from adjacent microvessels, tumor cells and connective tissue elements were considered as a single countable microvessel [12].

Statistical Analysis

Statistical analysis was performed with SPSS V. 17 (statistical package for social sciences) and Excel 2007 programs. Continuous variables were expressed as mean±SEM (standard error of the mean), while categorical variables were expressed as numbers and percentages.

Statistical relations between two categorical variables were tested using Chi-square or Fisher exact tests. Relations between categorical and continuous variables were tested using unpaired t-test and ANOVA. Values were considered statistically significant when p-value < 0.05.

Results
Clinicopathological parameters
The clinicopathological parameters of chronic cervicitis, LSIL (Fig. 1), HSIL (Fig. 2) and carcinoma (Fig. 3) cases included in the present study are summarized in Tables 1.
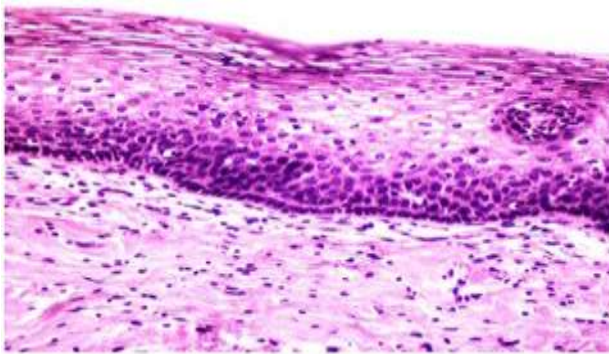
Fig. 1. Low grade squamous intraepithelial lesion shows dysplastic cells (high N/C ratio with hyperchromatic nuclei) limited to the lower one thirds of the epithelium, (H&E), (20x).
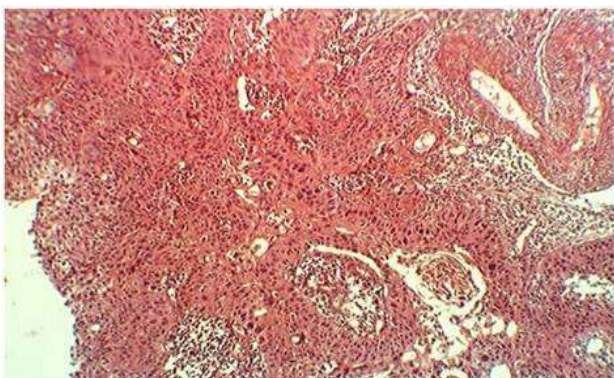
Fig. 3. Moderately differentiated non-keratinizing squamous cell carcinoma composed of irregular islands of tumor cells associated with abundant eosinophilic cytoplasm with individual keratinization and prominent nuclear pleomorphism, (H&E), (10x).

p53 immunohistochemical expression
p53 was not expressed immunohistochemically in all studied chronic cervicitis cases and expressed in 6 (25%) cases of LSIL, 17 (60.7%) cases of HSIL and 43 (81.1%) cases of cervical carcinomas, with significant increase in its expression with increasing severity of the lesions (p < 0.001). Regarding p53 staining index (SI), the majority of cases of carcinoma 30 out of 53(56.6%) showed high SI (6-8) (Fig. 4), while only 6 out of 28 cases of HSIL (21.4%) were with high SI (6-8). Regarding LSIL, the majority of cases 18 out of 24 (75%) were negative for p53 and the rest of the cases showed low SI (Fig. 5).
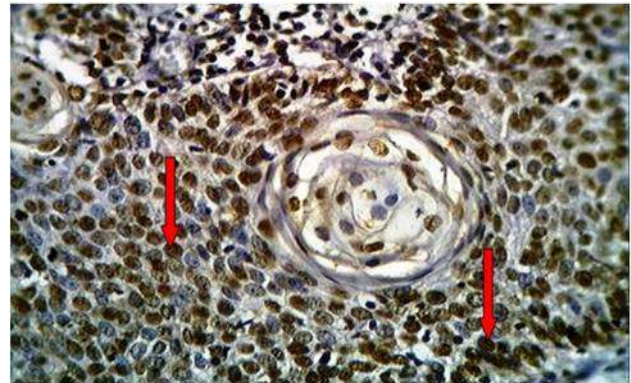
Fig. 4. Well differentiated squamous cell carcinoma of the uterine cervix stained immunohistochemically with anti-p53 monoclonal antibody showing positive brown nuclear staining (arrows) with moderate intensity (2) , high percentage (4) and SI of 6, (40X).
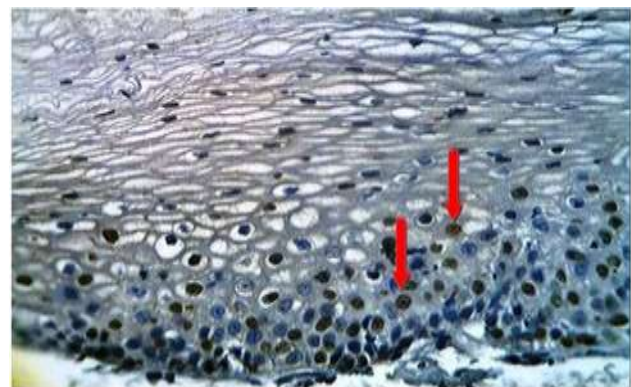
Fig. 5. Low grade squamous intraepithelial lesion of the uterine cervix stained immunohistochemically with anti-p53 monoclonal antibody showing positive brown nuclear staining (arrows) with moderate intensity (2) , moderate percentage (3) with SI of 5, (40X).

Table 1. Clinicopathological parameters of chronic cervicitis, low grade and high grade squamous intraepithelial lesion and invasive cervical carcinoma cases

Parameters		Values
Histopathological diagnosis	Chronic cervicitis	22
	LSIL	24
	HSIL	28
	Invasive carcinoma	53
Age (Mean±SEM) and range (years)	Chronic cervicitis	38.27±2.53 (22-62)
	LSIL	38.46±2.3 (22-62)
	HSIL	43.32±1.92 (27-63)
	Invasive cervical carcinoma	44.13±1.4 (29-70)
Histopathological types of invasive cervical carcinoma	Adenocarcinomas	12 (22.64%)
	Adenosquamous carcinomas	3 (5.66%)
	Squamous cell carcinomas	38 (71.7%)
Grade of invasive cervical carcinoma	Well-differentiated	9 (16.98%)
	Moderately-differentiated	28 (52.83%)
	Poorly-differentiated	16 (30.19%)
Stage of invasive cervical carcinoma(pathological FIGO staging system)*	I	13 (29%)
	II	24 (53%)
	III	8 (18%)

LSIL = low grade squamous intraepithelial lesion, HSIL = high grade squamous intraepithelial lesion, *8 out of 53 carcinoma cases were lacking information about the stage (6 cases were punch biopsies and 2 cases were cone biopsies).

Table 2. Frequency distribution of chronic cervicitis, low grade and high grade squamous intraepithelial lesion and invasive cervical carcinoma cases according to immunohistochemical expression and scoring index of p53.

Frequency of p53 expression and SI (0,2-8)	Chronic cervicitis No. (%)	LSIL No. (%)	HSIL No. (%)	Carcinoma No. (%)
Positive	0 (0%)	6 (25%)	17 (60.7%)	43 (81.1%)
Negative (0)	22 (100%)	18 (75%)	11 (39.3%)	10 (18.9%)
2	0 (0%)	2 (8.3%)	5 (17.9%)	4 (7.5%)
3	0 (0%)	1 (4.2%)	2 (7.1%)	1 (1.9%)
4	0 (0%)	2 (8.3%)	3 (10.7%)	5 (9.4%)
5	0 (0%)	1 (4.2%)	1 (3.6%)	3 (5.7%)
6	0 (0%)	0 (0%)	1 (3.6%)	7 (13.2%)
7	0 (0%)	0 (0%)	3 (10.7%)	10 (18.9%)
8	0 (0%)	0 (0%)	2 (7.1%)	13 (24.5%)
Total	22	24	28	53
p value		<0.001		

LSIL = low grade squamous intraepithelial lesion, HSIL = high grade squamous intraepithelial lesion, SI = scoring index

Table 3. Association of p53 immunohistochemical expression with clinicopathological parameters of squamous intraepithelial lesions, and invasive cervical carcinomas

Clinicopathological parameter		Positive p53	Negative p53	p value
Age (years)	SIL	40.3±2.22	37.45±2.08	0.335
(mean±SEM)	Invasive carcinoma	37.7±1.73	42±2.68	0.266
Histopathological	Adenocarcinoma	12 (100%)	(0%)	
type of invasive	Adenosquamous	3 (100%)	(0%)	0.046
carcinomas	Squamous	28 (73.7%)	10 (26.3%)	
Grade of invasive	Well-differentiated	8 (88.9%)	1 (11.1%)	
carcinoma	Moderately-differentiated	23 (82.1%)	5 (17.9%)	0.718
	Poorly-differentiated	12 (75%)	4 (25%)	
Pathological	I	10 (76.9%)	3 (23.1%)	
stage of invasive	II	19 (79.2%)	5 (20.8%)	0.686
carcinoma	III	7 (87.5%)	1 (12.5%)	

SILs: squamous intraepithelial lesion

Table 4. Frequency distribution of chronic cervicitis, low grade and high grade squamous intraepithelial lesion and invasive cervical carcinoma cases according to immunohistochemical expression and scoring index of bcl2.

Frequency of bcl2 expression and SI (0,2-8)	Chronic cervicitis No. (%)	LSIL No. (%)	HSIL No. (%)	Carcinoma No. (%)
Positive	13 (59.1%)	16 (66.7%)	16 (57.1%)	32 (60.4%)
Negative (0)	9 (40.9%)	8 (33.3%)	12 (42.9%)	21 (39.6%)
2	5 (22.7%)	4 (16.7%)	4 (14.3%)	9 (17%)
3	3 (13.6%)	3 (12.5%)	3 (10.7%)	4 (7.5%)
4	2 (9.1%)	3 (12.5%)	2 (7.1%)	5 (9.4%)
5	1 (4.5%)	2 (8.3%)	3 (10.7%)	3 (5.7%)
6	1 (4.5%)	2 (8.3%)	2 (7.1%)	4 (7.5%)
7	0 (0%)	1 (4.2%)	2 (7.1%)	5 (9.4%)
8	1 (4.5%)	1 (4.2%)	0 (0%)	2 (3.8%)
Total	22	24	28	53
p value		0.999		

LSIL = low grade squamous intraepithelial lesion, HSIL = high grade squamous intraepithelial lesion, SI = scoring index

The differences in frequency of distribution of cases with positive and negative expression of p53 of the studied cases and the distribution of cases according to the SI of p53 are highly significant (p < 0.001) as shown in table 2.

There was no significant association between age and IHC expression of p53 in studied SILs and carcinomas (p = 0.335; p = 0.226, respectively). In invasive cervical carcinoma, p53 expression was not statistically different according to grade and FIGO pathological stage, however, the current study revealed a significantly higher IHC expression of p53 in adenocarcinomas and adenosquamous carcinomas cases compared to squamous cell carcinomas (p = 0.046) as shown in table 3.

bcl2 immunohistochemical expression

Statistical analysis showed no significant difference in IHC expression and SI of bcl2 and

in different study groups, (p= 0.999) (Fig. 6 and 7 and table 4).

Regarding clinicopathological parameters, the present work revealed non-significant association of bcl2 expression with age of patients in both SILs and invasive carcinomas (p = 0.37) and (p = 0.873), respectively. bcl2 expressions was not statistically different according to histopathological types, grade and FIGO pathological stage of invasive cervical carcinomas (p = 0.696; p = 0.449; P = 0.915, respectively (Table 5).

Fig. 6. Moderately differentiated squamous cell carcinoma of the uterine cervix stained IHC with anti-bcl2 monoclonal antibody showing positive brown cytoplasmic staining (arrows) with moderate intensity (2), high percentage (5) and SI of 7, (40X).

CD34 immunohistochemical expression

From statistical point of view there was a highly significant increase in MVD (detected by IHC expression of CD34) with increasing severity of cervical lesion (p < 0.001). The mean MVD in chronic cervicitis, LSIL, HSIL (Fig. 8) and carcinomas (Fig. 9-11) were 3.01±0.32; 9.57±1.59; 16.94±1.13; 55.51±2.15, respectively.

The present series showed no significant correlation between age and MVD (detected by IHC expression of CD34) in both studied SILs and carcinoma cases (r = -0.228, p = 0.103; r = -0.061, p = 0.665, respectively).

Adenosquamous carcinomas and adeno-carcinomas (Fig. 10) revealed significantly higher MVD compared to squamous cell carcinomas (Fig. 9) (p = 0.01). Regarding grade of carcinoma, there was a significant increment

in MVD with decreasing the degree of differentiation (p < 0.001) as shown in fig. 9-11. MVD was increasing significantly according to FIGO pathological stage of carcinomas (p = 0.012) as seen in table 6.
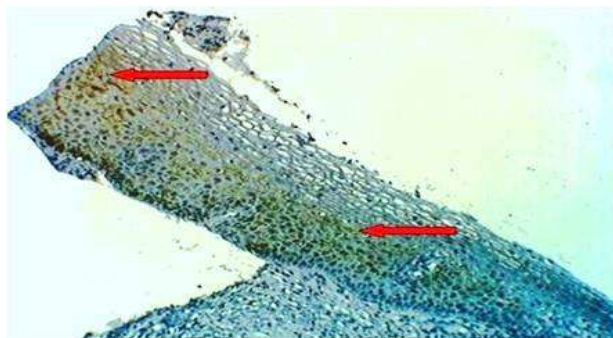
Fig. 7. Low grade squamous intraepithelial lesion of the uterine cervix stained IHC with anti-bcl2 monoclonal antibody showing positive brown cytoplasmic staining (arrows) with weak intensity (1), moderate percentage (3) and SI of 4, (4X).

Fig. 8. Low grade squamous intraepithelial lesion of the uterine cervix stained IHC with anti-CD34 monoclonal antibody showing positive brown cytoplasmic staining of endothelial cells of microvessels (arrows) with moderate increase in microvessl density in the connective tissue underneath basement membrane of the dysplastic epithelium (40X).

Discussion

The present study shows lack of p53 IHC expression in all studied chronic cervicitis cases, with significant increase in its expression from LSIL through HSIL to cervical carcinomas. This result is comparable to the literatures which stated that the p53 was not expressed in non-neoplastic cervical lesions with increase in

its expression with increasing severity of the lesion from LSIL through HSIL to carcinoma [10,11,14,15]. In the current work, actually it could not be confirmed which type of p53 protein (mutant or wild) since the antibody used in the study can detect both types.

Taking the age in consideration, this study revealed non-significant association between age and IHC expression of p53 in SILs and carcinomas .This result agrees with that obtained by other Iraqi study done by Baythoon *et al* [16] and with a study done by Koyamatsu *et al* [17].
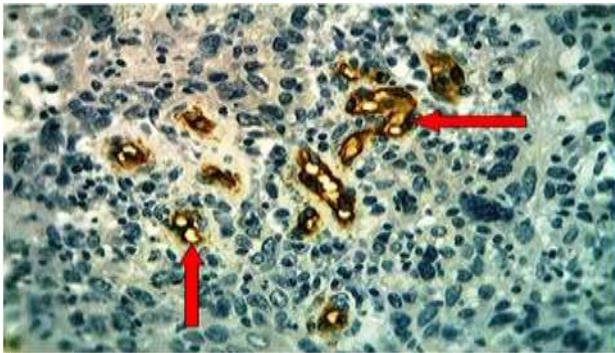
Fig. 9. Well differentiated squamous cell carcinoma of the uterine cervix stained IHC with anti-CD34 monoclonal antibody showing positive brown cytoplasmic staining of endothelial cells of microvessels (arrows) with moderate increase in the intratumoralmicrovessl density (MVD) (40 X).
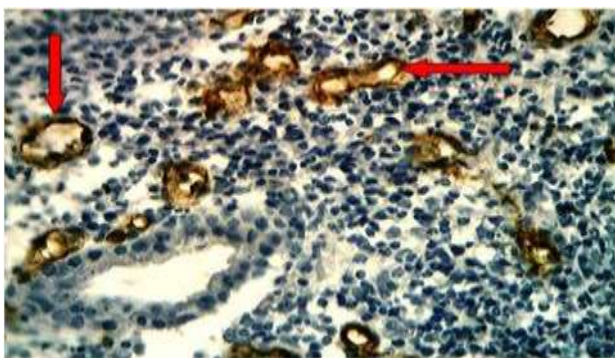
Fig. 10. Moderately differentiated adenocarcinoma of the uterine cervix stained IHC with anti-CD34 monoclonal antibody showing positive brown cytoplasmic staining of endothelial cells of microvessels (arrows) with high intratumoralmicrovessl density (MVD) (40 X).

Concerning the histopathological types of cervical carcinoma, this study shows a statistically significant higher IHC expression of p53 in adenocarcinomas and adenosquamous carcinomas compared to that of squamous cell carcinomas. This result is in accordance with other Iraqi study done by Baythoon *et al* [16] and with other studies done by Cheah and Looi [18] and Abdelall *et al* [11].
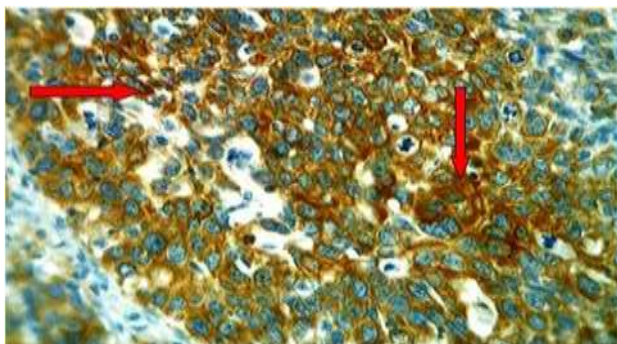

Fig. 11. Poorly differentiated squamous cell carcinoma of the uterine cervix stained IHC with anti-CD34 monoclonal antibody showing positive brown cytoplasmic staining of endothelial cells of microvessels with marked increase in the intratumoralmicrovessl density (MVD) (arrows) (10 X).

The higher IHC expression of p53 in adenocarcinoma compared to that of squamous cell carcinoma may be due to higher frequency of mutations in adenocarcinoma [18]. Most mutations induce conformational changes causing over-expression of p53 protein, stabilizing it and rendering it detectable by IHC analysis [19]. It has been suggested that p53 over-expression represents an adverse prognostic factor [20]. Since p53 expression in adenocarcinoma is significantly higher than that of squamous cell carcinoma of the cervix, this would contribute to the less favorable prognosis of the former than the latter [21].

Regarding the histopathological grade of cervical carcinomas, the current study shows non-significant difference in p53 IHC expression among different grades of studied cervical carcinoma. This result is in agreement with other Iraqi study done by Baythoon *et al*

[16] and with other studies worldwide [22-24]. The present study also showed that IHC expression of p53 was not significantly associated with pathological stage of invasive cervical carcinoma. This result is comparable to that obtained by other studies [3,14,22,24].

The current study recorded non-significant difference in IHC expression of bcl2 in studied groups. This result is in agreement with other studies [10,22,25-28]. In a study done by Grace *et al* [15], a significant increase in IHC expression of bcl2 with increasing severity of cervical lesions from mild dysplasia to carcinoma had been observed. This difference is possibly due to different sample size, sensitivity and specificity of different antibodies used, and different modes of scoring systems and interpretations of the results.

This research reported non-significant association between age and IHC expression of bcl2 in both SILs and carcinoma cases. This result agrees with study done by Wootipoom *et al* [24].

Taking the histopathological type of cervical carcinoma in consideration, the current work found non-significant relation between histopathological types of cervical carcinoma and bcl2 immunoreactivity. This finding goes with that obtained by Tjalma *et al* [29].

Regarding cervical carcinoma cases, the present study showed non- significant association of grade and pathological stage to the IHC expression of bcl2. This result is supported by other studies [22,24].

MVD is a commonly used as a measure of angiogenesis. Measuring MVD requires labeling the vessels to be counted using antibodies against any of the antigens naturally expressed by endothelial cells like Factor-VIII, CD31, CD34 and CD105 [31]. In the present study, CD34 was selected based on its superior sensitivity, with detection of a greater number of microvessels in cervical tumors compared to other antibodies [32]. MVD is considered a significant prognostic factor that correlates with increased metastasis and worse prognosis in many tumor types [33].

Table 5. Association of bcl2 immunohistochemical expression with clinicopathological parameters of squamous intraepithelial lesions, and invasive cervical carcinomas

Clinicopathological parameter		Positive bcl2	Negative bcl2	p value
Age (years) (mean±SEM)	SIL	37.62±1.78	40.45±2.74	0.37
	Invasive carcinoma	38.31±2.05	38.81±2.21	0.873
Histopathological type of invasive carcinomas	Adenocarcinoma	7 (58.3%)	5 (41.7%)	
	Adenosquamous	1 (33.3%)	2 (66.7%)	0.696
	Squamous	24 (63.2%)	14 (36.8%)	
Grade of invasive carcinoma	Well-differentiated	7 (77.8%)	2 (22.2%)	
	Moderately-differentiated	15 (53.6%)	13 (46.4%)	0.449
	Poorly-differentiated	10 (62.5%)	6 (37.5%)	
Pathological stage of invasive carcinoma	I	9 (69.2%)	4 (30.8%)	
	II	14 (58.7%)	10 (41.3%)	0.915
	III	5 (62.5%)	3 (37.5%)	

SILs = squamous intraepithelial lesion

Table 6. Association of MVD (detected by CD34 immunohistochemical expression) with histopathological type, grade and pathological stage of invasive cervical carcinomas

Parameter		MVD Mean ± SEM	p value
Histopathological type	Adenocarcinoma	65.09 ± 3.7	
	Adenosquamous	67.57 ± 7.8	0.01
	Squamous	51.53 ± 2.44	
Grade	Well-differentiated	37.69 ± 3.54	
	Moderately-differentiated	52.7 ± 2.22	< 0.001
	Poorly-differentiated	70.44 ± 2.43	
Pathological stage	I	46.47 ± 4.05	
	II	59.74 ± 3.11	0.012
	III	64.19 ± 3.82	

MVD = microvessel density, SILs = squamous intraepithelial lesion

This study recorded a significant increase in MVD (detected by IHC expression of CD34) with increasing severity of cervical lesion from chronic cervicitis through LSIL and HSIL to carcinomas. This result is concordance with other studies, which also used CD34 for detecting MVD [34-36].

Stepan et al [37] also noticed a significant increase in MVD (detected by IHC expression of CD105) with increasing severity of cervical lesion from normal cervix through LSIL and HSIL to carcinoma. In a study done by Dellas et al [31], in which CD31 was used for detecting MVD, a significant increased in MVD from benign cervical lesions through preinvasive lesions to invasive cervical carcinomas had been identified. The loss, or inactivation, of wild type p53 has been reported to indirectly promote tumor angiogenesis by up-regulation of angiogenesis promoting protein, VEGF and down-regulation of a potent angiogenesis inhibitor, thrombospondin-1 (TSP-1), providing rationale for the contribution of angiogenesis to cervical cancer early in carcinogenesis [34,38].

The present series found non-significant correlation between age and MVD (detected by IHC expression of CD34) in studied SILs and carcinoma cases. This finding is in accordance with other studies [39,40].

Concerning cervical carcinoma types, the current study showed a significant higher MVD in adenosquamous carcinomas and adenocarcinomas compared to squamous cell carcinomas. This result is in agreement with other studies [39,41]. In a study done by Vieira et al [42], a higher MVD had been identified in squamous cell carcinoma. This difference may be attributable to different sample size, different percentage of each type of carcinoma and different methods used to assess MVD.

The current work showed a significant higher MVD in poorly differentiated carcinomas and lowest value was detected in well differentiated carcinomas. This result agrees with other studies [37,39,41].

Regarding pathological FIGO stage of cervical carcinomas, the present research showed a significant higher MVD with increasing pathological FIGO stage of cervical carcinoma cases. This result is similar to that found by other study done by Landt et al [43].

The majority of studies trying to find a correlation between angiogenesis and outcome in cervical cancer report conflicting results, but most have concluded that more extensive tumor angiogenesis is associated with higher rates of tumor recurrence following treatment and poorer survival in cervical cancer [40, 44]. In SILs,

many studies demonstrate a significant increased in MVD with increasing severity of the lesions [31, 34- 36].

In conclusion, P53 plays an important role in the progression of the severity of intraepithelial cervical lesions. Thus, testing this marker in dysplastic cervical lesions might improve the accuracy, precision and sensitivity of cervical lesions diagnosis. bcl2 plays no role in cervical neoplastic transformation and has no utility to differentiate premalignant from malignant lesions of the uterine cervix. MVD is sequentially increased from LSIL through HSIL, and then into invasive carcinoma and can be utilized as ancillary marker for the risk of malignant transformation of cervical intraepithelial lesion. Intratumoral quantification of MVD in cervical carcinoma reflects the grade and pathological stage of the tumor so that it may be a useful additional prognostic factor.

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Author contributions
All authors contributed to this manuscript. They coordinated study subject recruitment, implementation and progress of this study, and helped with data interpretation and manuscript organization and editing.

Conflict of Interest
The authors have no conflicts of interest

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Evaluation of Intraplacental Villous Microvascular Density and Vascular Surface Area in Pregnancy Induced Hypertension and Its Correlation to Newborn Body Weight

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Abstract

Background	Preeclampsia is a major problem in obstetric practice; it is considered one of the largest causes of maternal and perinatal morbidity and mortality, and one of the most important causes of intrauterine growth restriction and low birth weight.
Objectives	To evaluate the intraplacental villous microvascular density and vascular surface area in pregnancy induced hypertension in term placentae, and its effect on newborn body weight.
Methods	A sample of 50 placentae divided into 25 normal term placentae (Control group) and 25 term placentae of pregnancy induced hypertension (preelampsia) considered as the (Test group). Fresh placental tissues were taken from the peripheral placental area, processed to paraffin blocks, stained with CD-34 (clone QBEnd-10) (DAKOCYTOMATION), assessment of the vascular density, and vascular area with image j software.
Results	Significant increase in vascular density, with a significant reduction in vascular area seen in placentae of preeclampsia compared to control group at p ≤ 0.05, in addition fibrosis with vascular degeneration and stenosis were evident in terminal villi, lead to avascular terminal villi in preeclampsia compared to control group. Significant reduction in newborn body weight in preeclampsia compared to control at p ≤ 0.05.
Conclusions	These results suggested that placenta adapt its structure to maintain its function, this adaptation reflected as an increase in vascular density that consequently occur as uteroplacental perfusion reduces due to maternal vasospasm, and the placenta becomes ischemic as gestation progresses due to hypoxia that affect terminal villi vasculature. Fibrin deposition, vascular degeneration, thickened vessels wall, and stenosis those together reduce the vascular area; these changes accordingly were reflected on newborn body weight in preeclampsia.
Key words	Preeclampsia, vascular density, vascular area, placenta.

List of abbreviation: PE = Pre-eclampsia.

Introduction

Preeclampsia (PE) is a heterogenic multisystemic disorder, of a sudden occurrence during the second half of pregnancy [1]. It is generally defined as the development of hypertension and proteinuria after 20 weeks of gestation in a previously normotensive woman [2]. The precise etiopathogenesis of PE remains to be a subject of extensive research, but it is believed that it is likely to be multifactorial, nevertheless, it is accepted that it is the presence of the placenta rather than the fetus, which is responsible for development of PE. The onset, severity, and progression of PE are significantly affected by the maternal response to placental derived factors and proteins [3].

Normal placental development requires continuous process of trophoblastic invasion of maternal endometrium as well as of

vasculature. The interstitial trophoblastic invasion causes proper anchorage to the endometrium whereas endovascular invasion leads to uterine spiral artery remodeling ultimately causing increased maternal blood flow and hence proper uteroplacental perfusion. The disease is associated with an increase in the vascular resistance at feto-placental interface and associated with changes seen in the fetal stem arteries [4].

Pregnancy complications like hypertension are reflected in placenta in a significant way both macroscopically and microscopically. Several studies have shown that utero-placental blood flow is decreased in pregnancy induced hypertension due to maternal vasospasm [5], that leads to fetal hypoxia and accordingly it may lead to fetal distress and fetal death [6]. Placental cells expressing CD-34 were mainly restricted to the embryonic vessels of placenta, blood in fetal vessels and vessel walls [7]. So it is used to identify placental vascular structures within placental villi.

The present study aimed to record the changes on the vascular density and vascular area in the placental villi from mothers with pregnancy induced hypertension, and its effect on birth weight of the new born babies in comparison to normotensive mothers. Since placenta is the mirror of maternal and fetal status, it could reflect the changes due to maternal hypertension.

Methods

A sample of 50 human placentae delivered by normal vaginal delivery, collected from the Obstetric and Gynecological Department at Al-Imamain Al-Kadhimain Medical City, with gestational age (38-40) weeks, taken after patient informed consent that had been signed by all participants, those samples fall into two groups:

1. Test group, include (25 placentae) delivered by mothers complaining from PE, which is defined according to International Society for the Study of Hypertension in Pregnancy [8] characterized by blood pressure greater than 140/90 mmHg measured on two occasions with 6 hours apart and protein urea more than 300 mg/24 hours in a previously normotensive women as defined by the at 35 week of gestation.

2. Control group that include the other 25 placentae that delivered to mother with normotensive and no protein urea.

All pregnancies with known vascular diseases as essential hypertension, and diabetes were excluded.

Tissue preparation

Immediately after delivery, placentae were cleaned from blood, five small tissue pieces were taken from the peripheral placental region (full depth cores of placental tissue), placed in 10 % neutral buffered formaldehyde as the proper fixative that is most commonly used in immunohistochemistry protocols, kept in the fixative for 3 days, cord and membranes were carefully removed, tissues proceed to paraffin blocks using routine histological techniques, 4 µm thickness sections taken from mid placental thickness to show villi in mid plane of intervillous space and placed on positively charged slides.

Immunohistochemical staining

Include DAKO monoclonal mouse anti human CD34 class II Clone QBEnd-10 Code No. M7165 stain with labeled streptavidin Biotin technique. The staining include: Deparaffinization, rehydration, Heat induced epitope retrieval incubation in peroxide blocking reagent, application of primary antibody, and biotinylated linker antibody, incubation with streptavidin/peroxidase and with 3,3-.di-amino benzidine, counter stain in Mayers haematoxylin, and mounting.

Newborn body weight measurements

This is measured in grams immediately after delivery for the control and test groups' babies at the premature infant unit at the Al-Imamain Al-Kadhimain Medical City.

Vascular counting

This done in sections that stained positive for CD-34 marker as the latter is expressed in vascular endothelial cells of the fetal capillaries, and blood cells within the vascular lumen. Vascularization was determined by counting of vascular density and surface area of vascular structures that stained positive for CD-34 by measuring their cumulative areas in each field [9].

Five random fields were selected from each placenta to measure the followings:

Microvascular density: done by counting the number of fetal blood vessels that stained positive with CD-34 (appear brown in color) in the placental villi, manual counting of vessels at high power fields sections at 400x magnification power, after background substraction option in the selected fields to make the positively stained vessels stand out. Making marks with image J 1.49 (as dot in the center of the marked vessels) to ovoid double counting of the vascular structures (Fig. 1A) Image J 1.49 is a public domain Java image processing program, it can display, edit, analyze, process, save and print images, can read many image formats. It is multithreaded, so time-consuming operations such as image file reading can be performed in parallel with other operations.
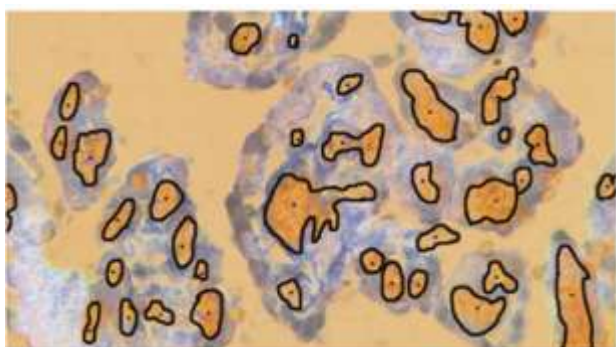
Fig. 1A. Marking of blood vessels in placental villi after background subtraction in the selected image that stained positive for CD-34 immunohistochemistry, multiple selection (by Dots) used to mark the center of each vessel at high power field to avoid double counting. image –J1.49 immunohistochemistry for CD-34, 400x.

The mean vascular surface area of fetal vessels (in pixels): done on slides stained positive with CD-34, with the use of software program image j 1.49, (Fig. 1B). It can calculate area and pixel value statistics of user-defined selections. Selections are of defined areas within an image. Area selections are created using the freehand selection tools. Area selections can be measured from drop down list (Analyze>Measure).
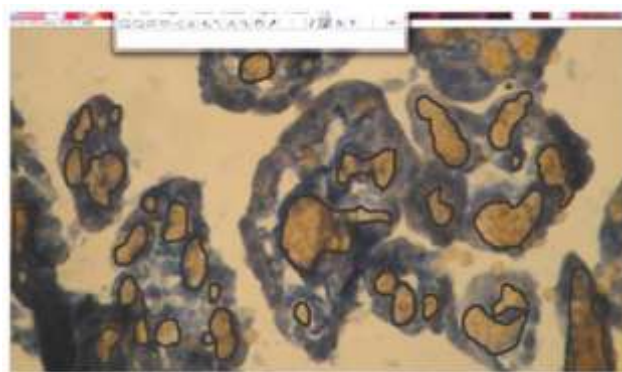
Fig. 1B. Image j 1.49 window showed the tool bar, the positively stained vessels in placental villi that appear in brown color were manually selected by free hand selection, area was calculated by adding the cumulative areas of stained vascular structures measured in pixels. image J 1.49 window for CD34 positive imunohistochemistry,400x.

Tissue sections measurements were examined using 400 x magnifications, area of interest were recorded Sony cyber shot digital camera.

Statistical analysis

The data were assessed by statistical package of social sciences (SPSS 17). The vascular measures would be compared between test group and control group using unpaired t-test. Newborn body weights were estimated for the test and control groups and compared using unpaired t-test. P value ≤ 0.05 considered statistically significant. All data were expressed as (mean ± SE).

Results

CD34 stain appear positive in control and test groups, its stain all vascular structures in placental villi even minute blood capillaries

that appear in brown color, including vascular wall and vascular lumen.

Placental villi showed extensive perivillous fibrin deposition (Fig. 2A) and intervillous fibrin deposition, with vascular degeneration presenting progressive fibrosis, and avascular terminal villi (Fig. 2B); vascular stenosis also can be seen in terminal vascular structures fig. 3. Thickening in the wall of blood vessels were seen in PE compared to normal term placentae (Fig. 4).
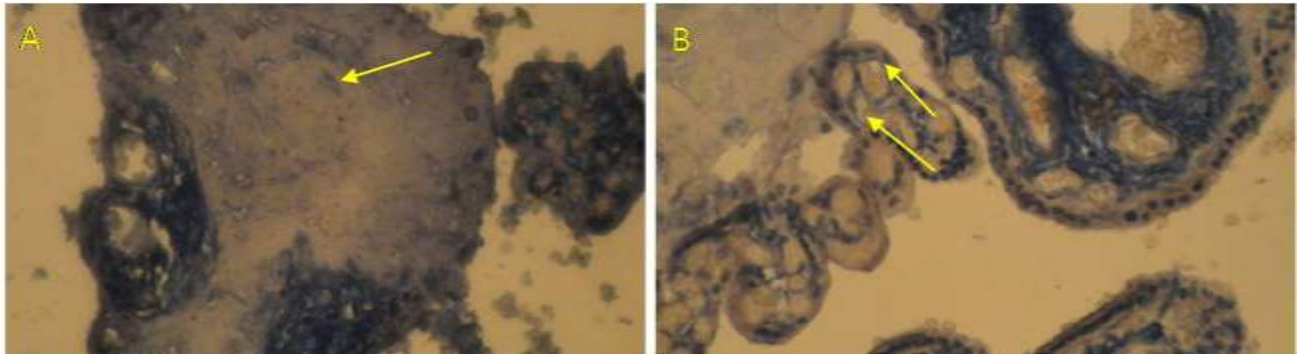
Fig. 2. Placenta of pregnancy induced hypertension at term showed extensive perivillous fibrin deposition (A) (black arrow), and intervillous (B) fibrin deposition (black arrows), immunohistochemistry for CD34- that showed brown positive color of fetal capillaries in placental villi, at 400x.

Increase in the mean ± SE of vascular density and reduction in (mean ± SE) of vascular area was significantly seen in PE, compared to normal placentae at term at $p \leq 0.05$ (Table 1 and Fig. 5).

Fig. 4. Thickened wall placental vessels in Preeclampsia in positively stained vessels that showed brown color for CD-34 immunohistochemistry at 400x.

Fig. 3. Terminal villi in Preeclampsia of term placenta showed a reduction the vascular area and absence in fetal vessels that stained in brown color positive for CD-34 immunohistochemistry, at 400x.

This reduction in blood vessel density in placental villi in PE cases compared to control placentae is associated with fibrinization, stenosis and thickening in vessel wall of the villi.

The decreased blood vessel lumen found in the mature intermediate villi and terminal villi, this may results in the complete absence of capillaries in the terminal villi in most of the placentae (Fig. 3-5). The new born birth weight ranged from 1800-2700 Gram in PE, compared to 2500-3500 Gram in control group, their mean ± SE was significantly reduced in PE compared to control group at $p < 0.05$ (Table 2).
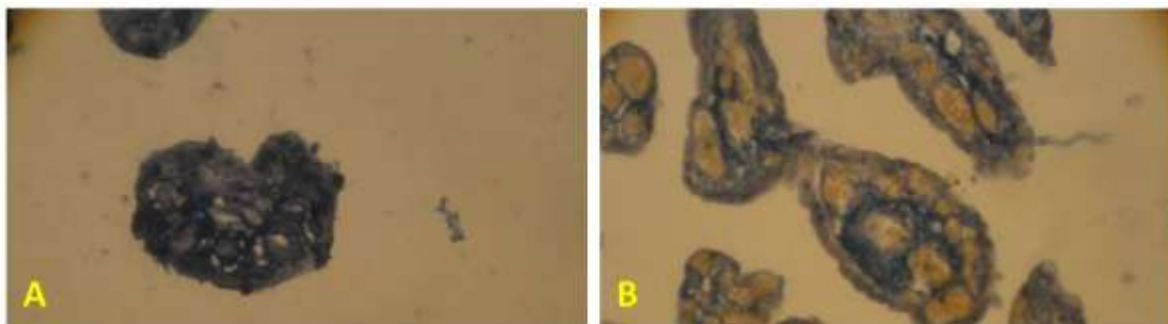
Fig. 5. Variations in vascular density and vascular area in placental villi that stained positive for CD-34 immunohistochemistry (A) PE, (B) normal term placenta, 400x.

Discussion

CD-34 showed an intense positive reaction in the vessels within the villous axis, tertiary villi, even minute capillaries were markedly demarcated, thus it allow us to precisely appreciate the vascular density and vascular area of these vessels. The CD34 protein is a member of a family of single-pass transmembrane sialomucin proteins that show expression on early hematopoietic and vascular-associated tissues [10].

Table 1. Variation in the vascular density and vascular area (in pixels) in normal term placentae and PE, with their statistical significance

Vascular measures	Normal placenta	Pre-eclampsia
VD	8±2.13	11.84±2.28*
VA (pixels)	121409±348.4	80506±237.9**

VD = vascular density, VA = vascular area* p = 0.008, ** p = 0.032

Table 2. Comparison of birth weight (in grams) of newborn between preeclampsia and control group

Birth weight (gm)	Normal placenta	Pre-eclampsia
mean ± SE	2924.35±260*	2343.6±237**
Range	(2500-3500)	(1800-2700)

* p = 0.005, ** p = 0.003

Pregnancies complicated by PE are reflected on the placenta both macroscopically and microscopically, although the placenta adapts well to the hypoxic condition in PE, these compensatory changes that occur are insufficient. Placental examination has clinical value in cases of PE and intrauterine growth retardation, both of which are associated with high perinatal morbidity and mortality accompanied with gross pathological changes in the placenta [11].

In the present study, comparing PE placentae to control placentae, the increase in vascular density; and reduction in vascular area of the PE placentae impedes normal placentation and pathologically results in massive microscopic changes in the placenta. Consequently, the resultant decreased perfusion could cause oxidative stress.

The cytotrophoblast cells invade into the uterine spiral arteries and transform them from small-caliber resistance vessels into high-caliber capacitance vessels capable of providing enhanced placental perfusion adequate for the growing fetus. For this transformation, a certain amount of hypoxia is needed to stimulate placental blood vessel formation. Until approximately 10 weeks of gestation, the embryo exists in a hypoxic environment with nutrients provided by the endometrial glands, however, prolonged durations of hypoxia or oxidative stress leads to poor placental

perfusion, which is the underlying pathogenesis of PE [12].

The reduction in vascular area measure found in placental villi that stained with CD-34 could be part of pathology involving the spiral artery that may extend to villous capillaries, since the invasion of uterine spiral arterioles by trophoblasts is limited to the superficial portions of the decidua, and 30-50% of these arterioles in the placental bed escape trophoblast remodeling [13,14].

It was found that the mean luminal diameter of uterine spiral arterioles in women with PE is less than one-third of the diameter of similar vessels from uncomplicated pregnancies [15].

While the increase in vascular density could be due to stimulation of vasculogenesis due to hypoxia, that consequently occur as uteroplacental perfusion reduces, and the placenta becomes ischemic as gestation progresses [16,17].

This fetal hypoxia as lead to morphological and histological changes in the placenta, leading to PE or PE-associated intrauterine growth retardation, which contributes to premature delivery and fetal death [18,19].

PE has also been related to an imbalance between pro- and anti-angiogenicfactors in maternal circulation [20], Placental vasculature includes specialized blood vessels and supportive structures that permit gas exchange and nutrient transfer from the maternal circulation to the fetus. A functional balance of pro-angiogenic (placental growth factor and vascular endothelial growth factor) and anti-angiogenic factors (soluble forms-like tyrosine kinase-1 and s-endoglin) is important for optimal placental formation [21].

In pregnancy, the placenta is the predominant source of angiogenic factors and the imbalance seen in PE is considered as response to placental hypoxia. Whether the angiogenic imbalance in PE is a maternal response independent of off springsize, remains uncertain [22].

However, it has been speculated that PE is an adverse manifestation of the mother's attempt to compensate for impaired fetal growth, and PE increases the risk of small gestational age in the offspring [23].

These results suggested that placenta adapt its structure to maintain its function, this adaptation reflected as an increase in vascular density that consequently occur as uteroplacental perfusion reduces due to maternal vasospasm, and the placenta becomes ischemic as gestation progresses due to hypoxia that affect terminal villi vasculature. Fibrin deposition, vascular degeneration, thickened vessels wall, and stenosis those together reduce the vascular area; these changes accordingly were reflected on newborn body weight in preeclampsia.

Author contributions
Dr. Kamona collected the samples and did cross sectional study of placenta, Dr. Al-Amily writ the research; and Dr. Al-Marsoummi analyzed vascular surface area of fetal vessels that stained with CD-34, by using software program image j and doing statistical analysis by SPSS 17 statistical analysis software.

Conflict of interest
The authors declare no conflict of interest.

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Effect of Prenatal Exposure to Ketamine on Newborn Rat Frontal Cortex: Immunohistochemical Study with TUNEL Test

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Abstract

Background   Ketamine as an analgesic drug is widely used to provide sedation for minor procedures. It was reported that the use of this drug causes deletion of large numbers of neurons from the developing brain.

Objectives   To investigate the effect of prenatal ketamine exposure on the newborn rat frontal cortex using immunohistochemical TUNEL test.

Methods   Seventy two pregnant rats were divided into three groups: I, II, and III (24 rat for each group), and exposed to ketamine at the 7th day, 11th day, and 18th day, of gestational age. Each group was subdivided into control subgroup A injected intraperitoneally with normal saline, and the subgroups B, C and D injected with intraperitoneal ketamine with 5 mg/kg, 10 mg/kg, and 20 mg/kg, respectively. Paraffin block sections of newborn rat frontal cortices were investigated by TUNEL test.

Results   Counting of fluorescent cells showed progressive increase in mean values with increased dose of ketamine injection and advancing age of prenatal exposure to ketamine. Significant variability was demonstrated between the control subgroup A and the other subgroups, and between the subgroups of each group, whereas, non-significant variability was observed between the counted mean differences values obtained for subgroup B in all groups (I, II, and III) compared to that of the control subgroup A.

Conclusion   Prenatal exposure to ketamine in a dose of 5mg/kg was a relatively non-toxic in all studied groups compared to the control subgroup. Apoptosis in frontal cortical tissue was involved in the mechanism of neuronal death caused by ketamine exposure during pregnancy.

Keywords   Frontal, cortex, prenatal, ketamine, neurotoxicity, immnuohistochemistry.

Introduction

There are wide varieties of symptoms 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 [1].

Ketamine is used in the surgical emergencies requiring anesthesia; it has been suggested that ketamine can be used safely for anesthesia in infants and children [2].

Considering the effect on developmental tissue, ketamine is proved to affect 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 [3].

The current study is formulated to investigate the neurotoxicity of prenatal exposure to ketamine in rat using the immune-histochemical in situ direct DNA fragmentation assay (TUNEL-based detection kit).

Methods

In this study, female Wistar rats (*Rattus Norvegius Albinus*) aged 4-6 weeks and

weighted between 150-250gm were brought from Baghdad University, Medical College Laboratory Animal House. The study was performed during the period from November 2013 to May 2014. The 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 [4].

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) as seen in table 1. Animals of these groups were exposed to ketamine at different gestational periods (at the 7th day, 11th day, and 18th 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 (5 mg/kg, 10 mg/kg, and 20 mg/kg, respectively). Female rats which were found to have no signs of pregnancy were excluded.

Pregnant rats of the experimental subgroups received intraperitoneal ketamine hydrochloride 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 7th, 11th and 18th 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 [5].

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

The TUNEL-based detection kit reagent was provided by abcam (code ab66108).The fluorescein-labeled DNA was observed with a fluorescent microscope (Polyvar).

Statistical evaluation of the number of apoptotic cells that were stained in a bright yellow color was done by counting these cells in equidistant 6 linear fields along the cortical layers at 400X magnification in all the subgroups of each group.

Results

The immunohistochemical TUNEL test of the frontal cerebral cortex revealed three different fluorescent colors (Fig. 1-3):

1. Yellow color representing the apoptotic cells (positive color).
2. Orange color for the non-apoptotic cells (negative color).
3. Green color, also a negative color.
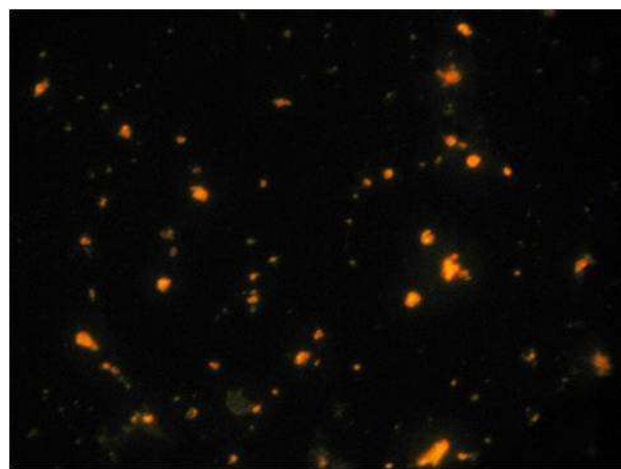
Fig. 1. Coronal section in frontal cerebral cortex from subgroup A (control) of group I showing the fluorescence activity of TUNEL test. 400X.

Counting of the apoptotic cells in group I showed mean value in subgroup B was (33 ± 3.7), in subgroup C was (49.3 ± 5.9), and that in subgroup D (83.6 ± 5.8) (Table 1).

Statistical analysis of the mean differences in group I showed significant variability between the control subgroup A and the subgroups B, C

and D (p < 0.001). ANOVA analysis of the mean differences of counting in group I showed that subgroup B varied significantly compared to subgroups C and D (p = 0.00 LSD). The mean difference of the counted values in subgroup C also varied significantly from subgroup D (p < 0.001).
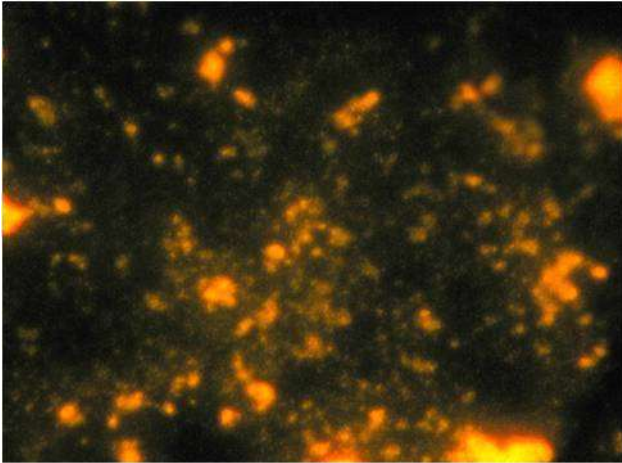
Fig. 2. Coronal section in frontal cerebral cortex from subgroup B (injected with 5mg/kg of ketamine) of group II showing the fluorescence activity of TUNEL test. 400X.
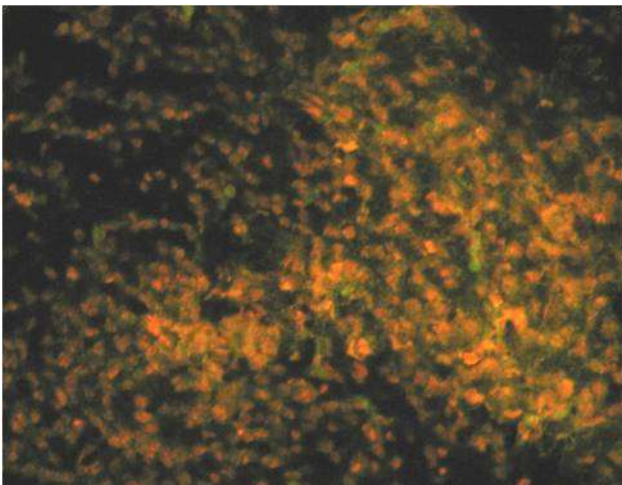
Fig. 3. Coronal section in frontal cerebral cortex from subgroup B (injected with 5mg/kg of ketamine)of group III showing the fluorescence activity of TUNEL test. 100X.

The mean values of subgroup D from group II showed the highest counting compared to subgroups B and C. The mean number of the positive fluorescent cells of subgroup D was

(104.5 ± 8.2), while that of subgroup B (53.8 ± 5.3) and subgroup C (80.3 ± 8.06) (Table 2).

Table 1. Mean number of apoptotic cells revealed by TUNEL test in the subgroups of group I.

Subgroup	mean±SD
A	21.5 ± 4.08
B	33.0 ± 3.7*
C	49.3 ± 5.9*
D	83.6 ± 5.8*

*p value ≤ 0.05 is considered statically significant. Dunnett t-test treats one group as a control, and compares all other groups against it.

Table 2. Mean number of apoptotic cells revealed by TUNEL test in the subgroups of group II.

Subgroup	mean±SD
A	39.1 ± 3.7
B	53.8 ± 5.3*
C	80.3 ± 8.06*
D	104.5 ± 8.2*

*p value ≤ 0.05 is considered statically significant. Dunnett t-test treats one group as a control, and compares all other groups against it.

There was statistically significant variability between the counting of the number of positively reacting cells in subgroup A (the control subgroup) compared to the treated subgroups C, and D and B (p < 0.001). The multiple comparison test of the mean differences in counting the number of positive cells showed that subgroup B varied significantly in comparison to subgroups C and D (p < 0.001). The mean differences of the counted values in subgroup C also varied significantly from subgroup D (p < 0.001).

The counted number of the bright yellow apoptotic cells was much higher in the cerebral cortices of the neonates from pregnant rats treated with 20mg/kg (subgroup D) in all groups (I, II, and III). The mean counting number of these positive fluorescent cells in subgroup B was (83.6 ± 6.6) and for subgroup C

(128.3 ± 14.9), while that for subgroup D was (175.8 ± 12.3) (Table 3).

Table 3. Mean number of apoptotic cells revealed by TUNEL test in the subgroups of group III.

Subgroup	mean±SD
A	54.8 ± 4.5
B	83.6 ± 6.6*
C	128.3 ± 14.9*
D	175.8 ± 12.3*

*p value ≤ 0.05 is considered statically significant. Dunnett t-test treats one group as a control, and compares all other groups against it.

There was significant variability between the counting number of positively reacting cells in subgroup A (the control subgroup) compared to the treated subgroups B, C, and D (p < 0.001).

The multiple comparisons analysis of the mean differences in counting the number of positive cells showed that subgroup B varied significantly in comparison to subgroups C and D (p < 0.001). The mean difference in the counted values of subgroup C was also significantly variable from subgroup D (p < 0.001).

Discussion

The effect of the prenatal ketamine exposure on the frontal cerebral cortex development was the concern of this study as many authors described neurobehavioral impairments seen after exposure of the developing rodent brain to anesthetic drugs [6].

The question whether anesthetic drugs can trigger neuroapoptosis in the developing non-human primate (NHP) brain was first addressed by Slikker and colleagues in the rhesus macaque brain [7]. These reports were supportive to the results of this study.

The number of the reactive cells was the least in subgroup (B) compared to subgroup (C) and (D). The presence of positively reacting apoptotic cells in the sections of the cerebral cortices of the control subgroup (A) is in congruence to the developmental history of the cerebral cortex reported in the literatures. It was documented that the developing cortical neuroepithelium generates more neurons than what is retained in the adult. Neuronal attrition occurs developmentally, by apoptotic cell suicide. The cell death is partly a consequence of competition for limited target-derived growth factors [8].

The number of neurons in developing rat brain is derived from early proliferative phase and a later phase of selective death of differentiated neurons. The neuronal precursor cells recognized in rat embryo before the 12th day of gestation and proliferate before the date of neuronal differentiation. It was reported that there is no change in cell number between the end of neurogenesis on the 15th day of gestation and birth [9].

The distribution of apoptotic cells in rat cerebral cortex at different developmental stages was described from embryonic day 16 to adulthood [10]. This description supports the hypothesis of this study that prenatal exposure to ketamine produces apoptotic changes in the rat frontal cortex.

The statistical analysis showed an attribute for considering the dose of 5 mg/kg as a relatively non-toxic dose in all the groups compared to the subcontrol group. This survival of the newborn rat cortices from the neurotoxic effect was also reported by one study suggesting that there are some cells in the cerebral cortex, which have the ability to resist the action of some neurotoxins. The cellular survival was suggested to be based on the equilibrium between the toxic action and the ability of living cells to protect themselves against cellular reactive oxygen species action [11]. Thus, our findings were parallel to the findings of other studies regarding the susceptibility of fetal brain to ketamine's apoptogenic action [12].

The progressive abundance of TUNEL positive cells in frontal cortical area of our subgroups in each group and indicates that apoptosis was

involved in the mechanism of neuronal death caused by ketamine exposure during pregnancy. This explanation is in agreement with the suggestion of previous studies [13].

The results of this study showed exaggeration of the apoptotic changes in the frontal cortical tissue by the effect of ketamine. This phenomenon was seen in previous reports that detailed the explanation of the tissue injury in the CNS following virus infection concluding that apoptotic changes are seen in specific regions with neuronal ability to undergo apoptosis [14].

The number of the positively reacting apoptotic cells counted with the TUNLE test showed a progressively increased mean values starting from control subgroup (A) followed by the subsequent groups B, C and D. This result is in agreement with the previous studies reporting progressive cortical cell apoptotic behavior in rat embryo reaching a prenatal peak at the 15th day of gestation [8].

In support to the results of prenatal exposure to ketamine on newborn rat frontal cortex demonstrated in this study, there are many drugs that have been described to trigger widespread apoptotic death of neurons in the developing animal brain [15-19]. It was concluded that susceptibility to the apoptosis reaches its peak during the developmental period of rapid synaptogenesis (also called the period of brain growth spurt). This period was reported in rodents to be primarily during the first 2 weeks after birth, but in humans, it extends from about mid-gestation to several years after birth [12]. All the recent human epidemiological studies pertaining to developmental anesthesia neurotoxicity have focused on full-term infants and children [20,21], the focus of future human research should be expanded to include third trimester fetuses and prematurely born infants.

The results of this study proved that neuronal apoptosis in rat by the effect of ketamine showed progressive peak during prenatal neurogenesis, and it is not only a phenomenon occurring if the rat was exposed to ketamine during the period of brain growth spurt.

The neuroapoptosis by the effect of drugs had been studied at both light and electron microscopic levels [22,23] and by immunohistochemical procedures including caspase-3 (AC3) immunohistochemistry [24]. TUNEL test has been used in this study for marking dying cortical cells to have a new focus on ketamine induced developmental neuroapoptosis. Many animal studies which investigated single or brief exposure to clinically relevant doses of commonly used anesthetics (ketamine, midazolam, propofol, isoflurane, sevoflurane, chloral hydrate) [25,26] showed supportive evidences to the results of this study.

This study concluded that prenatal exposure to ketamine in a dose of 5 mg/kg was a relatively non-toxic in all studied groups compared to the control subgroup. Apoptosis in frontal cortical tissue was involved in the mechanism of neuronal death caused by ketamine exposure during pregnancy.

Authors contributions:
Dr. Gaeb (a PhD candidate) performed the laboratory research work. Dr. Mobarak (supervisor) performed the interpretation of the results. Dr. Jaffar (supervisor) performed the production of the results.

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

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Effect of Chitosan and Dextrin Combination on Experimentally-Induced Thermal Injury in Rabbits

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Abstract

Background	Burn is a major health problem, life threatening with a high mortality and morbidity; Chitosan and its formulations are used as a topical dressing in wounds and burns management due to its nontoxic, hemostatic, healing stimulant, antimicrobial, biocompatible and biodegradable properties as well as its vehicle use to deliver biopharmaceuticals, antimicrobials and growth factors into tissue.
Objective	To evaluate the effects of chitosan-dextrin combination on induced burn in rabbits.
Method	Forty domestic male rabbits, weighing 1250-1750 kg were divided into five groups, each of eight animals: AH group: apparently healthy rabbits, BWT group: left with no treatment, AR group: treated with *Aqua Rosea*, AG-S group: treated with silver sulfadiazine cream and CH-D group: treated with chitosan – dextrin combination; all animals (except AH group) wereinduced burn and treatedtopically on burned area once daily for 28days. Tissue levels ofvascular endothelial cell growth factor (VEGF), tumor necrosis factor alpha (TNF-α) and skin histological examination.
Results	Histopathological evaluation showed enhances inflammatory response, vascularization, granulation tissue formation, and collage deposition due to the appropriate regulation of TNF-α and VEGF.
Conclusion	Topical use of chitosan – dextrin combinationshowed effective and enhance wound healing activities.
Key words	Thermal injury, burn, chitosan, dextrin.

List of abbreviations: AR = *Aqua Rosae*, Ag-S = Silver sulfadiazine, B.v = blood vessel, BWT = Burned animal with no treatment, CH-D = Chitosan – dextrin combination, ELISA = Enzyme linked immunosorbant assay, Pg = Pico gram, s.c = Subcutaneous, TNF-α = tumor necrosis factor alpha, VEGF = vascular endothelial growth factor.

Introduction

Burn is common universal problem that may lead to ugly scarring, serious handicapping. It can severely affect not only the skin but the whole body [1] because it is a coagulative necrosis of tissue and the damage depth is depending on the temperature and the duration of the exposure to the causative agent as heat, electricity, light, chemicals, friction or radiation. It affects the integrity of the skin because it is a barrier that protects the body from the external invasion (*e.g.*, microbial aggressions) [2].

Burns areserious problem in both the developed and developing world represented as physical and psychological assault on patients and considered a diverse and great challenge to medical staff [3].

Chitosan is modified natural carbohydrate polymer that produced commercially by deacetylation of chitin which found naturally in the skeleton of invertebrates, insects and some algae usually find in cell walls of squids, crabs, shrimps, crayfish and oysters as well as fungi cell wall [4]. Chitosan is a wound healing accelerator and it enhance every stage of healing because of improvement the functions of inflammatory cells like macrophages,

polymorphonuclear leukocytes and fibroblasts and increase the tensile strength of wound. These effects enhanced according to deacetylation degree, molecular weight and the state of chitosan [5].

Dextrin is produced by an enzyme called amylase in human and can be produced from a wide variety of starch, such as wheat, rice, corn, potato, and tapioca [6]. Dextrin act as formulation aidfor producing a desired texture in product, stabilizer and thickener forproducing viscous solutions or dispersions to enhance consistency and stabilize emulsions [7]. The current study was performed to investigate healing effect of chitosan – dextrin combination against burn injury.

Methods

Forty domestic male rabbits, weighing 1250-1750 kg were divided into five groups each of eight animals; they were housed in animal house of Al-Naharain Collage of Medicine. Before starting the study, the animals were left for 48 hours to acclimatize to the animal room conditions of controlled temperature, allowed free access to water and food.

Thermal injury was done by a metal bar (20*20*100) mm, heated in boiling water and preserved in equilibrium for about 15 min. with the present ofthermometer and the animals were anesthetized using ketamine: xylazine (22-50 mg/kg: 2.5-10 mg/kg IM) and put the bar for about 45 seconds on their shaved back [8]. The experimental protocol is as follows:

AH group: apparently healthy rabbits, BWT group: induced burn animal with no treatment, AR group: induced burn animals with treatment with *Aqua Rosea*, AG-S group: induced burn animals with treatment of silver sulfadiazine, and CH-D group: induced burn animals with treatment of chitosan – dextrin combination; each treatment was given topically on burned area once daily for 28 days.

In preparation of Chitosan Dextrin combination, Chitosan solution was prepared by dissolving of chitosan in distilled water containing of glacial acetic acid with the present of a magnetic stirre [9]. Chitosan solution was prepared by dissolving 0.1 g of chitosan in 50 ml of distilled water containing 1 ml of glacial acetic acid with the aid of three hours stirring by magnetic stirrer for maximum solubility then the addition of 30 g dextrin with continuous stirring and finally mixing with 20 g *aqua rosea* to get cream constancy.

At the end the animals have been sacrificed by ether on day 29, also skin tissue were divided in two parts for homogenation and staining to determine tissue levels of vascular endothelial cell growth factor (VEGF), tumor necrosis factor alpha (TNF-α) by and histological examination.

Principle of the assay of TNF-α andVEGFa quantitative sandwich enzyme immunoassay technique ELISA; where antibodies specific for TNF-α and VEGF have been pre-coated onto a microplate. Samples and standards and are pushed into the wells and the TNF-α contents are bound by the immobilized antibody; then removing the unbound substances, adding a biotin conjugated antibody to the wells, washing, adding avid in conjugated Horseradish Peroxidase to the wells, washing again, adding a substrate solution to the wells and color would appear in proportion to the amount of TNF-α and VEGF bound in the first step. The color must be stopped and the intensity of it is measured at 450 nm.

Preparation of skin tissue forhistological examination by fixation in 10% formalin and processed according to Bancroft and Stevens [10].

Statistical analysis was performed using SPSS-21 andDescriptive statistics were formulated as mean and standard error of mean (mean±SEM). One Way Analysis of Variance (ANOVA) and t-test was used to assess and the difference was considered significant when p value was equal to or below 0.05 [11].

Results

CH-D group showed a significant reduction in the levels of TNF-α in skin tissue homogenate (Figure 1), in addition to the significant elevation of VEGF in skin tissue homogenate

compared to other groups (p < 0.05), while BWT, AR and AG-S animal groups showed non-significant difference on TNF-α and VEGF level in skin tissue homogenate (p > 0.05) but significantly different comparing with AH group (Figure 2). In BWT and AR animal groups showed non-significant difference on all study parameters (p > 0.05), and according to histopathological examination of skin, CH-D group better inflammatory response, granulation tissue and fibrosis (Figure 7), when compared with other study groups: AH group (Figure 3), BWT group (Figure 4), AR group (Figure 5) and AG-S group (Figure 6).

Fig. 1. Tissue TNF-α levels (pg/ml) of study groups.

AH = apparently healthy group, BWT = burned without treatment group, AR = treated with *Aqua rosea* group, AG-D = treated with Silver sulfadiazine group, CH-D = chitosan- dextrin treatment group.

Discussion

Burn is one of the most widespread injuries, where an oxidation process associated with biological and metabolic alterations; the pathophysiology and histopathology of thermal burn in animals is very similar to that in humans [12]. Many studies have demonstrated the therapeutic properties of chitosan because of its biodegradability that makes it dissolves with time when used in wound [5] and the addition of dextrin to aqueous solvents as formulation aid and thickening agent to get desired texture as well as enhance consistency [7].

Wound healing is a physical rebuilding process, molecules for wound repair are secreted by fibroblasts and others present at the wound site. VEGF is a critical cytokine that exhibits chemoattractant properties [13]. Angiogenesis is an important factor in proliferative phase of wound healing and forsupplying nutrients and oxygen needed for skin regrowth thus VEGF is one of the most potent proangiogenic growth factors in the skin [14], wound repair is a process that granulation tissue gradually replaces necrotic tissue and abnormal expressions of VEGF is involved in this process [15]. Reduced levels of endogenous growth factors and diminished angiogenesis are contributory factors for impaired wound healing. VEGF is the most potent angiogenic growth factor, which accelerates healing [16].

Chitosan stimulated inflammatory cells, endothelial cells, newly formed blood vessels, reticular – collagen fibers and VEGF in the wound healing area,chitosan accelerates granulation tissue formation and accelerate the wound healing through increasing VEGF secretion in all stages of wound healing process, activates fibroblasts in the granulation tissue, makes them proliferate and increase extracellular matrix production [17].
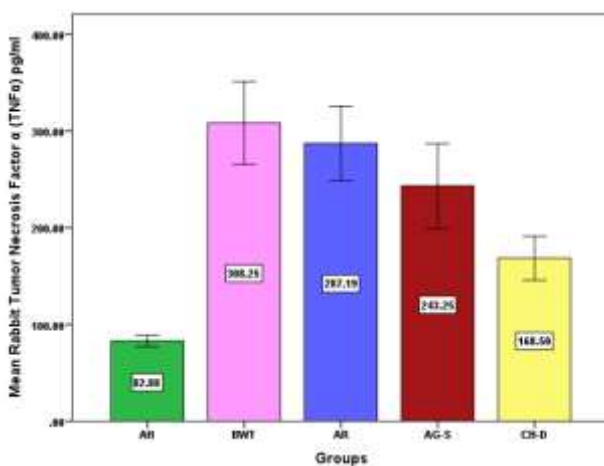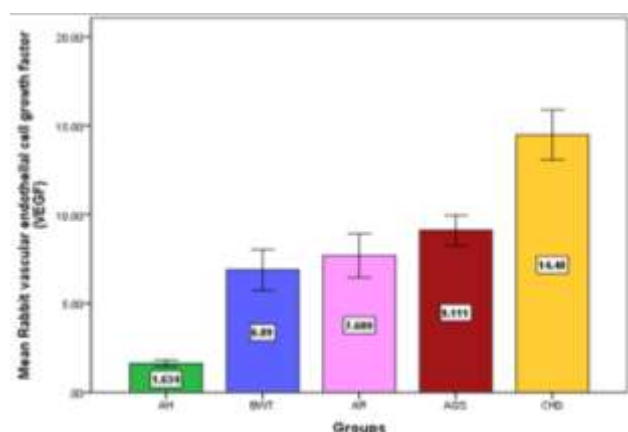

Fig. 2. Tissue VEGF levels (pg/ml) of study groups.

AH = apparently healthy group, BWT = burned without treatment group, AR = treated with *Aqua rosea* group, AG-D = treated with Silver sulfadiazine group, CH-D = chitosan- dextrin treatment group.

Fig. 3. Light microscopic section of rabbit skin tissue of AH group (the apparently healthy) showing normal skin tissue: epidermis (blue arrow), dermis, and hypodermis and subcutaneous. H&E (40X), (s.c) subcutaneous.
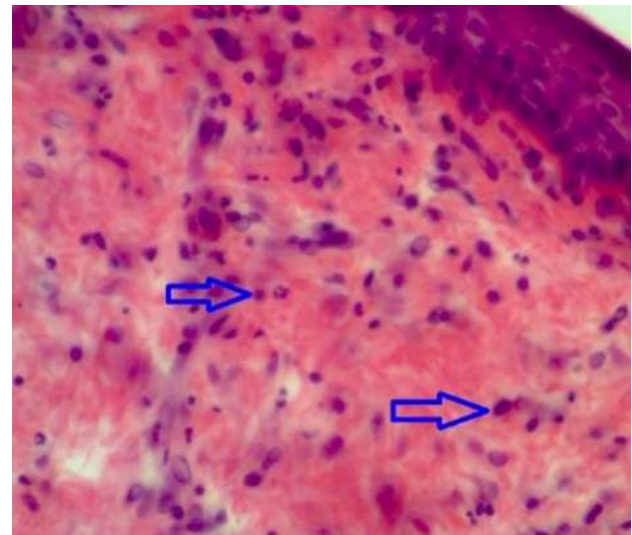
Fig. 5. Light microscopic section of rabbit skin tissue of AR group (*Aqua rosea* treatment) showing focal burning layer of epidermis with damage to dermis and hypodermis layers with certain inflammatory reaction (blue arrow). H&E (40X).

Fig. 4. Light microscopic section of rabbit skin tissue of BWT group (burned without treatment) showing burning skin layers with discontinuation of epidermis ,no skin appendages in the dermis layer and ulceration (blue arrow). H&E (40X).
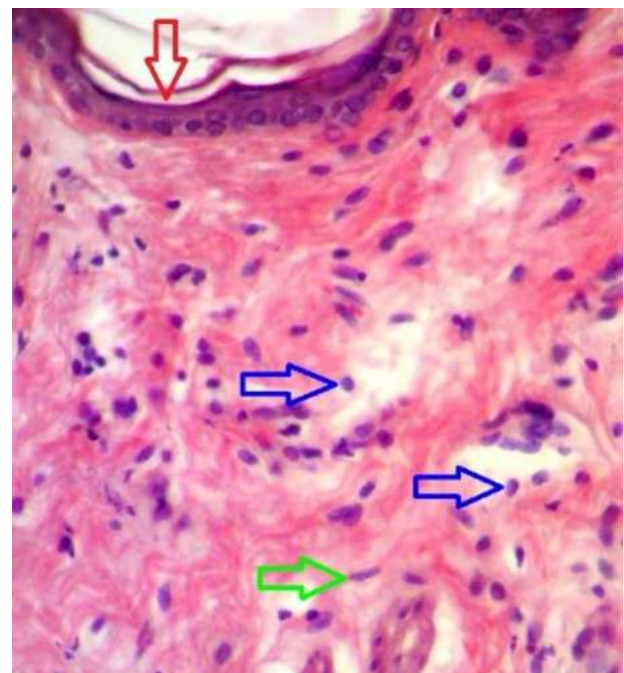
Fig. 6. Light microscopic section of rabbit skin tissue of AG-S group (Silver sulfadiazine treatment) showing more inflammatory (blue arrow) with mild reactive fibroblast (green arrow), re-epithelialization (red arrow) and collagen fiber. H&E (40X).
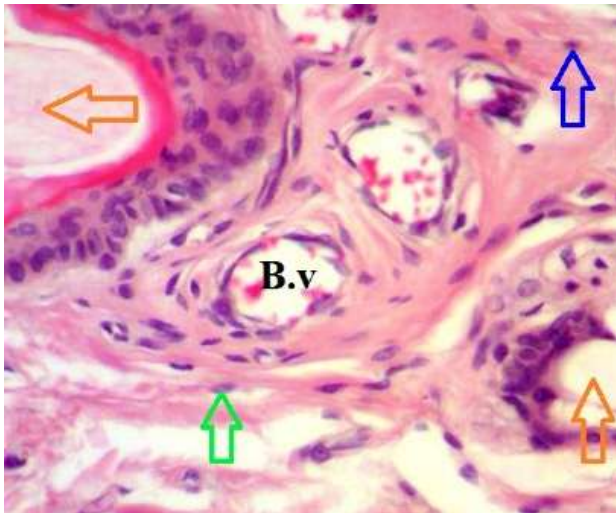
Fig. 7. Light microscopic section of rabbit skin tissue of CH-D group (combination of chitosan –dextrin treatment) showing more inflammatory (blue arrow) with reactive fibrous tissue formation with present of fibroblast cells (green arrow), hair follicles (orange arrow) and abundant granulation tissue (formation of new capillaries). H&E (40X). (B.v) blood vessel.

The physiopathological events following thermal injury related to acute inflammatory reactions in which hyperactive macrophages are primed to stimulate the down regulation or up regulation of certain inflammatory cytokines [18] and abnormal levels of tumor necrosis factor alpha, has been reported both systemically and locally in burn patients [19]. Low levels of TNF-α promote wound healing indirectly but high levels of TNF-α delay wound healing [20], up-regulated TNF-α during the inflammatory phase should decreased for the rapid wound healing response,moreover, a prolonged increase in levels of TNF-α may has a role in the development of multiple organ failure after thermal injury [21]. The current study found that chitosan-dextrin combination treatment animals groups had the lowest skin tissue TNF-α level when compared with other groups and these result suggested that chitosan promote wound healing via decreasing of up-regulated TNF-α levels as

tableand increasing of up-regulated VEGF levels.

Author contributions
Noor is a researcher who has done the technique of this work and conducted the writing of manuscript. Dr. Abdulkareem and Dr. Bahaa participated in supervision and in scientific review of the manuscript.

Conflict of interest
The authors declare no conflict of interest.

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Chlamydia Pneumoniae: The Potential Cause of Multiple Sclerosis

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Abstract

Background Multiple sclerosis is an autoimmune disease, its etiology until know is unknown. Many studies suggested that the environmental risk factors one of them is *chlamydia Pneumonia*e may play a role in the initiation of the disease.

Objectives To investigate the relationship between previously *Chlamydia Pneumoniae* infection and multiple sclerosis disease initiation

Methods Sixty patients with multiple sclerosis (30 newly and 30 previously diagnosed), their ages ranged from 13 to 58 years were enrolled in the present study. They attended seeking for treatment or for the diagnoses of multiple sclerosis in outpatient clinic at the Medical City, Baghdad Teaching Hospital, Baghdad in the period from December 2014 till March 2015. In addition, thirty healthy volunteers their gender and ages were matched with patients group were participated as a control. We measured the anti *chlamydia Pneumonia*e IgG by ELISA technique**.**

Results The *chlamydia Pneumonia*e positivity in the multiple sclerosis patients was considerably higher than the control group but the variation was not significant ($p > 0.05$) and there was no difference between the previously and newly diagnosed multiple sclerosis patients.

Conclusion There is no statistically significant relationship between previously *chlamydia Pneumonia*e infection and MS disease.

Keywords Multiple sclerosis, *chlamydia Pneumonia*e

List of abbreviation: MS = multiple sclerosis, Cpn = Chlamydia Pneumoniae, CNS = central nervous system, Ig = mmunoglobulin, RRMS = relapsing remitting multiple sclerosis, SPMS = secondary progressive multiple sclerosis, PPMS = primary progressive multiple Sclerosis.

Introduction

Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease of the central nervous system characterized by demyelination and axonal loss [1,2].

MS is a multifactorial disease, which results from complex interactions between susceptibility genes and environmental factors [3]. In the MS pathogenesis, *Chlamydia pneumonia* (Cpn) has been getting increased attention as a possible cause of MS [4].

Cpn is found to be common in a variety of neurological disorders and not only in MS. It is possible that MS patients might be less able to clear the organism from the CNS [5].

The ability of the Cpn to persist in monocytes and macrophages in tissues for long periods, circumvents the mechanisms of bactericidal and oxidative stress, activate the endothelial cells with production of adhesion molecules and cytokine overproduction has suggested that it may participate in the development or progression of certain acute and chronic inflammatory diseases of the CNS [6].

The role of Chlamydia in the pathogenesis of neurobehavioral disorders or mental is

uncertain and will require further confirmation [7].

The objective of this study is to investigate the relationship between previously Chlamydia Pneumoniae infection and multiple sclerosis disease initiation.

Methods

Case control study was done which involved; sixty patients with MS their ages were range from 13 to 58 years. They were attended for seeking treatment or attended for new diagnoses in the MS outpatient clinic at Baghdad teaching medical city in the period which extended from December 2014 to March 2015.

The diagnosis of each case was established according to MC Donald criteria (2010) done by a neurologist and confirmed by MRI. Patients were subjected to questionnaire about name, age, gender, smoking, family history, medication and clinical signs.

Patients were divided into two groups, group I on treatment and group II as newly diagnosed patients. This study was approved by the Ethical Committee of College of Medicine/Al-Nahrain University and all samples were obtained with informed consent in accordance with the teaching hospital of medical city declaration.

Three ml of blood was collected from each patient and control included in this study then centrifuged and the serum was separated and stored at $2\text{-}8^oC$ until used. Human immunoglobulin G (IgG) anti-Cpn was measured with an enzyme-linked immunosorbant assay (ELISA) (Nova Tec – Germany).

Statistical analysis

Analysis of data was carried out using the available statistical package of SPSS-22 (Statistical Packages for Social Sciences- version 22). Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values). Statistical significance was considered whenever the P value was equal or less than 0.05.

Results

Table 1 distributes MS and control subjects according to their age and gender and table 2 distributes them according their family history of MS and smoking habits. The MS patients were distributed on three subgroups according to type of the disease, 55 (91.6%) of patients were with relapsing remitting MS (RRMS) RRMS type and 3 (5%) of patients were with secondary progressive MS (SPMS) type while 2 (3.3%) of patients with primary progressive MS (PPMS) type. Moreover, there were no significant differences between treated and newly diagnosed MS patients regarding to the presence of the respiratory signs (Table 3).

Serum level of IgG anti *Chlamydia Pneumonae* antibodies

The results of ELISA concerned with the IgG positivity against Chlamydia for studied groups are described in table 4. This study revealed no significant difference in the percentage of IgG positivity between patients group and healthy control group (p > 0.05), out of 60 patient, 19 were positive (31.7 %) for IgG anti- Chlamydia, while out of 30 healthy control, 4 were positive (13.3 %). Furthermore, there were no significant differences (p > 0.05) in the percentage of IgG positivity between two patients groups (Fig. 1).

Table 1. Distribution of multiple sclerosis patients and healthy controls according their age and gender

Age (years)	Multiple sclerosis N = 60	Control group N = 30
<30	21 (35%)	11 (36.7%)
30-39	20 (33.3%)	8 (26.7%)
40-49	13 (21.7%)	9 (30.0%)
>50 years	6 (10.0%)	2 (6.7%)
Mean±SD (Range)	34.5±10.6 (13-58)	34.7±10.1 (18-53)
Males	25 (41.7%)	8 (26.7%)
Females	35 (58.3%)	22 (73.3%)

Table 2. Distribution of multiple sclerosis patients according to family history and smoking habitat

Parameter		Multiple sclerosis		
		New cases	Treated cases	Total
Family history	Positive	3 (10%)	3 (10%)	6 (10%)
	Negative	27 (90%)	27 (90%)	54 (90%)
Smoking	Yes	3 (10%)	5 (16.7%)	8 (13.3%)
	No	27 (90%)	25 (83.3%)	52 (86.6%)

Table 3. Distribution of multiple sclerosis patients according to clinical features

Parameter		Multiple sclerosis		
		New cases	Treated cases	Total
Multiple sclerosis Type	RRMS	28 (93.3%)	27 (90.0%)	55 (91.6%)
	PPMS	1 (3.3%)	1 (3.3%)	2 (3.3%)
	SPMS	1 (3.3%)	2 (6.7%)	3 (5.0%)
Medications type	IFN -b1		25 (83.3%)	
	Rebif		3 (10.0%)	
	Avonex		2 (6.7%)	
Respiratory signs	Yes	10 (33.3%)	13 (43.3%)	23 (38.4%)
	No	20 (66.7%)	17 (56.7%)	37 (61.1%)

RRMS = relapsing remitting multiple sclerosis, SPMS = secondary progressive multiple sclerosis, PPMS = primary progressive multiple sclerosis.

Table 4. The positivity of studied groups for anti *Chlamydia Pneumonae* IgG antibodies

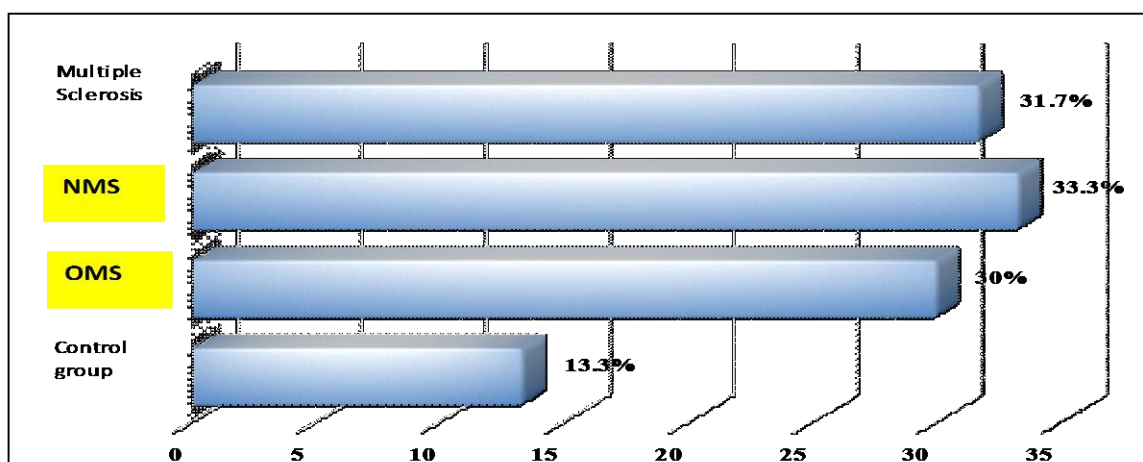| IgG anti-Chlamydia pneumoniae Abs	Multiple sclerosis N = 60	Control group N = 30
Positive (> 11.0)	19 (31.7)	4 (13.3)
Negative (< 11.0)	41 (68.3)	26 (86.7)

Fig. 1. Chlamydia Pneumoniae positivity in studied groups

Table 5. The relation between IgG anti *Chlamydia Pneumonae* antibodies positivity with demographic and clinical factors in the studied groups

| Feature | | IgG anti *Chlamydia Pneumonae* positivity | | | |
| | | Multiple sclerosis N = 60 | | Control group N = 30 | |
		No	Mean±SD	No	Mean±SD
Age (years)	<30	21	8.09±4.91	11	5.63±4.11
	30-39	20	7.40±5.76	8	5.66±3.23
	40-49	13	9.41±6.26	9	9.30±2.81
	>50	6	10.00±1.90	2	7.20±2.12
Gender	Male	25	9.48±5.78	8	8.01±4.22
	Female	35	7.51±4.83	22	6.42±3.47
MS Type	RRMS	55	8.26±5.37	-	-
	PPMS	2	13.10±2.69	-	-
	SPMS	3	6.50±3.97	-	-
Family history	Positive	6	10.23±3.88	-	-
	Negative	54	8.12±5.41	-	-
Smoking	Yes	8	11.25±7.01	-	-
	No	52	7.88±4.91	-	-
Medications type	IFN -b1	25	8.34±4.84	-	-
	Rebif	3	5.20±6.35	-	-
	Avonex	2	17.30±3.25	-	-
Respiratory signs	Yes	23	7.62±5.57	-	-
	No	37	8.78±5.13	-	-

Discussion

Over the past 10 years, a many of reports have found a possible relationship between Cpn infection and CNS diseases including MS, and other variety of neurobehavioral disorders. Many of researchers whom investigate the relationship between Cpn and MS examined the serum of the patients after disease occurrence and other examined prospectively.

The Results from studies on Cpn in MS patients have led to discordant results due to the lack of an efficient and standardized method for detection of Cpn [8]. In a comparative trial, one research group distinguished Cpn in the majority of MS patients but not in controls whereas three other researchers did not detect Cpn in any sample [9].

In present study, out of sixty MS patients there were nineteen seropositive for IgG anti Cpn and four persons from healthy control out of thirty also was seropositive. The differences were considerably high but not significant the p- value was (0.060) and OR (3.01) (0.92-9.85). The study, which is done on US army personnel and used samples of blood collected prior to the onset of the disease, it found no association between Cpn and the risk of RRMS when compared to healthy controls adjusting for latitude of residence at time of entry into active duty and education level [10].

Also the current study agrees with Villoslada et al [11], but disagree with Sriram et al [12] which was strongly connect between the Cpn and MS. Their study referred to that 97% polymerase chain reaction assays confirmed the presence of Cpn outer membrane protein gene in the CSF versus 18% of other neurological disease controls and 86% of MS patients had increased CSF antibodies to Cpn elementary body antigens by ELISA. This result is never repeated

in subsequent researches, Sriram et al study depended on the detection of Cpn in the CSF of patients by PCR technique and detection of IgG against Cpn by ELISA, which is differ from the current study [12].

In another study, an association between Cpn infection and development of MS was found, this association was mostly attributable to a strong association with risk of progressive MS. Overall, the titer of anti – Cpn IgG in the serum were elevated in women with MS. The titer of Cpn-specific IgG antibody were elevated in women which were suffering from progressive MS as compared with controls, but did not differ between RR MS cases and controls [13]. In the current study, PPMS was 6.6% and SPMS 10% of all patients whom enrolled in this study so if the progressive MS patients were more than this ratio the differences in the anti-Cpn IgG titer between the patients and control may be more than know. This is one of the probabilities.

Finally, some studies suggest a role of Cpn only as a CNS innocent bystander secondary effect, encouraged by excessively active chronic inflammation operating in MS. Others suggest a role of Cpn as a cofactor in development and progression of the disease in a subset of MS patients, the past infection with Cpn may play a role as a cofactor in a part of patients but not in all of them. Although the current results did not show significant deference between the patients and control group, yet, the difference was considerable [14].

In conclusion, our study revealed no statistically significant relationship between previously Cpn infection and MS disease.

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Author contribution

Khaliel collects the patient data and did the laboratory work and Dr. Abbas write the article.

Conflict of interest

No conflict of interest

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Effects of Cytochrome p-450 Inducer and Inhibitor Antiepileptic Drugs on Lipid Profile in Normal and Diabetic Rabbits

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Abstract

Background	Anticonvulsants drugs are a diverse group of pharmacological agents used in the treatment of epileptic seizures are known to inhibit or activate cytochrome p-450 enzymes that play a crucial rule in the metabolic process.
Objective	To investigate any possible differences in the effect of antiepileptic drugs; enzyme inducer (carbamazepine) and enzyme inhibitor (lamotrigine) on lipid profile in normal and diabetic rabbits depending on its effect on cytochrome p-450 enzymes.
Methods	Fifty four healthy domestic rabbits of both sexes weighing 0.5-2.5 kg were studied. They were divided into group A (standard) received tolamatrigine and carbamazepine without induction of diabetes and group B (received the same regimen) with induction of diabetes. Lipid profile was tested in the two groups.
Results	Carbamazepine-treated group showed a significant increase in the lipid profile at day 20 of treatment compared to day 5 in comparison with induced and non-induced control groups and control group in normal and diabetic rabbits. Lamotrigine showed less effect on lipid profile; and in non-diabetic treated groups it showed a non-significant change in lipids level as compared to the control group.
Conclusions	Anti-epileptics drugs that possess an enzyme inducing effect as carbamezpin tend to induce high lipids profile in comparison to lamotigine.
Keyword	Lamotrigine, carbamazepine, anticonvulsants, glucose, lipid.

List of abbreviation: CYP450 = cytochrome P450, AEDs = new antiepileptic drugs, CBZ = carbamazepine, FBG = fasting blood glucose, TC = total cholesterol, HDL-C = high-density lipoprotein cholesterol, LDL-C = low-density lipoprotein cholesterol, VLDL-C = very low-density lipoprotein cholesterol

Introduction

Cytochrome P450 (CYP450) is a group of enzymes present in every type of cell in the body except red blood cells and skeletal muscle cells. They are important in metabolizing substances normally present in the body such as steroids, fat-soluble vitamins, fatty acids, prostaglandins, and alkaloids. The P450 enzymes also detoxify drugs and a great number of environmental pollutants, such as carcinogens present in tobacco smoke and charcoal-broiled meat, polychlorinated biphenyls, and dioxin. CYP450, which is the main enzyme involved in the drug metabolism, was published in 1958. Since then, it has become clear that CYP450 is a superfamily of enzymes with more than 200 family members. The CYP450 enzyme system is responsible for the metabolism of a wide range of endogenous and exogenous substrates [1,2].

By catalyzing the first steps in different pathways of cholesterol degradation, CYP450 play key roles in cholesterol homeostasis [3].

Although many new antiepileptic drugs (AEDs) have been introduced over the past 15 years, the consensus first choice for focal seizures has

traditionally been carbamazepine (CBZ) which exhibits a potent induction of the CYP450 enzyme system [4].

CYP450 enzymes are known to figure prominently in numerous important aspects of metabolism as in lipid metabolism on the other hand also an inhibition of CYP450 enzymes has shown to affect the metabolic process and so affect the levels of lipid profile [5].

The aim of the study is to investigate any possible differences in the effect of AEDs on lipid profile depending on its effect on CYP450 enzymes.

Methods
Fifty four healthy domestic rabbits of both sexes weighing 0.5-2.5 kg were used in the present study. They were supplied from Center of Technical Institution, Al-Nahrin University. They housed one per cage, which is provided with a wire mesh floor. They were kept in a well-controlled hygienic environment and water was given *ad libitum*.

Animal design
Animals were allocated into group A (standard) received lamotrigine (lamictal®) 25 mg/kg (GsK, Poland) and CBZ (Tegertol ®) 90 mg/kg (Novartis, Switzerland) and group B (received the same regimen) plus they were subjected into induction of diabetes.

Induction of Diabetes
The rabbits were injected with alloxan monohydrate dissolved in sterile saline (0.9% NaCl) at a single dose of 150 mg/kg intraperitoneally. The baseline fasting blood glucose (FBG) was determined before intraperitoneal administration of alloxan. After 6 hr of alloxan administration, 5% glucose solution was infused orally in the feeding bottle for a day to overcome the early hypoglycemic phase as a result of acute massive pancreatic release of insulin. Hyperglycemia was confirmed by elevated serum glucose level, determined at 3rd day post-induction. The rabbits that became hyperglycemic (FBG level

around 200-250 mg/dl) and stable were include in the study [6].

Measurement of serum lipid profile
The level of lipid profile was measured at day 5 and day 20 post-treatment.

Serum total cholesterol determination
Serum total cholesterol (TC) was estimated according to the method of Allain (1974) where a readymade kit is used for this purpose, based on the oxidation of cholesterol, which resulted in the formation of H_2O_2, and when the latter is reacted with phenol, a red colored quinonimine was formed and the intensity of color was measured at 505 nm and compared with standard cholesterol solution [7].

Serum triglyceride determination:
Serum triglyceride (TG) levels were determined according to the method of Fossati and Prencipe (1982) and a readymade kit was utilized for this purpose, based on enzymatic oxidation of the glycerol-3-phosphate, which is generated from the hydrolysis of TG moiety. The oxidation process resulted in the formation of H_2O_2, which is measured spectrophotometrically as indicated before [8].

Determination of serum high and low density lipoprotein cholesterol
Serum high-density lipoprotein cholesterol (HDL-C) levels were estimated according to the method of Burstein et al (1970) through which low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C) was determined calorimetrically by measurement of light absorbance at 505 nm, using a readymade kit for this purpose [9].

Result
The TC level of the control group was 67.66 ± 4.5 mg/dl at day 5 and 68.6 ± 4.5 mg/dl at day 20 of the study. The CBZ-treated group showed significant difference (p < 0.05) when compared to control group with 3.8 % increment in the total mean value of

cholesterol. On the other hand, lamotrigine-treated group demonstrated a non-significant difference when compared to the control group plus 0.8 % increment in the total mean value of cholesterol.

The TC in CBZ-treated group was 68.3 ± 2.5 mg/dl at day 5 and increased to 73 ± 2.6 mg/dl at day 20 (p < 0.05). The percentage of change equals to 6.8 %. Moreover, the TC in the lamotrigine-treated group was 69.0 ± 3.0 mg/dl at day 5 and 68±5 mg/dl at day 20. The percentage of change was 1.4% with no significant differences between day 5 and day 20 of the study (Table 1).

Table 1. Cholesterol level at day 5 and day 20 of the study for the control group and treated groups

Days of treatment	Blood cholesterol level (mg/dl)		
	Control group	Treatment group	
		Carbamazepine	Lamotrigine
5	67.66 ± 4.50	68.33 ± 2.51	69.00 ± 3.00
20	68.66 ± 4.0	73.0 ± 2.64	68.0 ± 5.00
Total	67.91 ± 3.50	70.50 ± 2.90	68.50 ± 3.7
*	---	p < 0.05	ns
**	---	p < 0.05	ns
†	---	3.8%	0.8%
‡	1.47%	6.8%	1.4%

* = treated Vs control group, ** results on day 5 vs day 20 in the same group. † = percentage of change between each treated group Vs control group, ‡ = percentage of change between day 5 and day 20 of the same group.

The TC level was 183 ± 3.6 mg/dl at day 5 and 200±5 mg/dl at day 20 of the study. The CBZ-treated group showed significant difference (p < 0.01) as compare to the control group with 6.6 % increment between the mean values whereas, lamotrigine-treated group show a non-significant change compared to the control group.

In the CBZ-treated group, TC level was 183±3 mg/dl at day 5 and increased to 220.66±4.04 mg/dl at day 20 (p < 0.001) with 20.2 % increment. Considering lamotrigine-treated group, TC level was 186 ± 2.0 mg/dl at day 5 and increased to 190.3 ± 2.3 mg/dl at day 20 (p < 0.01) with 2.3% increment (Table 2).

Table 2. Cholesterol level at day 5 and day 20 of the study for the standard control and treated diabetic groups

Days of treatment	Blood cholesterol level (mg/dl)		
	Control group	Treatment group	
		Carbamazepine	Lamotrigine
5	183.00 ± 3.6	183.0 ± 3.00	186.0 ± 2.00
20	200.00 ± 5.0	220.6 ± 4.04	190.3 ± 2.3
Total	188.33 ± 7.91	200.91 ± 16.04	188.03 ± 3.42
*	---	p < 0.01	ns
**	---	p < 0.001	p < 0.01
†	---	6.6%	-0.1%
‡	9.2%	20.2%	2.3%

* = treated Vs control group, ** results on day 5 Vs day 20 in the same group. † = percentage of change between each treated group Vs control group, ‡ = percentage of change between day 5 and day 20 of the same group.

The TG level of the standard control group was 85 ± 5 mg/dl at day 5 and 86 ± 3 mg/dl at day 20 of the study. CBZ-treated group showed significant difference ($p < 0.001$) as compared to the control group with 51.6 % increment between the total mean values. Meanwhile, Lamotrigine-treated group showed non-significant changes in comparison to the control group with only 0.16% increment. CBZ-treated group demonstrated significant increment ($p < 0.001$) from day 5 (86.0 ± 1.0 mg/dl) to 160±5 mg/dl on day 20, with 86% increment within the same group. Moreover, lamotrigine-treated group show no change in the blood TG level between day 5 and day 20 (86.0 ± 3.0 mg/dl versus 85.3 ± 3.04 mg/dl) with only 0.8 % increment (Table 3).

Table 3. Triglyceride level at day 5 and day 20 of the study for the standard control and treated groups

Days of treatment	Blood triglyceride level (mg/dl)		
	Control group	Treatment group	
		Carbamazepine	Carbamazepine
5	85.0 ± 5	86.00 ± 1	86.00 ± 3
20	86.00 ± 3	160.00 ± 5	85.30 ± 3.04
Total	85.25 ± 2.9	129.25 ± 31.7	85.34 ± 2.31
*		$p < 0.001$	ns
**		$p < 0.001$	ns
†		51.6%	0.16%
‡	1.17%	86%	-0.8%

* = treated Vs control group, ** results on day 5 Vs day 20 in the same group. † = percentage of change between each treated group Vs control group, ‡ = percentage of change between day 5 and day 20 of the same group.

The TG level of the positive control group was 133.6 ± 4.04 mg/dl at day 5 and 153.6 ± 3.2 mg/dl at day 20 of the study. CBZ-treated group showed significant difference ($p < 0.01$) as compared to the control group with 23% increment between the total mean values. Meanwhile, Lamotrigine-treated group showed non-significant changes in comparison to the control group with only 0.15% decrement. CBZ-treated group demonstrated significant increment ($p < 0.01$) from day 5 (130.3 ± 2.5 mg/dl) to 246 ± 5.2 mg/dl on day 20, with 88.7% increment within the same group. Moreover, lamotrigine-treated group showed increased blood TG level significantly ($p < 0.01$) between day 5 and day 20 (135.3 ± 2.5 mg/dl versus 161 ± 4.5 mg/dl) with only 18.9 % increment (Table 4).

The LDL level of the standard control group was 46 ± 3 mg/dl at day 5 and 45 ± 1 mg/dl at day 20 of the study. Lamotrigine-treated group showed non-significant changes in comparison to the control group with only 0.4% increment; whereas, CBZ-treated group showed significant difference ($p < 0.05$) as compared to the control group with 33.7 % increment between the total mean values. Meanwhile, CBZ-treated group demonstrated significant increment ($p < 0.001$) from day 5 (45.0 ± 2.0 mg/dl) to 60.2 ± 0.72 mg/dl on day 20, with 33.7% increment within the same group. Moreover, lamotrigine-treated group show no change in the blood LDL level between day 5 and day 20 (44.33 ± 4.5 mg/dl versus 45.8 ± 0.2 mg/dl) with -0.6% decrement (Table 5).

The HDL level of the standard group was 32.6 ± 1.5 mg/dl at day 5 and 33.6 ± 0.5 mg/dl at day 20 of the study. Lamotrigine-treated group showed non-significant change in comparison to the   control group with -2.4 % decrement; whereas, CBZ-treated group showed a significant difference ($p < 0.05$) as compared to

control group with 6.2% increment between the total mean values. Furthermore, CBZ-treated group demonstrated non-significant difference in HDL level at day 5 (32.0 ± 2.0 mg/dl) versus 37 ± 4 mg/dl at day 20 with 15%

increment. Similarly, lamotrigine-treated group showed non-significant differences (33.0 ± 2.0 mg/dl at day 5 versus 32.0 ± 0.4 mg/dl at day 20) with 2.6 % decrement (Table 6).

Table 4. Triglyceride level at day 5 and day 20 of the study for the standard control and treated diabetic groups

Days of treatment	Blood Triglyceride level (mg/dl)		
	Control group	Treatment group	
		Carbamazepine	Lamotrigine
5	133.66 ± 4.04	130.33 ± 2.51	135.33 ± 2.51
20	153.66 ± 3.21	246.00 ± 5.29	161.0 ± 4.58
Total	144.33 ± 8.45	177.50 ± 46.10	144.08 ± 10.79
*		$p < 0.01$	NS
**		$p < 0.01$	$p < 0.01$
†		23%	-0.15%
‡	8%	88.7%	18.9%

* = treated Vs control group, ** results on day 5 Vs day 20 in the same group. † = percentage of change between each treated group Vs control group, ‡ = percentage of change between day 5 and day 20 of the same group.

Table 5. Low-density lipoprotein level at day 5 and day 20 of the study for the standard control and treated groups

Days of treatment	Blood low-density lipoprotein level (mg/dl)		
	Control group	Treatment group	
		Carbamazepine	Lamotrigine
5	46.00 ± 3	45.0 ± 2	44.33 ± 4.5
20	45.00 ± 1	60.2 ± 0.72	45.8 ± 0.2
Total	45.5 ± 2.71	50.8 ± 6.45	45.2 ± 2.07
*		$p < 0.05$	ns
**		$p < 0.001$	ns
†		11.6%	-0.6%
‡	-2%	33.7%	- 3.38%

* = treated Vs control group, ** results on day 5 Vs day 20 in the same group. † = percentage of change between each treated group Vs control group, ‡ = percentage of change between day 5 and day 20 of the same group.

The LDL level was 100.3±4.5 mg/dl at day 5 and 120±5 mg/dl at day 20 of the study. The CBZ- and lamotrigine-treated groups showed non-significant difference as compare to the control group. CBZ-treated group demonstrated significant increment ($p < 0.001$) in LDL level (98.6 ± 4 mg/dl at day 5 as compared to 144.6 ± 5 mg/dl at day 20) with 46.6 % increment between the mean values Likely, lamotrigine-

treated group show significant increment ($p < 0.01$) in LDL level (102 ± 2.6 mg/dl at day 5 versus 124 ± 1.1 mg/dl at day 20 with 22.4% increment (Table 7).

The HDL level of the control group was 27.3±4.04 mg/dl at day 5 and 20±5 mg/dl at day 20 of the study. The CBZ-treated groups showed significant difference ($p < 0.05$) as compare to the control group with 20.5%

increment. CBZ-treated group showed HDL level of 28 ± 3 mg/dl at day 5 and increased significantly (p < 0.05) as compared to 31 ± 4.5 mg/dl at day 20 with 11.7 percent change. Lamotrigine-treated group demonstrated significant decrement (p < 0.05) in HDL level (26.6 ± 3.5 mg/dl at day 5 versus 19.8 ± 0.76 mg/dl at day 20 with 25.4 % percent change (Table 8).

Table 6. High-density lipoprotein level at day 5 and day 20 of the study for the standard control and treated groups

Days of treatment	Blood high-density lipoprotein level (mg/dl)		
	Control group	Treatment group	
		Carbamazepine	Lamotrigine
5	32.6 ± 1.5	32.00 ± 2.0	33.0 ± 2
20	33.66 ± 0.57	37.00 ± 4.0	32.23 ± 0.4
Total	33.41 ± 0.99	35.50 ± 3.17	32.55 ± 1.67
*		p < 0.05	ns
**		ns	ns
†		6.2%	-2.4%
‡	3%	15%	-2.6%

* = treated Vs control group, ** results on day 5 Vs day 20 in the same group. † = percentage of change between each treated group Vs control group, ‡ = percentage of change between day 5 and day 20 of the same group.

Table 7. Low-density lipoprotein level at day 5 and day 20 of the study for the standard control and treated diabetic groups

Days of treatment	Blood low-density lipoprotein level (mg/dl)		
	Control group	Treatment group	
		Carbamazepine	Lamotrigine
5	100.33 ± 4.5	98.66 ± 4.04	102.00 ± 2.64
20	120.0 ± 5	144.6 ± 5.03	124.93 ± 1.1
Total	111.41 ± 8.7	114.75 ± 19.63	112.31 ± 10.05
*		p < 0.05	ns
**		p < 0.001	p < 0.001
†		2.9%	0.8%
‡	19.6%	46.6%	22.4%

* = treated Vs control group, ** results on day 5 Vs day 20 in the same group. † = percentage of change between each treated group Vs control group, ‡ = percentage of change between day 5 and day 20 of the same group.

Discussion

Animal data showed that a particular enzyme of CYP450, CYP51A1, catalyzes the conversion of lanosterol into cholesterol intermediates. When these intermediates build up through inhibition of the enzyme, they in turn inhibit the rate-limiting step of cholesterol synthesis, 3-hydroxy-3- methylglutaryl-coenzyme A reductase (HMG-CoA reductase), and slow the synthesis of cholesterol [7]. It follows that induction of CYP51A1 should therefore increase cholesterol production through metabolism of these intermediates and reduced feedback inhibition. The increased activity of the cholesterol synthesis rate limiting (enzyme HMG-CoA reductase) may also lead to increase in LDL-C levels [3].

On the other hand CYP450 enzymes play a role in the synthesize of polipoprotein A (the main HDL lipoprotein particle) leading to increased concentration of HDL-C level in the blood [10].

There is a strong evidence that drugs assumed to have an inhibitory or stimulatory effect on CYP450 enzymes had an effects on lipid profile, like ketoconazole which is potent CYP450 inhibitor that has been shown to reduce cholesterol production in studied animal and this effect is documented clinically by the finding that patients taking valproate, (CYP450-inhibiting properties) have lower TC levels than controls [11].

Table 8. High-density lipoprotein level at day 5 and day 20 of the study for the standard control and treated diabetic groups

Days of treatment	Blood high-density lipoprotein level (mg/dl)		
	Control group	Treatment group	
		Carbamazepine	Lamotrigine
5	27.33 ± 4.04	28.00 ± 3	26.6 ± 3.51
20	20.00 ± 5	31.33 ± 4.5	19.83 ± 0.76
Total	24.33 ± 4	29.33 ± 3.33	24.07 ± 3.71
*		$p < 0.05$	ns
**		$p < 0.05$	$p < 0.05$
†		20.5 %	-0.01 %
‡	-26.7%	11.7%	-25.4%

* = treated Vs control group, ** results on day 5 Vs day 20 in the same group. † = percentage of change between each treated group Vs control group, ‡ = percentage of change between day 5 and day 20 of the same group.

These findings suggest that serum lipids parallel to the activity of the CYP-450 enzyme system, so that treatment with a CYP450 inducing agent increases lipids, and upon withdrawal of the drug lipids return to the baseline [12].

In the present study the drugs that had been chosen has different effect on CYP450 from enzyme induction (CBZ) to enzyme inhibitor (lamotrigine) and so it is suspected to differ in their effect on lipid profile. Research found that AEDs which induces the CYP450 will alter the metabolism in a variety of ways that may become apparent when the patients are started on or taken off these drugs [13].

Mintzer et al [14] covered this possible effect in his researcher he found that patients switched from enzyme-inducing agent phenytoin to the non-inducing drugs levetiracetam will experience a sizable drop in serum lipids, along with changes in other serologic indices of vascular risk.

Other researcher Abou-Khalil [15] found that epileptic patients switched from an enzyme inducer (CBZ) to non-inducer drug like lamotrigine shows significant increase and produces rapid and clinically significant amelioration in several serological markers of vascular risk.

Compared to the control group, CBZ-treated patients may have higher lipid levels than controls; at least one such study measured before and after treatment found that CBZ produces significant elevation of lipids in blood [11].

Studying the correlation between CBZ and high lipid profile level and its relation to lipid profile and so on atherosclerosis has been done also by Brämswig et al [16]. The investigators concluded that significant lipid alterations were observed in CBZ-treated patients with epilepsy in comparison with healthy control subjects.

These results compatible with the results obtained in the present study as it was found that CBZ-treated group showed significant

increase in blood TC, TG, LDL, and HDL levels both in cases of day 5 versus day 20 results of the study and when comparing the results with the control group in normal and diabetic rabbits.

On other hand, lamotrigine exhibited less effect on lipid profile, in non-diabetic treated groups it showed a non-significant change in lipids levels in compared to the control group.

In the diabetic-treated group, it showed a significant increase in TC, TG, HDL levels from day 5 to day 20 in comparison to the same group where as in comparison to the positive control group it showed non-significant increase in all lipid profiles levels. These results reflect no significant effect of lamotrigine in both normal and diabetic rabbits while the increase in diabetic group from day 5 to day 20 was similar to the increase in the positive control group this estimate that the increase is due to the effect of diabetes on lipid profile rather than the effect of drug itself.

We conclude that AEDs that possess an enzyme inducing effect as CBZ tend to have high lipids profile in comparison to lamotrigine (AEDs with no enzyme induction effect). This is very important in both normal and diabetic condition as AEDs can be given to non-diabetic patient or to diabetic one for treatment of neurological and psychological diseases. These effects could be vital for patient suffering from other diseases as cerebrovascular accident, angina, heart failure and hyperlipidemia

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Author contribution
Dr. Abdul-Bari collected the data and analyzed it; Dr. Al-Jawad arranged it and supervised the study; and Dr. Kadhim interpreted and arranged drafting of this paper.

Conflict of interest
There is no conflict of interest that could be perceived.

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Evaluation of Leptin in Sera and Follicular Fluids of Infertile Women Undergoing Intra-Cytoplasmic Sperm Injection and Their Effects on Pregnancy Outcome

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Abstract

Background   Leptin may serve as the critical link between the body's adipose tissue and the hypothalamo-pituitary axis, thus it is considered a possible link between nutrition and reproduction.

Objective   To evaluate leptin in the serum and follicular fluid and its effect on fertilization rate and pregnancy outcome in infertile female patients undergo intra-cytoplasmic sperm injection cycle.

Methods   Seventy four infertile women who agreed to participate in the study were selected randomly from those attending the Fertility Centre in Al-Sader Teaching Hospital, Holly Najaf. Hormonal analysis was done for serum and follicular fluid leptin hormone at the day of ovum pickup.

Result   Serum and follicular fluid leptin hormone levels were increased above its normal cutoff level according to the kit used. There was a relationship between the fertilization rate and leptin in serum and follicular fluid. Fertilization rate was significantly ($p = 0.049$) different depending on the serum leptin level while it has nothing to do with follicular fluid leptin hormone. On the other hand, follicular fluid leptin significantly ($p = 0.04$) affect pregnancy outcome.

Conclusion   Leptin provides possible impact on oocyte and/or embryo quality leading to impaired endometrial bed preparation that may be involved in pregnancy failure.

Keywords   Leptin, ferilization rate, pregnancy outcome.

List of abbreviation: BMI = body mass index, IVF = in vitro fertilization, ICSI = intracytoplasmic sperm injection, OR = ovarian response, FF = follicular fluid, FR = fertilization rate, hCG = human chorionic gonadotrophin, FSH = follicle stimulating hormone, LH = luteinizing hormone, E2 = estradiol, PN = pronuclei, ET = embryo transfer, PR = pregnancy rate, ART = assisted reproductive technologies.

Introduction

Fertility is strongly dependent on the presence of a critical amount of total fat [1]. It is known that a drastic reduction in the size of the adipose reserve, as it happens in over trained athletes or in pathological situations, is associated with amenorrhea and infertility, and this association persists until the body mass index (BMI) returns to normal values [2]. This was classically interpreted as the result of the existence of a permissive signal produced by the adipose tissue: the "critical weight hypothesis," originated from the observation that the age at menarche is more closely related to body weight than to chronological age [3,4].

Despite its primary role in the regulation of body weight, it is becoming clear that leptin exerts widespread and unanticipated actions on other endocrine systems, linking the adipose tissue with the hypothalamus and exerting a regulatory role on many

neuroendocrine systems, such as the growth hormone axis, the thyroid hormone axis, and the reproductive axis [5,6]. Leptin receptors and leptin mRNA have been identified in the human hypothalamus and ovary, and leptin mRNA and protein production have been discovered in ovarian granulosa cells, oocytes, and early cleavage stage embryos [7,8].

During in vitro fertilization (IVF) /intra-cytoplasmic sperm injection (ICSI), a high relative leptin increase is associated with adiposity and a reduced ovarian response (OR) [9]. These observations support the possibility that high leptin concentrations might reduce ovarian responsiveness to gonadotropins. Leptin might explain in part why obese individuals require higher amounts of gonadotropins than lean subjects to achieve ovarian hyperstimulation; thus, leptin is considered a possible link between nutrition and reproduction [4].

Many studies have demonstrated adverse effects of leptin on IVF outcomes, including inhibition of ovarian follicular development and steroidogenesis [10,11]. However, some have reported no adverse effects [12,13]. Therefore, up to the present, the research community has failed to reach consensus on this issue.

The aim of our study is to study the level of serum and follicular fluid (FF) leptin hormone and its relation to fertilization rate (FR) and pregnancy outcome in infertile patients undergo ICSI cycle.

Methods

A hospital-based cohort study was conducted to determine the levels of leptin with FR in ICSI cycle. Seventy four infertile women agreed to participate in this research were selected randomly from those attending the Fertility Centre in Al-Sader Teaching Hospital, Al-Najaf Holly City during the period from March 2013 to November 2013. The study was approved by the Institute Review Board of the College of Medicine, Al-Nahrain University.

The mean age for infertile female patients was 31.41±5.45years. Infertility due to a female cause was present in 39 (52.7%) and to a male cause in 35 (47.3%) of the cases. Primary infertility was present in 57 (77%) whereas only 17 (23%) patients have secondary infertility. The duration of infertility for the entire patients group (8.11±3.94 years).

Those women with visible ovaries on ultrasound (model AMIB7, Canada), no uterine fibroid, uterine anomaly or ovarian cyst measuring ≥ 20 mm in diameter, negative screening tests for hepatitis B and C, as well as for human immune deficiency viruses, inability to achieve pregnancy in a period of ≥ 12 months despite regular unprotected intercourse, and no matter the cause of infertility was female or male factor were selected for the study.

All the participants were asked to come back on cycle day 2 for complete medical evaluation.

Treatment protocols and ovarian monitoring

Single injection of gonadotropin-releasing hormone (GnRH) agonist, Decapeptyl (Diphereline; 0.1 mg, Beaufond, Ispenpharma-France) was administered subcutaneously at CD 2; thereafter, results of follicle stimulating hormone (FSH), luteinizing hormone (LH) and estradiol (E2) were monitored. If FSH serum level below 10 IU/ml, LH serum level below 8 IU/ml and E2 below 50 pg/ml, then the patient follow treatment by short protocol.

Fifty seven infertile patients enrolled in the short induction protocol. They are given 0.1 mg/day of Decapeptyl as a morning dose and purified 75 IU/ml FSH either as highly purified urinary FSH (Metrodin-HD serono, Switzerland) or recombinant DNA technology prepared agent Gonal-f (serono, Switzerland) as an evening dose subcutenously or intra-muscularlly. The dose of FSH in each regimen can be adjusted according to hormonal results, age and response of folliculogenesis.

Seventeen patients who have high FSH, LH or E2 level or with endometrial thickness more

than 10 mm at CD2 follow long type of induction protocol. They administered GnRH agonist, Decapeptyl CR (tiptorelin; Ferring, Germany 3.75 mg/ampule) subcutaneously at cycle day 21 of the menstrual cycle. Then FSH, LH and E2 level is checked 10-14 days after injection or after the cycle started; if FSH < 10 IU/ml and LH < 8 IU/ml, E2 level below 50 pg/ml and endometrial thickness < 5 mm, then Gonal-f or metrodin HP given daily for 6 days under strict indication.

The patients were monitored for follicular growth and endometrial thickness by serial transvaginal ultrasound and serum E2 from the 6th day of stimulation with gonadotropins.

Titration of FSH (upward or downward) is based on the response of folliculogenesis; when at least 2 dominant follicles of 17 mm in diameter in each ovary is ready, ovulation is triggered by hCG-pregnyl 5000-10,000 IU (Serono S.P.H, Italy) intramuscularly, to stimulate the women natural LH surge which stimulate the final growth and maturation of the oocyte. Thirty six hours following hCG trigger, follicular aspiration is done by transvaginal guidance.

Women who develop > 20 follicles measuring > 10 mm in diameter or having E2 level > 3000 pg/ml (ovarian hyper stimulation); in addition, those who develop at least 3 follicles measuring 18 mm following 14 days of FSH treatment, or E2 level < 200 pg/ml, (poor responder) were excluded from the study.

Under general or local anesthesia, ovum pick up was done through transvaginal aspiration usually timed 34-36 hours following hCG [pregnyl 5000-10,000 IU (Serono S.P.H, Italy)] and carried out via ultrasound guidance. Those patients eligible for ICSI cycle were scheduled for oocyte pick up after programmed ovulation induction.

Ten ml of FF samples were collected in plain tubes at the day of ovum pick up between 08:00-10:00 am. The FF first received by the embryologist for oocytes pick up and leaves the reminder fluid for further hormonal assay. At the sametime, 10 ml of venous blood were collected in plain tubes. Both samples left at least 15 minutes at room temperature before centrifugation at 3000 rpm (Rotofix 32A, Germany) for 10 minutes for measurement of leptin hormone (Beckman Coulter, Germany) by sensitive Enzyme Linked ImmunoSorbant Assay technique "ELISA" (BioTek, USA).

Once oocyte was received by the embryologist, denudation after identification, then examination of corona-complex.

After insemination, fertilized oocytes must be examined 16-20 hours for the presence of two round nuclear structures, the male and female pronuclei (PN). Pronuclei must be scored within the appropriate time span, before they merge and are no longer visible. This ensures only normal zygotes with two pronuclei (2PN's) are cultured for embryo transfer (ET). Usually, the laboratory will assign a grade for each embryo to identify the best quality embryos that are then selected for embryo transfer or cryopreservation.

The selection criteria or grading systems must be applied for all cleavage stages from day 2-4 to allow selection of the most viable embryos. Embryo development was evaluated approximately every 24 h. Slow dividing, non-dividing (arrested) or fragmenting embryos are selected against. Compacting embryos on day 3 that have closely apposed cell membranes are selected for. Standard morphological criteria used in evaluating embryo quality include the rate of division judged by the number of blastomeres, size, shape, symmetry, and cytoplasmic appearance of the blastomeres, and the presence of a nucleate cytoplasmic fragment. The quality of each embryo is assessed using the following grading system.

Grade I: Excellent-quality embryos with equally sized blastomeres without or with up to 10% a nucleate fragment.

Grade II: Embryos with 10-20% fragmentation considered good

Grade III: Fair-quality embryos with fragmentations between 20-50%.

GradeIV: Embryos with > 50% fragmentations and with unequally sized blastomeres were said to be poor-quality embryos.

The total number of pregnancies was detected by counting how many ladies have increasing positive serum hCG (more than 20 IU/ml) at least 15 days after ET. Clinical pregnancy was determined by observation of gestational sac with fetal heartbeats on transvaginal U/S 6-7 weeks of gestation. In general, pregnancy rate (PR) was defined as the number of pregnant ladies after ICSI divided by the whole number of patients under went ICSI cycles multiplied by 100.

Data Analysis

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) version 18. Categorical variables were presented as frequencies and percentages. Continuous variables were presented as mean and standard deviation 95% confidence interval. Pearson's correlation coefficient was used to study the relation between two continuous variables. A P value of ≤ 0.05 was considered as significant.

Results

Seventeen (23.0%) infertile patients had positive pregnancy test while 57 (77%) showed negative pregnancy test. Forty eight (64.9%)

patients have a FR of ≥ 50 % and 26 (35.1%) have FR of < 50% while the mean FR was 0.61±0.30 (Table1).

Leptin level was ≥ 7.36 ng/ml in 79.1% and < 7.36 ng/ml in 20.9% of infertile patients.

Table 1 and fig. 1 illustrate the relationship between the FR and leptin in serum and FF. FR was significantly (p = 0.049) different depending on the serum leptin level while it has nothing to do with FF leptin hormone. On the other hand, FF leptin significantly (p = 0.04) affect pregnancy outcome.

Discussion

In our study, the effects of serum and FF leptin levels on pregnancy outcomes were evaluated in short protocols of assisted reproductive technologies (ART). Both serum and FF leptin hormone levels were increased. These findings were in harmony with the results of Al-Bderi [14] who demonstrated that serum and FF leptin hormone levels were significantly high in infertile women. On the other hand, FF leptin levels were lower than simultaneous serum leptin levels, which contradict the findings of Ergenoğlu et al [6]. Also we could not establish a relationship between serum leptin levels and pregnancy outcomes, similar to Gürbüz et al [15] and Ergenoğlu et al [6] but contrary to Yang and Huang [16].

Table 1. The differences in fertilization rate by serum and follicular fluid leptin hormones

| Leptin hormone		No.	Fertilization Rate (Mean ± S.D)	t-test	*P* value
Serum	≥ 7.36 ng/ml*	9	0.44 ± 0.28	2.115	0.049*
	< 7.36 ng/ml	34	0.66 ± 0.31		
Follicular fluid	≥ 7.36 ng/ml	14	0.66 ± 0.35	0.651	0.518
	< 7.36 ng/ml	29	0.59 ± 0.30		
Leptin hormone		No.	Pregnancy outcome (Mean ± SD)	t-test	*P* value
Serum	Positive	11	16.14 ± 7.23	0.445	0.659
	Negative	32	14.75 ± 9.44		
Follicular Fluid	Positive	11	8.47 ± 3.24	2.122	0.040*
	Negative	32	13.45 ± 7.50		

* cutoff point of hormone according to kit used
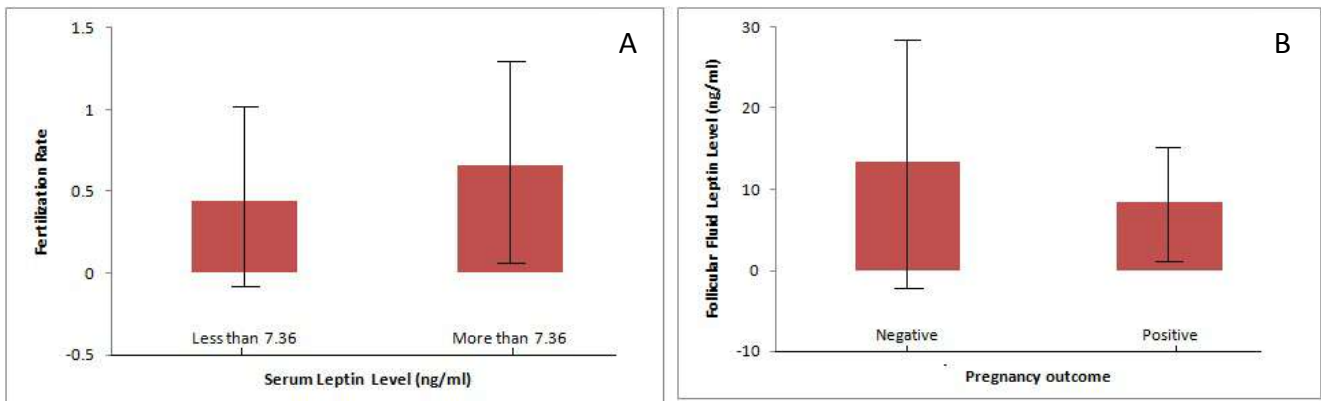
Fig. 1. (A) Relation of serum leptin level to fertilization rate and (B) follicular fluid leptin to pregnancy outcome

One of the major highlights of the present findings includes adverse impact of high FF leptin on pregnancy outcome. These patients who became pregnant from ICSI had lower mean FF concentrations of leptin than patients who did not become pregnant. This is probably due to that leptin can affect follicular development through a central (hypothalamic-pituitary) and end organ (ovary and endometrium) effects [17]. Our findings were also reported by Mantzoros et al [18]; Brannian et al [19] and Chakrabarti et al [1] but in contrast to the findings of Chang et al [20] who demonstrated that the FF leptin concentration is not significantly related to oocyte maturity and corresponding embryo development.

Additionally, Anifandis et al [11] considered the possible mechanisms by which leptin affects fertility and reduce pregnancy success are direct inhibitory action of high leptin levels on the ovaries that lead to ineffective follicular maturation,embryo quality and response. However, others failed to prove those associations [21,22].

As well, during IVF\ICSI, relatively high leptin is associated with adiposity, reduced OR, and low PR [3,4]. Some reports indicated that elevated serum and FF leptin levels may be used as predictive markers of ART failure [18]. In contrast, Agarwal et al [23] considered blood and FF leptin as non-suitable markers of oocyte maturation, embryo quality or ICSI outcome.

Taken together, these observations provide possible indication of the impact of elevated leptin on oocyte and/or embryo quality leading to impaired endometrial bed preparation that may be involved in pregnancy failure in women with elevated leptin response and consequent pregnancy outcome. The small population size limits the statistical power to judge the precise correlation.

In the present study, the effects of serum and FF leptin levels on pregnancy outcomes were evaluated in short protocols of assisted reproductive technologies (ART).

Both serum and FF leptin hormone levels were increased above its normal level. These findings were in harmony with the results of Al-Bderi [14] who demonstrated that serum and FF leptin hormone levels were significantly high in infertile women. On the other hand, FF leptin levels were lower than simultaneous serum leptin levels, which contradict the findings of Ergenoğlu et al [6]. Also, establish a relationship between serum leptin levels and pregnancy outcomes, similar to Gürbüz et al [15] and Ergenoğlu et al [6] but contrary to Yang and Huang [16].

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Author contribution
Dr. Farhood collects the data and follow the patients, Dr. Hamdan and Al-Salihi supervise the study and write the paper and revise it.

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

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Serum HDL in Patients with Ischemic Stroke - A Case Control Study

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Abstract

Background   Elevated high-density lipoprotein cholesterol (HDL-C) levels have been shown to be protective against cardiovascular disease. However, the association of specific lipoprotein classes and ischemic stroke has not been well defined.

Objectives   To evaluate the association between HDL-C and ischemic stroke in men and women, and to compare the results with a control sample of the same age group.

Methods   A hospital based case-control study was done comparing serum HDL-C levels in 48 ischemic stroke patients to 50 controls recruited from Al-Imamain Al-Kadhemain Medical City, all patients and control had negative past medical history, negative history for smoking and alcohol. Serum lipid profile was determined in all of them.

Results   This study showed that regarding the serum HDL-C levels, 41 (85.4%) patients were found to have low levels (less than 40 mg/dl) while among the control group, only 19 (38%) have shown low serum HDL-C level.

Conclusion   The study showed that there is a significant association between the low level of serum HDL-C cholesterol and the risk of ischemic stroke in this population.

Keywords   cholesterol, lipoproteins, HDL-cholesterol, ischemic stroke

List of Abbreviation: HDL-C: High-density lipoprotein-cholesterol, VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, CVD: cardiovascular disease.

Introduction

Stroke is the third most common cause of death in developed world after cancer and cardiovascular disease (CVD). It is the most common cause for severe physical disability [1]. Stroke is defined by the world health organization as the clinical syndrome of rapid onset (usually seconds or minutes) of focal (or global, as in subarachnoid hemorrhage) cerebral deficit, lasting more than 24 hours or leading to death, with no apparent cause other than a vascular one [2].

Strokes can be classified into two major categories: ischemic and hemorrhagic [3]. Ischemic strokes are those that are caused by interruption of the blood supply [4]. Eighty percent of total strokes in the United States are due to an ischemic event which could be thrombosis, embolism or systemic hypoperfusion [5].

Hyperlipidemia or hyperlipoproteinemia involves abnormally elevated levels of any or all lipids and/or lipoproteins in the blood [6]. Fatty acid consumption was associated with the risk of stroke, in general, long-chain saturated fatty acids (14 or more) tend to increase risk for CVD and cerebrovascular accident [7].

High-density lipoprotein cholesterol (HDL-C) is one of the five major groups of lipoproteins, and it is the smallest in the molecular size. HDL-C carries many lipid and protein species, several of which have very low concentrations but are biologically very active. HDL-C help to inhibit oxidation, inflammation, activation of the endothelium, coagulation, and platelet aggregation. All these properties may contribute to the ability of HDL-C to protect from atherosclerosis [8].

There is overwhelming evidence for a strong independent, inverse relation between levels of HDL-C and coronary heart disease [9]. However, their association with cerebrovascular disease is not clear. Indeed, HDL-C is not usually mentioned as a risk factor for ischemic stroke [10]. The association between coronary artery disease and cerebrovascular disease can be ascribed to a common pathophysiological antecedent, atherosclerosis. Many patients with clinically apparent or silent myocardial ischemia have coexistent cerebrovascular disease.

Atherosclerotic lesions tend to develop first in the aorta, then in the coronary arteries, and later in the cerebrovascular and peripheral circulation [11]. Serum lipid levels have been related to carotid artery atherosclerosis in a variety of ultrasonographic and angiographic studies [12], but their relation to stroke is unclear. A negative association between HDL-C levels and risk of stroke or transient ischemic attacks has been found in several, although not all, case-control studies [13].

The purpose of this study was to examine the association of specific lipoprotein class (serum HDL) and ischemic stroke among men and women.

Methods

It is a case control study held at Al-Imamain Al-Kadhemain Medical City to evaluate the association between serum HDL-C levels and the occurrence of ischemic stroke.

From November 2013 to March 2015, 48 patients aged 43 to 89 years (21 males, 27 females) admitted to the Department of Medicine at the Al-Imamain Al-Kadhemain Medical City in Baghdad for acute ischemic stroke with negative past medical history were included in this study.

All of them presented with a focal neurological deficit of sudden onset. Transient ischemic attacks (complete regression of all neurological deficits in less than 24 hours) and strokes were both included.

Patients with vascular risk factors other than hyperlipidaemia were excluded from entry to the study by asking patients whether a doctor had ever told them that they had hypertension, angina or myocardial infarction (heart attack, coronary thrombosis), previous stroke, diabetes, or other disorders. They were also asked for details of any regular medical treatment including antihypertensive treatment, 12-lead ECG was recorded at rest. Serum lipid profile was determined in all patients.

Patients with hemorrhagic transformation were excluded during data collection.

Clinical examination and cerebral CT scan without contrast injection were performed in all of them after admission.

These information were obtained from the patients themselves if they were able to communicate or from their relatives if they were unable to talk.

Fifty healthy persons of the same age group from staff of the hospital were randomly recruited during the same period, asked whether a doctor had ever told them that they had hypertension, angina or myocardial infarction (heart attack, coronary thrombosis), stroke, diabetes, or other disorders. They were also asked for details of any regular medical treatment including antihypertensive treatment, Serum lipid profile was determined in all of them.

Data Analysis

Lipid profile was done for them for the purpose of this study; a serum sample after 12 hours of overnight fasting was taken to the lab for the

measurement the Total serum cholesterol, triglycerides, and HDL-cholesterol, using enzymatic colorimetric method.

Statistical Analysis

Analysis of data was carried out using the available statistical package of SPSS-17 (Statistical Packages for Social Sciences-version 17 "PASW" Statistics) for determination of statistical significance among different variables. A descriptive statistics like mean together with analytic statistics, have been done when appropriate. A *P* value of less than 0.05 was considered as significant.

Results

The study included 48 (patients with ischemic stroke and negative past medical history), and 50 (healthy persons served as control).

The frequency of ischemic stroke among patients was more in females 27 (56.25%), with only 21 male patients (43.75%), while the control group was chosen with equal male: female ratio (Table 1).

The frequency of ischemic stroke was also more frequent among the age older than 65 years with 35 cases (72.9%) while 13 cases (27%) were found to be younger than this age, In the control group the age difference was less prominent with 26 patients (52%) were older than 65 years and 24 patients (48%) younger than this (Table 2).

Regarding the serum HDL-C levels, 41 (85.4%) patients were found to have low levels (less than 40 mg/dl) while among the control group, only 19 (38%) have shown low serum HDL-C level (Table 5, Figure 1).

Table 1. Gender distribution among cases and control groups

Sex	Cases	%	Controls	%	*P* value
Male	21	43.75%	25	50%	0.5
Female	27	56.25%	25	50%	

Table 2. Age distribution in the cases and control groups

Sex	Cases	%	Controls	%	*P* value
> 65 years	35	72.9%	26	52%	0.03
≤ 65 years	13	27%	24	48%	

Table 3. The relationship between age and sex among cases

Sex	Male	Female	Total	*P* value
> 65 years	14	21	35	
≤ 65 years	7	6	13	0.39
Total	21	27	48	

Table 4. The frequency of the presenting symptoms among cases

Symptoms	Frequency	Percentage
Left sided weakness	16	33.0%
Right sided weakness	20	41.6%
Slurred speech	12	25.0%

Table 5. The serum HDL-C levels among cases and control groups

Serum HDL	Cases	%	Controls	%	*P* value
≤ 40 mg/dl	41	85.4%	19	38%	
> 40 mg/dl	7	14.5%	31	62%	< 0.0001

Odd Ratio= 9.56
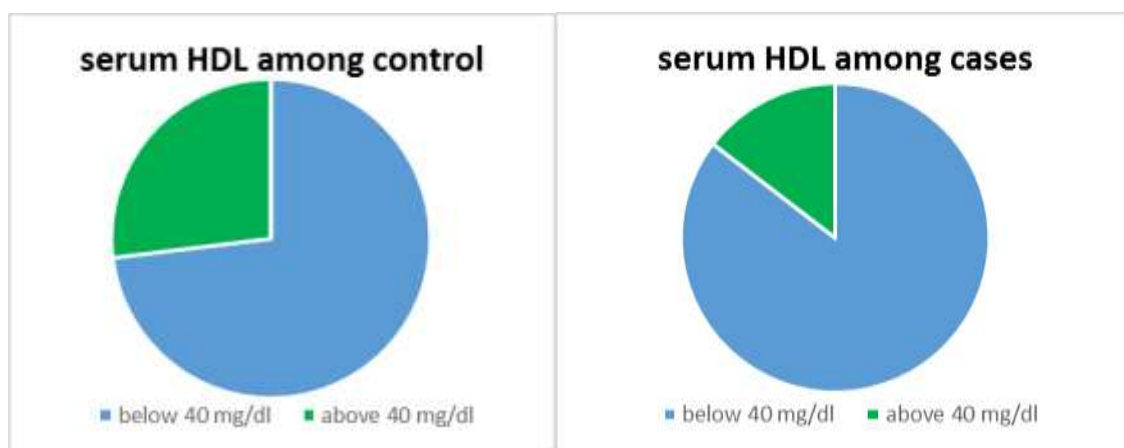
Figure 1. HDL-C level

Discussion

It has been shown in this case-control study, which is done on 48 ischemic stroke patients with negative past medical history and 50 control persons that the number of female patients was 27 compared to 21 male patients but this sex difference among cases wasn't statistically significant. It could be due to the small sample examined in this hospital based study since previous studies have indicated that Men have a higher risk for stroke. It has also revealed that 35 cases were older than 65 years while only 13 were younger than this age, and this is statistically significant difference among age group between patients with ischemic stroke and the control group ($P = 0.03$)

These results were consistent with Senelick et al that showed that 95% of strokes occur in people aged 45 years and older and two-thirds of strokes occur in those over the age of 65 years [14]. They are also consistent with Al-Mahdawi et al study in which there was statistically significant difference among age group older than 55 years with ischemic stroke ($P = 0.04$), this indicates that old age is an important risk factor in the development of stroke [15].

Regarding the level of serum HDL-C, this study showed that 85.4% of the patients were having

low levels while only 38% of the control group were having such results taking into consideration that the control group were healthy, this may point to the importance of serum HDL-C levels in patients with ischemic stroke. The Northern Manhattan Stroke study has demonstrated a protective effect of greater HDL-C level for ischemic stroke in an elderly, multiethnic population of men and women and that HDL-C levels showed more protection for atherosclerotic stroke than non-atherosclerotic infarction but was significantly protective against both subtypes [16]. The Veterans Affairs–High density lipoprotein cholesterol Trial (VA-HIT) showed an independent protective effect of HDL-C level for stroke and other vascular outcomes over the 5 years of the study [17].

Prospective cohort studies generally support an inverse association between HDL-C levels and the risk of ischemic stroke [18-20] and this was also consistent with the findings of the inverse association between HDL-C and the risks of total and ischemic stroke in both men and women in Yurong Zhang et al study [21].

However, the relationship between abnormalities of serum lipids and stroke has been less clear than for coronary artery disease [22]. Some prospective cohort studies including the Framingham Heart Study have found no association between total serum cholesterol or HDL-C level and cerebral infarction [23]; this could be because many of those studies did not differentiate between cholesterol components and the different stroke subtypes [24].

In this hospital based case-control study, there is a significant association between the occurrence of ischemic stroke and the low levels of serum HDL-C cholesterol.

Author Contribution
Dr. Al-Khazraji: research idea & discussion. Dr. Mudhahir: cases collection & writing. Dr. Hassan: reviewing of literatures of this & other researches.

Conflict of Interest
The authors disclose no any financial & personal relationships with other people or organizations that inappropriately influence (bias) outwork.

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HLA-DRB allels, IL-10 and Vitamin D level: Potential Impact on Multiple Sclerosis

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Abstract

Background	Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease with unknown etiology. Variation in the HLA-DRB1 gene is the potent genetic risk factor for promoting MS. Other agents relate with an increased risk of developing MS include the cytokines levels such as interleukin-10 (IL-10) and the vitamin D (vit. D) deficiency.
Objectives	To study a possible role of HLA-DRB1, vit. D deficiency as a risk factor for MS development and to estimate the level of IL-10 in the serum of MS patients and its role in disease initiation or progression.
Methods	Sixty MS patients, of them 30 were newly diagnosed with an age range between 13 and 58 years were included in this study, in addition to thirty healthy volunteers their gender and age matched with patients group serve as a control group. Blood samples collected to assess serum levels of vit. D and IL-10 by Enzyme-Linked Immunosorbant Assay (ELISA) and for DNA extraction, which used in the HLA-DRB1 2 digit genotyping.
Results	The HLA-DRB1 genotyping revealed that the HLA-DRB1*15 frequency was higher but statistically insignificant in the MS patients as compared with healthy control group. IL-10 level was significantly lower in MS patients on treatment than the control group. On the other hand, vit. D in the newly diagnosed MS patients was significantly different from the control group (higher in the control group), but there was no variance with MS patients on treatment, the level of vit. D in the studied groups was less than global value.
Conclusion	In MS patients the frequency of HLA-DRB1*15 was higher than control group but the difference was not significant. In addition, the level of IL-10 and vit. D may have a role in the development of MS.
Keywords	Multiple sclerosis, human leukocyte antigen, IL-10

List of Abbreviation: MS = multiple sclerosis, HLA = human leukocyte antigen, IL-10 = interleukin 10, vit. D = vitamin D.

Introduction

Multiple sclerosis (MS) is a complex autoimmune disease, not referable to a single genetic or environmental factor [1]. Several diseases with features common to MS are associated with certain human leukocyte antigen (HLA), especially autoimmune diseases [2]. HLA system provides a set of genetic loci their proteins play important role in immune response. Several studies indicated an association between the alleles of the HLA-DRB1 and MS. In northern European-descended populations, association with this gene was identified only within families that carried the HLA DRB1*1501 allele [3]. The family history of MS is a strong known risk factor, individual has an approximately 1-in-750 (0.1%) chance of developing MS. The first-degree relatives of the person with MS have approximately 2.5% to 5% risk factor to develop MS, while the identical twins of

patients with MS have a 25% chance of developing the disease [4].

Since MS is an autoimmune disease, there may be important role of cytokines such as interleukin-10 (IL-10) in MS development or course of the disease. High levels of Th1 cytokines are mostly obvious during experimental autoimmune encephalomyelitis and MS relapse, but there are high levels of Th2 cytokines during remission in MS patients [5].

Vitamin D (vit. D) can modulate the innate and adaptive immune responses where its deficiency is associated with increased autoimmunity as well as an increased susceptibility to infection. There is growing epidemiologic evidence linking vit. D deficiency and autoimmune diseases like MS, diabetes mellitus, and rheumatoid arthritis (6).

The objective of this study was to study a possible role of HLA-DRB1, vit. D deficiency as a risk factors for MS development and to estimate the level of IL-10 in the serum of MS patients and its role in disease initiation or progression.

Methods

A present case control study involved sixty MS patients with an age range from 13 to 58 years who were seeking treatment or those attending for the diagnosis in the MS Outpatients' Clinic at Baghdad Teaching Medical City in the period from December 2014 to March 2015. The diagnosis of each case was established according to MC Donald criteria done by a neurologist and confirmed by MRI and sometime by oligo-clonal band test in the CSF.

Patients were divided into two groups; group I on treatment and group II as newly diagnosed patients. This study was approved by the Institutional Review Board of the College of Medicine, Al-Nahrain University, and all samples were obtained with informed consent in accordance with the Teaching Hospital of Medical City declaration.

Five ml of blood were collected from each patient and control, divided into 3 ml for serum separation and 2 ml for DNA extraction then stored at -20 °C until used.

DNA extraction:

DNA Extraction Kit (Genaid) is optimized for genomic, mitochondrial and virus DNA purification from whole blood (fresh blood and frozen blood) and tissue.

Detection of vit. D in the serum (Eurouimmun – Germany)

This ELISA test kit is designed for the in vitro determination of 25-OH vit. D in human serum or plasma samples.

Detection of human IL-10 (Ray Bio – USA)

Human IL-10 ELISA kit is an in vitro enzyme-linked immune-sorbentassay for the quantitative measurement of human IL-10. This assay employed an antibody specific for human IL-10 coated on a 96- well plate. Standards and samples are pipetted into the wells and IL-10 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human IL-10 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of IL-10 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

HLA-DRB1 genotyping (Fujirebio – Belgium)

The HLA-DRB1 genotyping is done by INNO-LIPA HLA-DRB1 amplification plus kit and INNO-LIPA HLA-DRB1 plus kit (which is line probe assay) by single sequencing probe, PCR-SSO method.

Statistical analysis

Analysis of data was carried out using the available statistical package of SPSS-22

(Statistical Packages for Social Sciences- version 22). Statistical significance was considered whenever the P value was equal or less than 0.05. The significance of difference of different means (quantitative data) were tested using Students-t-test for difference between two independent means or Paired-t-test for difference of paired observations (or two dependent means), or ANOVA test for difference among more than two independent means. The significance of difference of different percentages (qualitative data) was tested using Pearson Chi-square test with application of Yate's correction or Fisher Exact test whenever applicable.

Result

The mean age of patients was 34.5 ± 10.6 years, and there was female predominance among patients, there were 35 (58.3%) of MS patients females, while 25 (41.7%) were males. Regarding distribution of patients according to family history the current data showed that (10.0%) of patients had positive family history of MS. No statistically significant association (p > 0.05) in age or gender existed between patients and control group.

The MS patients were distributed on three subgroups according to type of the disease, 55 (91.6%) of patients were with relapsing remitting type and 3 (5%) of patients were with secondary progressive type while 2 (3.3%) of patients with primary progressive type. The HLA genotyping was done for (48) patients and (30) controls, the result referred to considerable difference (but not significant $P > 0.05$) between the patients and control regarding DRB1 *15, table (1), 12 out of 48 patients were DRB1 *15 positive and 3 out of 30 controls were positive (P value 0.102). The DRB1 *11 was high in both groups (18 in MS group and 11 in control) but no significant difference between them. Also DRB1 *13 was high in the 2 groups without significant difference (12 in MS 11 in controls).

Table 1. HLA-DRB1 genotypes distribution among cases and controls

Allel	Multiple sclerosis		Control		OR	95% CI for OR	*P* value
	Positive	%	positive	%			
DRB1*01Allel	2	4.2	2	6.7	0.609	0.081-4.568	0.626
DRB1*03Allel	14	29.2	13	43.3	0.538	0.208-1.397	0.201
DRB1*04Allel	10	20.8	4	13.3	1.711	0.484-6.044	0.401
DRB1*06Allel	-	-	1	3.3	-	-	-
DRB1*07Allel	11	22.9	7	23.3	0.977	0.331-2.880	0.966
DRB1*08Allel	3	6.3	1	3.3	1.933	0.192-19.49	0.570
DRB1*09Allel	2	4.2	-	-	-	-	-
DRB1*10Allel	2	4.2	2	6.7	0.609	0.081-4.568	0.626
DRB1*11Allel	18	37.5	11	36.7	1.036	0.403-2.666	0.941
DRB1*13Allel	12	25.0	11	36.7	0.576	0.214-1.548	0.272
DRB1*14Allel	2	4.2	2	6.7	0.609	0.081-4.568	0.626
DRB1*15Allel	12	25.0	3	10.0	3.0	0.770-11.69	0.102
DRB1*16Allel	2	4.2	-	-	-	-	-
Blank	6		3				

OR = odd ratio, CI= confidence interval of OR

The present study showed significant decrease ($P < 0.05$) in the serum level of IL-10 among MS patients (1.49 pg/ml) as compared with healthy control group (1.71 pg/ml). Similarly, the vit. D

level was insignificantly less in patients group (13.02 ng/ml) as compared to 15.31 ng/ml of the control group (Table 2). Considering the IL-10 level, the current result revealed no significant differences between healthy controls and newly diagnosed MS patients, while it was significantly less (P = 0.009) in the treated MS patients versus healthy control group. Similarly, its level was significantly decreased (P = 0.028) in the treated versus

newly diagnosed MS patients. Interestingly, comparing the three studied groups showed significant difference in mean of vitamin D level between control group and newly diagnosed patients (Table 3).

Likewise, no significant differences between healthy control and MS patients on treatment. In addition, the present study showed no significant correlation between IL-10 and vit. D, (Table 4).

Table 2. Interleukin-10 and vitamin D levels in patients with multiple sclerosis and control group

Parameter	Multiple sclerosis N = 60			Control group N = 30			*P* value
	Mean ± SD	SE	Range	Mean ± SD	SE	Range	
IL-10 (pg/ml)	1.49±0.40	0.054	0.60-3.10	1.71±0.56	0.114	1.10-3.40	0.049*
Vit. D (ng/ml)	13.02±5.89	0.794	4.50-35.50	15.31±6.01	1.203	4.60-31.00	0.114

IL-10 = interleukin-10, Vit. D = vitamin D, *Significant difference using student-t-test between two independent means at 0.05 level

Table 3. Interleukin-10 and Vit. D levels in the serum of MS sub groups and controls

Group	IL-10 (pg/ml)			Vit. D (ng/ml)		
	Mean ± SD	SE	Range	Mean ± SD	SE	Range
Newly MS	1.60±0.44	0.080	1.10-3.10	11.04±4.32	0.831	4.50-18.80
Treated MS	1.37±0.32	0.062	0.60-2.30	14.93±6.61	1.248	5.20-35.50
Control group	1.71±0.56	0.114	1.10-3.40	15.31±6.01	1.203	4.60-31.00
P value		0.009*			0.005*	
P value		0.028‡			0.013‡	
P value		0.022†			0.014†	

IL-10 = interleukin-10, MS = multiple sclerosis, * = significant difference using student-t-test comparing treated MS patients with the control group, ‡ = significant difference using student-t-test comparing treated MS patients versus newly diagnosed MS patients, † = significant difference using ANOVA test among three independent means.

Table 4. Correlation between IL-10 and vitamin D in MS subgroups

Group	r	p
MS (n=60)	-0.110	0.434
Newly MS (n=30)	-0.051	0.801
Treated MS (n=30)	-0.011	0.957

* IL-10 (pg/ml), vit. D (ng/ml)

Discussion

Current result revealed higher frequency of DRB1 *15 allele in MS group as compared with control, the difference was statistically not significant but considerable which is agreed

with Chao et al [7], Hossein et al [8] and disagree with Disanto et al [9]. As it is known the common HLA alleles are different between the populations depend on the ethnic group, migration and other factors. The allele

frequency of HLA-DRB1*15 is found to be around 33-36% among Norwegian MS patients, significantly higher than control [10]. In most European studies on MS populations there is an association to the HLA-DRB1*1501 allele [11]. It has been suggested that there is another HLA DRB1 allele associated with the increase susceptibility for MS, the most determined additional risk allele is HLA-DRB1*03 [12]. In Sardinia, the HLA-DRB1*03 is significantly associated with MS while HLA-DRB1*1501 has a low frequency [13]. It is estimated that the HLA class II association accounts for 20-60% of the genetic susceptibility in MS [14].

In addition, the current results revealed that the IL-10 concentration in treated MS patients was significantly less than healthy control, this result correspond with other studies Hasheminia et al [15] and Inoge et al [16]. The level of IL 10 in treated MS patient with interferon beta was less than of newly diagnosed MS patients this result agree with Inoge et al [16] and disagree with Oèzenci et al [17] who referred that the untreated patients had lower numbers of IL-10 secreting blood MNC(mono nuclear cell) compared with the control group. Dimisianos et al [18] referred that the IL-10, IL-4 and IL-6 not affected by IFNβ treatment, so, the significantly decrease of IL-10 in the treated patient may be not due to the treatment, but may be during the disease progression there will be depletion in the IL-10 which lead to relapses recurrence. Peelen et al [19] mentioned that IL-10 not affected with treatment in MS patients.

Interleukin 10 was first identified as a molecule that limits inflammation and supports humeral immune responses. IL-10 deficient animals develop lethal inflammation of the intestine, which can be relieved by ectopic administration of IL-10. Deficiency or aberrant expression of IL-10 can enhance inflammatory response to microbial challenge but also lead to development of a number of autoimmune diseases [20]. IL-10 has been also implicated in a number of other inflammatory animal models, including experimental autoimmune encephalomyelitis [21]. Thus, impaired IL-10 expression or signaling can enhance clearance of pathogens during an acute infection but also exaggerate inflammatory response resulting in exacerbated immunopathology and tissue damage [22].

Other studies have shown that increased IL-10 levels in spinal cord correlate with EAE remission, and exogenous administration of IL-10 effectively ameliorated EAE when targeted directly to the CNS. Similarly, lower production of IL-10 in humans appears to be a risk factor for MS, as accumulative data have shown that MS patients had lower IL-10-secreting T cell frequency than controls. Overall, experimental findings indicate that IL-10 has an important disease suppressor function in both EAE and MS [23-24].

In the present study, the level of vit. D in serum of patients and healthy controls was less than 30 ng/ml except one patient and one control. There was no significant deference's between healthy group and patients this result consistent with Al-Mahdawi et al [25]. Grau-López et al [26] showed no differences in the level of vit. D in healthy control and patients in summer and no relationships were found between plasma 25(OH) D concentrations and clinical or radiological variables. Present result disagree with Van der et al [27] and Soilu-Hänninen et al [28] who mention that the serum levels of vit D were significantly lower in patients than in controls .

Its worthy to mention that the level of vit. D in serum of newly diagnosed patient was significantly less than the healthy control. The explain of this result one of two probability: the first one is that the newly diagnosed patients usually have the first relapse maximally before 4-6 weeks and a lot of studies refer to that the concentration of the vit. D decreased during and after short period of the relapse according to Al-Mahdawi et al [25]. Also researchers working in Tasmania reported an inverse relationship between the relapses rate and estimated serum 25(OH) D [29].

Second explanation that the low level of vit. D is one of the environmental risk factors, which involved in the initiation of the disease, and the level of the vit. D in MS patients on treatment more than newly diagnosed patients may due to their listening to their doctor's advising to improve their nutrition or due to consumption of some of the nutritional complementary, which contain vit. D.

Many reports of vitamin D deficiency predicting development of autoimmune disease in the future have been published for MS, and other autoimmune disease like diabetes mellitus and rheumatoid arthritis [30].

Also in current study, there was a significant relationship between low levels of vit. D and the gender, the result revealed that the women were significantly lower than men in the serum level of vit. D; this agree with Issa, 2007 [31] who found that the vit. D level is significantly lower in women's serum also found variation in vit. D level among seasons. This work is disagreeing with Johnson et al [32]. This difference in vit. D levels between men and women in Iraqi population may due to social religious reasons and behaviors which is impose on women to cover all the body and as a result they will exposed to the sun light less than men, or due to repeated pregnancy which is led to vit. D depletion in the body. Also there is another explanation; that the difference is due to the normal physiology of males and females, the hormonal changes during the normal Menstruation may play a role.

In conclusion, the result of current study revealed that the HLA-DRB1*15 in MS patients was common but its cant be considered as a susceptible allele. As well as the IL-10 deficiency may play an important role in MS disease progression and its continuous deficiency may lead to increase disease activity. In addition, vit. D deficiency may play a role as cofactor for disease initiation or its role may restrict during relapses.

Author Contribution
Dr. Abbas supervisor the study; Dr. Khaliel did the laboratory work and write the article; and Dr. Shaheed examined the patients and consultant.

Conflict of Interest
No conflict of interest

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The Protective Effects of Felodipine on Methotrexate-Induced Hepatic Toxicity in Rabbits

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Abstract

Background	Methotrexate is folic acid antagonist, used in the therapy of various types of diseases. Oxidative stress and inflammation have the major role in methotrexate toxicity.
Objective	To study the protective effects of felodipine against methotrexate-induced hepatotoxicity in rabbits.
Methods	Twenty four rabbits divided randomly into three groups. Group I was left without treatment, group II received a dose of 20 mg/kg methotrexate (MTX) intraperitoneally from 3rd day for three successive days and group III received a dose of 0.5 mg/kg felodipine orally for 7 days in addition to MTX therapy similar to group (2). On 8th day the following parameters (liver enzymes, liver tissue homogenate of glutathione, malondialdehyde and tumor necrosis factor-α) were monitored. As well as assessment of histological changes on liver tissue sections after scarification.
Results	Administration of felodipine significantly decreased the elevated levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), malondialdehyde and tumor necrosis factor-alpha. It also elevated glutathione levels significantly, with improvement of histological features related to MTX exposure in group III compared with group II.
Conclusion	Felodipine can protect hepatic tissue against MTX-induced hepatotoxicity.
Keywords	Hepatotoxicity, Methotrexate, Felodipine, Glutathione, Malondialdehyde, Tumor Necrosis Factor-alpha.

List of abbreviation: ALP = Alkaline phosphatase, ALT = Alanine Aminotransferase, AST = Aspartate aminotransferase, DHFR = Dihydrofolate reductase, D.W = Distilled water, DNA=Deoxyribonucleic acid, E=Eosin, HAI = Histological Activity Index, H = Hematoxylin, GSH=Glutathione, iNOS = Cytokine-induced nitric oxide synthase, L-type = Long-Lasting calcium channels, MDA = Malondialdehyde, MTX = Methotrexate, NADP = Nicotinamide adenosine diphosphate, NADPH = Nicotinamide adenosine diphosphate hydrogen, NO = Nitric oxide, NOS = Nitric oxide synthase, ROS = reactive oxygen species, SD = Standard deviation, SPSS = Statistical Package for social Science, TNF-α=Tumor necrosis factor alpha

Introduction

The liver plays a major role in transforming and clearing chemicals which lead to increase its susceptibility to the toxicity from these agents; drugs are important causes of liver injury, more than 900 drugs, toxins, and herbs have been attributed to cause hepatic injury [1].

Methotrexate (MTX) is one of the folic acid antagonists, which is widely used in the therapy of various types of diseases [2]. It is used in high doses for different types of malignancies such as breast and lung carcinomas in addition to leukemia, while low doses used to treat inflammatory conditions and in the treatment of various autoimmune diseases including rheumatoid arthritis, juvenile idiopathic arthritis and psoriasis [3,4]. Moreover it is the first choice treatment for ectopic pregnancy [2,5]. MTX induced toxicity appears to be a consequence of the interaction of many factors that include the length of treatment, dosing schedule, type of disease, patient risk factors and the presence of genetic

and molecular apoptotic factors [2,6,7]. Methotrexate's therapeutic and toxic effects are a result of its capability to limit DNA and RNA synthesis by inhibiting dihydrofolate reductase (DHFR) and thymidylate synthetase that are essential for DNA synthesis [5,6]. This blocking in the synthesis of nucleic acids, certain amino acids and consequently proteins might lead to damage of organelles and plasma membranes of the hepatic parenchymal cells and interfering with their function and allowing leakage of enzymes [8]. Under normal conditions, NADPH is used by glutathione reductase to maintain the reduced state of cellular glutathione, which is a well-known as an important cytosolic antioxidant protecting against reactive oxygen species (ROS). It has been established that the cytosolic NAD (P)-dependent dehydrogenases [9] and NADP malic enzyme are repressed by MTX, indicating that the drug could reduce the availability of NADPH in cells via inhibiting pentose cycle enzymes [10]. MTX may also depress nucleic acid metabolism, due to the interference with the pentose phosphate shunt. Therefore, the significant reduction in glutathione levels promoted by methotrexate leads to a reduction of efficiency of the antioxidant enzyme defense system, which lead to sensitizing the cells to ROS [9]. Thus, the damaging effect of methotrexate is partly due to its direct toxic effect via increasing ROS production [6].

Taking into consideration the relationship between glutathione and the toxic effects of methotrexate, interest has focused on compounds that have the ability to work as antioxidants [11]

Felodipine is a calcium channel antagonist belongs to the second generation dihydropyridines; its targeting long lasting (L-type) calcium channels [12,13] and used in treatment of essential hypertension, or prophylaxis of angina [13,14] and symptomatic relief in raynaud's disease [15]. It is considered as a chain-breaking antioxidant [16] according to Janero et al who reported a direct effect of

calcium antagonists on the lipid peroxidation of cardiac membranes [17].

This study was designed to evaluate whether the hepatotoxic effects caused by administration of methotrexate could be prevented or amileroated by concomitant felodipine treatment.

Methods

This prospective randomized controlled study was conducted from January 2014 through May 2014 at the Department of Pharmacology, College of Medicine, Al-Nahrain University. Experimental protocols were approved by the Institutional Review Board (IRB).

Twenty four healthy, domestic rabbits aged 3-4 months and weighing (600-1300) gm. of both sexes were studied. Before starting the experiment, rabbits were left for 72 hours to acclimatize to the animal room conditions and were maintained on an environment of controlled temperature with 12 hours light/dark cycle with free access to food and tap water. They were divided randomly into three groups, each group including eight animals. Group I as a (Negative control): rabbits were left without treatment. Group II as (Positive control): rabbits were given MTX injection (Ebwe, Australia) as an intraperitonial dose of (20 mg/kg) [18] from 3rd day of the experiment for three successive days. Group III (felodipine + MTX): rabbits were given felodipine tablet (Astrazeneca, Sweden) in a dose of 0.5 mg/kg orally once daily [19] for 7 days, and then MTX was given intraperitonially in a dose of (20 mg/kg) similar to group 2. At the end of experiment, the rabbits were subjected to blood collection under anesthesia by ether inhalation, the blood collected directly from the heart, centrifuged to get serum, which stored at -20°C for biochemical analysis.

After sacrification, the liver tissue were excised by thoracic section, two portions was isolated one of them were fixed in 10% formalin for 24 hours and embedded in paraffin blocks and underwent cutting by microtome into 5 mm thick sections, in addition to staining with

hematoxylin-eosin (H-E) stains. These sections were examined by Olympus CH-2 light microscope by histopathologist. The other portion was mobilized into the cooling box quickly to prevent autolysis and homogenization was done by rinsed the liver piece with chilled phosphate buffer saline (1X PBS) at 4 °C, blotted with filter paper and weighed. Half gram of liver tissue was homogenized in 5 ml of (1X PBS) utilizing tissue homogenizer [20] for 1 minute at 4 °C, then after two freeze thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g at 4°C. The supernatant was obtained and stored at -20°C for the assay of reduced glutathione, malondialdehyde and tumor necrosis factor alpha levels in the tissue.

Chemicals:

Reagent kits for assay of transaminases were purchased from BioMerieux-France, ALP assay kit was purchased from Biolabo Sa France, total bilirubin assay kit was obtained from Randox-United Kingdom, and kits for total protein and albumin were purchased from Linear Chemicals – Spain. Reagent ELISA kits for determination of tissue malondialdehyde (MDA), reduced glutathione (GSH) and tumor necrosis factor alpha (TNF-α) were purchased from Cusabio - China. The work was done in accordance with the method prescribed in each diagnostic kit.

Histological Analysis:

Score of liver damage severity was semi quantitatively assessed using the modified Histological Activity Index [21] 'Modified HAI' (table 1).

Statistical Analysis

Statistical analysis was performed by using computer program SPSS -19. Crude data was analyzed to obtain mean and standard deviation (SD). Student paired *t- test* was used to compare between two groups. *P* of ≤ 0.05 was considered significant and *P*-value of ≤ 0.001 considered as highly significant. The histological score comparison between each group were done by *Chi square* test [22].

Table 1: Modified Histological Activity Index (Modified HAI) grading: necro-inflammatory scores [21]

	SCORE
A. Periportal or periseptal interface hepatitis (piecemeal necrosis)	
Absent	0
Mild(focal, few portal areas)	1
Mild\moderate(focal, most portal areas)	2
Moderate(continuous around<50% of tracts or septa)	3
Severe(continuous around>50% of tracts or septa)	4
B.Confluent necrosis	
Absent	0
Focal confluent necrosis	1
Centrolobular necrosis in some areas	2
Centrolobular necrosis in most areas	3
Centrolobular necrosis +occasional portal-central (P-C)bridging	4
Centrolobularnecrosis+ multiple P-C bridging	5
Panlobular or multilobular necrosis	6
C.Focal (spotty) lytic necrosis, apoptosis and focal inflammation	
Absent	0
One focus or less per 10× objective	1
One to four foci per10× objective	2
Five to 10 foci per 10× objective	3
More than 10 foci per 10× objective	4
D.Protal inflammation	
None	0
Mild, some or all portal areas	1
Moderate, some or all portal areas	2
Moderate/marked, all portal areas	3
Marked, all portal areas	4
Maximum possible score for grading	**18**

Results

Analysis of t-test revealed a highly significant increase (*P* ≤ 0.001) in the level of S. ALP, S. AST, tissue MDA and TNF-α in positive control group II (MTX treated) in comparison with negative control group I, in addition to significant decrease (P ≤ 0.05) in the level of S. total protein, S. albumin in group II compared with group I. It is also observed that group II showed statistically highly significant decrease (*P* ≤ 0.001) in S. GSH than group I. The level of S. ALT show significant increase (*P* ≤ 0.05) in group II also unlike the level of S. total bilirubin (table 2). Comparison between group II rabbits with group III (after concomitant use of felodipin) revealed statistically significant decrease (P ≤ 0.05) in the level of S. ALP, S. ALT and S. AST in the latter group with no significant difference in the level of S. total protein, S. albumin and S. total bilirubin in both

of them. There is a high significant increase ($P \leq 0.001$) in the level of T. GSH while high significant decrease ($P \leq 0.001$) in the level of

tissue MDA and TNF-α in group III rather than group II (table 3).

Table 2: Comparison between –ve control group (Group I) and +ve control group (Group II) in relation to S. Total Protein, S. Albumin, S. Total Bilirubin, S. ALP, S. ALT, S. AST, T. GSH, T. MDA and T. TNF-α.

Parameter	Group I -ve control (Not treated) N=8 Mean±SD	Group II +ve control (MTX treated) N=8 Mean±SD	*P* value
S. Total Protein (g/dl)	5.63±0.91	4.28±0.47	0.0023[*]
S. Albumin (g/dl)	2.63±0.35	2.1±0.21	0.0029[*]
S. Total Bilirubin (mg/dl)	0.11±0.11	0.12±0.13	0.878
S. ALP (U/l)	59.25±6.02	128.13±22.52	< 0.0001[**]
S. ALT (U/l)	49.25±11.02	183.75±97.54	0.0017[*]
S. AST (U/l)	42.25±6.5	232.75±116.25	0.0004[**]
T. GSH (nmol/l)	35.76±3.6	12.33±0.63	< 0.0001[**]
T. MDA (ng/l)	122.28±0.69	135.2±4.2	< 0.0001[**]
T. TNF-α (pg/l)	85.53±3.73	170.89±14.8	< 0.0001[**]

* significant difference at $P \leq 0.05$ ** highly significant difference at $P \leq 0.001$

Table 3: Comparison between +ve control group (Group II) and MTX + Felodipine treated group (Group III) in relation to S. Total Protein, S. Albumin, S. Total Bilirubin, S.ALP, S.ALT, S. AST, T. GSH, T. MDA and T. TNF-α.

Parameter	Group II +ve control (MTX treated) N=8 Mean±SD	Group III (MTX+Felodipine) N=8 Mean±SD	*P* value
S. Total Protein (g/dl)	4.28±0.47	4.66±0.32	0.0762
S. Albumin (g/dl)	2.1±0.21	2.24±0.15	0.1592
S. Total Bilirubin (mg/dl)	0.12±0.13	0.14±0.12	0.798
S. ALP (U/l)	128.13±22.52	91.88±20.55	0.0046*
S. ALT (U/l)	183.75±97.54	94.0±17.22	0.0225*
S. AST (U/l)	232.75±116.25	115.5±23.85	0.0143*
T. GSH (nmol/l)	12.33±0.63	20.83±1.51	< 0.0001**
T. MDA (ng/l)	135.2±4.2	125.66±4.57	0.0007**
T. TNF-α (pg/l)	170.89±14.8	122.73±10.88	< 0.0001**

* significant difference at $P \leq 0.05$ ** highly significant difference at $P \leq 0.001$

There is also a statistically significant increase ($P \leq 0.05$) in the "modified HAI" scoring in group II when compared with group I (table 4). The histopathological examination of negative control group (group I) reveals normal hepatic tissue, no portal or periportal inflammation, necrosis and fibrosis as shown in figure 1, while there was a significant loss in hepatic

architecture in positive control group (group II) demonstrated as portal inflammation with periportal interface hepatitis (piecemeal necrosis), centrilobular necrosis and bridging necrosis as in figure 2. However; this score showed a statistically significant decrease ($P \leq 0.05$) in group III when compared with group II (table 5). The histopathological examination of

MTX+felodipine treated group (group III) reveals significant restoration of hepatic architecture with mild portal inflammation of mononuclear cells infiltrate as shown in figure 3.

Table 4: Comparison of histopathological changes (by modified HAI scoring) between –ve control group (Group I) and +ve control group (Group II).

Score	Group I (-ve control) (Not treated) N=8		Group II (+ve control) (MTX treated) N=8		P value
	No.	%	No.	%	
0	8	100	0	0.0	0.003*
4	0	0.0	4	50.0	
5	0	0.0	2	25.0	
6	0	0.0	1	12.5	
8	0	0.0	1	12.5	

* Denote significant difference at P ≤ 0.05

Table 5: Comparison of histopathological scores between +ve control group (Group II) and MTX+felodipine treated group (Group III)

Score	Group II (+control) (MTX treated) N=8		Group III (MTX+felodipine treated) N=8		P value
	No.	%	No.	%	
1	0	0.0	4	50.0	0.015*
2	0	0.0	3	37.5	
3	0	0.0	1	12.5	
4	4	50.0	0	0.0	
5	2	25.0	0	0.0	
6	1	12.5	0	0.0	
8	1	12.5	0	0.0	

* Denote significant difference at P ≤ 0.05

Discussion

The results of the present study indicate that MTX lead to oxidative tissue damage by increasing lipid peroxidation and consequently inflammation in the liver tissue and decreasing the level of antioxidant enzymes. Also, increased AST, ALT and ALP with decreased

levels of total protein and albumin, which considered as biochemical indicators of liver damage; the histopathological findings support this. Subsequent results were shown that felodipine provided significant protection from the effects of MTX on the liver. The damage of liver tissue after MTX exposure is a well known phenomenon, and the clear sign of hepatic injury is the leakage of hepatic enzymes into plasma.

Definitely, both the biochemical parameters and histological manifestations supported a diagnosis of liver damage. The elevated levels of serum enzymes of ALT, AST and ALP in MTX-treated rabbits indicate the increased permeability, damage or necrosis of hepatocytes [23], these findings have been agreed with other studies [27,29].

The decreased levels of serum total proteins were due to the dissociation of polyribosomes from endoplasmic reticulum and also due to defects in protein biosynthesis [24]. Consequently, albumin level reduced as it represents the larger portion of serum proteins and due to increased renal loss of albumin secondary to MTX induced nephrotoxicity, in agreement with Jwied in 2009 and Rizvi in 2012 who reported that liver disorders are related to a decrease in the serum levels of total proteins [25,26].

Figure 1: Section of liver tissue of group I (control group) on day 8 of the experiment shows normal hepatic tissue, no portal or periportal inflammation, necrosis and fibrosis. H & E stain, (40X). H: hepatocyte, Hc: hepatic cord, S: sinusoid, Pv: portal vein, Bd: bile duct.

It is well known that oxidative stress plays an important role in the tissue damage due to MTX [7,27,28]. The extent of severity caused by MTX-associated liver injury was linked to both the dose and the treatment interval [27]. The significant lowering in glutathione (GSH) levels induced by MTX as expressed above could produce a reduction of effectiveness in the antioxidant enzyme defense system and increased sensitivity of the cells to ROS [9]. MDA was a stable metabolite of the free radical caused lipid peroxidation cascade [29]. It is used usually as a marker of oxidative stress and destroying of lipid layers [30]. As described above, methotrexate leads to lipid peroxidation via significant elevations in MDA levels.

The lipid peroxidation mediated by oxygen-free radicals was thought to be an important cause of destruction and damage to the cell membranes and was suggested to be a contributing factor of the development of MTX- mediated tissue damage [29].

The free-radicals were seem to trigger the accumulation of leukocytes in the tissues involved, and thus exacerbate tissue injury indirectly through the activation of neutrophils. It has been exposed that activated neutrophils secrete enzymes and liberate oxygen radicals [31] also free radicals have a direct damaging effects on these tissues [27]. Moreover, it has been determined that methotrexate leads to histological damage including portal inflammation with centrilobular necrosis. The histological alterations may occur though methotrexate oxidative properties. These results are confirmed with other previous studies [23,27] with difference in the severity due to the difference in the duration of the toxicity induction.
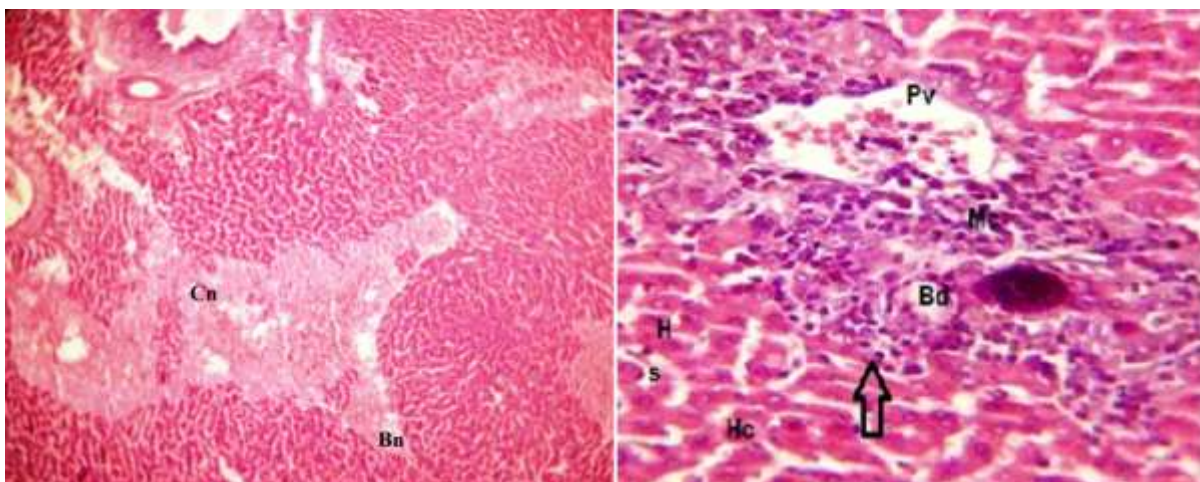
Figure 2: Sections of liver tissue of group II (MTX 20 mg/kg) on day 8 of the experiment shows portal inflammation with periportal interface hepatitis (piecemiel necrosis) (arrow on the right) and centrilobular necrosis (Cn) and bridging necrosis (Bn) (on the left). H & E stain, (40X) on right & (20X) on left. H: hepatocyte, Hc: hepatic cord, S: sinusoid, Pv: portal vein, Bd: bile duct, Mc: mononuclear cells infiltrate.

The current study reported that the administration of felodipine with MTX lowered the levels of MDA significantly and exhibited a marked elevation in the level of GSH in the hepatic tissue as compared to MTX group. This observation increase thoughts that felodipine showed antioxidant properties [32] by acting as lipophilic chain-breaking antioxidant.

Calcium antagonists including felodipine have also been shown to prevent glutathione loss [33], and this might be a possible mechanism too. Felodipine inhibited cytokine-induced nitric oxide production (iNOS) and nitric oxide synthase (NOS) mRNA induction. In addition, felodipine inhibited cytokine-induced superoxide production both in the presence and absence of an (NOS) inhibitor, suggesting

that it acted as a superoxide scavenger. The attenuation of intracellular calcium by felodipine can also modulate free radicals and inflammatory mediators production [34].
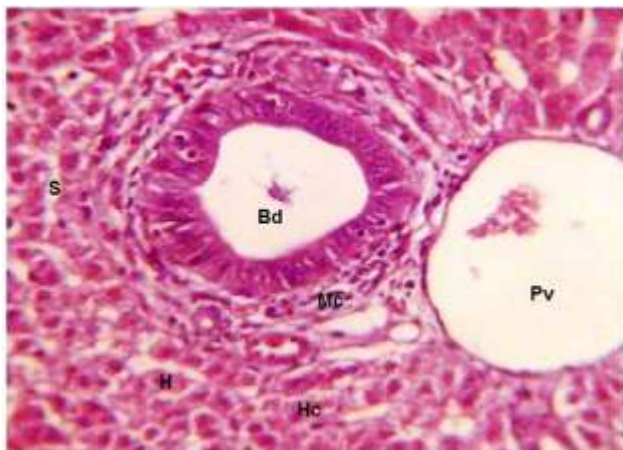
Figure 3: Section of liver tissue of group III (MTX 20 mg/kg +felodipine 0.5mg/kg) on day 8 of the experiment, shows mild portal inflammation. H & E stain, (40X). H: hepatocyte, Hc: hepatic cord, S: sinusoid, Pv: portal vein, Mc: mononuclear cells infiltrate.

Monocyte-derived macrophages were rich in nicotinamide-adenine-dinucleotide phosphate (NADPH) oxidaselead to amplifying the generation of (ROS) reactive oxygen species [35]. Again, ROS production was associated with intracellular calcium (iCa) elevation, protein kinase C activation and NADPH oxidase activation, leading to a vicious cycle of inflammation and progressive necrosis and fibrosis [36]. These results suggest that the antioxidant and consequently anti-inflammatory properties of felodipine may have beneficial effects in protecting against cellular damage caused by lipid peroxidation. The histological picture of the group treated with felodipine showed mild portal inflammation, which is a reversible damage as a result of the antioxidant and inflammatory modulation effects that reported above, which prevent further damage. Matsubara et al in 2010 reported that the hisopathological picture of the cardiac and kidney tissue were improved after felodipine had been administered, indicating the protective effects of felodipine on the myocardium and kidney by decreased perivascular inflammation and myocardial necrosis and fibrosis in aldosterone-high salt intake hypertensive unnephrictomized rats [37].

In conclusion, felodipine seems to have hepatoprotective activity through its favorable effects on liver function tests, oxidative stress, TNF-α and histopathological scores.

Author contributions
The first author involved in the collection of samples, arrangement and writing of the study under supervision of the second and third authors, histopathological grading done by the third author.

Conflict of interest
There are no any financial and personal relationships that could bias this work.

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Human Papilloma Virus Types 16 and 18 in a Sample of Iraqis Patients Presented with Oral Cancer

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Abstract

Background Oral squamous cell carcinoma is the most common malignant neoplasm of oral mucosa. Human papilloma (HPV) virus cause a broad scope of diseases from benign to invasive tumors, types 16 and 18 classified as carcinogenic to humans.

Objective To assess the occurrence rate of human papilloma virus genotypes in oral cancer patients and their association with various risk factors.

Methods Fifty five (55) unstimulated whole saliva samples were collected from 35 histopathologically confirmed patients with oral cancer and 20 apparently healthy subjects were enrolled in this study. Genomic DNA was extracted from exfoliate cells to amplify HPV-DNA using HPV-L1 gene sequence primers by polymerase chain reaction method, the viral genotyping was performed using direct sequencing method.

Results The mean age of patients group was 52.23±13.73 years, while in healthy subjects group was (50.55±12.5) years. Risk of smoking was highly significant with odds ratio 60.79 and a 95% confidence interval of 3.40-1086.71. However, the risk of alcoholism was significant with odds ratio 27.77 and a 95% confidence interval of 1.51-511.27. Forty-six percent (16/35) of oral cancer patients were positive for detection of HPV-DNA ($P < 0.0002$). The most frequent HPV genotypes in patients group was HPV-18 accounting for (31%) of cases ($P < 0.05$). The rate of HPV was significantly higher among younger ages (< 50 years) with $P = 0.042$. In addition, the rate of HPV was higher with other variables (male, tongue tissue, grade I differentiation, squamous cell carcinoma) with no significant association ($P = 0.273$, $P = 0.739$, $P = 0.173$, and $P = 0.700$ respectively).

Conclusion Human papilloma virus types 16 and 18 may be a risk factor for oral cancer independent of alcohol and tobacco.

Keywords OC, HPV, OSCC, PCR, Direct sequencing

List of abbreviation: OSCC= Oral squamous cell carcinoma, HPV= Human papilloma virus, OC= Oral cancer, DNA= Deoxyribonucleic acid, L1= late gene 1 (capsid), PCR= Polymerase chain reaction

Introduction

Oral cancer (OC) accounted for 300,000 cases (2.1% of the world total), with two thirds occurring in men. Worldwide, 145,000 deaths occurred (1.8% of the world total), of which 77% were in the less developed regions [1]. The established etiological factors of OC included cigarette smoking and heavy alcohol abuse; however, a growing group of patients, including young adults and women, have no known tobacco or alcohol exposure have been emerged, therefore; possible viral etiologic factors such as oncogenic human papilloma virus (HPV) have been proposed [2]. High-risk HPV-16 and 18, as etiologic agents of anogenital carcinomas, have been firmly

established in the literatures and due to morphological similarities and epitheliotrophic nature of HPV as well as HPV's oncogenic potential, a link between OC and HPV seemed logical [3]. International Agency for Research on Cancer (IARC) has assigned HPV as an independent risk factor since 2007 and that 30%-50% of oral squamous cell carcinoma (OSCC) has been associated with HPV-16 [4]. Over 200 HPV types have been identified in many different human lesions, being categorized as low- and high risk HPVs, depending on their potential to lead the epithelium to carcinogenesis. In the oral cavity, low-risk HPV types 6 and 11 are the most prevalent in benign lesions, as the high-risk types 16 and 18 are respectively the most found in malignant ones. The viral genome of HPV can be sectioned into three parts: an early (E) region, which encodes proteins necessary for viral replication and transcription; a late (L) region, which encodes structural proteins of the viral capsid (L1 and L2); and a non-coding region segment, which contains elements that regulates the viral deoxyribonucleic acid (DNA) replication and transcription [5]. By definition, the nucleotide sequences of the E6, E7, and L1 of a new HPV type should be no more than 90% homologous to the corresponding sequences of known HPV types. HPVs have further been classified into subtypes, when they have 90% to 98% sequence similarity to the corresponding type; and variants, when they show more than 98% sequence homology to the prototype [6]. The estimates of overall HPV prevalence from a meta-analysis study by Petrick et al., (2014), which reported an overall summary HPV prevalence of 29.0% (95% CI: 25.1–33.1%) [7]. The rise in incidence is mostly occurring in individuals aged 40-55 years, without environmental risk factors, and is associated with persistent infection with high-risk HPVs, HPV positive (+) OSCC patients tend to be younger than HPV negative (-) ones. HPV-16 is the most common genotype found in almost 90% of the HPV positive (+) oropharyngeal cancers [8].

The aim of the current study is to detection of the HPV genotypes as an independent risk factor in oral cancer patients.

Methods
Patients:
This case-control design study was approved by the Committee of Ethical Standards in the College of Medicine, Al-Nahrain University and underwent to the terms of Ethical Considerations of the Iraqi Ministry of Health. Thirty five (35) newly diagnosed patients (24 males and 11 females) were histopathologically confirmed with OC by two independent pathologists; these patients were attended to maxillofacial surgery clinic of Ghazi Al-Hariri for Specialized Surgery Hospital in Baghdad were enrolled in this study during the period from April 2014 till April 2015. The inclusion criteria for this study were a) presence of oral cavity cancer (including oral tongue, floor of mouth, gingival, lips, buccal mucosa); b) no previous head and neck cancer; c) no prior oncological therapy. Twenty (20) samples were taken from healthy volunteers whom attend to private clinic for routine dental were collected as control group. The samples processing and DNA extraction was done in Medical Legal Institute/Ministry of Health. Viral detection, PCR and genotyping were done in Central Public Health Laboratory/Public Health Directorate/Ministry of Health.

Saliva Samples:
Up to 5 mL of un-stimulated whole saliva samples taken from each subject and collected in a 50 mL centrifuge tube, which remains on ice while collecting them. The samples were centrifuged at 2,500 rpm for 15 min at 4 °C to spin down exfoliated cells, the saliva supernatant were discarded. Cell pellets were stored at -80 °C until further processing [9].

Genomic DNA extraction:
Viral DNA was extracted from frozen 200 µl of saliva samples (cell pellet) by using AccuPrep® Genomic DNA extraction kit

(Cat# K-3032) according to the manufacturer's guideline (Bioneer, Korea). The concentration and purity of the extracted DNA was quantified by the use of NanoDrop^{TM} 2000 Spectrophotometer instrument following the manufacturer's instructions (Thermo Scientific, USA). The ratio of A260/A280 of wavelength absorbance is calculated, a ratio of ~1.8 was generally accepted as "pure" for DNA [10].

PCR analysis:
HPV-DNA was detected using conventional PCR for HPV-L1 primers (conserved L1 gene in HPV types). Alignments were obtained from the GenBank online BLAST server. HPV-DNA was amplified by PCR assay using primers were designed by using the complete sequence of HPV-L1 gene (GenBank: JX316023.1) as previously demonstrated by Agoston *et al.*, (2010) [11]. *Forward 5'-ACTGGAAAGGTGCTTGTACC-3'* and *Reverse 5'-ACAGGGTTCACAGCCAACAA-3'*, amplicon size 321bp. AccuPower® PCR PreMix Kit (Cat# K-2012) was used to prepare mastermix according to manufacturer's instructions (Bioneer, Korea) as follows: 5 µl of template DNA, 1.5 µl of (10 pmol from both primers), and 12 µl of PCR water. The 20 µl reactions were incubated in Thermocycler (MyGene, Korea). PCR thermocycler condition consisted of initial denaturation incubation at 95 °C for 5 minutes followed by 30 cycles at 95 °C incubations for 30 seconds (denaturation), 58°C incubations for 30 seconds (annealing), and at 72 °C incubation for 30 seconds (extension), finally incubation at 72 °C for 5 minutes for the final extension. Amplification products were analyzed in 2% polyacrylamide gel.

Sequencing analysis:
Amplification products were purified by EZ-10 Spin Column DNA Gel Extraction Kit (Cat# BS353) following the manufacturer's instructions (Biobasic, Canada). Genotyping of HPV was based on direct sequencing PCR fragments by AB DNA sequencing system performed by Bioneer Company in Korea.

Statistical analysis
Mean values were compared using independent samples t-test. Chi-square and Fischer Exact tests were used to study association between any two categorical variables. Correlation coefficient was used to evaluate correlation between numeric variables (e.g. age) and or ordinal nominal variables. Odds ratio statistic was used to assess risk. P values of less than 0.0001 and less than 0.05 were considered highly significant and significant respectively.

Results
In the present study, the mean age of patients was 52.23±13.73 years with a range of (17-70) years, there was a male predominance among patients group with a proportion of (69%) in comparison to (31%) for female patients (Table 1). Male to female ratio was 1.8:1. Twenty one (21) patients out of 35 were smoker with a proportion of 60%. Meanwhile, alcoholism was reported in only 10 patients accounting for 28.5% where none in the control group were smokers or drinkers (Table 2). Sixteen (16) patients out of 35 had positive HPV-DNA accounting for (46%) while none of the apparently healthy subjects were positive for HPV-DNA. This difference was statistically highly significant ($P < 0.0002$). The approximate odds ratio was 34.69 with a 95% confidence interval of 1.95 to 618.66 as in (Figure 1). The predominant HPV genotype was HPV-18 accounting for (31.43%) and (11.43%) for HPV-16 (Figure 2). Mean age of patients with positive HPV infection was significantly lower than negative HPV infection, 47.13±13.01 years versus 56.53±13.13 years, the rate of HPV was significantly higher among younger ages (< 50 years) with $P = 0.042$ as in (Figure 3). Regarding association of HPV infection with other variables: male predominance, tongue tissue, grade I

differentiation, and squamous cell carcinoma subtype were higher in rates with no significant associations were found with HPV ($P > 0.05$).

There were no significant association between tobacco smoking and/or alcoholism with HPV infection ($P > 0.05$) (Table 3).

Table 1. Demographic data of patients and control subjects

Characteristics		Patients group No=35	Control group No=20	P-value
Age (years)	Mean±SD*	52.23±13.73	50.55±12.5	0.654
	Range	17-70	24-74	
Gender	Female	11 (31%)	7 (35%)	
	Male	24 (69%)	13 (65%)	1.000
	Total	35 (100%)	20 (100%)	

*SD= Standard deviation; NS= Not significant

Table 2. Risk factors associated with oral cancer patients

Risk factor	Patients group		Control group		P-value	Odds ratio	95% confidence interval
Smoking	Smoker	21 (60%)	Smoker	0			
	Non-smoker	14 (40%)	Non-smoker	20	< 0.0001*	60.79	3.40-1086.71
	Total	35 (100%)	Total	20 (100)			
Alcoholism	Drinkers	10 (29%)	Drinkers	0			
	Non-drinkers	25 (71%)	Non-drinkers	20	0.009**	27.77	1.51-511.27
	Total	35 (100%)	Total	20 (100)			

*P value highly significant (< 0.001); **P significant (< 0.05).

Discussion

The HPV average rate in OC was 25%, ranging from (<5-100%), in small studies and (1.4-48.8%) in larger ones based on ethno-geographic area, sample size, HPV DNA detections method in tissue and classification of head and neck subsites [12-14].

Current results revealed that (46%) of patients with OC had HPV-DNA which suggest a significant association between them, the results are consistence with similar case-control studies in Iran obtained by (Sahebjamee et al., 2009; Kermani et al., 2012; and Tabatabi et al., 2015) they reported

detection of HPV with rate of (40.9%, 42.8%, and 43.9%) respectively among OC cases [15-17]. PCR amplification is a method that consists of multiplying DNA sequences exponentially, making the detection of HPV DNA in human saliva samples easier and more sensitive than other methods, which may explains high rate of HPV-DNA in this study.

In regard to HPV genotypes, the predominant genotype was HPV-18, which is concurrent with a study by Kermani et al., (2012) who reported that rate of HPV-18 was (28.6%) followed by HPV-16 with (14.3%) of cases in

Iran [16]. According to many studies, HPV-16 is the most common high risk HPV in HPV related OC [13-15,17,18]. However, the results of few studies have indicated that other high risk HPV types can be the commonest [16,19]. It is difficult to determine the parameters, which make one

genotype to be the most predominate due to variation in those parameters such as the type of samples, preparation of samples, sensitivity of the methods used, status of the disease, and geographical regional differences.
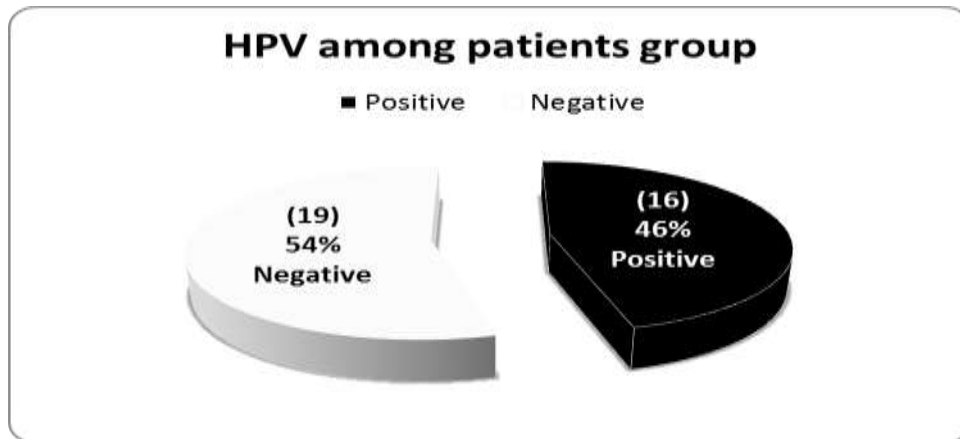
Figure 1. Human papilloma virus among patients group
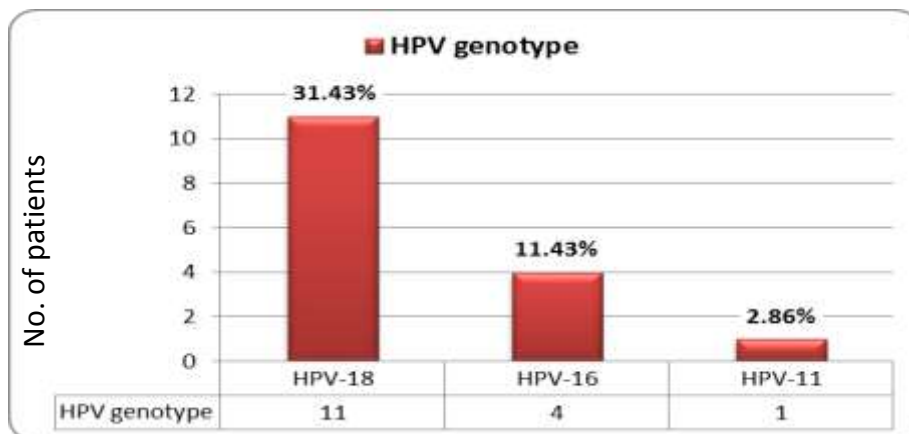
Figure 2: Human Papilloma virus genotypes among patients group
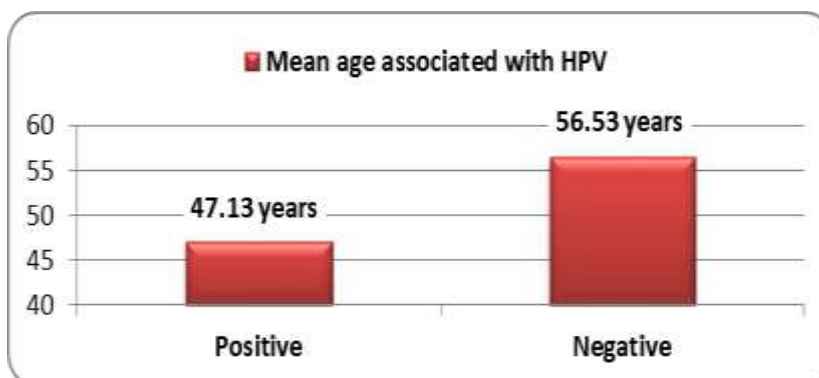
Figure 3. HPV positive cases association with mean age in patients group

Table 3. HPV association with demographic and clinico-pathological parameters

Variables		HPV-positive	HPV-negative	Total	*P* Value
Gender	Male	9 (56.25%)	15 (79 %)	24	
	Female	7 (43.75%)	4 (21%)	11	0.273
	Total	16	19	35	
Tumor location	Tongue	7 (43.75%)	7 (36.85%)	14	
	Others	9 (56.25%)	12 (63.15%)	9	0.739
	Total	16	19	35	
Tumor differentiation	Grade I	13 (81.25%)	11 (58%)	24	
	Grade II	2 (12.5%)	2 (10.5 %)	4	
	Grade III	1 (6.25%)	6 (31.5%)	7	0.173
	Total	16	19	35	
Histological subtypes	SCC	11 (68.75%)	15 (79%)	26	
	Others	5 (31.25%)	4 (21%)	9	0.700
	Total	16	19	35	
Smoking tobacco	Smokers	9 (56.25%)	12 (63%)	21	
	Non-smokers	7 (43.25%)	7 (37%)	14	0.739
	Total	16	19		
Alcohol drinking	Drinkers	6 (37.5%)	4 (21%)	10	
	Non-drinkers	10 (62.5%)	15 (79%)	25	0.454
	Total	16	19	35	

In the current study, patients ages with positive HPV was significantly lower than that of patients with negative HPV. This result was in agreement with Kermani *et al*., (2012) whom demonstrated that mean age of patients with positive high risk was 42.17±5.03 years with age range (35-50) years [16]. Results of the current study disagree with Sahebjamee *et al*. (2009) and Tabatabai *et al*. (2015) according to them, the rate of HPV in older people above 50 years was higher than those aged below 50 years [15,17]. Tonsil and oropharyngeal cancers increased in male predominance over the last 30 years, despite a decline in smoking, which may be linked to the increasing proportion of HPV positive cancers, may be due to changes in sexual activity [20].

In relation to gender, in this study there was male predominance with (69%). This finding was parallels with the results obtained by (Tabatabai *et al*., 2015; Kreimer *et al*., 2011) [17,18]. A possible reason for higher OC ratio in men could be due to higher consumption of tobacco and alcohol products.

Lateral border of tongue representing (43.75%) with HPV positivity was recorded in the present study, which comes in accordance with findings by Kermani *et al.*, (2012) who found that tongue with a proportion of (40%) than other sites [16]. Tongue is preferred topographical location for the OC, an observation in this study come is compatible with the results of previous Iraqi study conducted by Al-Sened *et al.*, (2009), which displayed that tongue was the predominant site in (42.4%) of cases [19]. In contrast to SahebJamee *et al*., (2009), in which, tongue with proportion of (45.45%) than other oral locations with HPV positivity [15]. A study by Kreimer *et al.,* (2011) found that there is site

specific predilection of high risk HPV toward non-keratinized tongue tissue [18].

HPV positivity was among the vast majority of well differentiated grade I tumors accounting for (81.25%; 13 out 16 patients) these results are in agreement with a recent study by Patil *et al.*, (2014) who investigate the correlation of HPV in histological grades of OSCC; well differentiated tumors were the most prevalent in tongue tissue followed by buccal mucosa [21]. The results disagree with Kermani *et al.*, (2012) in which majority of patients had advanced stage of the disease grade III with proportion of 64% [16]. Some studies have shown the relevance of HPV positive OSCC and more advanced grade of tumors and nodal metastases. Possible explanation about the current results is the small size of studied group and the bias toward grade I tumors come with fact the tongue site are histologically diagnosed as grade I tumors mostly.

There was no correlation between cigarette smoking and/or alcohol consumption with HPV positive tumors had been found in the current study. These results can be interpreted as that both smoking and/or alcohol consumption association with HPV-related OC are independent risk factor for the OC, however; Gillison *et al.*, (2008) discover a weak correlation with the use of marijuana [22]. There is some controversy over the impact of smoking and alcohol consumption on HPV infection in OSCC; it had suggested that additive effects of smoking on HPV positivity in oral cavity and oropharyngeal SCC, and that HPV seropositive smokers have a higher risk for developing OSCC [23,24].

In conclusion, Human papilloma virus types 16 and 18 may be a risk factor for oral cancer independent of alcohol and tobacco.

Acknowledgment

Author contributions

Al-Malkey collected the samples, conducted the experimental aspects of the study, and writes the manuscript; Abbas put the concepts of the study design, revised and approved the final version of the manuscript; Yassen did general consultation.

Conflict of interest

Authors declare no conflict of interest

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Molecular Identification of *Giardia lamblia* Genotypes Isolates from Children with Diarrhea

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Abstract

Background	Infection with *Giardia lamblia* (*G. lamblia*) parasite regarded as the most important causative agent for diarrhea, and a major public health problem.
Objectives	Molecular identification and characterization of *G. lamblia* genotypes and association with gender, age and presence of different clinical signs.
Methods	One hundred children with diarrhea were included. Fecal samples were taken from them during the period from May 2014 to February 2015. The age range was 2 months to 18 years. All stool samples were examined by microscopic examination, multiplex real time polymerase chain reaction and nested PCR.
Results	Among 42 fecal samples from patients with giardiasis diagnosed by multiplex real time PCR, amplification of triosphosphate isomerase gene of *G. lamblia* was successful among 25/42 (59.52%) samples. However, the amplification of these samples showed that 7 (28%) contained genotype A and 18 (72%) samples contained genotype B; genotype B was more prevalent than A in males 11/18 (61.11%) and females 7/18 (38.89%) respectively. Regarding age, the results showed that no differences in distribution of genotypes were found statistically among different age groups of patients. Regarding to clinical aspects, the rates of abdominal pain, weight loss, anorexia and fever of giardiasis genotype B are higher than the genotype A.
Conclusion	*G. lamblia* genotype B is the most frequent genotype among children with diarrhea. Also, the presence of the clinical aspects is genotype specific.
Keywords	*Giardia lamblia*, children with diarrhea, and molecular identification.

List of Abbreviations: *Giardia lamblia* = *G. lamblia,* triosephosphate isomerase = tpi, Polymerase Chain Reaction = PCR and Multiplex Real-Time PCR = Multiplex RT PCR.

Introduction

The protozoan *Giardia* is an intestinal parasite that can infect many species in the animal kingdom including mammalian, avian, reptilian, domesticated animals, and human [1]. *Giardia spp*. is unique in their possession of two nuclei that are identical in appearance, DNA content, transcription and time of replication. There are 5 chromosomes per haploid genome. The genome shows little evidence of heterozygosity, supporting that *Giardia* is sexual [2].

In human, it can cause gastrointestinal infections ranging from mild to severe as well as chronic disease [3]. *Giardia lamblia* (*G. lamblia*) is typically characterized in human by diarrhea, steatorrhea, maldigestion, abdominal cramps, bloating, malabsorption and weight loss. Person-to-person transmission occurs by hand-to-mouth transmit of cysts from the feces of a person infected with *Giardia*. Outbreaks of *Giardia* infections in families and institutions, such as day care centers and nursing homes, especially those with diapered children, have been associated with fecal-oral route [4].

Microscopic diagnosis of this protozoan is neither sensitive nor specific. Studies have found that excretion of trophozoites or cysts in the feces can be intermittent and therefore could lead to missed infections due to the low numbers of the diagnostic stages in the feces [5], other diagnostic methods have been developed such as the Enzyme-linked immunosorbent assay, culture and the Polymerase Chain Reaction (PCR). However, molecular methods like PCR are used to classify *G. lamblia* into assemblages (genotype) and subassemblages (subgenotype). Most studies use tests depend on one or more of genetic loci: Small SubUnit Ribosomal RNA, elongation factor 1 alpha, triosephosphate isomerase (tpi), glutamate dehydrogenase and β-giardin genes. However, the use of a various gene, or even a various set of PCR primers, can occasionally assign the same isolate to a different assemblage [6].

A large amount of data has shown that *G. lamblia* should be considered a species complex whose types show little variation in their morphology still can be assigned to at least eight distinct assemblages (A to H) depend on genetic analyses [7]. The assemblages A and B are important in human infection; they also can infect other mammals [8]. Interestingly, several reports have suggested a role of the assemblages for the presence and the severity of *G. lamblia* infections [9,10]. However, some studies found a stronger relationship with assemblage B [11,12]. Also, other study showed that assemblage B exhibited more extensive association with persistent symptoms, while assemblage A was found in connection with intermittent diarrhea [13].

The present study was conducted to do molecular identification and characterization of *G. lamblia* genotypes and association with gender, age and presence of different clinical signs.

Methods

Patients and samples: One hundred patients were included in the current study, who were examined and interviewed by pediatric physician and attend to the Parasitology Laboratory in Al-Imamain Al-Kadhimain Medical City in Baghdad, suffering from diarrhea with different gastrointestinal complaints by asked them about different clinical aspects.

General fecal samples were taken during the period from May 2014 to February 2015. The age range was 2months to 18 years.

Stool samples examinations
1-Macroscopical Examination
Samples were noticed in terms of consistency, color, odor and presence of blood and mucus.

2- Microscopical Examination
-Direct Method
From each fecal sample, smears with normal saline and lugol's iodine were prepared. Two direct smears were examined from each fecal sample, by preparing two clean dry microscope slides, one with normal saline and the other with lugol's iodine agents. By using clean wood stick, the fecal specimen was touched in various sites, especially where streaks of blood or pus were noticed, then mixed thoroughly with each drop of normal saline and lugol's iodine agents on the prepared slides, then each slide was covered with a cover slip. The smear was examined thoroughly under the low (x10) and high (x40) powers of the microscope.

3. Molecular study
DNA-extraction
The DNA extraction of *G. lamblia* was performed according to the manufactures protocol of AccuPrep® Stool DNA extraction Kit provided by Bioneer/Korea.
Multiplex Real-Time PCR
Direct, qualitative detection and differentiation of *G. lamblia* was performed according to the manufacture's protocol of RIDA®GENE Parasitic

Stool Panel is a multiplex RT- PCR kit provided by R-Biopharm/Germany.

Determination of *G. lamblia* genotypes

A nested PCR was performed to amplify the tpi gene, for the primary PCR, a PCR product of 605 bp was amplified by using primer set forward primer AL3543 and reverse primer AL3546 designed by Sulaiman *et al*., (2003) [14].

PCR amplification mixture was performed in 20 µl final volume with 2 µl of template DNA in PCR PreMix (1 U of Taq polymerase, 250 µM each of deoxynucleoside triphosphate (dNTP), {dATP, dCTP, dGTP, dTTP}, 10 mM Tris-HCl, 30 mM KCl, 1.5 mM $MgCl_2$, stabilizer and tracking dye), 1 µl of each primer, 16 µl distilled water.

The thermocycling conditions were as follows:

- ***An initial denaturation step:*** -95°C for 5 min
- ***35 cycles:*** -94°C for 45 s
  - -50°C for 45 s
  - -72°C for 60 s
- ***Final extension step:*** -72 °C for 10 min

The secondary round was performed as separate PCR reactions for each genotype. Underwent further amplification using a set of separate A [15] and B [16] assemblage specific primers Presence of mixed infection was detected by visualizing the occurrence of bands in the agarose gel 1.5%, at 332 bp for assemblage A amplified using primer sets forward primer AssAF and reverse primer AssAR and at 400 bp for assemblage B amplified using primer set forward primer AssBF and reverse primer AssBR as shown in Table 1.

PCR amplification mixture was performed in 50 µl final volume with 10 µl of primary PCR product as a template DNA in PCR PreMix (2.5 U of Taq polymerase, 250 µM each of deoxynucleoside triphosphate (dNTP), {dATP, dCTP, dGTP, dTTP}, 10 mM Tris-HCl, 30 mM KCl, 1.5 mM $MgCl_2$, stabilizer and tracking dye), 4 µl of each primer, 32 µl distilled water.

The thermocycling conditions were as follows:

- ***An initial denaturation step:*** -94°C for 10 min
- ***35 cycles:*** -94°C for 45 s
  - -64°C for 45 s
  - -72°C for 60 s
- ***Final extension step:*** -72 °C for 10 min

The amplified products were analyzed by electrophoresis in 1.5% agarose gel stained with 0.5 mg/mL ethidium bromide.

Table 1. Primers used in the present study for amplification of fragments of the *G. lamblia* tpi gene.

Primary PCR Round		
Primer	Sequence 5'-3'	Product
AL3543	AAA TIA TGC CTG CTC GTC G	605 bp
AL3546	CAA ACC TTI TCC GCA AAC C	
Secondary PCR Round		
Primer	Sequence 5'-3'	Product
Genotype A		
AssAF	CGC CGT ACA CCT GTC	332 bp
AssAR	AGC AAT GAC AAC CTC CTT CC	
Genotype B		
AssBF	GTT GTT GTT GCT CCC TCC TTT	400 bp
AssBR	CCG GCT CAT AGG CAA TTA CA	

Questioner performance

A questionnaire was prepared, asking patients (or their parents) about their clinical symptoms and habitation, besides other questions about hygienic habits.

The study was approved by the Ethical committee of the College of Medicine, Al-Nahrain University.

Statistical analysis

The Statistical Analysis System (SAS, 2012) was used to show the influence of different factors in study parameters. The Chi-square- x^2 test was used to compare between percentages. The lower level of accepted statistical significant difference is below or equal to ($p \leq 0.05$), and the high significant difference is below or equal to ($P \leq 0.001$) [17].

Results

Identification of *G. lamblia* genotypes

Among 42 fecal samples from patients with giardiasis diagnosed by multiplex RT PCR, amplification of tpi gene of *G. lamblia* was successful among 25/42 (59.52%) samples. However, the amplification of these samples showed that 7 (28%) contained genotype A (Figure 1) and 18 (72%) samples contained genotype B (Figure 2). Statistically, significant differences appeared in the distribution of genotypes among giardiasis patients at $p \leq 0.05$ as shown in table (2).
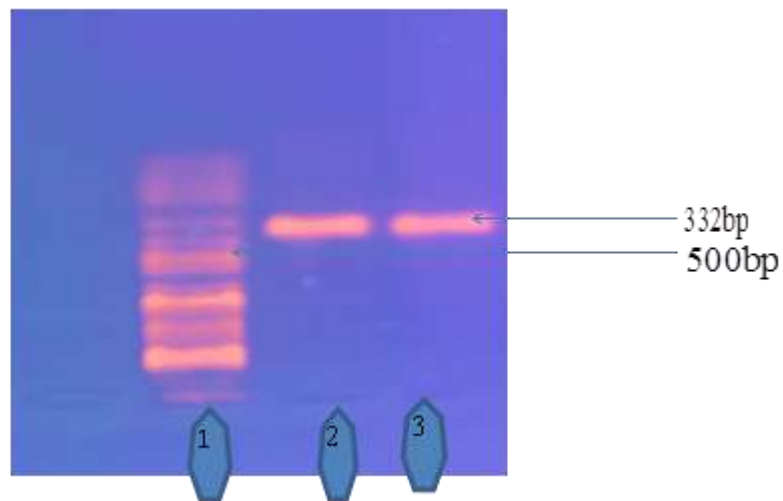
Figure 1. Agarose electrophoresis of PCR amplification for tpi gene (332bp). Lane 1 represents DNA ladder (100bp), Lane 2, 3 represent PCR product of genotype A from examined samples. Fragments were resolved on 1.5% agarose gel and visualized by ethidum bromide staining.
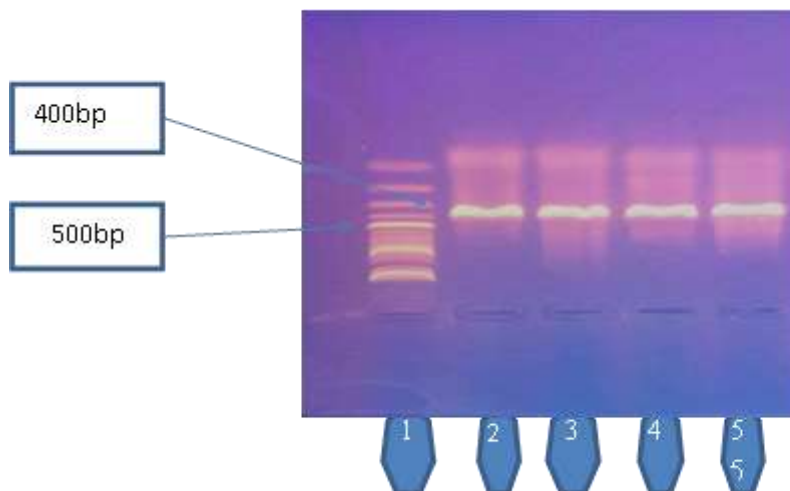
Figure 2. Agarose electrophoresis of PCR amplification for tpi gene (400bp). Lane 1 represents DNA ladder (100bp), Lane 2,3,4,5 represent PCR product of genotype B from examined samples. Fragments were resolved on 1.5% agarose gel and visualized by ethidum bromide staining.

hemoglobin, bile salts, polysaccharides from mucus, bacteria, and food degradation product} [19,20].

In the present study, *G. lamblia* assemblage A and assemblage B were never detected together, whereas a mixture of these assemblages has been reported previously in a other studies [21,22].

The predominance of genotype B in patients with diarrhea in present study is agreement with recently study in Baghdad of AL-Obaidi (2014) who showed that (75%) genotype B and (15.62%) genotype A while (9.37%) mixed genotype [23].

However, *Giardia* genotype B was detected to be the only genotype identified in both diarrheal and non-diarrheal children. The

significance of this finding is that, children are getting infected via, human-to-human transmission, as the genotype B has been found to be exclusively anthroponotic [24,25]. Among Saudi children, all assemblage B reported was associated with diarrhea [26]. Guinea-Bissau showed predominance of genotype B in diarrheal cases [27].

This may be related to that the most studied genotype B isolates, differs from genotype A isolates in a diversity of biological important ways; host infectivity, metabolism and growth requirements [28]. Beside, in human volunteer studies, B consistently infected normal volunteers, while A was unable to stimulate infections. B was more pathogenic than A in gerbils [29].

Table 5. Correlation between clinical aspects of *G. lamblia* genotype A and genotype B

Clinical aspects	A	%	B	%	*P* value
Abdominal pain	1/7	14.29	15/18	83.33	0.003
Weight loss	1/7	14.29	13/18	72.22	0.008
Flatulence	3/7	42.86	8/18	44.44	0.942
Fatigue	3/7	42.86	10/18	55.56	0.568
Anorexia	1/7	14.29	12/18	66.67	0.018
Fever	2/7	28.57	13/18	72.22	0.045
Vomiting	0/7	0.00	6/18	33.33	0.079

The diversity of *G. lamblia* genotype among the studied populations could be related to different modes of transmission in each area, comprising human to human, foodborne, waterborne or zoonotic transmissions. It has been also found that these variation in the prevalence of genotypes may be attributed to the geographical location, but this finding could indicate a possible risk of waste exposure to the origin of drinking water, and in addition the life style of the studied population who may be in close contact with animal wastes, especially in rural regions [30].

It has been known that genotype A is most often responsible for zoonotic transmission with wide range of animals presenting as reservoir hosts. Although genotype B is most likely transmitted from human to human, it has

been reported in some animals and may represent a zoonotic potential as well [31]. However, assemblage B isolates are more infectious [29,32].

Interestingly, identification of different genotypes that contribute to disease enables differentiation in host specificities, transmission methods and sources of infection [33]. Therefore, it may lead to control on the parasitic infections.

The present study showed that *G. lamblia* of both genotypes A and B more prevalent among males than females. Statistically, no significant difference between assemblages' distribution and gender was found. Similar findings were reported by Anthony *et al*. in 2007, which showed no significant difference between genotypes prevalence and gender in

Philippines [34]. On the other hand, Mohammed Mahdy *et al*. in 2009 showed that females were at two fold higher risk of acquiring giardiasis of assemblage B compared to males [35]. Also, the study showed that children age range from 2 to 5 and 6 to 11 years were at higher risk of being infected with genotype B. This finding was consistent with Mohammed Mahdy *et al*. (2009) and Sadek *et al.* (2013) that detected, this age group as a high risk group for giardiasis [35,36]. This result was also in agreement with worldwide report suggesting that giardiasis is one of the serious health problems among population of younger age groups [37]. However, this may be related to the fact that children are susceptible to both genotypes with variability in predominance from one region to other. The susceptibility could be related to the practicing inappropriate personal hygiene. Lacking effective immunity has also been postulated to explain age specific manner.

The significant differences between genotypes and clinical symptoms of gastroenteritis (abdominal pain, weight loss, anorexia and fever). The results correlate genotype B with high rates of symptomatic giardiasis than genotype A, at the same time, the current study found that assemblage B of *G. lamblia* presents with all kinds of clinical features ranging from mild to severe. Similarly, Mohammed Mahdy *et al*. (2009) and Pelayo *et al*. (2008) showed that genotype B was more significantly among symptomatic patients [35,38]. This is due to alternations in the nutrient absorption caused by *G. lamblia,* and the association between *G. lamblia* genotype A and B in the development of clinically overt diarrhea and other gastrointestinal symptoms [39].

In contrast, other studies reported a significant association between assemblage A and the presence of symptoms and genotype B and asymptomatic giardiasis [9,40]. On the other hand, Eligio-Carcia *et al*. (2002) reported there was no correlation between digestive manifestations and genotypes [41]. While other study showed that genotype A and B exhibit no

apparent differences in virulence, suggesting that host factors more than type of *G. lamblia* genotypes play a dominant role in determining the clinical symptoms of the infection [42].

Recent advances suggest that variability in *Giardia* strains, host nutritional status, the composition of microbiota, co-infecting enteropathogens, host genetically determined mucosal immune responses, and immune modulation by *Giardia* are all relevant factors influencing disease manifestations after *G. lamblia* infection [43].

This study concluded that G.lamblia genotype B is the most frequent genotype among children with diarrhea. Also, the presence of the clinical aspects is genotype specific.

Author contributions
Dr. Al-Bashier put the study concept and design; Dr. Mohamed did the physical examination and diagnosis; and Hussein collect stool samples, did the laboratory analyses and preparation of the manuscript.

Conflict of Interest
The authors declare no conflict of interest.

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Hyponatremia in a Group of Iraqi Patients with Stroke

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Abstract

Background   Hyponatremia is a common electrolyte disorder encountered in patients of stroke, which is usually either due to inappropriate secretion of antidiuretic hormone (SIADH) or cerebral salt wasting syndrome (CSWS). Making an accurate diagnosis is important because the treatment of each condition is quite different.

Objectives   We conducted this study to determine the incidence and etiology of hyponatremia in patients of stroke.

Methods   A cross sectional study of 100 patients diagnosed to have stroke (50 ischemic, 50 hemorrhagic) based on clinical history, examination and brain images. Patients were evaluated for hyponatremia (serum sodium < 135 mmol/L) during the second week of their admission.

Results   Out of 100 patients, 17 patients had hyponatremia, all Patients with serum sodium level in hyponatremic range were limited to patients with hemorrhagic stroke, while no patients with ischemic stroke had a low serum sodium level, this difference was significantly different, P-value was < 0.001.

Conclusion   Incidence of hyponatremia in our study population was 17%, all Patients with hyponatremia were limited to patients with hemorrhagic stroke, in patients of hyponatremia; 82% were having SIADH and 18% were having CSWS.

Keywords   Hyponatremia, ischemic stroke, hemorrhagic stroke, syndrome of inappropriate antidiuretic hormone, cerebral salt wasting syndrome**.**

List of Abbreviations: SIADH = syndrome of inappropriate anti-diuretic hormone, CSWS = cerebral salt wasting syndrome, ADH = antidiuretic hormone, Na^+ = Sodium.

Introduction

Stroke is the third most common cause of death in developed world after cancer and cardiovascular disease; it is the most common cause for severe physical disability [1]. Stroke is defined by the World Health Organization as the clinical syndrome of rapid onset (usually seconds or minutes) of focal (or global, as in subarachnoid hemorrhage) cerebral deficit, lasting more than 24 hours or leading to death, with no apparent cause other than a vascular one [2]. Strokes can be classified into two major categories: ischemic and hemorrhagic [3].

Hyponatremia is a common electrolyte disorder encountered in patients of neurological disorders such as stroke, subarachnoid hemorrhage, and meningitis, which is usually either due to syndrome of inappropriate secretion of antidiuretic hormone (SIADH) or cerebral salt wasting syndrome (CSWS) [4].

Hyponatremia can be a vexing problem for those who care for critically ill neurologic patients. Although seemingly simple at first glance, the accurate diagnosis and effective treatment can be complex [5].

CSWS first described by Peters et al in 1950, and it is defined by the development of extracellular volume depletion due to a renal sodium transport abnormality in patients with

intracranial disease and normal adrenal and thyroid function [6].

Complications of CSWS include symptomatic hyponatremia and dehydration; CSWS is considered a definite clinical entity and may be more common than perceived [7].

CSWS usually develops in the first week following a brain insult. Its duration is usually brief (spontaneously resolves in 2-4 weeks), although it can last for several months [8].

The SIADH is the most common cause of euvolemic hyponatremia in hospitalized patients. The syndrome is defined by the hyponatremia and hypo-osmolality that results from inappropriate, continued secretion and/or action of antidiuretic hormone (ADH) despite normal or increased plasma volume, which results in impaired water excretion[9].

The ADH promotes the reabsorption of water from the tubular fluid in the collecting duct, the hydro-osmotic effect, and it does not exert a significant effect on the rate of sodium (Na^+) reabsorption.

A second action of ADH is to cause arteriolar vasoconstriction and a rise in arterial blood pressure, hyponatremia in this syndrome is a result of an excess of water and not a deficiency of Na^+ [10].

SIADH consists of hyponatremia, inappropriately elevated urine osmolality (>100 mmol/kg), and decreased serum osmolality in a euvolemic patient. SIADH should be diagnosed when these findings occur in the setting of otherwise normal cardiac, renal, adrenal, hepatic, and thyroid function [11].

In SIADH, there is a persistent production of ADH despite body fluid hypotonicity and an expanded effective circulatory volume so that the negative feedback mechanism that normally controls ADH fails and ADH continues to be released [12].

Other factors known to stimulate ADH secretion, such as hypotension, severe pain, nausea, and stress [5].

Hyponatremia that meet the criteria for the diagnosis of SIADH develop on an average of seven days following the brain insult [13].

Neurologic complications in SIADH occur as a result of the brain's response to changes in osmolality. Hyponatremia and hypo-osmolality lead to acute edema of the brain cells. The rigid calvaria prevent expansion of brain volume beyond a certain point, after which the brain cells must adapt to persistent hypo-osmolality. However, a rapid increase in brain water content of more than 5-10% leads to severe cerebral edema and herniation and is fatal [14]. Hyponatremia can be aggravated by the hospitalization and may be secondary to the administration of hypotonic intravenous fluids [15-17].

Irreversible neurologic damage and death may occur when the rate of correction of Na^+ exceeds 0.5 mmol/L/hr for patients with severe hyponatremia. At this rate of correction, osmolytes that have been lost in defense against brain edema during the development of hyponatremia cannot be restored as rapidly when hyponatremia is rapidly corrected. The brain cells are thus subject to osmotic injury, a condition termed osmotic demyelination [14,18].

Certain factors such as hypokalemia, severe malnutrition, and advanced liver disease predispose patients to this devastating complication [19].

Differentiation of SIADH from CSWS can be difficult because both can present with hyponatremia and concentrated urine with natriuresis [6]. Making an accurate diagnosis is important because the treatment of each condition is quite different.

Vigorous salt replacement is required in patients with CSWS, whereas fluid restriction is the treatment of choice in patients with SIADH. Although most physicians are familiar with SIADH, they are much less familiar with CSWS. This review emphasizes the need for CSWS to be included in the differential diagnosis of hyponatremia in a patient with central nervous system disease. Distinguishing between these two disorders is of crucial importance because therapy indicated for one disorder but used in the other can result in negative clinical consequences [20,21]. The aim of this study is to

determine the frequency of hyponatremia in patients of stroke admitted in the hospital.

Methods

This study was across sectional survey on the patients who admitted to the Neurology Ward of Al-Imamain Al-Kadhimain Medical City from January 2012 to April 2012.

The study included 100 patients diagnosed to have stroke (50 patients diagnosed to have ischemic stroke and 50 patients diagnosed to have hemorrhagic stroke) on the basis of clinical history, examination and brain image.

A questionnaire was prepared to collect data from the patients it included information about the age, sex, presentation, past medical history, brain image findings, and this information was obtained from the patients themselves or from their relatives.

All the patients were assessed for hyponatremia (serum sodium < 135 mmol/L) volume status, packed cell volume, blood urea /serum creatinine ratio, urinary sodium was also measured during the second week of their admission.

All those patients who had a history of intake of drugs that can cause hyponatremia, gastroenteritis, head trauma, brain tumor, hemorrhagic infarction, bronchogenic carcinoma, leukemia, lymphoma and recent surgery were excluded from this study.

SIADH was differentiated from CSWS by intravascular volume depletion in CSWS (elevated packed cell volume and possibly increased blood urea/serum creatinine ratio) despite a urine sodium concentration that is not low.

The data was analyzed using Statistical package for social science (SSPS) software. Data were presented as frequency and percentage. Comparison between groups using Chi-square test.

Results

The study conducted on 100 patients, 50 patients with ischemic and 50 patients with hemorrhagic stroke their ages ranged from 42 to 83 years

Mean of patients' age with hemorrhagic stroke was not significantly different from that of patients with ischemic stroke. It was 63.4±9.92 years in patients with hemorrhagic stroke while it was 60.94 ± 6.42 years in patients with ischemic stroke (Fig. 1).
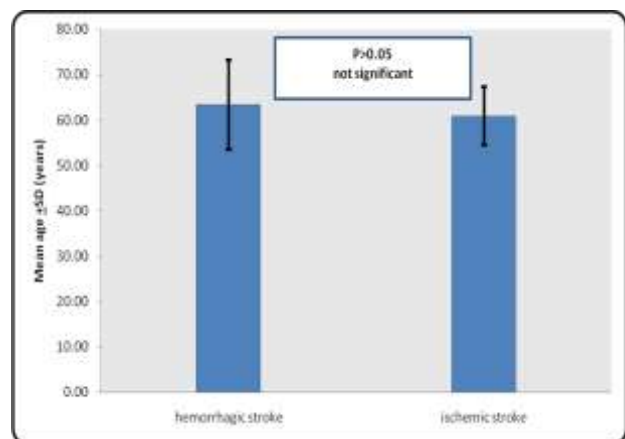
Fig. 1. Comparison of mean age between patients with hemorrhagic stroke and patients with ischemic stroke

Although patients younger than 55 years of age were more frequent in patients with ischemic stroke, 26% versus 16%, there was no statistical significance. Both types of strokes were more frequent in male patients than female patients. Male to female ratio was 1.08:1 in hemorrhagic patients and 1.27:1 in ischemic patients. Hypertension was the main risk factor in hemorrhagic group, accounting for 72%. It was significantly more frequent in hemorrhagic group than ischemic group, 72% versus 40% respectively. This difference was significant with a P-value of 0.001. Other risk factors, diabetes mellitus, structural cardiac disease and/or arrhythmia, previous stroke, smoking, and alcoholism, were not significantly different (Table 1).

Table 1. Risk factors in stroke patients

Basic Characteristic and risk factors	Hemorrhagic stroke		Ischemic stroke		p value
	No.	%	No.	%	
Gender (M:F)	26:24	52:48	28:22	56:44	0.688
Hypertension	36	72	20	40	0.001
Diabetes mellitus	12	24	13	26	0.817
Previous stroke	3	6	5	10	0.461
Structural cardiac disease and/or arrhythmias	6	12	8	16	0.695
Smoking	10	20	11	22	0.806
Alcoholic	1	2	1	2	1.000

The presentation of patients was the same in both groups, in form of disturbed level of consciousness, focal neurologic deficit, headache and visual disturbances.

Convulsion was encountered only in hemorrhagic group. None of presentation was significantly different when compared in both groups.

Mean serum sodium was significantly lower in hemorrhagic stroke than in ischemic stroke. It was 134.56 ± 5.77 mmol/L in hemorrhagic group, while it was 139.7 ± 3.16 mmol/L in ischemic group ($P < 0.001$) as shown in fig. 2.
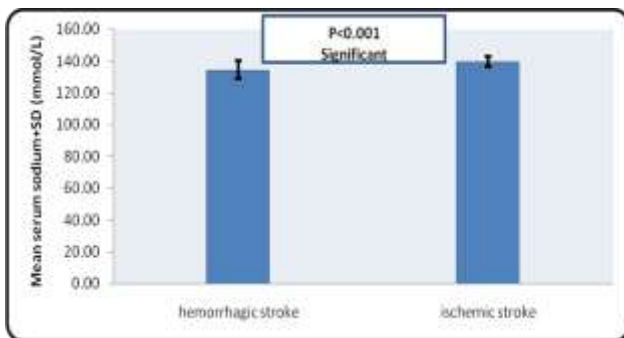
Fig. 2. Comparison of mean serum sodium between patients with hemorrhagic stroke and patients with ischemic stroke

Seventeen patients with serum sodium level in hyponatremic range (< 135 mmol/L) were limited to patients with hemorrhagic stroke, while no patients with ischemic stroke had a low serum sodium level. This difference was significantly different ($P < 0.001$) as shown in table 2.

Out of 17 Patients with serum sodium level in hyponatremic range 14 patients (82%) had SIADH and 3 patients (18%) had CSWS. This difference was significant ($P < 0.001$) as shown in table 3.

Discussion

Hyponatremia in patients with an acute CNS disease is the most common electrolyte disturbance encountered in neurological wards. Hyponatremia in stroke is usually of the hypoosmolal type caused either by SIADH or CSWS.

Out of 100, 17% of the patients who included in this study found to be hyponatremic, about 70% of the hyponatremic patient presented with disturbed consciousness, and 47% have CT scan showing intraventricular extension of the intracranial hemorrhage, this fact may be related to the severity of hemorrhage and increased intracranial pressure that in turns increase the release of ADH [22,23].

17 Patients with serum sodium level in hyponatremic range (< 135 mmol/L) were limited to patients with hemorrhagic stroke, while no patients with ischemic stroke had a low serum sodium level, this difference was significantly different. Out of 17 Patients with serum sodium level in hyponatremic range 14 patients (82%) had SIADH and 3 patients (18%) had CSWS. This difference was significant.

Table 2. Frequency of patients with hyponatremia in hemorrhagic and ischemic groups

Hyponatremia	Hemorrhagic stroke		Ischemic stroke		p value
	No.	%	No.	%	
Yes	17	34	0	0	
No	33	66	50	100	< 0.001
Total	50	100	50	100	

Table 3. Frequency of hyponatremia according to the cause

Hyponatremia	No.	%	P-value
SIADH	14	82	
CSWS	3	18	< 0.001
Total	17	100	

This is compatible with the study in Brussels, Belgium, 131 patients were studied retrospectively during 2008-2009, patients developed CSWS in 12.7%, of whom 7.9% within 1 week from admission and 4.8% after one week [24].

Another study, out of 1000 patients, 353 had hyponatremia. Out of this 353 patients, 238 (67%) had SIADH and 115 (33%) had CSWS. SIADH was seen in 83 patients who had an ischemic stroke and 155 patients of hemorrhagic type. CSWS was found in 38 patients with ischemic stroke and 77 patients with hemorrhagic ones [25]. This may be due to small sample size this current study.

In one study; hyponatremia in the acute stroke stage was seen in 11.6% of cases and has been found to be a predictor of 3-year mortality in patients with acute first-ever ischemic stroke [25].

Another study in Stanford University School of Medicine, Kao and Vavao included 316 patients from 2004 to 2007, they found that 59% of patients were hyponatremic, 35.4% were categorized with SIADH and 22.9% with CSWS [26].

One study performed previously in critically ill adult neurological patients found hyponatremia in 1-15% of the patients, which was associated with a mortality increase of 7-60% [27].

Therefore close monitoring of serum sodium must be done in all hospitalized stroke patients especially those with hemorrhagic stroke and it is of paramount importance that the physician differentiates between SIADH and CSWS because of the disparate nature of the treatments of these two entities. Improper treatment can worsen the underlying condition and may result in poor neurological outcomes.

Conflict of Interest
The authors disclose no any financial and personal relationships with other people or organizations that inappropriately influence (bias) outwork.

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