Iraqi Journal of Medical Sciences

IRAQI JMS

المجلة العراقية للعلوم الطبية

Volume 15, Number 2, 2017
April-June

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

Volume 15 (2) 2017
DOI: 10.22578/IJMS.15.2.

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

IRAQI JOURNAL OF MEDICAL SCIENCES

Editorial Board Members

Iraqi Journal of Medical Sciences

Aims and Scope

Iraqi Journal of Medical Sciences is published by College of Medicine, Al-Nahrain University. It is a quarterly multidisciplinary medical journal. High quality papers written in English, dealing with aspects of clinical, academic or investigative medicine or research will be welcomed. Emphasis is placed on matters relating to medicine in Iraq in particular and the Middle East in general, though articles are welcomed from anywhere in the world.

Iraqi Journal of Medical Sciences publishes original articles, case reports, and letters to the editor, editorials, investigative medicine, and review articles.

Mission and Vision

Mission of Iraqi JMS

To establish rapid review processes aiming to puplish scientific papers that help to augment knowledge and highlight discoveries in the field of medical sciences to be a world wide forum in assisting the distribution of medical reasearches to career readers

Vision of Iraqi JMS

To be pioneer national medical journal interesting in increasing the understanding of diseases and treatment.

All correspondence and subscription information requests should be addressed to:
The Editor of **Iraqi Journal of Medical Sciences**
College of Medicine
Baghdad, Iraq
Tel. + 964 7717516090
P.O.Box 70044, Kadhimiya, Baghdad, Iraq.
E-mail: iraqijms@colmed-alnahrain.edu.iq
http://www.iraqijms.net

Iraqi JMS FORMAT

INSTRUCTION TO AUTHORS

Iraqi Journal of Medical Sciences (Iraqi JMS) is a periodic, peer-reviewed journal published quarterly by College of Medicine, Al-Nahrain University. Iraqi JMS publishes manuscripts in all fields of health and medicine written in English.

Types of Contributions: Original articles, review articles, case studies, editorials, medical education, history of medicine, ethics, practical points, medical quiz, conferences, meetings and letters to the Editor.

Manuscripts:

- Submission of a manuscript implies that is not being considered for publication anywhere.
- The author should provide the following:

A. A document officially state that the current work was carried out at the site, which provides the certification. The document should be signed by the highest authorized member at that location.

B. Document stated clearly that his current work is in agreement with the medical ethics provided either from the local ethical committee in the place where he did his work or from the Ministry of Health, Department of Training and Improving skill - Research and Educational facilities, the approval has to be stated separetly in the method section.

C. Publication fees are 80,000 IDs in addition to 20,000 IDs for checking of plagiarism. Other extra fees will be taken for extra pages (6000 dinars for each additional page (more than six pages) and up to 24000 IDs only).

- Manuscripts submitted to Iraqi JMS are subject to editorial evaluation and revision by three referees after being checked electronically for any plagiarism.
- The format of IJMS complies with the uniform requirements for manuscripts submitted to Biomedical Journals, published by the International Committee of Medical Journals Editors (ICMJE) (Vancouver, British Colombia, 1979) and its last update in October 2001, available on the web site www.icmje.org.
- Manuscript should be typewritten font size 14, double spaced on size A4 (29.5x21 cm) paper with wide margins and line- numbered. Page should be numbered consecutively. One original and three photocopies including figures, tables, and photographs should be submitted. Begin each of following sections on separate page in the following sequence: Title page, abstract and keywords, text, acknowledgments, references, tables, and legends for illustration.
- Manuscript and figures will not be returned to the authors whether the editorial decision is to accept, revise or reject.
- Manuscripts must be accompanied by a covering paper signed by all authors that the paper has not been published in and will not be submitted to any other journal if accepted in Iraqi JMS.

- The title page should contain (a) title of the manuscript, (b) names of each author (first name, middle initial and family name) including highest academic degree, (c) official academic and/or clinical title and affiliation (d) name and address of the institution where the work was done (e) name and address (E-mail if available) of the author to whom correspondence should be sent.
- Authors can also submit the scientific publication through the official Iraqi JMS web site at (http://submit.Iraqijms.com/). Users must register when accessing the Iraqi JMS online submission system for the first time, by clicking on "Register." Three steps are involved in obtaining a personal account.

Abstract: Manuscript should include an abstract of not more than 250 words. Structured abstract typed on a separate sheet and consist of background, objective, method, results, and conclusion.

Keywords: Three to ten keywords should be provided on the same page as the abstract in English. As far as possible, be selected from the National Library of Medicine, Medical Subject Headings.

Manuscript format: It should be divided into the following parts: introduction, methods, results and discussion.

References: All references should be listed in consecutive numerical order by English numerical, in the order of citation in the text **and each reference must be followed with its DOI link.** Once a reference is cited all subsequent citations should be to the original number.

Examples

1. Standard Journal Article: use et al when the number of authors exceeds 3.
Halliwell B, Gutteridge JMC. Oxygen toxicity, Oxygen radicals, transition metals and disease. Biochem J. 1984; 219(1): 1-14.
2. Books: Mann JI, Pyorala K, Teuscher A. Diabetes in epidemiological perspective. London: Churchill Livingstone; 1983. p. 1-5.
3. Chapter in book: Phillips SJ, Whisnant JP. Hypertension and strock. In: Laragh JH, Brenner BM. editors. Hypertension: Pathophysiology, diagnosis, and management. 2nd ed. NewYork: Raven Press; 1995. p. 465-78.

- **How to find DOI for the references of your submitted article to Iraqi Journal of Medical Sciences (IJMS)**

1. First, click on this link http://www.crossref.org/guestquery/
2. Go to "search on article title"
3. Fill in the author name and the title of the reference
4. Copy and paste the found DOI (if any: as some references have no DOI) to the end of each reference in the reference list in your article to be submitted to IJMS.

That's it !!

Tables: Each table should be typed on a separate page double-spaced, including all headings, number all tables with Arabic numerals and include a short title. Vertical lines between columns are to be avoided.

Figures: All figures must be suitable for reproduction without being retouched or redrawn. Photographs must be supplied as glossy black and white prints. The top of the figures should be indicated clearly.

Legends: Captions for figures must be typed; double spaced, and must not appear on the figure.

Acknowledgments: Collate acknowledgments in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Conflict of interest: All authors must disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work. **Example** of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications\registrations, and grants or other funding. See also http://www.elsevier.com\conflictsofinterest .
Please complete and upload the conflict of interest and author declaration form with your manuscript.

Author contributions: Each author is required to declare his or her individual contribution to the article: all authors must have materially participated in the research and\or article preparation, so roles for all authors should be described. The statement that all authors have approved the final author's article should be true and included article in the disclosure.

Role of the funding source: You are requested to identify who provided financial support for the conduct of the research and\or preparation of the article and to briefly describe the role of the sponsor (s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source (s) had no such involvement then this should be stated.

List of abbreviation: Any abbreviations used should be listed after the abstract and defined at first use in the main body of the article. Use only widely accepted and conventional abbreviations. Avoid abbreviations in the title and abstract.

Proof Reading will be done by the secretarial office of the journal. The principal author will receive a copy of the journal. The authors are responsible for accuracy of all statements, data, and references included in the manuscript.

- After the manuscript has been accepted for publication, authors are required to supply the final version of the manuscript on CD in MS word version 6 or later.

Iraqi Journal of Medical Sciences

A Medical Journal Encompassing All Medical Specializations

Issued Quarterly

CONTENTS

Iraqi Journal of Medical Sciences

A Medical Journal Encompassing All Medical Specializations

Issued Quarterly

CONTENTS

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

Surgical Training and Qualification in North America: Review and Comparison

Faiz Tuma *MD, MEd, FRCSC, FACS*

Associate Professor of Surgery at Central Michigan University, USA

Abstract

Training and competency certification for the specialty of general surgery in North America have many similarities and some differences between the two countries (Canada & USA). The work and learning environment in both countries are very similar leading to many similarities in training and certification. Accreditation of the training centers and structured residency programs with carefully designed curriculum are the core requirements for specialization. The difference is in the accrediting and supervising institutions. Similarly, is the certification process. The Royal College of Physicians and Surgeons of Canada is responsible for both accrediting training centers and conducting the certification exams. While in the US, more than one organization is involved in the two processes. This variation may lead to different standards and quality of training. This difference is difficult to evaluate.

Keywords Surgery, Training, Qualification, North America

Citation Faiz Tuma. Surgical training and qualification in North America: review and comparison. Iraqi JMS. 2017; Vol. 15(2): 106-107. doi: 10.22578/IJMS.15.2.1

List of abbreviation: ACGME = Accreditation Council for Graduate Medical Education, ABSITE = American Board of Surgery In-Training Examination, CE = Certifying Exam, QE = Qualifying Exam, MOC = Maintenance of Certification, RCPSC = The Royal College of Physicians and Surgeons of Canada

Surgical training and qualification in North America consists of training in an accredited surgery program and passing a board exam. In US, the Accreditation Council for Graduate Medical Education (ACGME) supervises the training programs. While the American Board of Surgery conducts the board exam process and certification. In Canada, both the accreditation and board certification are supervised and conducted by one entity, The Royal College of Physicians and Surgeons of Canada (RCPSC).

Training in accredited surgery program is essential for the board certification in both US and Canada. There are many residency programs of variable sizes and capacity to train on surgery. In Canada, all surgery residency programs are affiliated with universities. In US, surgery programs can be either academic – affiliated with university – or community based. Each of the two setting (academic and community based training) has its own pros and cons.

In both Canada and the US, training generally follows a predesigned, structured, and approved curriculum in a residency program. In the US, there are 277 accredited general surgery residency programs and 8,102 on duty hours for the year 2017-2018 according to the official site of ACGME. While in Canada, there are 17 accredited general surgery programs. Six of these programs are in the province of Ontario, the most populated province in the country.

During the program, residents work and train in various blocks or rotations, both in the core general surgery and the related subspecialties. The duration of each block (rotation) is usually 1-3 months in length. In these blocks (rotations), residents work in teams of about 2-4 supervising surgeons and 1-3 residents. They are evaluated during and at the end of each block in addition to the evaluation at the end of each academic year. In the US, there is a yearly written examination as well. This is called the American Board of Surgery In-Training Examination (ABSITE). This exam includes both clinical and basic sciences. While in Canada, there is a primary written exam conducted by the RCPSC. It should be undertaken during the first 2-3 years of training. It is mostly for basic sciences.

In the US, the board exam process consists of two separate parts. The first part is the written exam – the Qualifying Exam (QE). This is conducted once a year, usually in August. Passing this exam allows entering the second part – the Certifying Exam (CE). CE is conducted multiple times during the year, usually around 5 times in various cities. Upon passing the CE successfully, the American Board of Surgery will issue the Board Certificate. This certificate is valid for 10 years. Re-examination every 10 years is a requirement to maintain certification. In Canada, the board exam consists of two parts but they are conducted independently of the result of each other. The written part is conducted during early June of each year; while the oral part is conducted during late June of each year independently of the written exam result. On passing both parts the RCPSC grants the board certificate.

The American College of Surgeons provides membership and educational services for its members. Board certification is not necessary for the membership. In Canada, the RCPSC provides educational, academic and maintenance of certification (MOC) services to its membership. Canadian Board certification is mandatory for the membership. MOC requires annual and five-yearly achievement of certain credit points in continuous medical and professional education. The Canadian Board is therefore valid for life. The American Board of Surgery requires recertifying (taking the board exam) every 10 years to maintain certification.

The common question is which system of training and certification is better, the Canadian system or the US system? There is no definite answer for this question. The answer depends mainly on the program of training and the ultimate goal of the surgeon. Both sides have structured training programs of variable strengths to ensure training quality. The US side relies more on the ACGME strict criteria of training due to the diversity of quality and sizes of programs. In Canada, the RCPSC relies more on the academic affiliation of all the programs with universities. All Canadian programs and training are recognized by the American Board of Surgery. But not all US programs are recognized by the RCPSC. Diversity of training and subspecialties are wider in the US than in Canada.

Large programs, especially the academic, offer more scholar and academic training than hands on. The high number of residents and fellows increases competition on available procedures for training. While in community and smaller programs there is much less completion and more available operative procedures for residents. Academically, larger programs tend to have multiple subspecialties and expertise for the diverse surgical cases. This increases the exposure and spectrum of training if hands on maintained at an acceptable level.

References

1. Accreditation Council for Graduate Medical Education. 2017. Retrieved from http://www.acgme.org/
2. American College of Surgeons. 2017. Retrieved from https://www.facs.org
3. Royal College of Physicians and Surgeons of Canada. 2017. Retrieved from http://www.royalcollege.ca/rcsite/home-e
4. The American Board of Surgery. 2017. Retrieved from http://www.absurgery.org/default.jsp?index

E-mail: faizt@hotmail.com

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

High Prevalence of John Cunningham Viruria in Renal Transplant Recipients

Mervit B. Jasim[1] *MSc*, **Ali J.H. Al-Saedi**[2] *FICMS*, **Mustafa R. Hussein**[2] *FICMS*, **Asmaa B. Al-Obaidi**[1] *PhD*, **Haider S. Kadhim**[1] *PhD*

[1]Dept. of Microbiology, College of Medicine, Al-Nahrain University, Baghdad, Iraq, [2]Center of Kidney Diseases and Transplantation, Medical City of Baghdad, Iraq

Abstract

Background John Cunningham virus (JCV) is one of the important viruses in immunocompromised patients. High JC viruria is seen in kidney allograft recipients; some of them with a polyomavirus associated nephropathy (PVAN) just like BK polyomavirus but to a lesser extent.

Objective To detect JCV viruria in a sample of Iraqi renal transplant recipients, and its association with renal function.

Methods A prospective study enrolled 71 renal transplant recipients (RTR) and 20 normal donors (ND) as controls. Urine samples were collected from all RTR and ND. Viral DNA was extracted from 1 ml urine samples, and then, JC virus DNA was detected and measured by Taqman quantitative real-time PCR.

Results Out of 71 RTRs, 31 (43.66%), and 2 out of the 20 (10%) controls had positive JC viruria. The mean JCV viruria was 6.8 x104, and 1.04x103 copies/ml for RTRs and controls respectively.

Conclusion There is a relatively high prevalence of JCV viruria in Iraqi RTR patients.

Keywords JC virus, renal transplantation, urine, real-time PCR.

Citation Mervit B. Jasim, Ali J.H. Al-Saedi, Mustafa R. Hussein, Asmaa B. Al-Obaidi, Haider S. Kadhim. High prevalence of John Cunningham viruria in renal transplant recipients. Iraqi JMS. 2017; Vol. 15(2): 108-115. doi: 10.22578/IJMS.15.2.2

List of abbreviation: BKV = BK polyomavirus, CMV = Cytomegalovirus, CSA = Cyclosporine A, D/R = Donor/recipient serostate, IS = immunosuppressive drugs, JCV = JC polyomavirus, MMF = Mycophenolate, PML = Progressive multifocal leukoencephalopathy, PVAN = Polyomavirus associated nephropathy, QRT-PCR = Quantitative real time polymerase chain reaction, RTR = Renal transplant recipients, TAC = Tacrolimus

Introduction

Human polyomaviruses have become important clinical entities, coincident with the development and use of more potent immunosuppressive agents. Polyomavirus-associated nephropathy (PVAN) is one of the important causes of graft dysfunction with a high rate of graft loss [(1)]. Two viruses among this group are well known for their association with nephropathy; those are BK and JC viruses [(2)].

The JC polyomavirus (JCV) is a small non-enveloped, with double-stranded circular DNA nucleic acid [(3)]. The virus has a high prevalence rate worldwide. Approximately 60-80% of adults in the United States have detectable antibodies against JC virus [(4)]. JC virus establishes lifelong latency in the kidneys, central nervous system, and hematopoietic progenitor cells [(5,6)]. It is considered the causative agent of progressive multifocal leukoencephalopathy (PML), which is a rare

disease characterized by the lytic infection of glial cells [7].

Infection by JCV has been observed in renal transplant recipient (RTR) as both nephropathy and/or PML. Renal transplant recipients have the highest risk of developing JCV associated nephropathy in comparison to other organ recipients [8,9].

Risk factors for PVAN are controversial and likely involve multiple determinants, but profound immunosuppression has been generally accepted as a key factor [9,10]. Low-level JCV replication and shedding are common in immunocompetent individuals, but in RTRs it is more common to observe high-level polyomavirus replication, as identified by decoy cell shedding. Moreover, progression from viruria to viremia precedes the development of histologically proven polyomavirus nephropathy by several weeks [11,12].

In Iraq, to the best of our Knowledge, there is no such study on JCV in RTRs, and few studies investigated viral infections in Iraqi renal transplants, including BK virus [13,14], human cytomegalovirus [15], Epstein Barr virus [16] and Human herpes virus-6 [17].

This study aimed to investigate the rate of occurrence of JCV in RTRs, by quantitative real time PCR in urine samples, and to correlate the level of JC viruria in RTRs with renal function test and the types of immunosuppressive regimens.

Methods

Study Population

A prospective study conducted from October 2015 to March 2016, seventy-one (71) renal transplant recipients RTRs were enrolled from the (Center of Kidney Diseases and Transplantation) in the Medical City of Baghdad, during their first post-transplantation period. A consent letter obtained from all patients and controls enrolled in the study. This study approved by the ethical Committee of the College of Medicine-Al-Nahrain University. Twenty (20) apparently healthy age and sex-matched normal donors enrolled in this study as controls.

Clinical and laboratory data were obtained from each patient. From all RTRs and controls, 5 ml urine samples collected and preserved in deep freeze for viral DNA extraction.

Two main Standard immunosuppressive regimens were mainly followed in RTRs; either the cyclosporine A (CSA), mycophenolate (MMF), and prednisolone, or the regimen that included tacrolimus (TAC) instead of CSA, in addition to MMF and prednisolone.

Viral DNA Extraction

For viral DNA extraction from the urine samples; Geneius™ Micro g DNA Extraction kit (Geneaid, England) was used. One ml urine sample was used in viral DNA extraction, according to the manufacturer protocol.

Real Time PCR for Measuring JC Viruria

For the quantitative detection of JCV; GeneProof PCR kit ISIN Version kit (England) is a Real-Time test, which is based on the principle of the so-called - “TaqMan” probe. Thirty µl of Master Mix were added into PCR tubes, and 10 µl of the (sample DNA, positive or negative controls, or standards) were added to the master mix. The final reaction volume was 40 µl. All components were kept at +2 °C to +8 °C during the PCR preparation. Real time PCR instrument used in this work was STRATAGENE MxPro QPCR (Agilent Technologies, USA). The thermal protocol for Geneproof PCR kit is composed of a two hold steps, and one amplification cycle. The real-time data is collected at the third step of the amplification cycle as demonstrated in table (1).

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

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

instructions, JCV DNA copies was calculated according to the following formula:

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

Table 1. JCV real time PCR amplification profile

Steps	Temperature	Time	Data collection	Cycle
1-hold	37 °C	2 min		**1**
2-hold	95 °C	10 min		**1**
3PCR	95 °C	5 s	FAM+HEX	**45**
	60 °C	40 s		
	72 °C	20 s		

Statistical analysis

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

Results

Among the 71 RTR; 61 (85.90%) were males, and 10 (14.10%) were females, their mean age was 38.54±8.01 years, ranging between 18 and 77 years. Statistically, there was no significant difference (P=0.117) between the mean of the RTRs and control group indicating that they were of a comparable age.

Quantitative real time PCR (QRT-PCR) run gave positive viruria in 31 out of 71 (43.7%) RTRs and 2 (10%) out of the 20 controls, which was significantly higher in RTRs, (P=0.007).

The mean of JCV viruria was 6.8 x104, and 1.04x103 copies/ml for RTRs and controls respectively, which is significantly higher in RTRs (P<0.001).

On the other hand, table (2) demonstrated that 15/25 (60%) of RTR with elevated serum creatinine values, were positive for JC viruria, which was statistically significant (P=0.048). In addition, on comparing JC viruria with the type of immunosuppression regimen, 21 out of the 31 viruria patients (67.7%), were on CSA regimen, and the remaining 10/31 (32.3%) were on TAC regimen, however, these results were statistically not significant (p= 0.146), table (2) demonstrate the clinical data demographic data of RTR patients in relation with JC viruria.

Discussion

There's a growing evidence on the high seroprevelance of JCV and its association with significant deadly diseases [18-20]. In this study, JCV was investigated in urine of RTRs using QRT-PCR, and 31 out 71 (43.66%) of RTRs had positive JCV viruria (JCV DNA in urine); the frequency was higher in comparison to other studies, ranging from 13.7 to 36.8% [21-24].

It is well known that all organ transplant recipients are immunocompromised subjects because of the chronic use of IS drugs, therefore these patients are subjected to develop reactivation of any latent viruses [25]. JCV is among the viruses that remains latent for the whole life of the infected person [5,6,8,26,27].

Table 2. Clinical and demographic characteristics of RTR patients in relation with JC viruria

		Viruria positivity					
		Present No. (%)	Absent No. (%)	Total	P value	RR	CI
Age groups	< 40 years	15 (42.86)	20 (57.14)	35	0.542		
	≥ 40 years	16 (44.44)	20 (55.56)	36			
Gender type	Female	4 (44.44)	5 (55.56)	9	0.616		
	Male	27 (43.55)	35 (56.45)	62			
Donor relation	Un related	19 (38.78)	30 (61.22)	49	0.163		
	Related	12 (54.55)	10 (45.45)	22			
PTP	< 6 months	16 (55.17)	13 (44.83)	29	0.084		
	≥ 6 months	15 (35.71)	27 (64.29)	42			
Serum Creatinine	Abnormal	15 (60)	10 (40)	25	**0.048**	1.72	1.02 -2.87
	Normal	16 (34.78)	30 (65.22)	46			
Creatinine clearance	Abnormal	19 (57.58)	14 (42.42)	33	0.495		
	Normal	12 (31.58)	26 (68.42)	38			
CMV IgG D/R	-/-	8 (34.78)	15 (65.22)	23	0.495		
	-/+	5 (45.45)	6 (54.55)	11			
	+/-	3 (75)	1 (25)	4			
	+/+	15 (45.45)	18 (54.55)	33			
CMV IgM D/R	-/-	30 (44.12)	38 (55.88)	68	0.416		
	-/+	0 (0)	1 (100)	1			
	+/-	0 (0)	1 (100)	1			
	+/+	1 (100)	0 (0)	1			
IS regimen	CSA	21 (50)	21 (50)	42	0.146		
	TAC	10 (34.48)	19 (65.52)	29			
UTI	None	25 (42.37)	34 (57.63)	59	0.431		
	UTI	6 (50)	6 (50)	12			

PTP: post-transplantation period, CMV: cytomegalovirus, D/R: donor/recipient serostate, IS: immunosuppression, CYC: cyclosporine, TAC: tacrolimus, UTI: urinary tract infection.

Studies on polyomavirus viruria have shown different patterns of shedding for each of these viruses in normal and immunosuppressed hosts (28,8,29,30). BKV viruria is rare in healthy individuals (0-6%) and significant BKV urinary replication and shedding are clearly dependent on disruptions of cellular immunity [8,28,29,31-33]. Thus, BKV viruria increases in HIV patients in whom viruria correlates with lower CD4 counts [8,29,30,34]. In contrast, asymptomatic JCV viruria commonly found in immunocompetent hosts (could reach up to 40%) [8,28,29,30,34] and the relationship of JCV viruria to immune dysregulation is less clear than that of BKV.

The fact that the JCV has the potential to cause renal disease albeit less commonly than BKV, is confirmed in sporadic cases [31,35]. Specifically, JCV-mediated nephropathy has been reported by Kazory et al., 2003 [36], and Wen et al., 2004 [2] in RTRs. Recently, a study showed that infection of primary human renal tubule epithelial cells with JCV and BKV results in divergent innate immune responses that control JCV but fail to control BKV [37].

In another study, Drachenberg et al., 2007 [21], they have found that one fifth of renal transplant recipients were shedding JCV developed biopsy-proven JCV nephropathy.

Results of the current study showed that 15 out of 25 (60%) RTRs who had high serum creatinine, were positive for JC viruria. These results are in agreement with that of other studies, which suggested a role of JCV in allograft nephropathy in RTRs just like BKV [3,21,38-40].

There was no significant association between JCV viruria and age, a result which is supported by other reports from Brazil (Melo et al., 2013) [41], Spain (Lopez et al., 2008) [42], Iran (Taheri et al., 2011) [40], (Bozorgi et al. 2012) [43] and USA (Agostini et al., 1996) [44].

Similarly, no significant difference was observed between different genders regarding JC viruria, which was the same as reports from Italy (Pagani et al., 2003) [45], Poland (Kmieciak et al., 2008) [46] and Serbia (Karalic et al. 2014) [47]. Although, 27 out 31 (87.09%) who had positive JC viuria were males, which is in consistence with other studies that found significantly higher frequency of the virus in males, in USA (Agostini et al., 1996) [43], and Japan (Zhong et al., 2007) [48].

The current study found no correlation between JCV viuria and CMV IgM and IgG donor/recipient (D/R) serostate. The frequency of CMV IgG D+/R+ was 15 out 31 JCV positive viuria, while CMV IgM D+/R+ was 1 out of 31 JCV positive viuria. A study on multiple sclerosis in Denmark showed that out of 123 patients, fifty-three patients (43.1%) were JCV negative and 70 (56.9%) positive. CMV-IgG antibodies were detected only in six patients, otherwise no IgM antibodies were detected [49].

Results of this study showed that the number of patients with early < 6 post-transplantation period was 16 out of 31 JCV positive viuria and number of patients with late post-trnsplantation period ≥ 6 were 15 out of 31 JCV positive viuria. Statistically, there' was no significant difference between JCV frequency and the post-transplantation period, which is consistent with the findings of others studies which showed that JC virus prevalence was consistent regardless of the time after renal transplantation [40,50].

The current study found no significant difference between the prevalence of JCV viruria and donor relation with recipient and this finding agrees with other study which showed no correlation to donor relatedness [51]. However, 19 out 31 (61.2%) positive patients had living unrelated donor, which could be explained by the fact that the most of the nephrologist increase the dose of IS drugs in patients who had allograft from unrelated donor due to increased risk of HLA-mismatch. This may activate stronger cell mediate-immunity response, and thus increase the risk of viral infections or reactivations [52].

Studies on BKV, showed that better HLA matching reduces the risk of kidney allograft rejection and the risk of viral nephropathy [53].

The mean and frequency of JC viruria in RTRs were significantly higher than in healthy controls ($p<0.007$). These results are consistent with the findings in Taiwan (Lai et al., 2008) [54]; (Yin et al. 2010) [55] and China (Hu et al., 2011) [56]. This could explain the role of immunosuppressive regimens used in RTRs. Immunosuppressive drugs were considered as risk factor for reactivation of JCV in RTRs which is documented by different studies [21,56].

Although in the current study there was no significant correlation between JCV and type of IS regimen, 21 out of 31 (67.7%) positive JC viruria were on CSA regimen, and that agrees with study by (Hu et al., 2011) [56] who showed CSA is a risk factor for JCV reactivation.

Some studies have reported that therapy containing MMF, or CSA or azathioprine significantly increase the risk of polyomavirus infection [25,57,58], while other studies have failed to find a correlation between the frequency of JCV viruria and the use of immunosuppressive drugs. [31,59]. However, in this study, CSA use was an important independent predictor of JCV infection.

One limitation of the present study was lack of confirmation of the detection by kidney biopsy because of unapproved protocol biopsy in our center.

In conclusion, the relatively high prevalence of JC viruria as compared with control group.

Acknowledgments

Authors would like to acknowledge all the staff members in the Center of Kidney Diseases and Transplantation, Medical City of Baghdad, for their cooperation in accomplishing this study

Author contributions:

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

Conflict of interest

Authors declare no conflict of interest.

Funding

There is no funding source for this research.

References

1. Hirsch HH, Knowles W, Dickenmann M, et al. Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients. N Engl J Med. 2002; 347(7): 488-96. doi: 10.1056/NEJMoa020439.
2. Wen MC, Wang CL, Wang M, et al. Association of JC virus with tubulointerstitial nephritis in a renal allograft recipient. J Med Virol. 2004; 72(4): 675-8. doi: 10.1002/jmv.20037.
3. Delbue S, Ferraresso M, Ghio L, et al. A review on JC virus infection in kidney transplant recipients. Clin Development Immunol. 2013; 2013: 926391. doi: 10.1155/2013/926391.
4. Padgett BL, Walker DL. Prevalence of antibodies in human sera against JC virus, an isolate from a case of progressive multifocal leukoencephalopathy. 1973; 127(4): 467-70. doi: 10.1093/infdis/127.4.467.
5. Arthur RR, Shah KV. Occurrence and significance of papovaviruses BK and JC in the urine. Prog Med Virol. 1989; 36: 42-61.
6. Schneider EM, Dörries K. High frequency of polyomavirus infection in lymphoid cell preparations after allogeneic bone marrow transplantation. Transplant Proc. 1993; 25(1 Pt 2): 1271-3.
7. Delbue S, Ferraresso M. JC polyomavirus infections in transplant patients. 2012; 2(3): 1000e114. doi: 10.4172/2161-0991.1000e114.
8. Hirsch HH, Steiger J. Polyomavirus BK. Lancet Infect Dis. 2003; 3(10): 611-23.
9. Hirsch HH. Polyomavirus BK nephropathy: a (re-) emerging complication in renal transplantation. Am J Transplant. 2002; 2(1): 25-30.
10. Comoli P, Binggeli S, Ginevri F, et al. Polyomavirus-associated nephropathy: Update on BK virus-specific immunity. Transpl Infect Dis. 2006; 8(2): 86-94. doi: 10.1111/j.1399-3062.2006.00167.x.
11. Drachenberg CB, Hirsch HH, Ramos E, et al. Polyomavirus disease in renal transplantation: Review of pathological findings and diagnostic methods. Hum Pathol. 2005; 36(12): 1245-55. doi: 10.1016/j.humpath.2005.08.009.
12. Drachenberg CB, Papadimitriou JC, Hirsch HH, et al. Histological patterns of polyomavirus nephropathy: Correlation with graft outcome and viral load. Am J Transplant. 2004; 4(12): 2082-92. 10.1046/j.1600-6143.2004.00603.x.
13. Al-Obaidi AB, Abd KH, Habib MA, et al. detection of Bk Polyomavirus using Real time PCR and urine Cytology In 99 Renal Transplant Recipients. Iraqi J Med Sci. 2015; 3(1): 131-41.
14. Al-Obaidi AB, Qasim BJ, Husain AG, et al. BK Polyomavirus-infected decoy cells in urine cytology specimens of renal transplant recipients. Iraqi J Med Sci. 2015; 13(1): 70-5.
15. Kadhim HS, Al-Obaidi AB, Abd KH, et al. BK polyomavirus and Cytomegalovirus Co-infections in renal transplant recipients : a single center study. Int J Adv Res. 2015; 3(1): 856-64.
16. Shams-aldein SA, Abdlameer AS, Al-obaidi A, et al. Detection of Epstein Barr virus in renal transplant recipients: two centers study. Iraqi J Med Sci. 2015; 13(2): 191-9.
17. Jasim HR. A study of viral load of HHV-6 DNA in samples of Iraqi patients during the first year after kidney transplantation. MSC thesis 2015. Microbiology, College of Medicine Al-Nahrain University, Iraq.
18. Åström K-E, Mancall EL, RIchardson EP. Progressive multifocal leuko-encephalopathy. Brain. 1958; 81(1): 93-111.
19. Brooks BR, Walker DL. Progressive multifocal leukoencephalopathy. Neurol Clin. 1984; 2(2): 299-313.
20. Houff SA, Berger JR. The bone marrow, B cells, and JC virus. J Neurovirol. 2008; 14(5): 341-3. doi: 10.1080/13550280802348222.
21. Drachenberg CB, Hirsch HH, Papadimitriou JC, et al. Polyomavirus BK versus JC Replication and nephropathy in renal transplant recipients: a prospective evaluation. Transplantation. 2007; 84(3). 323-30. doi: 10.1097/01.tp.0000269706.59977.a5

22. Costa C, Bergallo M, Sidoti F, et al. Polyomaviruses BK- And JC-DNA quantitation in kidney allograft biopsies. J Clin Virol. 2016; 44(1): 20-3. doi: 10.1016/j.jcv.2008.08.006.

23. Randhawa PS, Finkelstein S, Scantlebury V, et al. Human Polyoma virus-associated interstitial nephritis in the allograft kidney. Transplantation. 1999; 67(1): 103-9.

24. Boldorini R, Omodeo-Zorini E, Suno A, et al. Molecular characterization and sequence analysis of Polyomavirus strains isolated from needle biopsy specimens of kidney allograft recipients. Am J Clin Pathol. 2001; 116(4): 489-94. doi: 10.1309/GAUE-92W7-ACDV-X46M.

25. Cukuranovic J, Ugrenovic S, Jovanovic I, et al. Review Article Viral Infection in Renal Transplant Recipients. ScientificWorldJournal. 2012; 2012: 820621. doi: 10.1100/2012/820621.

26. Dörries, K. Latent and persistent Polyomavirus infection. In: Khalili K, Stoner GL. Human Polyomaviruses: molecular and clinical perspectives (eds). New York, USA: John Wiley & Sons, Inc.; 2001. doi: 10.1002/0471221945.ch10.

27. Qiao L, Qu Q, Jiang X. Clinical significance of quantitative and qualitative detection of BK and JC virus in blood and urine of renal transplantation recipients. Pakistan J Med Sci. 2016; 32(2): 435-9. doi: 10.12669/pjms.322.8978.

28. Polo C, Pérez JL, Mielnichuck A, et al. Prevalence and patterns of polyomavirus urinary excretion in immunocompetent adults and children. Clin Microbiol Infect [Internet]. 2016; 10(7): 640-4. doi: 10.1111/j.1469-0691.2004.00882.x.

29. Doerries K. Human Polyomavirus JC and BK persistent infection. In: Ahsan N (ed). Polyomaviruses and human diseases. New York, NY: Springer; 2006. p. 102-16. doi: 10.1007/0-387-32957-9_8.

30. Knowles WA. Discovery and epidemiology of the human Polyomaviruses BK virus (BKV) and JC virus (JCV). In: Ahsan N (ed). Polyomaviruses and human diseases. New York, NY: Springer; 2006. p. 19-45. doi: 10.1007/0-387-32957-9_2.

31. Randhawa P, Uhrmacher J, Pasculle W, et al. A comparative study of BK and JC virus infections in organ transplant recipients. J Med Virol. 2005; 77(2): 238-43. doi: 10.1002/jmv.20442.

32. Tsai RT, Wang M, Ou WC, et al. Incidence of JC viruria is higher than that of BK viruria in Taiwan. J Med Virol. 1997; 52(3): 253-7.

33. Kitamura T, Yogo Y, Kunitake T, et al. Effect of immunosuppression on the urinary excretion of BK and JC Polyomaviruses in renal allograft recipients. Int J Urol. 1994; 1(1): 28-32.

34. Markowitz RB, Thompson HC, Mueller JF, et al. Incidence of BK virus and JC virus in human immunodeficiency virus-infected and -uninfected subjects. J Infect Dis. 1993; 167(1): 13-20.

35. Racusen LC, Halloran PF, Solez K. Banff 2003 Meeting Report: New Diagnostic Insights and Standards. Am J Transplant. 2004; 4(10): 1562-6. doi: DOI: 10.1111/j.1600-6143.2004.00585.x.

36. Kazory A, Ducloux D, Chalopin JM, et al. The first case of JC virus allograft nephropathy. Transplantation. 2003; 76(11): 1653-5. doi: 10.1097/01.TP.0000090749.42791.14.

37. Assetta B, De Cecco M, O'Hara B, et al. JC Polyomavirus infection of primary human renal epithelial cells is controlled by a type I IFN-induced response. MBio. 2016; 7(4): pii: e00903-16. doi: 10.1128/mBio.00903-16.

38. Kusne S, Vilchez RA, Zanwar P, et al. Polyomavirus JC urinary shedding in kidney and liver transplant recipients associated with reduced creatinine clearance. J Infect Dis. 2012; 206(6): 875-80. doi: 10.1093/infdis/jis469.

39. Kijpittayarit S, Razonable RR. JC Virus Infection After Transplantation: Beyond the Classic Progressive Multifocal Leukoencephalopathy? Gastroenterol Hepatol (NY). 2007; 3(1): 74-6.

40. Taheri S, Kafilzadeh F, Shafa M, et al. Original Article Comparison of polyomavirus (BK virus and JC viruses) viruria in renal transplant recipients with and without kidney dysfunction. J Res Med Sci. 2011; 16(7): 916-22.

41. Melo FA, Bezerra AC, Santana BB, et al. JC polyomavirus infection in candidates for kidney transplantation living in the Brazilian Amazon Region. Mem Inst Oswaldo Cruz. 2013; 108(2): 145-9.

42. López V, Gutiérrez C, Burgos D, et al. Prospective study of infection and nephropathy due to BK and JC Polyomavirus in 76 kidney transplant recipients. Transplant Proc. 2008; 40(9): 2927-9. doi: 10.1016/j.transproceed.2008.08.098.

43. Bozorgi SM, Tahaei SME, Mohebbi SR, et al. Molecular prevalence of JC virus in Tehran, Iran. Gastroenterol Hepatol Bed Bench. 2012. 5: 84-9.

44. Agostini HT, Ryschkewitsch CF, Stoner GL. Genotype profile of human polyomavirus JC excreted in urine of immunocompetent individuals. J Clin Microbiol. 1996; 34(1): 159-64.

45. Pagani E, Delbue S, Mancuso R, et al. Molecular analysis of JC virus genotypes circulating among the Italian healthy population. J Neurovirol. 2003; 9: 559-66. doi: 10.1080/13550280390241269.

46. Kmieciak D, Dębicki S, Trzeciak WH. Occurrence rate and genotype distribution of the JC virus (JCV) in a sample from the Polish population. J Med Virol. 2008; 80: 1079-83.

47. Karalic D, Lazarevic I, Knezevic A, et al. Distribution of JC virus genotypes among serbian patients infected with HIV and in healthy donors. J Med Virol. 2014; 86(3): 411-8. doi: 10.1002/jmv.23796.

48. Zhong S, Zheng HY, Suzuki M, et al. Age-related urinary excretion of BK polyomavirus by nonimmunocompromised individuals. J Clin Microbiol. 2007; 45: 193-8. doi: 10.1128/JCM.01645-06.

49. Atyabi SR, Bouzari M, Kardi MT. John cunningham (JC) virus genotypes in kidney transplant recipients,

rheumatoid arthritis patients and healthy individuals in Isfahan, Iran. J Med Virol. 2017; 89(2): 337-44. doi: 10.1002/jmv.24626.
50. Auer M, Borena W, Dorothee HL, et al. Correlation between Anti-JCV and Anti-CMV Antibodies (P4.037). Neurology. 2015; 84(14): Supplement P4.037.
51. Funahashi Y, Kato M, Fujita T, et al. Prevalence of Polyomavirus positivity in urine after renal transplantation. Transplant Proc. 2014; 46(2): 564-6. doi: 10.1016/j.transproceed.2013.09.053.
52. Lopez V, Gutierrez C, Sola E, et al. Does JC Polyomavirus cause nephropathy in renal transplant patients? Transplant Proc. 2010; 42(8): 2889-91. doi: 10.1016/j.transproceed.2010.07.061.
53. Mengel M, Marwedel M, Radermacher J, et al. Incidence of polyomavirus-nephropathy in renal allografts: influence of modern immunosuppressive drugs. Nephrol Dial Transplant. 2003; 18(6): 1190-6.
54. Lai NS, Lu MC, Lee MC, et al. Detection of polyomavirus JC genotype from transplant patients by capillary electrophoresis: comparison to fragment length polymorphism analysis. J Formos Med Assoc. 2008; 107(3): 239-44. doi: 10.1016/S0929-6646(08)60142-4.
55. Yin WY, Lu MC, Lee MC, et al. A correlation between polyomavirus JC virus quantification and genotypes in renal transplantation. Am J Surg. 2010; 200(1): 53-8. doi: 10.1016/j.amjsurg.2009.03.017.
56. Hu JH, Zhao H, Huang YP, et al. Opportunistic posttransplantation virus infections in renal transplant recipients. Transplant Proc. 2011; 43(10): 3715-9. doi: 10.1016/j.transproceed.2011.07.024.
57. Sachdeva MS, Nada R, Jha V, et al. The high incidence of BK polyoma virus infection among renal transplant recipients in India. Transplantation. 2004; 77(3): 429-31. doi: 10.1097/01.TP.0000113163.02039.30.
58. Brennan DC, Agha I, Bohl DL, et al. Incidence of BK with tacrolimus versus cyclosporine and impact of preemptive immunosuppression reduction. Am J Transplant. 2005; 5(3): 582-94. doi: 10.1111/j.1600-6143.2005.00742.x.
59. Koralnik IJ, Boden D, Mai VX, et al. JC virus DNA load in patients with and without progressive multifocal leukoencephalopathy. Neurology. 1999; 52(2): 253-60.

Correspondence to Mervit B. Jasim
E-mail: mvt.msc89@gmail.com
Received Oct. 24th 2016
Accepted Jan. 2nd 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

The Effects of Dexamethasone on Tibia Development of Local Chick-Embryo. I: Computer-Assisted Morphometric Study

Wathiq Q.S. Ali[1] *MSc*, **Mohammad O. Selman**[1] *FICMS*, **Insaf J. Al-Hasson**[2] *PhD*, **Imad M. Al-Ani**[3] *PhD*, **Anam R. Al-Salihi**[4] *PhD*

[1]Dept. of Applied Embryology, High Institute of Infertility Diagnosis and ART, Al-Nahrain University, Baghdad, Iraq, [2]Al-Kindy College of Medicine, Baghdad University, Baghdad, Iraq, [3]Dept. of Basic Medical Sciences, Kulliyyah of Medicine, International Islamic University Malaysia, [4]Al-Nahrain University, Baghdad, Iraq

Abstract

Background Dexamethasone is a glucocorticoid as a member of the steroidal anti-inflammatory and immunosuppressant. It has well documented effects on skeletal structures osseous and cartilaginous, commonly used to treat or control diseases.

Objective To evaluate by histomorphometric study the effects of dexamethasone on the embryogenesis of long bones in chick embryos.

Methods Forty-eight fertile chick eggs of *Gallus gallus domesticus*, were used. The eggs were divided into 2 groups; control and treated groups of 24 eggs each, these groups were subdivided into 4 subgroups (n=6 eggs). On day 10 of incubation, the control group was injected with 25 µl of distilled water while the treated group was injected with 25 µl of distilled water contained 8 µg dexamethasone. In the next days (11, 12, 13, and 14 of incubation), 12 chick embryos were sacrificed in each day. A computer-assisted morphometric/ image analysis (Motic Image Plus version 2.0ML), was used to measure length, area, perimeter of tibiae, and the area and perimeter of the perichondral osseous collar of cross section in mid-diaphyseal zone of these bones.

Results These bones of chick embryos treated with dexamethasone, suffered shortening and retardation in length, weight, area and perimeter throughout the period of this study, decline area and perimeter to the perichondral osseous collar in the mid-diaphyseal zone.

Conclusion Dexamethasone given at day 10 of incubation caused tibial bones growth retardation at development stages 11, 12, 13, and 14-days; this was observed in the measured parameters: bone length, area, perimeter and weight.

Keywords Bones, chick-embryo, dexamethasone, histomorphometry

Citation Wathiq Q.S. Ali, Mohammad O. Selman, Insaf J. Al-Hasson, Imad M. Al-Ani, Anam R. Al-Salihi. The Effects of dexamethasone on tibia development of local chick-embryo. I: computer-assisted morphometric study. Iraqi JMS. 2017; Vol. 15(2): 116-128. doi: 10.22578/IJMS.15.2.3

List of abbreviations: POC = Perichondral osseous collar

Introduction

The chick embryos (*Gallus gallus domesticus*) are very good models for the study of early vertebrate embryogenesis and later organogenesis [(1)]. Furthermore, the ease of in vivo experimental manipulation is one of the main factors that have made the chick embryo an important animal in developmental research [(2)]. In addition, the poultry models have been proven as a valuable model for human skeletal defects [(3)]. The developmental phase between 4 and 9 days of incubation is characterized by rapid

changes in the wings, legs, and visceral arches [3]. Detailed morphological sequence of events occurring in long bone development from Hamburger-Hamilton stage 32 through stage 44 "7.5-18 days" and 2 days post hatching; the detailed patterning of osteoblasts, osteoid, mineral and vasculature were observed at the mid-diaphysis of the tibia [4]. Dexamethasone is potential glucocorticoid steroid exhibiting both anti-inflammatory and immunosuppressant properties, it is on the WHO model list of essential medicines, the most important medications needed in a basic health system [5]. It is widely used to treat many chronic inflammatory diseases and autoimmune conditions, such as rheumatoid arthritis and bronchospasm [6]. It also can be used postoperatively to reduce pain, wound infection, nausea and vomiting [7]. However, glucocorticosteroids has diverse effects on various systems of the body. Glucocorticoid excess also inhibited osteoblast activities leading to the development of osteopenia and osteoporosis [8]. Glucocorticoids impair the replication, differentiation and function of osteoblasts and induce the apoptosis of mature osteoblasts and osteocytes leading to suppression of bone formation [9].

The present study was conducted to evaluate by histomorphometric study, the effects of dexamethasone drug on embryogenesis of long bone in chick embryo.

Methods

Egg collection

This study was conducted using fertilized chick eggs of *Gallus gallus domesticus* chick mothers taken from the Public Authority for Agricultural Research, Ministry of Agriculture, Iraq.

Incubation of eggs

The eggs were swabbed by gauze with 70% ethanol, incubated in egg incubator with automated turning motor of the egg rack, and a stable temperature of 38 °C, and humidity about 55-60% with regulation circulated fresh air. Turning of eggs was done 8 times/day at 90° about its longitudinal axis.

Experimental design

Forty-eight fertile chick eggs were randomly divided into two groups; control and experimental (24 eggs per group) each group was subdivided into 4 subgroups of 6 eggs. At day 10 of incubation, control group were injected with 25 µl of distilled water while experimental group were injected with 25 µl of distilled water containing 8 µg of dexamethasone through a hole in the flatter zone of the egg (air cell). Subgroup "A" eggs were opened at day 11, subgroup "B" eggs were opened at day 12, subgroup "C" eggs were opened at day 13, while subgroup "D" eggs were opened at day 14 of total incubation, of control and experimental groups.

The legs (left and right) were removed from each embryo; tibia bone was skinned carefully under dissecting microscope, avoiding any damage or break by using skinning techniques under dissecting microscope. The weight of each tibia bone was taken with digital electric balance. The whole length of was measured by putting the bone at the beginning of ruler picture, the morphometric system was calibrated by comparison with this ruler measurement (Motic Image Plus 2.0ML), then taken the measurement of the length, area and perimeter to all bones, that will be studied in this study. Tibias were fixed in 10% formal saline, dehydrated through graded alcohols, cleared twice with xylene and embedded in paraffin wax. The mid-diaphyseal zone of tibia was sectioned by microtome (7 µm), to obtain the serial sections of the bone in this area then sprouted these sections on slides with the aid of water bath at 37 °C; all of these slides were stained with haematoxiline and eosin. Final examination of these sections at 4X, and serial images of these serial sections were taken by microscope with camera with TV-Based computer (micros), the best 3 images of each bone (N= 96) cross sections (N= 288 images) were entered in the morphometric system

(Motic) in the laptop software to take the measurement of the outside or total Area (A1) and total perimeter (P1), so the area (A2) and Perimeter (P2) of the internal bone cavity, the remain space between two area and two perimeters is the perichondral osseous collar (POC).The experimental animal protocol was approved by Institute Review Board of the College of Medicine, Al-Nahrain University.

Statistical analysis

Data were analyzed using, two-way classification with interaction (ANOVA) within SAS statistical Program (version 9.1/ 2010. USA). Means were compared by t-test at P<0.05 level of significant. when the result appears equal to or more than the LSD value, that mean a significant difference, but if the result appears less than the LSD value, that mean the difference in the result is statistically not significant, with keeping the P value on <0.05. Microsoft office excel 2007 programs, was used to illustrate the figure. Data were expressed as Means ± Standard Error of means.

Results

General observations

In the present study, dexamethasone injection on the 10th day of incubation in the air cell of the fertile chick eggs produced retardation on the ossification processes in the mid-diaphyseal zone of the tibia bone (Perichondral Osseous Collar), and in the four bone parameters that have been studied like: length, area, perimeter, and the weight in compare with the control group.

Perimeter measurement of tibia bone

No significant difference between the control (26.58±0.91) and treated (25.18±0.76) subgroups. On day 11, the t-test between them was 1.4 mm, this value was less than LSD value (LSD=2.52) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (32.03±0.3) and treated (24.63±0.51) subgroups; the t-test between them was 7.4 mm, this value was more than LSD value (LSD=2.52) when the P-value was constant at (P<0.05). On day 13, there was a significant difference between the control (36.33±1.65) and treated (27.43±0.48) subgroups; the t-test between them was 8.9 mm, this value was more than LSD value (LSD=2.52) when the P-value was constant at (P<0.05). Also on day 14; a significant difference was observed between the control (46.87±0.99) and treated (28.59±0.84) subgroups; the t-test between them was 18.28 mm, this value was more than LSD value (LSD=2.52) as in (Fig. 1).

Surface area measurement of tibia bone

Figure 2, showed no significant difference between the control (12.02±0.86) and treated (11.54±0.68) subgroups on day 11, the t-test between them was 0.48 mm^2, this value was less than LSD value (LSD=2.66) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (17.64±0.39) and treated (10.95±0.50) subgroups, the t-test between them was 6.69 mm^2, this value was more than LSD value (LSD=2.66) when the P-value was constant at (P<0.05). On day 13, there was a significant difference between the control (22.39±1.83) and treated (12.41±0.34) subgroups, the t-test between them was 9.98 mm^2, this value was more than LSD value (LSD=2.66) when the P-value was constant at (P<0.05). Also on day 14, there was a significant difference between the control (34.29±1.27) and treated (13.15±0.67) subgroups, the t-test between them was 21.14 mm^2, this value was more than LSD value (LSD=2.66) when the P-value was constant at (P<0.05). Concerning the difference between the means of control subgroups at different days, the results showed a significant linear increase difference in the bone area, while the differences between the means of treated subgroups at different days, the results showed no difference in the area as shown in (Fig. 2).

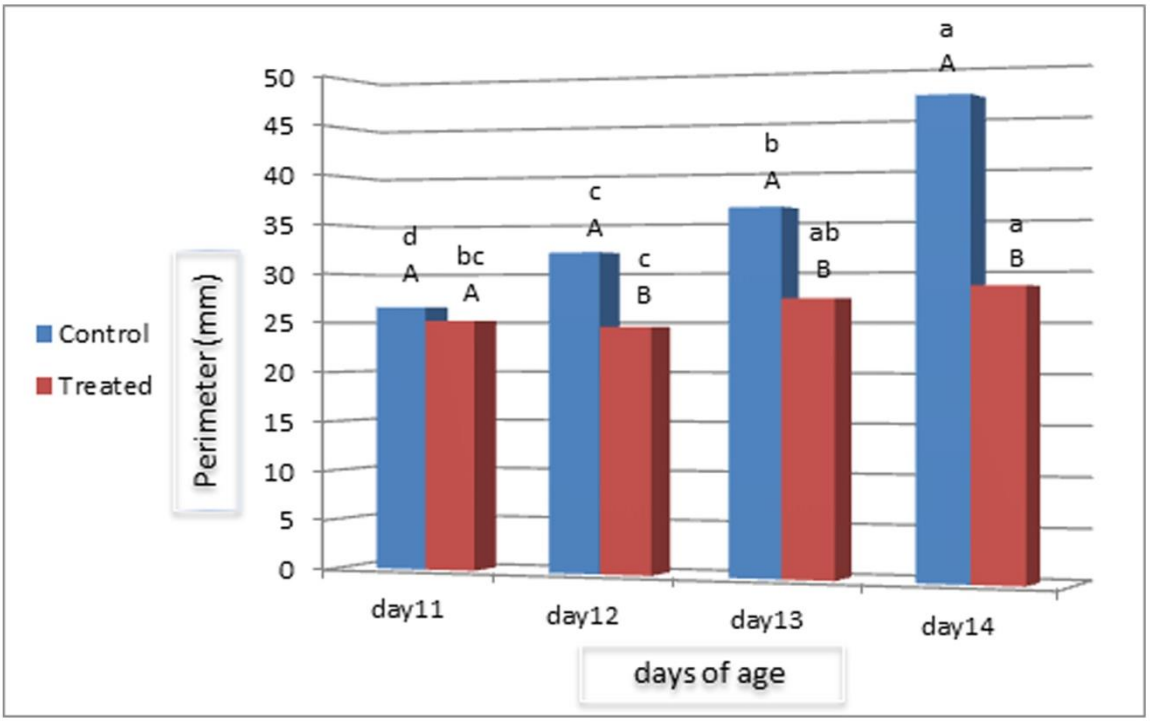

Figure 1. Comparison between the mean of growth of the Perimeter of the 12 tibia bone of chick embryos in the control and treated subgroups from day 11 to day 14 of incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between any two days differ significantly. Means with same small common letter in between any two days differ insignificantly.

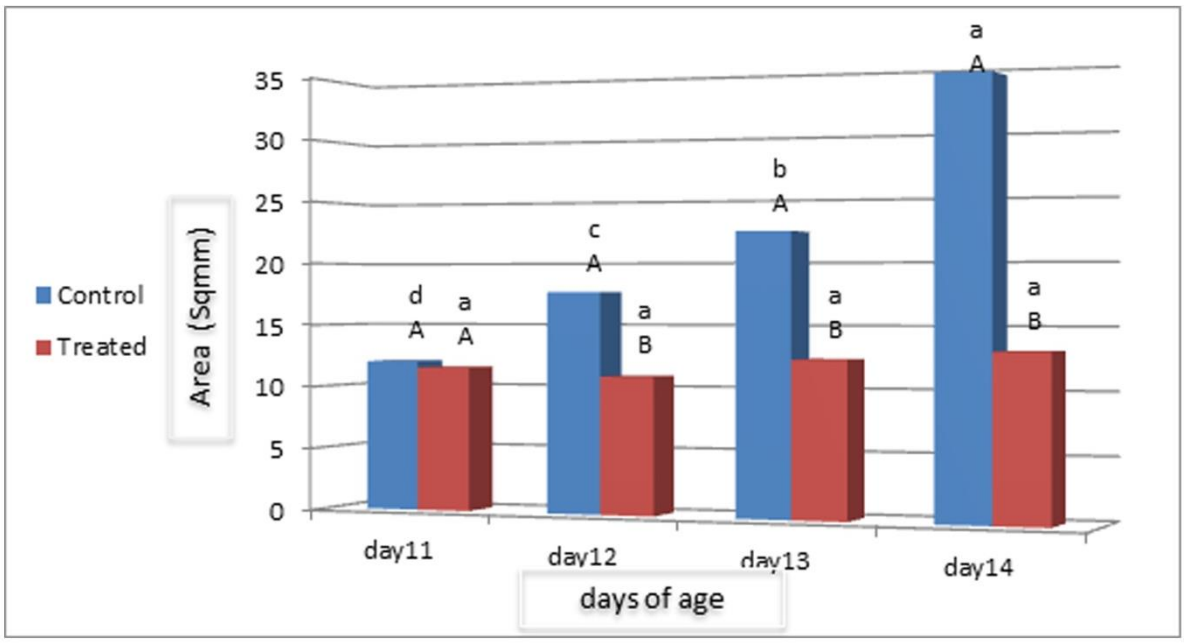

Figure 2. Comparison between the means of Area growth of 12 tibia bones of the control and treated subgroups from day 11 - 14 of incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between any two days differ significantly

Weight measurement of tibia bone

No significant difference between the control (0.0098±0.0009) and treated (0.0072±0.68) subgroups on day 11 (Fig. 3), the t-test between them was 0.0026 g, this value was less than LSD value (LSD=0.0037) when the P-value was constant at (P<0.05). On day 12, there was a significant reduction in the weight between the control (0.016±0.0003 g) and treated (0.0077±0.0005 g) subgroups the t-test between them was 0.0083 g, this value was more than LSD value (LSD=0.0037) when the P-value was constant at (P<0.05). On day 13, there was a significant increase in the weight between the control (0.024±0.002) and treated (0.010±0.0002) subgroups, the t-test between them was 0.014 g, this value was more than LSD value (LSD=0.0037) when the P-value was constant at (P<0.05). Also on day 14, there was a significant reduction in the weight between the control (0.045±0.002) and treated (0.013±0.0006) subgroups, the t-test between them was 0.032g, this value was more than LSD value (LSD=0.0037). Concerning the difference between the means of control subgroups at different days, the results showed a significant linear increase differences in the bone weigh, while there were no significant differences between the means of treated subgroups on day 11 with the day 12 and 13, so between 13 and 14, but it's a significant between day 14 and day 11, 12 (Fig. 3).

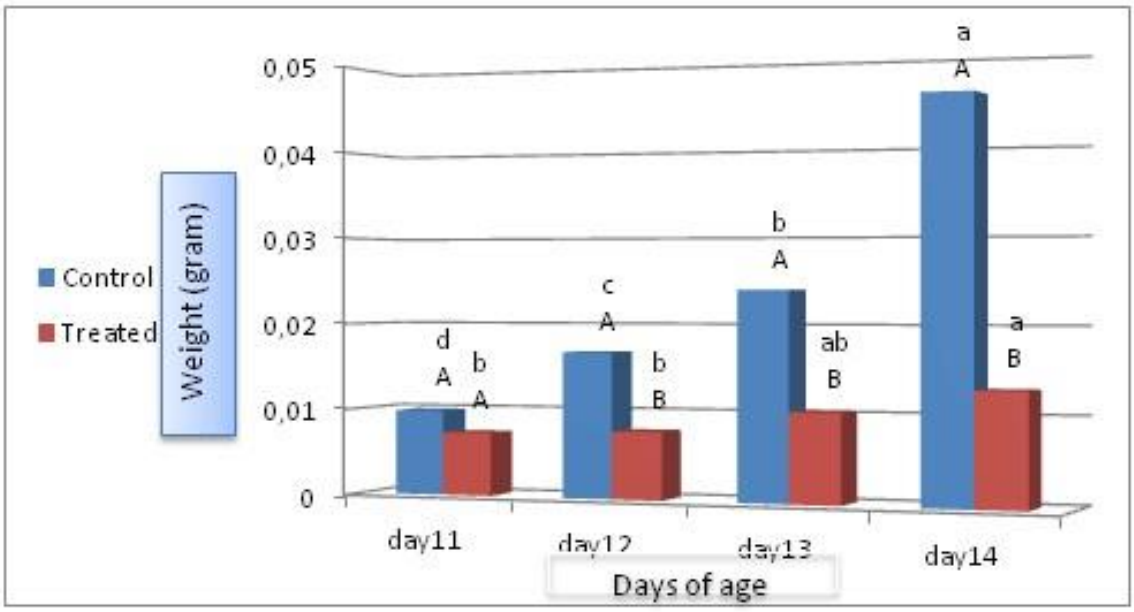

Figure 3. Comparison between the means of growth of the weight of the 12 tibia bone of chick embryos in the control and treated subgroups from day 11 to day 14 of incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between two days differ significantly. Means with same small common letter in between any two days differ insignificantly

Tibia bone Length measurement

No significant difference between the control (10.11±0.35) and treated (9.51±0.28) subgroups was seen on day 11, the t-test between them was 0.6 mm, this value was less than LSD value (LSD= 0.932) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (12.22±0.14) and treated (9.38±0.21) subgroups, the t-test between them was 2.84 mm, this value was more than LSD value (LSD= 0.932) when the P-value was constant at (P<0.05). On day 13, there was a significant difference between the control (13.77±0.53)

and treated (10.50±0.18) subgroups, the t-test between them was 3.27 mm, this value was more than LSD value (LSD= 0.932) when the P-value was constant at (P<0.05). On day 14, there was a significant difference between the control (18.62±0.43) and treated (10.87±0.31) subgroups, the t-test between them was 7.75 mm, this value was more than LSD value (LSD= 0.932) when the P-value was constant at (P<0.05), (Fig. 4). Concerning the difference between the means of control subgroups at different days, the results showed a significant linear increase differences in the bone length, while there were no significant differences between the means of treated subgroups on day 11 with the day 12, so between 13 and 14, but it's a significant between day11 and day13, 14, so between day 12 and day 13, 14, it's a significant (Fig. 4).

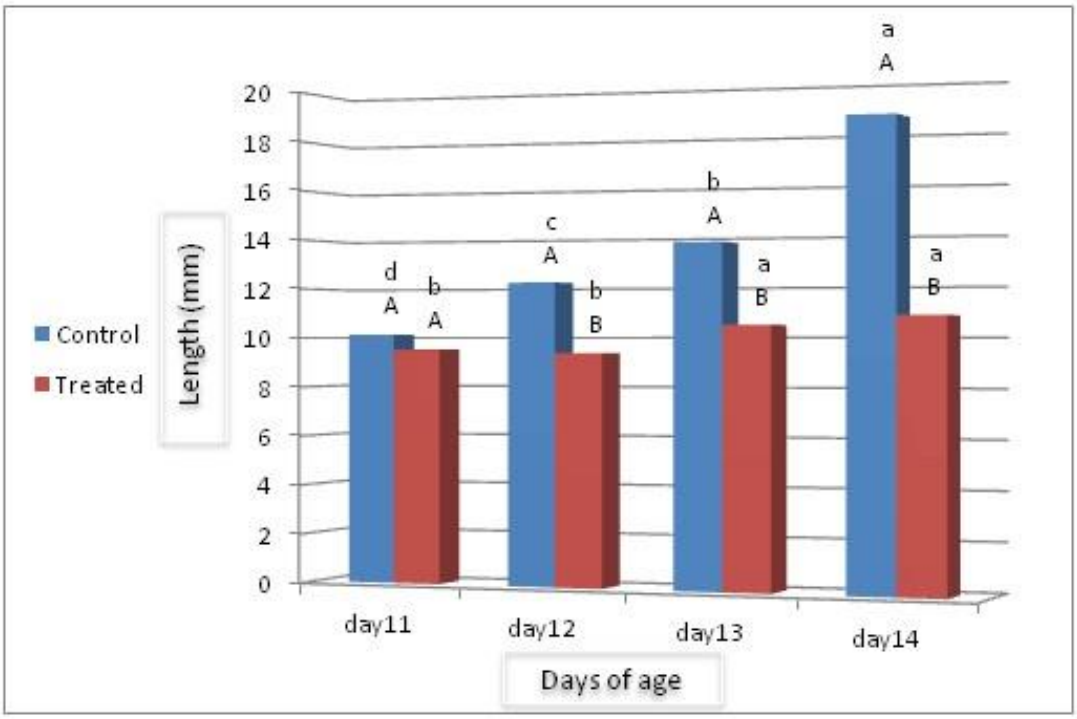

Fig. 4. Comparison between the means of growth of the length of the 12 tibia bone of chick embryos in the control and treated subgroups from day 11 to day 14 of incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between two days differ significantly

Cross section of tibia bone measurements

1. External Perimeter measurement of cross section (P1)

No significant difference between the control (3.56±0.10) and treated (3.40±0.10) subgroups was observed on day 11, the t-test between them was 0.16 mm, this value was less than LSD value (LSD= 0.252) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (4.31±0.05) and treated (3.29±0.07) subgroups, the t-test between them was 1.02 mm, this value was more than LSD value (LSD= 0.252) when the P-value was constant at (P<0.05). On day 13, there was a significant difference between the control (4.69±0.15) and treated (3.73±0.04) subgroups, the t-test between them was 0.96 mm, this value was more than LSD value (LSD= 0.252) when the P-value was constant at (P<0.05). On day 14, there was a significant difference between the control (5.42±0.06) and treated (3.71±0.04) subgroups, the t-test between them was 1.71 mm, this value was more than LSD value (LSD= 0.252)

when the P-value was constant at (P<0.05), (Fig. 5). Concerning the difference between the means of control subgroups at different days, the results showed a significant linear increase difference in the external perimeter of cross section (P1), while there were no significant differences in the means of treated subgroups on day 11 with the day 12, so between 13 and 14, but between day11 and day13, 14, as well between day 12 and day 13, 14, it's a significant (Fig. 5).

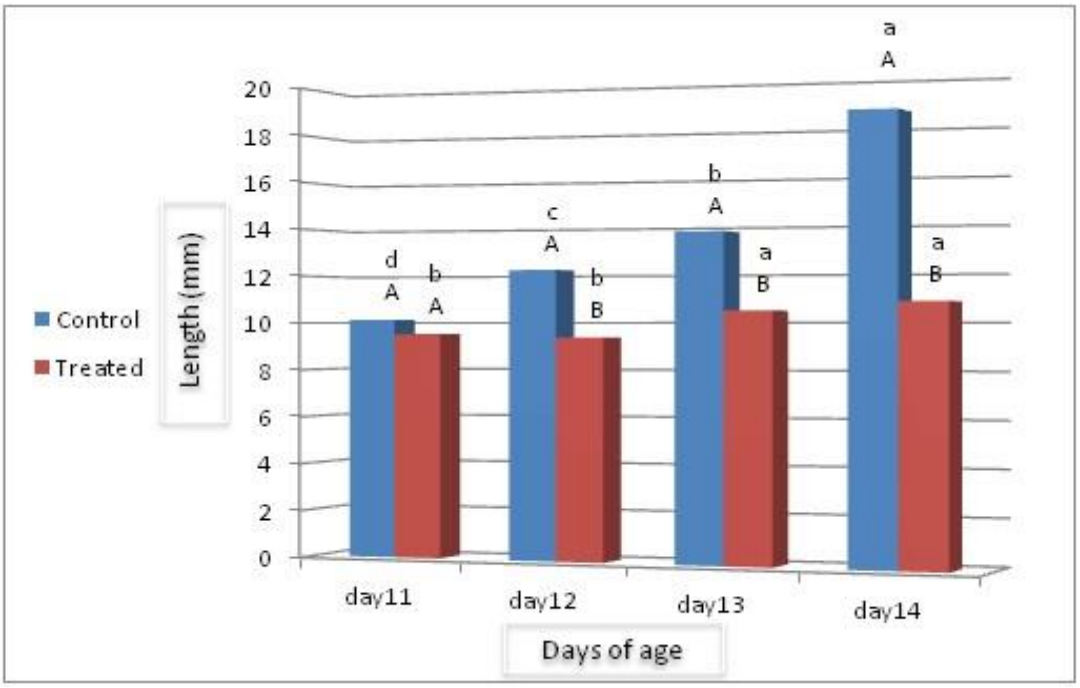

Figure 5. Comparison between the means of grow of the external perimeter (P1) of the cross section in mid- diaphyseal zone of the12 tibia bones of chick embryos in the control and treated subgroups from day 11 to day 14 of the incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between two days differ significantly

2.Internal Perimeter measurement of cross section (P2)

No significant difference between the control (2.14±0.06) and treated (2.19±0.06) subgroups on day 11 (Fig. 6), the t-test between them was 0.05 mm, this value was less than LSD value (LSD=0.155) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (2.34±0.03) and treated (2.00±0.08) subgroups, the t-test between them was 0.34 mm, this value was more than LSD value (LSD= 0.155) when the P-value was constant at (P<0.05). On day 13, there was no significant difference between the control (2.22±0.05) and treated (2.29±0.02) subgroups, the t-test between them was 0.07 mm, this value was less than LSD value (LSD= 0.155) when the P-value was constant at (P<0.05). On day 14, there was a significant difference between the control (2.40±0.05) and treated (2.14±0.02) subgroups, the t-test between them was 0.26 mm, this value was more than LSD value (LSD= 0.155) when the P-value was constant at (P<0.05). Concerning the difference between the means of control subgroups at different days, the results showed a variable difference in the bone Internal perimeter of cross section (P2), it increased significantly on day 12, reduced insignificantly on day 13 than that of day 12 and increased on day 14 than other days. While there was a significant difference between the means of

treated subgroups at day 11and 12, as well as between day 12 and 13, but between day11,13 so day13, 14, and day 12,14, was no significant, also between day14 and day 11, 12 and 13 (Fig. 6).

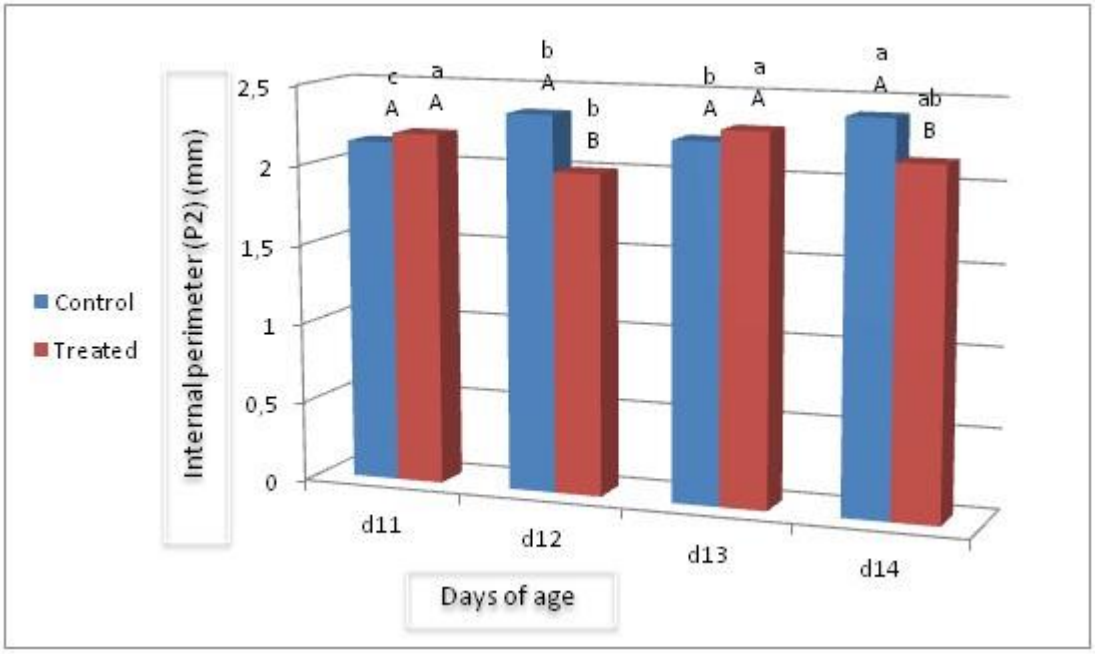

Figure 6. Comparison between the means of growth of the internal perimeter (P2) of the cross section in mid-diaphyseal zone of the 12 tibia bones of chick embryos in the control and treated subgroups from day 11 to day 14 of the incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between two days differ significantly. Means with same small common letter in between any two days differ insignificantly

Surface area of POC measurement

No significant difference between the control (2.20±0.14 mm) and treated (1.85±0.13 mm) subgroups on day 11 (Fig. 7), the t-test between them was 0.35 mm^2, this value was less than LSD value (LSD= 0.552) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (3.65±0.11 mm) and treated (1.75±0.10 mm) subgroups, the t-test between them was 1.9 mm^2, this value was more than LSD value (LSD=0.552) when the P-value was constant at (P<0.05). On day 13, there was a significant difference between the control (4.71±0.42 mm) and treated (2.41±0.06 mm) subgroups, the t-test between them was 2.3 mm^2, this value was more than LSD value (LSD=0.552) when the P-value was constant at (P<0.05). On day14, there was a significant difference between the control (6.82±0.21 mm) and treated (2.52±0.09 mm) subgroups, the t-test between them was 4.3 mm^2, this value was more than LSD value (LSD=0.552) when the P-value was constant at (P<0.05).Concerning the difference between the means of control subgroups at different days, the results showed a significant linear increase differences in the bone Area of POC while, there was no significant differences in the bone area of POC of means of treated subgroups on day 11 with the day 12, so between 13 and 14, but between day11 and day13, 14, as well between day 12 and day 13, 14, it's a significant (Fig. 7).

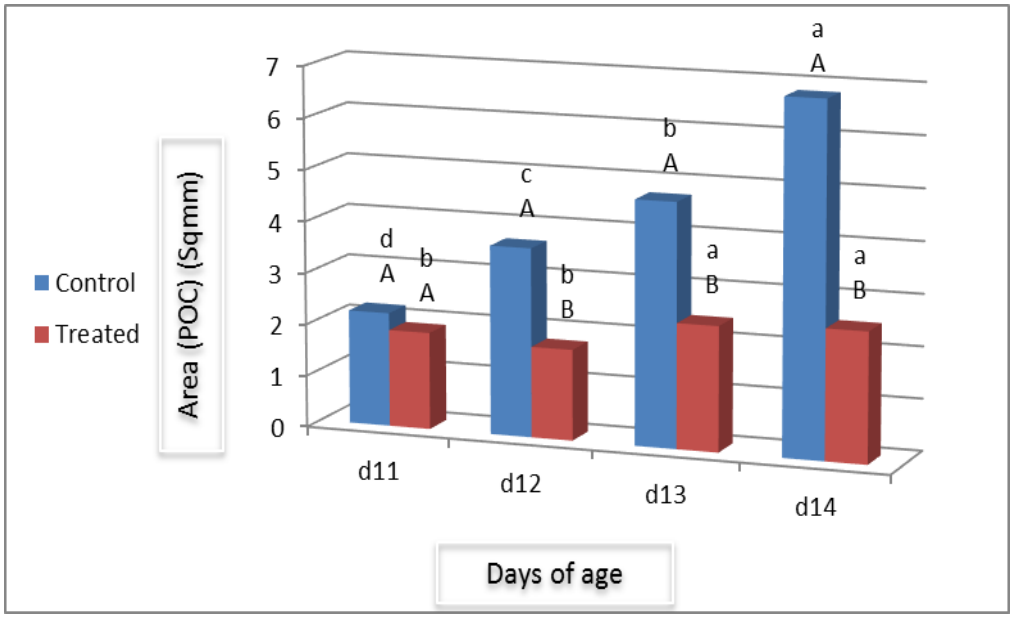

Fig. 7. Comparison between the means of grow of the Area of the perichondral osseous collar [A (P.O.C)] cross section in mid-diaphyseal zone of the 12 tibia bones of the control and treated subgroups from day 11 to day 14 of the incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between two days differ significantly

Perimeter of POC measurement

No significant difference in the POC perimeter between the control (1.42±0.05) and treated (1.20±0.05) subgroups on day 11 (Fig. 8), the t-test between them was 0.22 mm, this value was more than LSD value (LSD= 0.212) when the P-value was constant at (P<0.05). On day 12, there was a significant difference between the control (1.97±0.04) and treated (1.23±0.04) subgroups, the t-test between them was 0.74 mm, this value was more than LSD value (LSD= 0.212) when the P-value was constant at (P<0.05). On day 13, there was a significant difference between the control (2.46±0.15) and treated (1.43±0.03) subgroups, the t-test between them was 1.03 mm, this value was more than LSD value (LSD= 0.212) when the P-value was constant at (P<0.05). On day 14, there was a significant difference between the control (3.03±0.08) and treated (1.56±0.04) subgroups, the t-test between them was 1.47 mm, this value was more than LSD value (LSD= 0.212) when the P-value was constant at (P<0.05). Concerning the difference between the means of control subgroups at different days, the results showed a significant linear increase differences in the bone perimeter of POC perimeter, except at day 11, while the difference between the means of treated subgroups at day 11 was not significant with the day 12, so between 12 and 13, also between 13 and 14, but is a significant between day 11 and day 13, 14, and between day 12 and day 14.

Discussion

Dexamethasone is a synthetic glucocorticoid that has been used clinically as an anti-inflammatory drug. Long-term therapy with dexamethasone or other steroids may cause or exacerbate osteoporosis [10]. Glucocorticoid stimulates osteoclast-mediated bone resorption and reduces osteoblast-mediated bone formation, which results in increased overall net bone resorption [11].

The present work has demonstrated a significant decrease in tibia length of chick embryos treated with dexamethasone; this is in agreement with previous study that observed shorter femora and humeri in newborn piglets

treated with dexamethasone during their prenatal and neonatal life in comparison with controls [12]. This may be due to the inhibition of osteoblasts development and the inhibition of bone specific osteocalcin (OC) gene expression which arrested trabecular bone formation and likely contributes to glucocorticoid-induced osteoporosis [10].

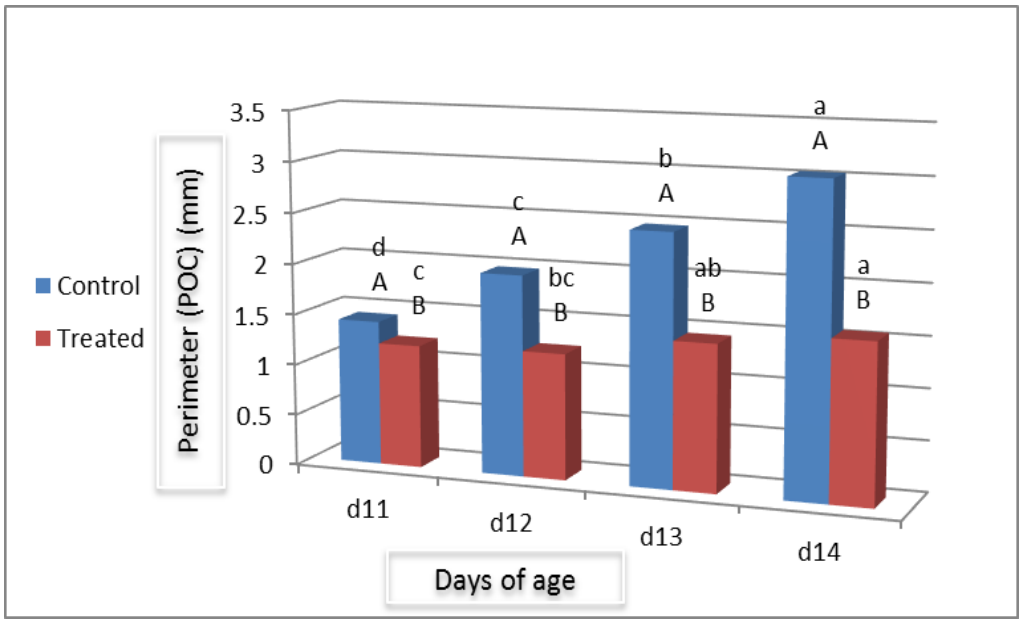

Figure 8. Comparison between the means of growth of the Perimeter of perichondral osseous collar [P (P.O.C)] cross section in mid-diaphyseal zone of the control and treated subgroups from day 11 to day 14 of the incubation period. Means with different capital letters in the same day differ significantly. Means with different small letter in between two days differ significantly. Means with same small common letter in between any two days differ insignificantly

The decrement in bone length may also be due to inhibition of protein and glycoprotein synthesis and reduction of the proliferating cells number in dexamethasone treated rats [13], it may also be due to the inhibition effect of the drug on the proliferation of the growth plate chondrocytes [14]. Dexamethasone administration in young male albino rat led to alteration in the structure of the epiphyseal plate growth with an observable reduction thickness, less frequent chondrocytes with wide matrix areas, thus corticosteroids might slow longitudinal bone growth and induced growth retardation [15]. Dexamethasone has been demonstrated to accelerate the deposition of calcium salts, inhibited the proliferation of chondrocytes, and increased apoptosis of chondrocytes and osteocytes that lead to shortening of the developing long bone of chick embryos [16]. The decline in bone formation in mice and humans receiving glucocorticoids is mediated by direct inhibition of osteoblast proliferation and differentiation and by an increase in the apoptosis rates of mature osteoblasts and osteocyte [17,18].

In the present investigation, decrease in the tibia bone weight was observed in the dexamethasone treated chick embryos; this is coinciding with Sultana [19], who observed a reduction in tibial weight in dexamethasone administrated immature female mice. Maternal treatment with dexamethasone decreased the weight of the tibia and led to thinning of articular and growth plate cartilages

and trabeculae thickness and reduced the serum GH concentration in male piglets [20]. Dexamethasone suppresses osteoblast function and bone morphogenetic protein "BMP" pathways by enhancing the expression of mRNA of BMP antagonists, and bisphosphonate and PTH exert pharmacologic effects [21].

The present study showed that dexamethasone caused a decrease in tibia bone area and perimeter of the bone as a whole from 11-day of incubation. Cross-sectional, cortical and trabecular areas were reduced by 30% in the hemimandible of dexamethasone treated female Sprague-Dawley rats during the growth phase, suggesting that the corticosteroid exerts a combined, negative action on bone geometry (mass and architecture) and volumetric bone mineral density of cortical bone [22]. Dexamethasone induced osteoporosis, growth retardation both in long bones and in the vertebral column and induced reduction in bone volume in the (three-week-old) mice [23]. Dexamethasone administration in both prenatal and neonatal life of the piglets led to reduction of volumetric bone density and mechanical and geometric properties of their bones [12].

In both controlled and treated groups, a morphometric system was organized to be able for compatible reading of the best 3 images cross section in the mid-diaphyseal zone of each bone, then comparison between the mean values of external and internal parameters, that gives us the idea of the development of the perichondral osseous collar that grow with the ossification process and affected this process with the retardation effects that occurred by used dexamethasone drug. So that the effect of this drug on the process of invasion to the connective and vascular tissue into the bone body. Greater of the differences between the external and internal perimeters, and area; refer to the greater of the perimeter and area of the collar.

The perichondral osseous collar in the mid-diaphyseal zone cross-section of tibia bone of the treated embryos of this study, showed a significant reduction when compared with normal control, however a significant increase in the area of perichondral osseous collar in the mid-diaphyseal zone cross-section of tibia bone during 13 and 14-day of incubation in embryos treated with dexamethasone of the present study when compared with those of days 11 and 12. This is coinciding with Gaytan et al. [24] who observed a rapid increase in cartilage volume from day 12 to day 13, with rapidly increased of the invading connective and vascular tissue volume from day 11 to day 14 whereas the rate of cartilage resorption increased until day 13 reaching perichondral osseous collar after this age. Treated embryos with dexamethasone showed a delay in the tibia longitudinal growth as well as in the growth of bone collar and have been related to the scarcity of resorptive cells found in the cartilage-marrow interface [25]. Perichondral tissues and blood vessels in particular influence chondrocyte maturation in a positive manner and may cooperate with hypertrophic chondrocytes in dictating the normal pace and location of the transition from cartilage to bone [26], the blood vessels of the dexamethasone treated rabbits have been detected to be irregular with disrupted endothelial cells and congested with blood that might be responsible for the delay of collar formation [27]. Morphological studies are in progress to observe the effect of dexamethasone administered during embryonic period on the histological processes changes in the bone development such as osteoblast proliferation, osteoclast activities, reduction in the bone collar thickness, impairment of matrix synthesis together with the negative effect on the whole-body mass as well.

In conclusion, the present investigation demonstrated that administration of dexamethasone caused tibial bones growth retardation at development, leading to reduction in the bone length, area, perimeter

and weight and retardation of the perichondral osseous collar.

Acknowledgments

The authors are indebted to the entire members of Applied Embryology and Physiology Laboratory in the High Institute of Infertility Diagnosis and ART, Al-Nahrain University for their help and support.

Author contributions:

A. Concept and design of the study: Dr. Selman, Dr. Al-Hasson, Dr. Al-Salih.
B. Acquisition of data: Ali, Dr. Selman, Dr. Al-Salih.
C. Analysis and/or interpretation of data: Ali, Dr. Al-Ani, Dr. Al-Hasson.
D. Drafting of the manuscript: Ali, Dr. Selman, Dr. Al-Hasson.
E. Revision of the manuscript for important intellectual content: Ali, Dr. Al-Ani, Dr. Al-Salih.

Conflict of interest

The authors have no conflicts of interest.

Funding

This research was sponsored by the High Institute of Infertility Diagnosis and ART, Al-Nahrain University, Baghdad, Iraq.

References

1. Alcântara D, Rodrigues MN, Franciolli AL, et al. Embryonic development of endoderm in chicken (Gallus gallus domesticus). Microsc Res Tech. 2013; 76(8): 803-10. doi: 10.1002/jemt.22232.
2. De Groef B, Grommen SV, Darras VM. The chicken embryo as a model for developmental endocrinology: development of the thyrotropic, corticotropic, and somatotropic axes. Mol Cell Endocrinol. 2008; 293(1-2): 17-24. doi: 10.1016/j.mce.2008.06.002.
3. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. 1951. Dev Dyn. 1992; 195(4): 231–72. doi: 10.1002/aja.1001950404.
4. Pechak DG, Kujawa MJ Caplan AI. Morphology of bone development and bone remodeling in embryonic chick limbs. Bone. 1986; 7(6): 459-72.
5. WHO Model list of essential medicines; 18th list April 2013. http://www.who.int/medicines/publications/essentialmedicines/en/index.html)
6. Straus DS, Glass CK. Anti-inflammatory actions of PPAR ligands: New insights on cellular and molecular mechanisms. Trends Immunol. 2007; 28(12): 551-8. doi: 10.1016/j.it.2007.09.003.
7. Coloma M, Duffy LT, White PF, et al. Dexamethasone facilitates discharge after outpatient anorectal surgery. Anesth Analg. 2001; 92(1): 85-8.
8. Barrett KE, Barman SM, Boitano S, et al. Ganong's Review of medical physiology, 25th ed. New York: McGraw-Hill Medical; 2015.
9. Canalis E, Mazziotti G, Giustina A, et al. Glucocorticoid-induced osteoporosis: pathophysiology and therapy. Osteoporos Int. 2007; 18(10): 1319-28. doi: 10.1007/s00198-007-0394-0.
10. Leclerc N, Noh T, Khokhar A, et al. Glucocorticoids Inhibit Osteocalcin Transcription in Osteoblasts by Suppressing Egr2/Krox20- Binding Enhancer. Arthritis Rheum. 2005 Mar;52(3):929-39. doi: 10.1002/art.20872.
11. Mitra R. Adverse effects of corticosteroids on bone metabolism: a review. PM R. 2011; 3(5): 466-71. doi: 10.1016/j.pmrj.2011.02.017.
12. Sliwa EA, Kowalik S, Tatara MR, et al. Effects of dexamethasone on physical properties and mineral density of long bones in piglets. Bull Vet Inst Pulawy. 2005; 49: 97-100.
13. Anneroth G, Bloom G. Structural changes in the incisor of cortisone treated rats. J Dent Res. ١٩٧٦; 45(2): 229-35. doi: 10.1177/00220345660450020301.
14. Smink JJ, Koedam JA, Koster JG, et al. Dexamethasone-induced growth inhibition of porcine growth plate chondrocytes is accompanied in level of IGF axis components. J Endocrinol. 2002; 174(2): 343-52.
15. Azmy MA, Abdallah MA. Morphology of young male Albino Rats' epiphyseal plate after dexamethasone administration. Life Sci J. 2013; 10: 2605-19.
16. Cheng X, Chen JL, Ma ZL, et al. Biphasic influence ofdexamethasone exposure on embryonic vertebrate skeleton development. Toxicol Appl Pharmacol. 2014; 281(1): 19-29. doi: 10.1016/j.taap.2014.09.014.
17. Weinstein RS, Nicholas RW, Manolagas SC. Apoptosis of osteocytes in glucocorticoid-induced osteonecrosis of the hip. J Clin Endocrinol Metab. 2000; 85(8): 2907-12. doi: 10.1210/jcem.85.8.6714.
18. Weinstein RS. Glucocorticoid-induced osteonecrosis. Endocrine. 2012; 41(2): 183-90. doi: 10.1007/s12020-011-9580-0.
19. Sultana K. Studies of the effects of Dexamethasone sodium phosphate on weight of bone (tibia) in mice. Pak J Pharm Sci. 1996; 9(1): 55-63.
20. Tomaszewska E, Dobrowolski P, Wydrych J. Postnatal administration of 2-oxoglutaric acid improves articular and growth plate cartilages and bone tissue morphology in pigs prenatally treated with dexamethasone. J Physiol Pharmacol. 2012; 63(5): 547-54.
21. Hayashi K, Yamaguchi T, Yano S, et al. BMP/Wnt antagonists are upregulated by dexamethasone in osteoblasts and reversed by alendronate and PTH:

potential therapeutic targets for glucocorticoid-induced osteoporosis. Biochem Biophys Res Commun. 2009; 379(2): 261-6. doi: 10.1016/j.bbrc.2008.12.035.

22. Bozzini C, Champin G, Alippi RM, et al. Effect of dexamethasone on mandibular bone biomechanics in rats during the growth phase as assessed by bending test and peripheral quantitative computerized tomography. Acta Odontol Latinoam. 2015; 28(1): 83-8. doi: 10.1590/S1852-48342015000100012.

23. Postnov P, De Schutter T, Sijbers J, et al. Glucocorticoid-Induced Osteoporosis in Growing Mice Is Not Prevented by Simultaneous Intermittent PTH Treatment. Calcif Tissue Int. 2009; 85(6): 530-7. doi: 10.1007/s00223-009-9301-3.

24. Gaytan F, Ranz FB, Aceitero J. Morphometric study of cartilage dynamics in the chick embryo tibia I. Methodology and tissue compartments in normal in normal embryos. J Anatomy. 1987; 154: 63-71.

25. Ranz FB, Aceitero J, Gaytan F. Morphometric study of cartilage dynamics in the chick embryo tibia. J Anatomy. 1987; 154: 73-9.

26. Yin B, Gentili C, Koyama E, et al. Antiangiogenic Treatment Delays Chondrocyte Maturation and Bone Formation during Limb Skeletogenesis. J Bone Miner Res. 2002; 17: 56-65. doi: 10.1359/jbmr.2002.17.1.56.

27. Kashmoola MA. Histopathological evaluation of skin wound in rabbits treated by systemic dexamethasone. J Bagh Coll Dentistry. 2007; 19(1): 58-61.

Correspondence to Dr. Imad M. Al-Ani
E-mail: imad_alani@yahoo.com
Received Sep. 18th 2016
Accepted Apr. 4th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

Ki-67 Immunohistohemical Expression in Prostatic Lesions

Inas A. Rasheed[1] *MSc*, **Alaa G. Hussein**[2] *FICMS*, **Mohanad M. AbdulGhany**[3] *MSc*

[1]Dept. of Pathology, [2]Dean of College of Medicine, Al-Nahrain University, Baghdad, Iraq, [3]Dept. of Pathology, Al-Kindy College of Medicine, Baghdad University, Baghdad, Iraq

Abstract

Background The cell proliferation marker, ki-67, is a nuclear and nucleolar protein, which can be detected during all active phases of the cell cycle (G1, S, G2, and mitosis), but absent from cell resting phases. Thus, it considered as an excellent marker for determination the cell growth fraction and it has been detected to be a useful marker in predicting the development of human tumors.

Objective To evaluate the immunohistochemical expression of the antigen ki-67 in benign, pre-malignant and malignant prostatic lesions.

Methods A cross section study of 115 paraffin embedded prostatic tissue blocks, 76 cases were benign prostatic hyperplasia (BPH), 9 cases were high grade-prostatic intraepithelial neoplasia (HG-PI N), and 30 cases were prostatic carcinoma (PCa). Sections from each block were prepared for immunohistochemical staining of ki-67.

Results Ki-67, semi-quantiative evaluation, revealed that the majority of BPH (88.2%) and HG-PIN (66.7%) presented weak positivity (+), on other hand, the majority of PCa (60.0%) presented moderate positivity (++), and 16.7% showed intense positivity (+++). For prostatic carcinoma (PCa), no significant association had found between Ki-67 and serum tPSA level, while a significant association with Gleason grade was found, the higher grade (≥7), the more intense positive immunolabeling for Ki-67.

Conclusion Significant differences between ki-67 immunolabeling and histological type of prostatic lesions, between BPH and HG-PIN, and prostatic carcinoma, which may have potential to evolve to malignancy.

Keywords BPH, HG-PIN, Ki-67, prostatic carcinoma, tPSA

Citation Inas A. Rasheed, Alaa G. Hussein, Mohanad M. AbdulGhany. Ki-67 Immunohistohemical expression in prostatic lesions. Iraqi JMS. 2017; Vol. 15(2): 129-134. doi: 10.22578/IJMS.15.2.4

List of abbreviations: BPH = Benign prostatic hyperplasia, HG-PIN = High-grade prostatic intraepithelial neoplasia, PCa = Prostatic carcinoma, PSA = Prostatic specific antigen

Introduction

The prostatic pathology is a common condition in the worldwide, and cause a considerable disability in elderly men, specially lower urinary tract symptoms, in form of urine retention, dripping, hesitancy, and others [1-3]. The prostatic glandular tissue starts proliferating after 40 years of age in 50% of men, and 80% by 70 years [3,4]. The proliferating prostatic tissue compresses the prostatic urethra, which lead to significant considerable disability with difficulty in passing the urine. The condition called benign prostatic hyperplasia (BPH) [4-6].

The prostate cancer is a common disease of elderly men worldwide, and ranks the fifth solid, non-cutaneous, non-hematological cancer in Iraq [7], it can be slow-growing, and identified in asymptomatic patients. Most of

the malignancies of the prostate arise from the glandular tissue, thus, the commonest type is adenocarcinoma [8,9]. About 70% of prostate cancer rise from the peripheral zone, 15-20%, and 10-15% arise in the central zone and transitional zone respectively [9-11]. Most of prostatic carcinoma are multifocal, with involvement of multiple zones [12,13].

Most of the patients with prostatic carcinoma are identified by screening in asymptomatic men, by assessment of serum level of prostatic specific antigen (PSA) and digital rectal examination [14,15]. In addition, prostatic cancer can be an incidental pathological finding when surgically removed to relieve obstructive urinary symptoms from BPH [15].

Intraepithelial proliferative lesions, an important lesion, is high-grade prostatic intraepithelial neoplasia (HG-PIN) which considered as premalignant condition for prostatic adenocarcinoma may identified incidentally by tissue biopsy, in which risk of malignant transformation ranging from 9-30% [15,16], or may coexist with underlying prostatic adenocarcinoma. Clinically, HG-PIN is not associated with elevated serum PSA level, and don`t produce abnormality in the prostatic texture or sized by digital rectal examination, unless prostatic carcinoma coexisted [16,17]. Early detection of HG-PIN lesions and follow up of the patients by 3-6 months' interval [16-18], by prostatic needle biopsy can contribute to eradicate early prostatic adenocarcinoma.

The nuclear expression of the Ki-67 protein is associated with cell proliferation, as it is present during all active phases of cell cycle (G1, S, G2, and mitosis), but absent from resting cells [19,20]. Therefore, Ki-67 protein is considered as an excellent marker for cell proliferation (growth fraction), and it is associated with a high mitotic count and high histological grade [21,22].

The objectives of this study were to evaluate the immunoexpression of the antigen ki-67 in benign, pre-malignant and malignant prostatic lesions, and distinguish the relation between ki-67 immunoexpression and serum tPSA level and Gleason grade for cases of prostatic carcinoma.

Methods

This was a cross sectional study approved by Institute Review Board of College of Medicine, Al-Nahrain University. The collection of samples last for the period from March, 2015 to February 2016, a total of 115 formalin fixed paraffin embedded prostatic tissue of which (76) cases were BPH, (9) cases were HG-PIN, and (30) of prostatic adenocarcinoma, were retrieved from the histopathology archive of Teaching Laboratory in Medical City, for the period from 2013 to February 2016.

All the clinicopathological parameters, which included age of the patient, preoperative total serum PSA, histopathological type and Gleason grade for cases of prostatic carcinoma, were obtained from patient`s admission case sheets and pathological reports. Any sample lacking the clinicopathological information was excluded from this study.

For each case, one representative (4 µm) section was stained with Hematoxylin and Eosin, and histopathological diagnosis was revised, other 4 µm section was placed on positively charged slide and stained immunohistochemically using three steps-indirect streptavidin method for monoclonal mouse antibodies including monoclonal mouse, anti-human, Ki-67 antigen, manufactured by Dako.

Interpretation of the results of immunohistochemical staining

Staining results of Ki-67: brown nuclear stain is considered positive. The percentage of cells positive for Ki-67 was scored semi-quantitatively, according to the number of stained cells observed as:

- Weak (marked as +), <25%
- Moderate (marked as ++), 25-75%
- Intense (marked as +++), >75%

Statistical analysis was performed with SPSS V.16, using chi-square, t-test with p value of <0.05.

Results

Semi-quantitative evaluation of the immunolabeling for ki-67 demonstrated that the majority of the BPH (88.2 %), and GH-PIN (66.7%) presented weak positivity (+), (Table 1). On other hand, the majority of prostatic carcinoma (PCa); (60.0%) presented moderate positivity (++), while intense positivity (+++) and weak positivity (+) were found in 16.7% and 23.3% of the cases respectively (Figure 1).

Table 1. Results of ki-67 immunoexpression among presented cases of paticular prostatic pathology

Ki-67 expression	BPH		HG-PIN		PCa	
	No.	%	No.	%	No.	%
Negative	5.0	6.6	1.0	11.1	0.0	0.0
Positive (+)	67.0	88.2	6.0	66.7	7.0	23.3
Positive(++)	3.0	3.9	2.0	22.2	18.0	60.0
Positive(+++)	1.0	1.3	0.0	0.0	5.0	16.7
Total	76.0		9.0		30.0	

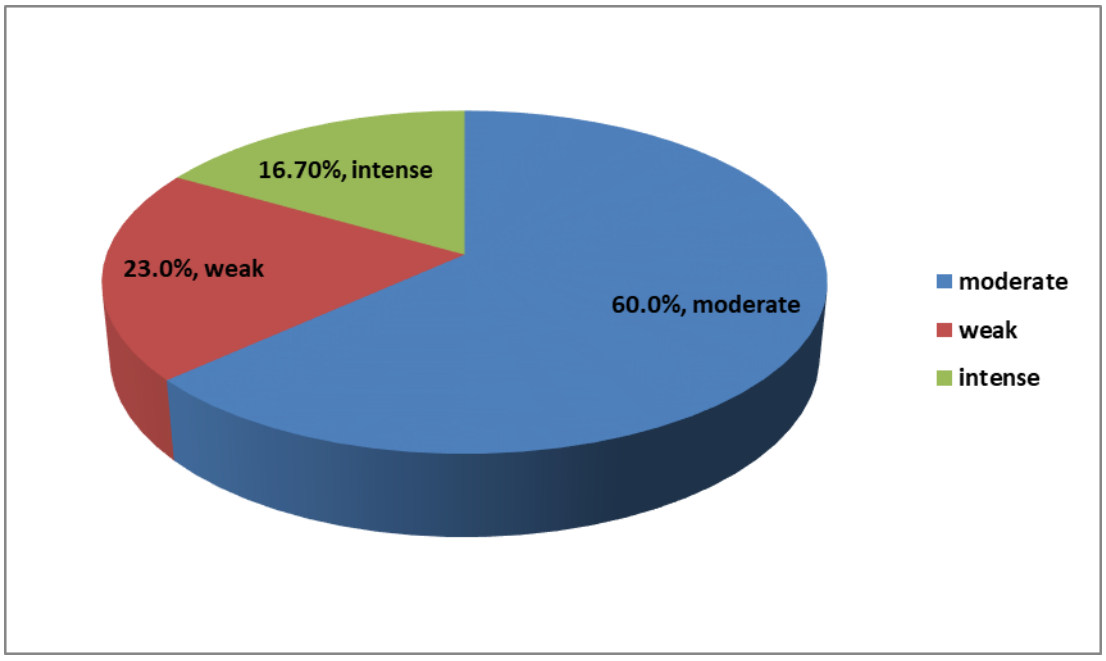

Figure 1. Ki-67 expression among presented cases of PCa

As shown in table (2), there was no significant association between Ki-67 positivity and serum total PSA levels, (p=0.32).

Regarding Gleason grade, there was a significant association between Ki-67 immunolabeling and higher grade. As shown in table (3), intense positive ki-67 immunoexpression was higher (25%) in grade 7 and lowest in grade 6 (zero%), (p=0.039).

Table 2. Ki-67 immunolebeling correlated to mean age and mean total serum PSA among presented cases of PCa

Ki-67 immunoexpression	Mean total S.PSA level (ng/ml)
Weak (+)	12.1±1.2
Moderate (++)	10.5 ±1.4
Intense (+++)	10.9±1.5

No significant association, p=0.32

Table 3. Ki-67 immunolabeling correlated to Gleason grade among presented cases of PCa

Gleason score	No.	Weak (+)		Moderate (++)		Intense (+++)	
		No.	%	No.	%	No.	%
6	4	2.0	50.0	2.0	50.0	0.0	0.0
7	8	1.0	12.5	5.0	62.5	2.0	25.0
8	10	2.0	20.0	6.0	60.0	2.0	20.0
9	8	2.0	25.0	5.0	62.5	1.0	12.5
Total	30.0	7.0		18.0		5.0	

Significant association, p=0.0276

Discussion

For current study, it has been showed that Ki-67 weakly expressed in majority of BPH and HG-PIN (88.2% and 66.7%, respectively). For prostatic adenocarcioma, the majority of the cases showed moderate positivity (60%), intense positivity was found in 16.7% of cases. No significant association was found between serum tPSA level and Ki-67 immunoexpression. Gleason score (≥7) were found to be associated with intense Ki-67 expression.

Mucci et al. in 2000 have demonstrated statistically significant increases in the expression of Ki-67 were seen from normal tissue to HG-PIN to prostatic carcinoma [23].

Munoz et al. in 2003, in a semi-quantative evaluation of Ki-67 immunolabeling in prostatic lesions, demonstrated that the majority of the BPH lesions (85.7%), and GH-PIN (72.0%), presented with weak positivity (+). On other hand, the majority of the PCa (62.9%), presented moderate positivity, with a significant correlation between Ki-67 immunolabeling and histological diagnosis. There were highly significant differences in ki-67 expression between BPH and PCa, and HG-PIN and PCa, with significant differences being found between Gleason grades and Ki-67 immunoreactivity. The immnoreactivity for ki-67 increase in accordance with the increase of the grade of histological lesion and the greater immunoreactivity being found in high Gleason grade [24].

Zhong et al. in 2008 revealed increased Ki-67 immunoexpression in prostatic carcinoma and BPH ($P<0.05$), relative to human normal prostatic tissues [25].

Sulik et al. in 2011 reported a significant association between Ki-67 expression in prostatic carcinoma and Gleason score (≥7), but no association with pre-operative PSA level [26].

Verma et al. in 2015 have found Ki-67 expression in 64% of the cases. All cases of well differentiated PCa (low Gleason grade) lack Ki-67 expression, while moderately and poorly differentiated PCa had showed ki-67 immunostaining. A significant correlation was found between Ki-67 positivity and increased Gleason`s grade, the higher Gleason grade, the higher ki-67 immunoreactivity [27].

Kaur et al. in 2016 demonstrated a significant association between ki-67 immunoexpression

and higher Gleason grade in prostatic carcinoma (≥7) [28].

Rajeswari in 2016 found a significant association between ki-67 immunostaining and Gleason grade, and can be used as a prognostic marker [29].

These studies support the findings of the present study that Ki-67 immunostaning significantly differs between benign, pre-malignant and malignant prostatic lesions, with a significant association of ki-67 expression and higher Gleason grade (≥7) in prostatic carcinoma.

This study concluded that there are significant differences between ki-67 immunoexpression and histological type of prostatic lesions, between BPH, HG-PIN, and prostatic carcinoma, which may have potential to evolve to malignancy. Also, Ki-67 protein immunolabeling is significantly associated with Gleason grade, but no significant association had been found with serum tPSA level.

Acknowledgments

Special thanks to all staff members of Pathology Department in, College of Medicine, Al-Nahrain University for their valuable help and advice.

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

All authors have no conflict of interest.

Funding

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

References

1. Briganti A, Capitaninio U, Suardi N, et al. Benign prostatic hyperplasia and its aetiologies. Europ Urol Suppl. 2009, 8: 865-71. doi: 10.1016/j.eursup.2009.11.002.
2. Chodak GW, Kim ED. Prostate Cancer. Medscape. 2015. http://emedicine.medscape.com/article/1967731-overview.
3. Kumar V, Abbas AK, Aster JC (eds). Robbin and Cotran Pathologic basis of disease. 8th ed. New Delhi, Saunders: 2010. p. 664-8.
4. Roehrborn CG, Mcconnell JD. Benign prostatic hyperplasia: etiology, pathophysiology, epidemiology and natural history. Campbell-Walsh Urology, 9th ed. Philadelphia: Saunders; 2007. p. 2727.
5. Untergasser G, Madersbacher S, Berger P: Benign prostatic hyperplasia: age related tissue remodeling Exp Gerontol. 2005; 40(3): 121-8. doi: 10.1016/j.exger.2004.12.008.
6. Lepor H. Pathophysiology of benign prostatic hyperplasia in aging male population. Rev Urol. 2005; 7(Suppl 4): S3–S12.
7. Al Hadithi RH. Histopathogical interpretation of prostatic autopsy in men over 50 years old in Baghdad. A FICMS thesis, Iraqi Commission for Medical Specialization in Pathology; 2003.
8. Bostwick D. Prospective origins of prostate carcinoma: PIN and atypical adenomatous hyperplasia. Cancer. 1996; 78(2): 330-6. doi: 10.1002/(SICI)1097-0142(19960715)78:2<330::AID-CNCR22>3.0.CO;2-W.
9. Rosai J. Rosai and Ackerman`s Surgical pathology. 10th ed. China; Mosby: 2011. p. 1368-88.
10. Sakr WA, Grignon DJ, Hass GP, et al. Epidemiology of high grade prostatic intraepithelial neoplasia. Pathol Res Pract. 1995; 191(9): 838-41.
11. Al-Saadi HA, Mahdi MS. Detection of prostate cancer using tumor markers and biopsy in outpatient in Kerbala city. Iraq. Int Res J Med Sci. 2013; 1(7): 17-21.
12. Matoso A, Arora K. Prostate microscopic mimics of prostate carcinoma adenoma/atypical adenomatous hyperplasia. Adenosis/atypical adenomatous hyperplasia. PathologyOutlines.com website. http://www.pathologyoutlines.com/topic/prostateadenosis.html.
13. Iczkowski K, Adenocarcinoma of peripheral ducts and acini. PathologyOutlines.com website. http://www.pathologyoutlines.com/topic/prostateadenoNOS.html.
14. Bostwick DG, Burke HB, Djakiew D, et al. Human prostate cancer risk factor. Cancer. 2004; 101(S10): 2371-490. doi: 10.1002/cncr.20408.
15. Akhter R, Reshi R, Dar ZA, et al. Histopathological study of prostatic lesions on needle biopsies with serum prostatic specific antigen. Int J Med Med Sci. 2014; 6(3): 87-91. doi: 10.5897/IJMMS2013.1014.
16. Wolf AM, Wender RC, Etzioni RB, et al. American cancer society guideline for early detection of prostate cancer: update 2010. CA Cancer J Clin. 2010; 60(2): 70-98. doi: 10.3322/caac.20066.
17. Mackinnon AC, Yan BC, Joseph LT, et al. Molecular biology underlying the clinical heterogenicity of prostate cancer. Arch Pathol Lab Med. 2009; 133(7): 1033-40. doi: 10.1043/1543-2165-133.7.1033.
18. Mesko S, Kupelian P, Demanes D, et al. Quantifying the Ki-67 heterogeneity profile in prostate cancer.

Prostate Cancer. 2013; 2013: 717080. doi: 10.1155/2013/717080.

19. Taneja SS, Morton R, Barnette G, et al. Prostate cancer diagnosis among men with isolated high-grade intraepithelial neoplasia enrolled onto a 3-year prospective phase III clinical trial of oral toremifene. J Clin Oncol. 2013; 31(5): 523-9. doi: 10.1200/JCO.2012.41.7634.

20. Bostwick D, Qian J, Ramani D. Immunohistochemistry of the prostate and bladder, testis, and renal tumors. In: Dabbs DJ (ed). Diagnostic immunohistochemistry. Philiadelphia: Churchil Livingstone; 2002. p. 407-34.

21. Madani SM, Ameli S, Khazaei S, et al. Frequency of ki-67 (MIB-I) and p53 expression among patients with prostate cancer. Indian J Pathol Microbiol. 2011; 54(4): 688-91. doi: 10.4103/0377-4929.91492.

22. Rioux-Leclercq N, Leray E, Patard JJ, et al. The utility of Ki-67 expression in the differential diagnosis of prostatic intraepithelial neoplasia and ductal adenocarcinoma. Hum Pathol. 2005; 36(5): 531-5. doi: 10.1016/j.humpath.2005.01.021.

23. Mucci NR, Rubin MA, Strawderman MS, et al. Expression of nuclear antigen Ki-67 in prostate cancer needle biopsy and radical prostatectomy specimens. J Natl Cancer Inst. 2000; 92(23): 1941-2.

24. Munoz G, Gomez F, Paz JI, et al. Ki-67 immunolabeling in premalignant lesions and carcinoma of the prostate. Histological correlation and prognostic evaluation. Euro J Histochemist. 2003, 47(2): 123-8.

25. Zhong W, Peng J, He H, et al. Ki-67 and PCNA expression in prostate cancer and benign prostatic hyperplasia Clin Invest Med. 2008; 31(1): E8-E15.

26. Sulik M, Maruszak K, Puchalska J, Misiukiewicz-Poć M. Expression of Ki-67 as a proliferation marker in prostatic carcinoma. Pol Ann Med. 2011, 18(1): 12-9.

27. Verma R, Gupta V, Singh J. Significance of p53 and ki-67 expression in prostate cancer. Urol Ann. 2015; 7(4): 488-93. doi: 10.4103/0974-7796.158507.

28. Kaur H, Paul M, Manjari M. Ki-67 and p53 immunohistochemical expression in prostate carcinoma: An experience from a tertiary care centre of North India. Ann Pathol Lab Med. 2016; 3(6): A509-16.

29. Rajeswari K, Meenakshisundaram K, Anushuya G, et al. Ki-67 as a prognostic marker in comparison with Gleason grading system in prostatic carcinoma. Indian J of Pathol Oncol. 2016; 3(1); 92-5.

Correspondence to Dr. Inas A. Rasheed
E-mail: drenas@gmail.com
Received Oct. 4th 2016
Accepted Feb. 12th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

Sero-Prevalence and Plasma Viral Load of Epstein Barr Virus among Iraqi Blood Donors

Amjad Q. Redha[1] *MSc,* **Asmaa B. Al-Obaidi**[2] *PhD*, **Haider F. Ghazi**[2] *PhD*, **Haider S. Kadhim**[2] *PhD*

[1]The Blood Donation Center, Al-Imamein Al-Kadhimein Medical City, Baghdad, Iraq, [2] Dept. of Medical Microbiology, College of Medicine, Al-Nahrain University, Baghdad, Iraq

Abstract

Background Epstein-Barr virus (EBV) is one of the most common latent viruses inside the humans' B-lymphocytes and it has been documented as a causative agent of many cancers. The virus may be transmitted when infected blood transfused to immunocompromised as well as immunocompetent individuals.

Objective To estimate the prevalence of EBV among apparently healthy blood donors by enzyme-linked immunosorbent assay (ELISA) and by quantitative real time polymerase chain reaction (RT-PCR).

Methods Four hundred fifty (450) blood donors were enrolled in this study. Plasma samples were screened by ELISA technique for detection of EBV viral capsid antigen (VCA-IgG). DNA extracted from 50 representative samples of these 450, and plasma EBV viral load was investigated by RT-PCR.

Results The overall sero-prevalence of EBV IgG was 79.8%, with a significantly higher prevalence among females than males. RT-PCR results were negative for all of the 50 representative samples.

Conclusion The high EBV sero-prevalence rates among Iraqi subjects raise the possibility of increasing the risk of EBV-associated malignant diseases.

Keywords Epstein-Barr virus, seroprevalence, VCA-IgG, real-time PCR, blood donors

Citation Amjad Q. Redha, Asmaa B. Al-Obaidi, Haider F. Ghazi, Haider S. Kadhim. Sero-prevalence and plasma viral load of Epstein Barr virus among Iraqi blood donors. Iraqi JMS. 2017; Vol. 15(2): 135-142. doi: 10.22578/IJMS.15.2.5

List of abbreviation: EBV = Epstein-Barr virus, ELISA = Enzyme-linked immunosorbent assay, LMP = Latent membrane protein, PTLD = Post-transplant lymphoproliferative disorder, RT-PCR = Real time polymerase chain reaction, TPHA = Treponema pallidum heamagglutination, VCA = Viral Capsid antigen

Introduction

Blood transfusion is still a significant mode of transmission of transfusion-transmissible infectious pathogens, given the need to determine sero-prevalence in the blood donors and to evaluate the residual risk in the blood recipients [(1,2)]. Epstein-Barr virus (EBV) infection is extremely common worldwide and approximately 90% of adults become antibody-positive before the age of 30 years [(3,4)]. Viral transmission is generally via saliva by kissing [(5)]. However, transmission via blood products, transplantation, and sexual transmission could also occur [(7-9)] and the risk of transfusion-transmitted infections is still considerable [(9,10)].

Previous studies reported that EBV infections might lead to severe morbidities and mortalities in healthy individuals [(12,13)]. Furthermore, the virus is causally linked to several malignancies, including Burkitt's lymphoma, Hodgkin lymphoma, nasopharyngeal carcinomas and leukemia [(13-19)].

Post-transfusion EBV infection is of concern in certain groups of immunocompromised individuals such as neonates, pregnant women, recipients of bone marrow and solid organ transplants and individuals with immuno-deficiency diseases especially in EBV sero-negative (susceptible) recipients [20-22].

In Iraq, to the best of our knowledge, there is no previous sero-prevalence study on EBV among healthy individuals. Thus, this study aimed to determine the sero-positivity of EBV in blood donors to establish basic knowledge for future studies.

Methods

This cross-sectional study was conducted from September 2015 to January 2016. Four hundred fifty (450) blood donors enrolled in the study including 400 males and 50 females who attended The Blood Donation Center in Al Imamein Al Kadhimein Medical City, and The National Blood Center. This study was approved by the Ethical Committee of the College of Medicine / Al-Nahrain University. Informed consent was obtained from all donors before taking samples.

Blood donors were all apparently healthy subjects, selected after responding to a panel of questions comprising a medical history. Healthy individuals aged between 18-63 years were eligible for blood donation. Donor selection was under the World Health Organization (WHO) guidelines to assessing donor suitability for blood donation. All samples were screened by enzyme-linked immunosorbent assay (ELISA) for hepatitis B virus surface antigen (HBsAg), and antibodies to HBc (core), (HIV1,2 Ab and HIV Ag), HCV and Treponema pallidum heamagglutination (TPHA) in the Blood Donation Centers as part of routine screening of donated blood units.

Three mL whole blood collected in EDTA-blood tubes and then plasma obtained by centrifugation of whole blood at 3,000 rpm for 10 min. The supernatant (plasma) was aspirated and stored at -40 °C until be used.

Measurement of the EBV IgG Viral Capsid antigen (VCA) Ab titer by ELISA

The anti-EBV VCA IgG Antibody ELISA Test Kit (DEMEDITEC/Germany) designed for the detection and the quantitative determination of specific IgG antibodies against EBV VCA in serum and plasma was used in this study. EBV VCA antigen bound on the surface of the microtiter strips. Diluted patient plasma or ready-to-use standards were pipetted into the wells of the microtiter plate. If the specimens contain antibodies to EBV VCA, a binding between the IgG antibodies of the plasma and the immobilized EBV antigen takes place. Then ready-to-use anti-human-IgG peroxidase conjugate was added, which will bind to the anti-human-IgG antibodies. After that the substrate (TMB) solution was added and then incubated at room temperature for 30 minutes. The development of a blue color in the wells indicates the presence of EBV IgG antibodies present in the specimens. The resulting color was measured spectrophotometrically at the wavelength of 450 nm. The concentration of the IgG antibodies is directly proportional to the intensity of the color.

Detection of EBV DNA using quantitative real time polymerase chain reaction (RT-PCR)

Fifty samples were subjected to viral DNA extraction and then RT-PCR for detection of EBV active viremia. These 50 samples randomly selected to be representative to all samples.

DNA was extracted from 200 µl of plasma using DNA-sorb-B (Sacace, Italy). DNA extraction steps included disruption/lysis of plasma sample, removal of the contaminants and recovery of the nucleic acid. The concentration and purity of the DNA were measured using the nucleic acid measuring instrument nanoDrop (England).

EBV Real-TM Quant Kit (Sacace, Italy) was used for the detection of LMP-gene in EBV genome. EBV LMP DNA amplification was detected on JOE (Yellow) channel, while the IC glob gene DNA amplification was detected on FAM

(Green) channel and exogenous Internal Control IC was detected on Rox (Orange) channel. The quantity of reactants for one reaction was 10 µL of PCR-mix -1 and 1.5 µL of PCR-mix-2 buffer and 0.5 µL of hot Start taq polymerase. DNA from sample/ standard/ positive or negative control was added to the mix. The final volume per reaction tube was 25 µL.

The RT-PCR instrument used in the study was STRATAGENE MxPro QPCR (Agilent Technologies, USA). The thermal protocol for Sacace Quantification Kit is composed of an initial denaturation for activation of the HotStarTaq DNA Polymerase at 95 °C for 15 min, followed by five cycles of thermal cycling 95 °C for 15 sec, and 60 °C for 20 sec, and 72 °C for 15 sec, and finally 40 cycles of 95 °C for 10 sec, and 60 °C for 40 sec, and 72 °C for 15 sec.

Statistical analysis

The Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA), version 20 was used for statistical analysis. EBV sero-positivity rates were calculated and compared according to different dependent variables. Differences were evaluated using the Chi-square test or Fisher exact test if there is 25% of cells less than expected count. P value of ≤ 0.05 was considered statistically significant.

Results

The results of EBV IgG anti-VCA titer were recorded as: negative, borderline, weak positive and positive according to the levels of Calibrator A, Calibrator B, Calibrator C and Calibrator D, respectively, according to kit instructions.

Results were also interpreted according to those instructions. Calibrator B with its concentration of 10 U/mL serves as cut-off value. If the value of the sample is higher than the cut-off +20% it was considered positive result, which represented 359/450 (79.8%) (Table 1).

The value below the cut-off– 20% was considered negative result which represented 18/450 (4%). Values with a range of +/-20% the cut-off were reported as borderline. As in relation to cut-off value equivocal samples which represented 73/450 (16.2%) were excluded from the study because they need further follow-up after 2-4 weeks to determine whether there are primary EBV infection or non-specific antibodies causing false positive.

Table 1. Frequency of EBV anti-VCA IgG antibody titer among blood donors

		Frequency	Percent
EBV IgG antibody titer	Positive	326	72.4
	Weak positive	33	7.3
	Border line	73	16.2
	Negative	18	4
Total		450	100%
EBV IgG antibody titer	Total positive	359/450	79.8%

Blood donors enrolled in this study included (88.9%) 400 males and (11.1%) 50 females. The results of this study showed higher rate of EBV positivity in females than in males with percentage 90% and 78.5%, respectively, which was statistically significant (P=0.036), as shown in table (2).

A significantly higher EBV sero-prevalence rate in those living in Baghdad than in those from other governorates, with percentage of 81.5% and 65.3%, respectively, (P=0.009), as shown in

table (2). This study showed that only one sample was positive for HBs-Ag, 14 for HBc-Ab, and three for TPHA. All samples were negative for anti HCV-Ab and HIV-Ab. There was no statistically significant association of EBV IgG, either with HBs-Ag, HBc-Ab or TPHA, P values were: 0.2, 0.6, and 0.5 respectively, none of the subjects had co-infections with any of these screened pathogens.

Quantitative real time PCR was conducted on 50 representative samples out of the 450 cases, according to the results of EBV VCA-IgG titers, to detect EBV viral load, using primers for EBV LMP-gene. The results of this study showed that all the 50 samples were negative for EBV LMP-gene.

Table 2. The association between EBV serology results and blood donors' descriptive data

Variable		IgG anti-VCA Negative N=91	IgG anti-VCA Positive N=359	Total	P value
Age groups	≤20 years	7 (26.9%)	19 (73.1%)	26	0.553 NS
	21-30 years	39 (22.7%)	133 (77.3%)	172	
	31-40 years	29 (19.1%)	123 (80.9%)	152	
	41-50 years	14 (17.3%)	67 (82.7%)	81	
	>50 years	2 (10.5%)	17 (89.5%)	19	
Gender type	Female	5 (10.0%)	45 (90.0%)	50	0.036 S
	Male	86 (21.5%)	314 (78.5%)	400	
Residence	Rural	12 (16.2%)	62 (83.8%)	74	0.22 NS
	Urban	79 (21.0%)	297 (79.0%)	376	
	Baghdad	74 (18.5%)	327 (81.5%)	401	0.009 S
	Governorates	17 (34.70%)	32 (65.30%)	49	
Blood groups	A	22 (17.46%)	104 (82.54%)	126	0.339 NS
	B	27 (19.57%)	111 (80.43%)	138	
	AB	5 (13.89%)	31 (86.11%)	36	
	O	37 (24.67%)	113 (75.33%)	150	
Occupation	Governmental employee	27 (20.0%)	108 (80.0%)	135	0.523 NS
	Private sector	54 (21.3%)	200 (78.7%)	254	
	Housewife	4 (11.1%)	32 (88.9%)	36	
	Student	6 (24%)	19 (76.0%)	25	
Cupping	No	67 (21.3%)	247 (78.7%)	314	0.223 NS
	Yes	24 (17.6%)	112 (82.4%)	136	
Travel	No	70 (21.7%)	252 (78.3%)	322	0.126 NS
	Yes	21 (16.4%)	107 (83.6%)	128	

NS: No statistical significance (p>0.05), S: Statistical significance (p<0.05).

Discussion

This study enrolled four hundred fifty (450) blood donors from the two main blood donation centers in Baghdad, and EBV sero-positivity among these blood donors was 79.8%, which is comparable to other results in the surrounding countries. For instance, sero-prevalence was reported among blood donors in Iran was 85% [24].

On the other hand, in Taiwan, Chen et al, 2015 reported that overall seropositive rate of EBV was (88.5%) [25]. In USA Balfour et al, 2013

reported that (90%) of healthy subjects had IgG antibodies against EBV viral capsid (VCA) antigen [26].

One explanation of the lower sero-prevalence reported in this study in comparison to other studies, whether in the surrounding countries or worldwide, is that the majority of studies showed that sero-positivity increases with age [3,25,27]. In this study, 350 out of 450 (78%) of subjects aged below 40 years old, i.e. in young age group, which could justify this slightly lower sero-prevalence rate.

Antibodies to EBV-Viral capsid Ag are the most commonly investigated Abs in sero-prevalence studies [28-31]. In the current study, survey of EBV was based on the presence of EBV IgG anti-VCA. Since anti-VCA IgG response has an important role in the detection of past infection, and some individuals might be anti-VCA IgM non-responders after acute primary infection [32], therefore, the current study was designed to detect anti-VCA IgG, and this has also been applied in many EBV sero-prevalence studies worldwide [5,30,33,34].

EBV causes a mild, self-limited infection in immunocompetent subjects. It is latent in B lymphocytes and can be transmitted through blood transfusion and usually presents as a clinical health hazard in high-risk recipients, such as immunosuppressed individuals [35-36].

Several studies suggested an association between blood transfusions and post-transplant EBV infection. The greatest risk is seen in EBV sero-negative patients receiving allografts from EBV-seropositive donors. Therefore, testing of donors will be helpful in the appropriate transfusion decision [37-38].

None of the blood donors enrolled in this study gave recent history of even minor flu-like illness or fatigue. In order for PCR to be effective in detecting the viral load it must be performed early during active infection before the immune system of the host eliminates the virus [39]. On the other hand, absence of viremia may be due to the very low viral load in these asymptomatic infected individuals (i.e. viral load could be below the detection limit) [40]. Although most individuals might be expected to carry EBV DNA in their lymphocytes, viral nucleic acid is not usually found in plasma in the absence of active EBV disease, since EBV is primarily B cell-associated, and plasma viremia is rare [41,42].

However, a study in Iraq on renal transplant subjects conducted by Shams-Aldin et al, 2015 in the same center of study and used the same EBV real time PCR kit on plasma samples, EBV viremia was detected in 19/57 (33%) of renal transplant recipients [43].

Gender type distribution among EBV sero-positive donors was significantly high in females than in males (P=0.036), a result that agrees with the findings of other studies. Crawford et al, 2002 and Chen et al, 2015 found that prevalence of EBV sero-positivity was significantly greater among women than men [6,25]. This difference is in accordance with the concept that women in general mount more vigorous antibody and cell mediated immune responses following infection or vaccination than men [37,44]. Thus, these antibodies could be detected more easily in women than in men.

Furthermore, the most common mode of EBV transmission for adults is via exposure to infected children [3]. Infected children actively excrete the virus in their saliva. In Iraq as this study observed, that women are at high risk of getting such childhood infectious diseases because of their higher contact rate with children than men, mainly mothers and teachers in kindergartens, daycares, and primary schools in which the vast majority are women teachers.

Most of previous studies showed that sero-positivity for EBV increases with age [3,25,27]. However, the current study revealed non-significant difference. Although similar result has been reported by some researches [45,46], this non-significant association of age could be explained by the fact that 40% of the participants are within 21-30 years old.

The study revealed significantly higher EBV sero-positivity among those living in Baghdad

as compared to those coming from other governorates (P=0.009). A finding that is mainly explained by the overcrowding conditions in Baghdad, in which there is higher crowding index as compared to other governorates. Such condition was frequently reported to be associated with high EBV sero-positivity [24,47]. On the other hand, the sample size from Baghdad was much more than other governorates (401 and 49 respectively) which, no doubt, influence the result of the investigation.

Collectively, these data suggest the high prevalence of anti-EBV IgG antibodies among Iraqi blood donors. This will raise the possibility of increasing the risk of EBV-associated malignancy. However, a further study to detect the prevalence of anti-EBV IgM antibodies among those donors before sounding the alarm.

Acknowledgments

Authors would like to acknowledge The Blood Donation Center in Al Imamein Al Kadhimein Medical City, and Iraqi National Blood Bank, for their cooperation in accomplishing this study.

Author contributions:

Redha: Collection of specimens, ELISA testing, DNA extraction, and real -time PCR, writing of the manuscript. Dr. Al-Obaidi: Supervision and performance of viral DNA extraction and real-time PCR run, writing of the manuscript. Dr. Ghazi: Supervision and performance of ELISA testing, and performance of all statistical analysis. Dr. Kadhim: Supervision of ELISA testing and final editing of the manuscript.

Conflict of interest

Authors declare no conflict of interest.

Funding

There is no funding source for this research.

References

1. Tao I, Bisseye C, Nagalo BM, et al. Screening of hepatitis G and epstein-barr viruses among voluntary non remunerated blood donors (VNRBD) in burkina faso, west africa. Mediterr J Hematol Infect Dis. 2013; 5(1): e2013053. doi: 10.4084/MJHID.2013.053.
2. Bembde AS, Mahajan NA, Bhale CP, et al. Prevalence of transfusion transmitted viral diseases among blood donors in MGM Medical College, Aurangabad, Maharashtra. Int J Health Sci Res. 2013; 3(1): 28-32.
3. Xiong G, Zhang B, Huang M, et al. Epstein-Barr virus (EBV) infection in Chinese Children: A retrospective study of age-specific prevalence. PLoS One. 2014; 9(6): e99857. doi: 10.1371/journal.pone.0099857.
4. Ok CY, Li L, Young KH. EBV-driven B-cell lymphoproliferative disorders: from biology, classification and differential diagnosis to clinical management. Exp Mol Med. 2015; 47(1): e132. doi: 10.1038/emm.2014.82.
5. Dowd JB, Palermo T, Brite J, et al. Seroprevalence of Epstein-Barr Virus Infection in U.S. Children Ages 6-19, 2003-2010. PLoS One. 2013;8(5):1-7.
6. Crawford DH, Swerdlow AJ, Higgins C, et al. Sexual history and Epstein-Barr virus infection. J Infect Dis. 2002; 186(6): 731-6. doi: https://doi.org/10.1086/342596.
7. Scheenstra R, Verschuuren E, De Haan A, et al. The value of prospective monitoring of Epstein-Barr virus DNA in blood samples of pediatric liver transplant recipients. Transpl Infect Dis. 2004; 6(1): 15-22. doi: 10.1111/j.1399-3062.2004.00044.x.
8. Adjei AA, Armah HB, Gbagbo F, et al. Seroprevalence of HHV-8, CMV, and EBV among the general population in Ghana, West Africa. BMC Infect Dis. 2008;8(1):111. doi: 10.1186/1471-2334-8-111.
9. Kaur P, Basu S. Transfusion-transmitted infections: existing and emerging pathogens. J Postgrad Med. 2005; 51(2): 146-51.
10. Rezvan H, Abolghassemi H, Kafiabad SA. Transfusion-transmitted infections among multitransfused patients in Iran: A review. Transfus Med. 2007; 17(6): 425-33. doi: 10.1111/j.1365-3148.2007.00794.x.
11. Tsai MH, Hsu CY, Yen MH, et al. Epstein-Barr virus-associated infectious mononucleosis and risk factor analysis for complications in hospitalized children. J Microbiol Immunol Infect. 2005; 38(4): 255-61.
12. Kimura H, Hoshino Y, Kanegane H, et al. Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. Blood. 2001; 98(2): 280-6.
13. Perret C. Methylation profile as a new tool for classification of hepatocellular carcinoma. J Hepatol. 2011; 54(4): 602-3. doi: 10.1016/j.jhep.2010.10.015.
14. Brooks GF, Carroll KC, Miller S, et al. Herpesviruses. In: Carroll KC, Hobden JA, Miller S, et al. (eds). Jawetz, Melnick, & Adelberg's Medical Microbiology. 27 ed. New York (NY): McGraw-Hill Education; 2016. p. 457-79.
15. Kang MS, Kieff E. Epstein-Barr virus latent genes. Exp Mol Med. 2015; 47(1): e131. doi: 10.1038/emm.2014.84.
16. Omer AR, Salih JI, Al-Nakshabandi AA. Frequency of blood-borne viral infections among leukemic patients in central Iraq. Saudi Med J. 2011; 32(1): 55-61.
17. Al-Mudallal SS, Al-Sinjery GM. Immunohistochemical expression of Epstein-Barr virus antigen latent

membrane Protein-1 and Bcl-2 in classical Hodgkin lymphoma. Iraqi J Med Sci. 2012; 10(3): 234-42.
18. Hanna BJ, Habib MA, Hussein AG. The expression of EBV versus apoptotic index in breast cancer. Iraqi Postgrad Med J. 2010; 9(4): 430-5.
19. Alaswad EA, Mohammed NA, Al-Rawak KJ. Determination of Epstein-Barr virus (EBV) DNA load as a biomarker to follow up EBV related Hodgkin's and Non Hodgkin's lymphoma patients using quantitative competitive polymerase chain reaction technique. Iraqi J Med Sci. 2011; 9(1): 61-73.
20. Sarmati L. HHV-8 infection in African children. Herpes. 2004; 11(2): 50-3.
21. Gasser O, Bihl F, Sanghavi S, et al. Treatment-dependent Loss of polyfunctional CD8+ T-cell responses in HIV-infected kidney transplant recipients is associated with herpesvirus reactivation. Am J Transplant. 2009;9(4):794-803. doi: 10.1111/j.1600-6143.2008.02539.x.
22. Babel N, Gabdrakhmanova L, Hammer M, et al. Induction of pre-transplant Epstein-Barr virus (EBV) infection by donor blood transfusion in EBV-seronegative recipients may reduce risk of post-transplant lymphoproliferative disease in adolescent renal transplant patients: report of two cases. Transpl Infect Dis. 2005;7(3-4):133-6. doi: 10.1111/j.1399-3062.2005.00110.x
23. Pourahamad M, Hooshmand F, Olyaee Nezhad S, et al. EBV seroepidemiology in married and unmarried women and men in Iran. Rep Biochem Mol Biol. 2014; 2(2): 94-7.
24. Chen CY, Huang KY, Shen JH, et al. A large-scale seroprevalence of Epstein-Barr virus in Taiwan. PLoS One. 2015;10(1): e0115836. doi: 10.1371/journal.pone.0115836.
25. Balfour HH, Odumade OA, Schmeling DO, et al. Behavioral, virologic, and immunologic factors associated with acquisition and severity of primary Epstein-Barr virus infection in university students. J Infect Dis. 2013; 207(1): 80-8. doi: 10.1093/infdis/jis646.
26. Elansary M, El Haddad H, Sharaf Eldin UA, et al. Seroprevalence and real-time PCR study of Epstein-Barr virus and the value of screening in pretransplant patients. Egypt J Intern Med. 2016; 28(1): 9-15. doi: 10.4103/1110-7782.182947.
27. Macsween KF, Crawford DH. Epstein-Barr virus—recent advances. Lancet Infect Dis. 2003; 3(3): 131-40.
28. Gärtner BC, Hess RD, Bandt D, et al. Evaluation of four commercially available Epstein-Barr virus enzyme immunoassays with an immunofluorescence assay as the reference method. Clin Diagn Lab Immunol. 2003; 10(1): 78-82.
29. Hess RD. Routine Epstein-Barr virus diagnostics from the laboratory perspective: Still challenging after 35 years. J Clin Microbiol. 2004; 42(8): 3381-7. doi: 10.1128/JCM.42.8.3381-3387.2004.
30. De Paschale M, Clerici P. Serological diagnosis of Epstein-Barr virus infection: Problems and solutions. World J Virol Febr World J Virol. 2012; 12(11): 31-43. doi: 10.5501/wjv.v1.i1.31.
31. Guerrero-Ramos A, Patel M, Kadakia K, et al. Performance of the architect EBV antibody panel for determination of Epstein-Barr virus infection stage in immunocompetent adolescents and young adults with clinical suspicion of infectious mononucleosis. Clin Vaccine Immunol. 2014; 21(6): 817-23. doi: 10.1128/CVI.00754-13.
32. Balfour HH, Sifakis F, Sliman JA, et al. Age-Specific prevalence of epstein-barr virus infection among individuals aged 6-19 years in the United States and factors affecting its acquisition. J Infect Dis. 2013;208(8):1286-93. doi: 10.1093/infdis/jit321.
33. Levine H, Balicer RD, Rozhavski V, et al. Seroepidemiology of Epstein-Barr virus and cytomegalovirus among Israeli male young adults. Ann Epidemiol. 2012; 22(11): 783-8. doi: 10.1016/j.annepidem.2012.06.099.
34. Gulley ML, Tang W. Using epstein-barr viral load assays to diagnose, monitor, and prevent posttransplant lymphoproliferative disorder. Clin Microbiol Rev. 2010;23(2):350-66. doi: 10.1128/CMR.00006-09.
35. Nishiwaki M, Fujimuro M, Teishikata Y, et al. Epidemiology of Epstein-Barr virus, cytomegalovirus, and Kaposi's sarcoma-associated herpesvirus infections in peripheral blood leukocytes revealed by a multiplex PCR assay. J Med Virol. 2006;78(12):1635-42. doi: 10.1002/jmv.20748.
36. Alfieri C, Tanner J, Carpentier L, et al. Epstein-Barr virus transmission from a blood donor to an organ transplant recipient with recovery of the same virus strain from the recipient's blood and oropharynx. Blood. 1996; 87(2): 812-7.
37. Lazda VA. Evaluation of Epstein-Barr virus (EBV) antibody screening of organ donors for allocation of organs to EBV serostatus matched recipients. Transplant Proc. 2006;38(10):3404-5. doi: 10.1016/j.transproceed.2006.10.066.
38. Trottier H, Buteau C, Robitaille N, et al. Transfusion-related Epstein-Barr virus infection among stem cell transplant recipients: A retrospective cohort study in children. Transfusion. 2012; 52(12): 2653-63. doi: 10.1111/j.1537-2995.2012.03611.x.
39. Gartzonika C, Vrioni G, Priavali E, et al. Utility of Real-Time PCR in the diagnosis of primary Epstein-Barr virus infection. J Med Microb Diagn. 2012 1: 118. doi:10.4172/2161-0703.1000118.
40. Suntornlohanakul R, Wanlapakorn N, Vongpunsawad S, et al. Seroprevalence of Anti-EBV IgG among various age groups from Khon Kaen Province, Thailand. 2015; 16(17): 7583-7.
41. Gärtner B, Preiksaitis JK. EBV viral load detection in clinical virology. J Clin Virol. 2010; 48(2): 82-90. doi: 10.1016/j.jcv.2010.03.016.
42. Qu L, Triulzi DJ, Rowe DT, et al. Stability of lymphocytes and Epstein-Barr virus during red blood cell storage. Vox Sang. 2007; 92(2): 125-9. doi: 10.1111/j.1423-0410.2006.00880.x

43. Shams-Aldein SA, Abdlameer AS, Al-Obaidi AB, et al. Detection of Epstein-Barr virus in renal transplant recipients: two centers study. Iraqi J Med Sci. 2015; 13(2): 191-9.
44. Beagley KW, Gockel CM. Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. FEMS Immunol Med Microbiol. 2003; 38(1): 13-22.
45. Pancharoen C, Mekmullica J, Chinratanapisit S, et al. Seroprevalence of Epstein-Barr virus antibody among children in various age groups in Bangkok, Thailand. Asian Pac J Allergy Immunol. 2001; 19(2): 135-7.
46. Libert N, Bigaillon C, Chargari C, et al. Epstein-Barr virus reactivation in critically ill immunocompetent patients. Biomed J. 2015; 38(1): 70-6. doi: 10.4103/2319-4170.132905.
47. Takeuchi K, Tanaka-Taya K, Kazuyama Y, et al. Prevalence of Epstein-Barr virus in Japan: Trends and future prediction. Pathol Int. 2006; 56(3): 112-6. doi: 10.1111/j.1440-1827.2006.01936.x.

Correspondence to Amjad Q. Redha
E-mail: amjadqassim1981@gmail.com
Received Sep. 28th 2016
Accepted Dec. 1st 2016

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

Cholelithiasis Following Bariatric Surgery: A New Approach to Deal with

Sajid H.A. Al-Helfy[1] *CABS, FICS*, **Qasim S. Almayah**[2] *PhD*, **Risala H. Allami**[3] *PhD*

[1]Dept. of Surgery, [2]Medical Research Unit, College of Medicine, [3]College of Biotechnology, Al-Nahrain University, Baghdad, Iraq

Abstract

Background Obesity and rapid weight loss induced by weight-reducing surgery are well recognized as a risk factor for the development of gallbladder stones. There is no standard policy whether to perform prophylactic cholecystectomy at the time of the bariatric operation or to give postoperative treatment to decrease the risk.

Objective To evaluate the incidence and risk factors of gallstone formation post-bariatric surgery. The results may help to decide how to deal with and follow up patients with post-bariatric surgery.

Methods A total of 120 patients who underwent weight-reducing operations were recruited for this study. Several factors expected to influence gallstone formation were recorded such as body mass index and excessive weight loss. Study population was followed up for 12 months postoperatively. Ultrasonography examination was performed for those who developed symptoms suggesting gallstone formation.

Results Twenty-six (21.7%) patients were found to develop gallstones. Of the studied risk factors, the percentage of excess weight loss, family history and carrying allele A of the variant rs670 were significant for predicting development of gallstone post-bariatric procedures.

Conclusion Based on the results of this study, it is reasonable to put an index for the risk of developing gallstone following bariatric surgery, and according to this index, the surgeon could decide whether to perform concomitant cholecystectomy along with the bariatric procedure or do not.

Keywords Bariatric surgery, cholelithiasis, single nucleotide polymorphism

Citation Sajid H.A. Al-Helfy, Qasim S. Almayah, Risala H. Allami. Cholelithiasis following bariatric surgery: a new approach to deal with. Iraqi JMS. 2017; Vol. 15(2): 143-150. doi: 10.22578/IJMS.15.2.6

List of abbreviations: BMI = Body mass index, BS = Bariatric surgery, EWL = Excess weight loss, GS = Gall stone, LRYGB = Laparoscope Roux-en-y gastric bypass, LSG = Laparoscopic sleeve gastrectomy, MO = Morbid obesity, SNPs = Single nucleotide polymorphisms

Introduction

Morbid obesity (MO) is a leading preventable cause of death worldwide. In 2014, World Health Organization estimated that 600 million adults and 42 million children were obese [(1)]. This condition is associated with 2-fold increase in mortality compared with the general population, and with an increased number of serious pathological conditions such as hypertension, type 2 diabetes mellitus, depression and gallstone (GS) [(2,3)]. Three main options are considered in the treatment of MO. These are lifestyle change, pharmacotherapy, and surgery. In the first two options, the weight loss usually not maintained, and up to 66-90% of patients regain weight after cessation of the changed lifestyle or treatment

[4,5]. On the other hand, bariatric surgery (BS) offers the only means of delivering substantial weight loss.

However, this marked efficiency of BS in treatment of MO is not without penalties; may be the most important of which is the increased incidence of GS regardless of the operation type. As early as 1983, Wattchow et al. [6] reported an increased likelihood of GS after Laparoscopic Roux-en-Y Gastric Bypass (RYGB). Shiffman et al. (7) evaluated the incidence of GS formation in 105 morbidly obese patients undergoing gastric bypass surgery. After 6 months follow up, 36% of the patients became suffering from cholelithiasis, a percentage which remained stable until 18 months. These facts impose intensive investigation to reduce or even eliminate such incidence of GS following BS.

Three approaches have been suggested to address this problem. The first one pioneered by O'Brien et al. [8] called for cholecystectomy for all patients with BS. Additional operation and hospitalization time as well as possible complications of the procedure are among main disadvantages of this approach. The second approach [9] involves waiting and performing cholecystectomy for those who develop symptomatic GS after BS. Finally, the third approach depends on the using of preventive drugs. In the last two approaches, there is a relatively high cost of follow up and medications. An alternative approach is the calculation of expected risk of GS formation in patients undergoing BS and deciding whether to do simultaneous cholecystectomy or not. Some risk factors were already addressed like excess weight loss (EWL) and found to positively affect GS formation [10]. Other factors like the effect of genetic were less studied.

Individual's genetic background is undoubtedly involved in GS formation either in obese or non-obese persons [11,12]. Certain single nucleotide polymorphisms (SNPs) in some genes were found to be associated with different diseases [13]. Of these, the SNPs rs670 in ApoA1 gene and rs351855 in FGFR4 gene were found to be associated with cholilithiasis. Dixit et al. [14] reported high risk for G allele of the SNP rs670 on GS disease in India, while Chen et al. [15] linked rs351855 with the aggravation of GS disease among Chinese population.

The current study aimed to evaluate the risk factors predisposing for gallstone formation in Iraqi patients undergoing BS in order to establish an index that help surgeon to decide how to deal with the problem of GS following BS.

Methods

Study Population

This is a prospective study of 120 MO-patients who underwent Laparoscopic Roux-en-Y Gastric Bypass (LRYGB) and Laparoscopic Sleeve Gastrectomy (LSG) at Al-Imamein Al-Kadhimein Medical City, Baghdad during the period from August 2013 to January 2015. Consent form explaining the objective and the scope of the study was obtained from each participant. Exclusion criteria were prior cholecystectomy, presence of GS by abdominal ultrasonography and refusal of participation in the study. During 12 months' post-operative follow up, the patients who were symptomatic for acute cholecystitis, acute cholangitis, abnormal and/or biliary pancreatitis as first presentation, were examined by abdominal ultrasonography along with liver function tests. Cholecystectomy was performed to those who developed gallstone during the follow-up period.

Data were collected by direct interview with each patient. These data included age, gender, preoperative body mass index (BMI), family history of GS, diabetes mellitus, and percentage of excess weight loss (%EWL), which was calculated according to Broca formula [16].

Blood samples were collected from each patient before the operation and kept in EDTA tubes. DNA was extracted from these samples using ready kit (gSYNCTM DNA Mini Kit Whole Blood Protocol/ Geneaid/ Korea) according to the manufacturer's instructions. The target sequence containing the SNPs rs670 in ApoA1 gene and rs351855 in FGFR4 gene were

amplified with specific primers using polymerase chain reaction technique (PCR).

The ApoA1 gene was amplified using the primers: forward 5'-AGGGAC AGA GCT GATCCT TGA ACT CTTAAG-3' and reverse 5'-TTAGGG GAC ACC TACCCGTCAGGA AGA GCA-3' [17]. Primers for FGFR4 gene were 5'-GACCGCAGCAGCGCCCGAGGCCAG-3' as forward primer and 5'-AGAGGGAAGAGGGAGAGCTTCTG-3' as reverse primer [18]. Each amplification was performed using 100 ng to 300 ng of genomic DNA in a volume of 25 µL using 12.5 pmol of each primer, 200 µM dinucleotide triphosphate, 15 mM magnesium chloride, 100 mM Tris (pH 8.0) and two units of Taq polymerase (Bioneer/ Korea). PCR conditions for ApoA1 involved an initial denaturation at 95°C for 5 min followed by 30 and with denaturation at 95 °C for 30s, annealing at 60 °C respectively for 45 s, extension at 72 °C for 60 s and final extension at 72 °C for 5 min. Almost similar conditions were applied for FGFR4 gene, but involved 35 cycles and the annealing temperature was 60 °C.

The PCR products were detected by 1% agarose electrophoresis and visualized under U. V light after staining with ethidium bromide. The amplified products were determined by comparison with a commercial 1000 bp ladder (Kappa Biosystem/USA). PCR products (435-bp and 168-bp for ApoA1 and FGFR4 respectively) were directly sequenced with ABI system (Macrogen/Korea).

Statistical analysis

Quantitative variables were expressed as means ± standard deviation (SD), while qualitative data were presented as absolute frequencies and proportions. Binary regression analysis was used to find out odds ratios (OR) with 95% confidence interval (CI) for categorical variables with respect to GS formation. Chi square test with 2x2 tables was employed to analyze the differences in proportions. Student t-test was used to compare means between two groups for quantitative variables. All analysis was performed with SPSS for windows software, version 16.0. A P-value of less than 0.5 was considered statistically significant.

Results

Demographic Characteristics of the Study Population

The demographic characteristics of the study population are shown in table 1.

Table 1. Demographic Characteristics of the study population

Parameter	Characteristics	Value
Age (years)	Mean	40.2
	Range	20-57
	SD	7.18
Gender	Male	44 (36.67%)
	Female	74 (63.33%)
BMI	Mean	41.19
	Range	37.1-43.9
	SD	6.62
Type 2 diabetes mellitus	Yes	12 (10%)
	No	108 (90%)
Family history of gallstone	Yes	7 (5.83%)
	No	113 (94.17%)
Bariatric Surgery	Roux-en-Y gastric bypass	14 (11.67%)
	Sleeve gastrectomy	106 (88.33%)

Incidence and risk factors of gallstones among MO-patients undergone BS

Out of 120 MO-patients underwent BS, 26 (21.6%) developed symptomatic GS diagnosed by transabdominal ultrasonography 2 to 12 months (mean 10 months) post BS. Elective cholecystectomy was performed by laparoscopic operation. Table 2 shows the association of different risk factors with the development of gallstone after BS. Only three of these factors (family history, %EWL and the SNP rs670) were significantly associated.

Five (19.23%) GS patients had family history of first or second relative with GS compared to 2 (2.13%) among patients with no GS with significant difference (OR=10.952, 95%CI = (1.987-60.371, P=0.005). Similarly, mean %EWL among GS and no GS patients were 24.6 and 21.65 with significant difference (P=0.028). The SNP rs670 had three genotypes; CC, CT, and TT (figure 1). In patients who developed GS, these genotypes account for 11 (42.31%), 9 (34.61%), and 6 (23.08%) respectively, compared to 61(64.89%), 29(30.86%), and 4(4.25%) respectively, in patients with no GS with significant differences for both heterozygous genotype (OR=8.318, 95%CI=2.013-34.37, P=0.003) and for homozygous mutant genotype (OR=4.833, 95%CI=1.112-21.014, P=0.036). Analysis of allele's frequencies of this SNP confirmed the significant association of T allele with the incidence of GS. The frequency of T allele between patients with and without GS was 40.38% and 19.68% respectively (OR=2.765, 95%CI=1.428-5.351, P=0.002).

On the other hand, each of the age, sex, BMI, operation type, DM and different genotypes of the SNP rs351855 did not show significant influence on the incidence of GS after BS. For age the two groups (GS and non GS patients) had very closed means of age (40.2 and 40.35 years respectively, P=0.488). Although female represented 73.08% among GS patients compared to 60.64 in non-GS patients, the difference was insignificant (OR=1.762, 95%CI=0.674-4.603, P=0.175).

Similar to rs670, the SNP rs351855 appeared with three genotypes which were CC, CT and TT (Table 2, figure 2). These genotypes represented 65.38%%, 26.93% and 7.69% respectively in patients with GS and 73.4%, 25.53% and 1.07% respectively in non GS patients. However, the differences were not significant.

At allelic level, the frequency of T allele in patients with GS was 21.15% compared with 13.83% in those with no GS with insignificant difference (OR=1.672, 95%CI=0.763-3.661, P=0.199).

Discussion

Bariatric surgery is known worldwide as a cost-effective treatment for MO patients. Cholecystitis and GS formation are the main unfavorable sequelae of this maneuver. Three approaches have been adopted to prevent or treat GS after BS. However, each of these approaches implies some disadvantages. In fact, the aim of this study is beyond determination of the incidence and risk factors associated with GS formation post-BS. That is because both the incidence and risk factors were well addressed, and there is almost a general agreement that only the %EWL is significant risk factor, and this what the current result revealed. However, for the best of our knowledge, it is the first time to investigate genetic risk factors in this regard. Thus, the study was intended to formulate an alternative approach to deal with GS formation in those patients. Although needs for maturation, this approach bases upon calculation of significant risk factors to get a value according which surgeon can take the decision whether to perform simultaneous cholecystectomy with BS or not. That means we have to establish a standardized language to evaluate these risks.

Out of nine studied risk factors, only 3 had significant association with the incidence of GS.

Table 2. Risk factors of gallstone formation following bariatric surgery

Risk factor		Gallstones 26 cases	No gallstones 94 cases	*P*-value	OR (95%CI)
Age (yr)	mean±SD	40.2±6.03	40.35±5.53	0.488	—
Sex	Male Female	7 (26.92%) 19 (73.08%)	37 (39.36%) 57 (60.64%)	0.175	1.762 (0.674-4.603)
BMI (Kg/m^2)	mean±SD	42.8±4.17	41.75±6.22	0.104	—
Operation	LRYGB LSG	4 (15.38%) 22 (84.15%)	10 (10.64%) 84 (89.36%)	0.357	0.655 (0.187-2.287)
Family History	No Yes	21 (80.77%) 5 (19.23%)	92 (97.87%) 2 (2.13%)	0.005	10.952 (1.987-60.371)
DM	No Yes	22 (84.62%) 4 (15.38%)	11 (11.7%) 83 (88.3%)	0.415	1.372 (0.398-4.727)
%EWL	Mean	24.6	21.65	0.028	—
rs670 Genotypes	CC CT TT	11 (42.31%) 9 (34.61%) 6 (23.08%)	61 (64.89%) 29 (30.86%) 4 (4.25%)	0.013 0.003 0.036	8.318 (2.013-34.370) 4.833 (1.112-21.014)
rs670 Alleles	C T	31 (59.62%) 21 (40.38%)	151 (80.32%) 37 (19.68%)	0.002	2.765 (1.428-5.351)
rs351855 Genotypes	CC CT TT	17 (65.38%) 7 (26.93%) 2 (7.69%)	69 (73.4%) 24 (25.53%) 1 (1.07%)	0.246 0.095 0.138	8.118 (0.695-94.866) 6.857 (0.539-87.279)
rs351855 Alleles	C T	41 (78.85%) 11 (21.15%)	162 (86.17%) 26 (13.83%)	0.199	1.672 (0.763-3.661)

SD: standard deviation; LRYGB: laparoscopic Roux-en-Y gastric bypass; LSG: laparoscopic sleeve gastrectomy; DM: diabetes mellitus; EWL: excessive weight loss

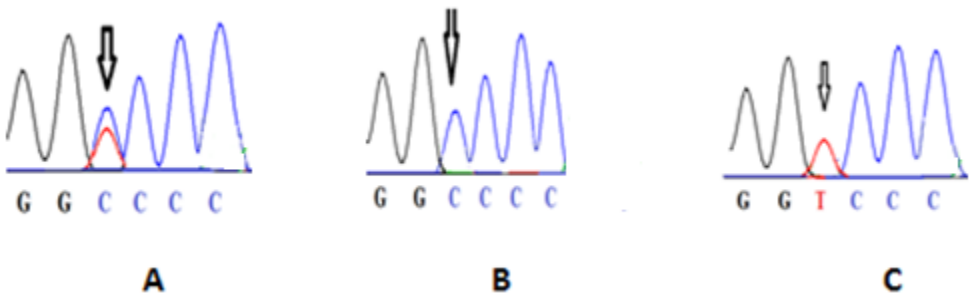

Figure 1. Different genotype patterns of the SNP rs670 (reverse strand). Heterozygous (CT) genotype (A), homozygous wild type (CC) genotype (B) and homozygous mutant (TT) genotype (C)

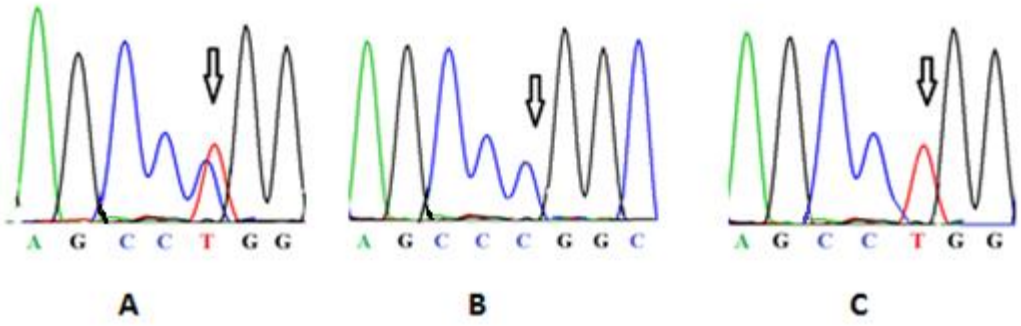

Figure 2: Different genotype patterns of the SNP rs351855; heterozygous (CT) genotype (A), homozygous wild type (CC) genotype (B) and homozygous mutant (TT) genotype (C)

For EWL percentage, there is no odds ratio because there is no reference EWL and it cannot be calculated at time of BS. However, the intensive investigations in this respect have determined the %EWL for every operation type. For example, after two years of LSG or LRYGB, the %EWL is 65-70% [16]. Body weight at the operation time does not seem to affect the GS stone formation as evidenced by the insignificant association of BMI with this disease, but this weight certainly influence %EWL. However, the range of this loss is very narrow and can be considered for every patient. It is the presence of other risk factors (genetics) that contribute the variation of GS incidence among different patients.

Family history can be easily obtained, and the risk of which can, also, be easily calculated through obtaining odds ratio. In normal population, studies revealed an increased frequency of cholelithiasis; nearly 3 times elevated risk in the relatives of GS patients [19]. The result of the current study showed that patient who has first or second-degree relative with GS exposes 10.95-fold risk of developing GS compared with patient without such relative. This implies predisposing factors among certain family mainly related to genetic components, similar dietary and other common lifestyles.

On the other hand, identification of different variants associate with the risk of GS is rather a hard task. With the rapid development of molecular technique, this task becomes less difficult. Fortunately, limited number of SNPs were reported to influence GS formation [14,20-22]. Of course, These SNPs, could be enrolled under genetic factors influencing family history, but not all family history is genetic, nor all family members carry the same variant. Therefore, study of these variants is important in putting an expectation for GS formation. Among the most studied gene in this respect is ApoA1 gene which encodes a major protein component of the HDL and a co-factor for lecithin cholestrolacyltransferase (LCAT). The later catalyzes the formation of plasma cholesterol ester [23]. Thus, the product of this gene is directly involved in GS formation because accumulation of the cholesterol ester in the gallbladder predispose for cholelithiasis [24]. The minor allele of the SNP rs670 (allele T) in ApoA1 gene was found to be associated with abnormal levels in serum lipids in several populations [25-27]. Furthermore, carriers of this allele were 1.7-times more chance to increase LDL-C than those who carry C allele.

In line with these results, the current study revealed that carrier of T allele has 2.76-fold risk of developing GS post BS compared to C allele carriers. Thus, patients who has a family history of GS and carries allele T of the SNP rs670 is highly predisposed to GS formation after BS, and therefore it is recommended in such patient to perform concurrent cholecystectomy with BS. Of course, the list of risk fact, especially SNPs, does not end at this point, and many SNPs could be added [27], but,

the present data strongly suggest a new approach for dealing with GS formation after BS through calculation of absolute risk. More studies with larger patients and other SNPs are needed to formulate such number. Then each significant risk factor will be given a value according to the obtained odds ratio. Summation of these values will result a number used by surgeon to decide how to deal with the problem of GS formation in patients with BS.

Acknowledgments
The authors highly appreciate the unlimited cooperation of patients, and the help from Medical Research Unit technicians.

Author contribution
Dr. Alhefy: work design and sample collection. Dr. Al-Mayah: Statistical analysis, DNA sequence analysis and writing. Dr. Allami: PCR, reviewing and writing of references.

Conflict of interest
The authors declare that they have no conflict of interest.

Funding
Self-funding.

References
1. WHO Fact Sheet. Overweight and Obesity. Available at http://www.who.int/mediacentre/factsheets/fs311/en, 2016.
2. Shiffman ML, Sugerman HJ, Kellum JH et al. Gallstones in patients with morbid obesity. Relationship to body weight, weight loss and gallbladder bile cholesterol solubility. Int J Obes Relat Metab Disord. 1993; 17(3): 153-8.
3. Bennett JM, Mehta S, Rhodes M. Surgery for morbid obesity. Postgrad Med J. 2007; 83: 8-15. doi: 10.1136/pgmj.2006.048868.
4. Padwal R, Li SK, Lau DC. Long-term pharmacotherapy for overweight and obesity: a systematic review and meta-analysis of randomized controlled trials. Int J Obes Relat Metab Disord 2003; 27(12): 1437-46. doi: 10.1038/sj.ijo.0802475.
5. Derosa G, Cicero AF, Murdolo G, et al. Efficacy and safety comparative evaluation of orlistat and sibutramine treatment in hypertensive obese patients. Diabetes Obes Metab. 2005; 7(1): 47-55. doi: 10.1111/j.1463-1326.2004.00372.x.
6. Wattchow DA, Hall JC, Whiting MJ, et al. Prevalence and treatment of gall stones after gastric bypass surgery for morbid obesity. Br Med J (Clin Res Ed). 1983; 286(6367): 763.
7. Shiffman ML, Sugerman HJ, Kellum JM, et al. Gallstone formation after rapid weight loss: a prospective study in patients undergoing gastric bypass surgery for treatment of morbid obesity. Am J Gastroenterol. 1991; 86(8): 1000-5.
8. O'Brien PE, Dixon JB. A rational approach to cholelithiasis in bariatric surgery: its application to the laparoscopically placed adjustable gastric band. Arch. Surg. 2003; 138(8): 908-912. doi: 10.1001/archsurg.138.8.908.
9. Hamad GG, Ikramuddin S, Gourash WF, et al. Elective cholecystectomy during laparoscopic Roux-En-Y-gastric bypass: is it worth the wait? Obesity Surg 2003; 13(1): 76-81. doi: 10.1381/096089203321136638.
10. Li VK, Pulido N, Fajnwaks P, et al. Predictors of gallstone formation after bariatric surgery: a multivariate analysis of risk factors comparing gastric bypass, gastric banding and sleeve gastrectomy. Surg Endosc. 2009; 23(7): 1640-4. doi: 10.1007/s00464-008-0204-6.
11. Wittenburg H, Lammert F. Genetic predisposition to gallbladder stones. Semin Liver Dis. 2007; 27(1): 109-21. doi: 10.1055/s-2006-960174.
12. Chuang SC, Hsi E, Lee KT. Genetic of gallstone disease. Adv Clin Chem 2013; 60: 143-85.
13. Al-Mayah QS and Chaloob FA. Association of IFN-γ (+874A/T) with Chronic Hepatitis B Virus Infection. Int J Advance Res 2014; 2(2): 192-5.
14. Dixit M, Choudhuri G, Saxena R, et al. Association of apolipoprotein A1-C3 gene cluster polymorphisms with gallstone disease. Can J Gastroenterol. 2007; 21(9): 569-75.
15. Chen Q, Li WJ, Wan YY et al. Fibroblast growth factor receptor 4 Gly388Arg polymorphism associated with the severity of gallstone disease in a Chinese population. Genet Mol Res. 2012; 11(1): 548-55. doi: 10.4238/2012.March.8.3.
16. Leuratti L, Khwaja HA, Kernigan DD. Evidence base for bariatric surgery. In: Agrawal, S. (ed). Obesity, bariatric and metabolic surgery. London: Springer; 2015. p 65-75.
17. Larson IA, Ordovas JM, Barnard JR, et al. Effect of apolipoprotein A-1 genetic variations on plasma apolipoprotein, serum lipoprotein and glucose levels. Clin Genet 2002; 61(3): 176-84.
18. Bange J, Prechtl D, Cheburkin Y, et al. Cancer progression and tumor cell motility are associated with the FGFR4 Arg allele. Cancer Res. 2002; 62: 840-7.
19. Hsing AW, Bai Y, Andreotti G, et al. Family history of gallstones and the risk of biliary tract cancer and gallstones: a population-based study in Shanghai, China. Int J Cancer. 2007; 121(4): 832-8. doi: 10.1002/ijc.22756.
20. Chen Q, Li WJ, Wan YY, et al. Fibroblast growth factor receptor 4 Gly388Arg polymorphism associated with severity of gallstone disease in a Chinese population.

Genet Mol Res. 2012; 11(1): 548-55. doi: 10.4238/2012.March.8.3.

21. Rudzinska K, Bogacz D, Kotrych D, et al. The APOB gene polymorphism in the pathogenesis of gallstone disease in pre- and postmenopausal women. Prz Menopauzalny. 2015; 14(1): 35-40. doi: 10.5114/pm.2015.49169.

22. Bora K, Pathak MS, Borah P, et al. Single nucleotide polymorphisms of APOA1 gene and their relationship with serum apoliprotein A-1 concentrations in the native population of Assam. Meta Gene. 2015; 7: 20-7. doi: 10.1016/j.mgene.2015.10.005.

23. Cucuianu M, Coca M, Hancu N. Reverse cholesterol transport and atherosclerosis. A mini review. Rom J Int Med. 2007; 45(1): 17-27.

24. Sandri L, Colecchia A, Larocca A, et al. Gallbladder cholesterol polyps and cholesterolosis. Minerva astroenterol Dietol. 2003; 49(3): 217-24.

25. Haase CL, Tybjaerg-Hansen A, Grande P, et al. Genetically elevated Apolipoprotein A-I, high-density lipoprotein cholesterol levels, and risk of ischemic heart disease. J Clin Endocrinol Metab. 2010; 95(12): E500-10. doi: 10.1210/jc.2010-0450.

26. Yin RX, Li YY, Lai CQ. Apolipoprotein Al/C3/A5 haplotypes and serum lipid levels. Lipids Health Dis. 2011; 10: 140. doi: 10.1186/1476-511X-10-140.

27. Toptas B, Gormuş U, Ergen A, et al. Comparison of lipid profiles with APOA1 MspI polymorphism in obese children with hyperlipidemia. In Vivo. 2011; 25(3): 425-30.

Correspondence to Dr. Qasim S. Almayah
E-mail: kasim19672003@yahoo.com
kasim19672003@colmed-alnahrain.edu.iq
Received Oct. 3rd 2016
Accepted Jan. 29th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

Study of Finger Print Patterns in Leprosy

Nagi A. Abdulla[1] *PhD*, **Salwa O. Bahalah**[2] *MD*, **Maysa S. Alnoban**[3] *MSc*, **AbdulBaset A. AlQbaty** *MBChB*

[1]Dept. of Paraclinic, Forensic Medicine Unit, [2]Dept. of Dermatology, [3]Dept. of Community Medicine Faculty of Medicine & Health sciences, Aden University, Yemen, [4]Taiz Leprosy Center, Yemen

Abstract

Background Dermatoglyphic is the study of epidermal ridge configuration on finger tips, palms and soles. Leprosy is an infectious disease due to *Mycobacterium Leprae*. Few report of dermatoglyphic patterns studies in patients with leprosy have shown that fingerprint patterns were also affected in leprosy.

Objective To identify the effect role of leprosy on of fingerprint patterns.

Methods A prospective case control study on 50 patients complaining from leprosy and 50 control group was conducted within 11 months period in order to study the patient fingerprint patterns.

Results In this study, male (74%) consisted high rate than female (26%) and majority of patients at age group ≥ 54 years old. The whorls and loop patterns were high in control group than cases with 36.3% and 48.9% respectively, while arch pattern was high in case group than control with 29.6% in case group. Arch patterns were high in little, ring and thumb fingers, while loop patterns were high in ring, index and thumb fingers than control one.

Conclusion The findings of this study were suggestive that there was an increase in the loop and arch patterns of individuals with leprosy and this was highly significant when compared to the control group in which the whorls pattern was higher, and to identify the patients with leprosy also to find if there are possible risk for future infection with leprosy by study the patterns of finger print.

Keywords Dermatoglyphic, fingerprints, leprosy.

Citation Nagi A. Abdulla, Salwa O. Bahalah, Maysa S. Alnoban, AbdulBaset A. AlQbaty. Study of finger print patterns in leprosy. Iraqi JMS. 2017; Vol. 15(2): 151-158. doi: 10.22578/IJMS.15.2.7

Introduction

Fingerprint (dermatoglyphic/ dactylography) is an impression of the friction ridge on all parts of the palms of the hands and soles of the feet; it came from two Greek words derma means (skin) and glyphs means (curves) [(1)]. Dermatoglyphic is highly individualistic and makes up the basis form for personal identification in forensic examinations; Galton classified dermatoglyphic depending upon their primary pattern as loops, whorls, arches, and compound as seen in figure (1) [(2)]. These dermal ridge differentiation takes place early in fetal development, between 13th to 19th weeks of intrauterine life, the medico-legal importance of these patterns are unique and remain unchanged throughout life [(3)]. The study of dermatoglyphic plays an important role and could be recognized as a powerful tool in the diagnostic features of certain psychological, medical, genetic, and congenital malformation [(4)] and its considered as a window of various diseases [(5)] including mongolism, rubella syndrome, congenital heart disease, selected neurological diseases, and other disorders [(6)].

Figure 1. Different patterns of fingerprint

Leprosy also known as Hansen's disease is a long term chronic granulomatous bacterial infectious disease that primarily affects the skin and peripheral nerves. This disease is caused by an obligate intracellular bacillus, *Mycobacterium Leprae* or *Mycobacterium Lepromatosis* [(7)]. Initially, infections are without symptoms and typically remain this way for 5 to 20 years, symptoms that develop include granulomas of nerves, respiratory tract, skin, and eyes [(8)]. This disease presents itself in two well defined stable and opposite poles (Lepromatous and Tuberculoid) and two unstable groups (Indeterminate and Dimorphic). The spectrum of presentation of the disease may also be classified as tuberculoid tuberculoid (TT), borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL), and lepromatous lepromatous (LL) [(7)].

Leprosy is spread between people, this is thought to occur through a cough or contact with fluid from the nose of an infected person, leprosy occurs more commonly among those living in poverty [(9,10)]. The clinical presentation and histopathologic changes depend on the immune status of the patient at the time of infection and over the nature course of the disease.

The diagnosis is currently based on 3 cardinal signs specified by the world health organization (WHO): hypopigmented or erythematous macules with sensory loss, thickened peripheral nerves, and positive acid-alcohol-last smear or skin biopsy [(11)]. The greatest risk factor for developing leprosy is contact with another case of leprosy; contacts of people with leprosy are five to eight times more likely to develop leprosy than members of the general population, however, conditions that reduce immune function, such as malnutrition, other illnesses, or host genetic differences, may increase the risk of developing leprosy [(9,12)].

The last epidemiology in leprosy, in 2015, the number of cases of leprosy was about 175,000 and the number of cases was 210,000 in 2013; 14 countries only recorded 95% of the globally reported leprosy cases, of this, India has the greatest number of cases (59%) followed by Brazil (14%) and Indonesia (8%) [(13)].

Despite effective treatment and education effort, leprosy stigma conditions to be problematic in endemic developing countries [(14)]. Modern multidrug therapy and new antibiotics of proven efficacy have made it possible to meet the WHO's targeted reduction in the incidence of *M. Leprae* infection to a single case per 10000 inhibitions in countries where the disease is endemic [(11)]. However, reports of dermatoglyphic patterns studies in patients with leprosy has been done by few workers [(6)] as studies by Enna et al. (1970) [(15)], Kapoor and Verma (1982) [(16)], Ghei et al. (1984) [(17)] have shown that finger print patterns were also affected in leprosy [(18)].

The objective of the current study was to determine whether the fingerprint patterns have a future role in identifying persons at risk of leprosy (to take preventive measurement from early against leprosy patients by study fingerprint patterns).

Methods

This study is a case control study (50 individuals complaining from leprosy and 50 healthy individuals as control group, which consisted of normal healthy individuals without any disease or congenital anomalies) conducted at the Out-Patient Clinic of Leprosy at Al-Gamhoria Teaching Hospital and in Leprosy Centers in Taiz and Hadhramout, from the period of February 2014 to January 2015. Information and consent was taken from the patients themselves, but in the case of children that consent was taken from parents by the authors at the clinic time. The materials that were used in this study are as follows:

- A clean plain glass plate (3x5 inch) with blue ink.
- White papers.
- Good lighting and hand magnifying lens.
- Detergent with towel for cleaning the ink from the hand.

To take finger prints, the following method was used: First, press and roll the finger firmly on the ink area, then press thoroughly to print record card (white paper). Next, label each print "left" and "right" for the hands, afterwards, label each fingerprint with "T" for thumb, "I" for index, "M" for middle, "R" for ring and "L" for little finger. Finally, all prints were analyzed by using a magnifying lens.

Statistical analysis

The collected data was analyzed by a computer facility, using microstate perform descriptive statistics of the investigated variables such as mean, range, frequency, percentage. A Chi square test (X2) was used for studying the association of categorical variables. The level of statistical significance was taken as $P < 0.05$ then presented in statistical tables.

Results

The study sample distributed into 50 persons as control group (without leprosy) and 50 persons as case group (with leprosy). The female consisted 26% of sample size while male appear with 74%. Age ≥54 years considered the dominant age group in this study, as seen in figures 2 and 3.

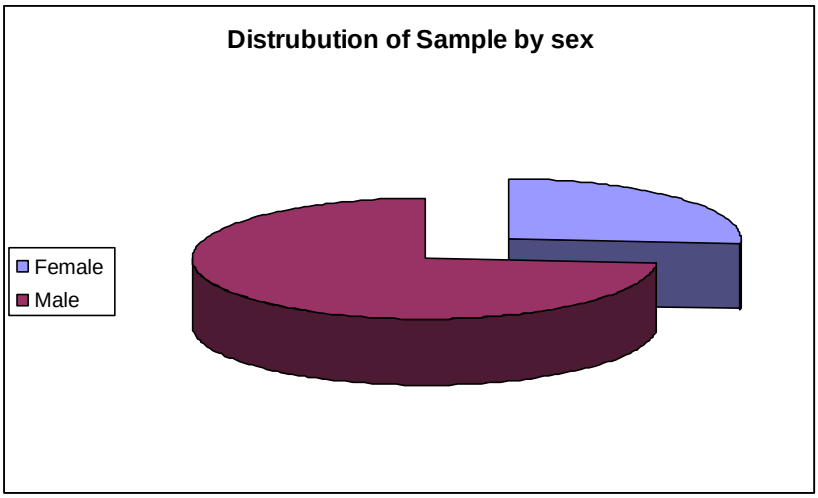

Figure 2. Distribution of sample studies according to sex

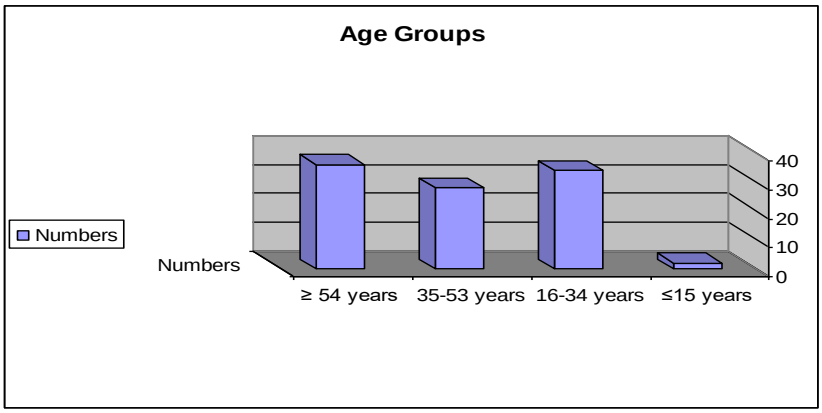

Figure 3. Distribution of sample according to age groups

It was found that the whorls pattern of fingerprint was more likely to appear in right index, right little in case group (person with leprosy) with odd ratio 0.130 (0.041-0.419) and 0.838 (0.260-2.695) respectively and arch pattern more likely to appear in case group in right thumb with odd ratio 0.474 (0.383-0.586), while the loop pattern also more likely in case group than control group in right middle fingers and right little with odd ratio 0.135 (0.056- 0.329) and 0.329 (0.132-0.824) respectively. The compound pattern appeared only in control group in right middle and thumb, as illustrated in table 1.

Table 1. Association between right hand finger print and both individual groups

Finger print	Control	Cases	P value	OR (95% CI)
Right little				
Arch	2 (12.5)	14 (87.5)	0.001	9.333 (1.004-43.681)
Whorl	41 (57.7)	30 (42.3)	0.766	0.838 (0.260-2.695)
Loop	7 (53.8)	6 (46.2)	0.015	0.329 (0.132-0.824)
Right ring				
Arch	5 (20)	20 (80)	0.005	6.000 (2.013-17.728)
Whorl	28 (100)	0 (0.0)	0.000	----------
Loop	17 (36.2)	30 (63.8)	0.009	2.912 (1.290- 6.571)
Right middle				
Arch	5 (33.3)	10 (66.7)	0.151	2.250 (0.709- 7.141)
Whorl	6 (19.4)	25 (80)	0.000	7.333 (2.652- 20.282)
Loop	38 (71.7)	15 (28.3)	0.000	0.135 (0.056- 0.329)
Compound	1 (100)	0 (0.0)	0.315	----------
Right index				
Arch	8 (34.8)	15 (65.2)	0.096	2.250 (0.854- 5.925)
Whorl	20 (83.3)	4 (16.7)	0.000	0.130 (0.041- 0.419)
Loop	22 (41.5)	31 (58.5)	0.071	2.077 (0.934- 4.615)
Right thumb				
Arch	5 (25)	15 (75)	0.022	0.474 (0.383- 0.586)
Whorl	14 (100)	0 (0.0)	0.826	----------
Loop	29 (45.3)	35 (54.7)	0.211	1.690 (0.740-3.857)
Compound	2 (100)	0 (0.0)	0.153	----------

Note: % taken by total row, *p value <0.05 (statistical significant), **Odd ratio (measure the risk)

This study showed that the loop pattern was more likely in case group in left middle and left little with odd ratio 0.141 (0.057-0.347) and 0.371 (0.158- 0.871) respectively. The whorls pattern was more likely in case group with odd ratio 0.091 (0.033- 0.253) for left ring and 0.169 (0.052- 0.548) for left index. The arch pattern was less likely in control group with odd ratio 0.490 (0.185- 1.300) for left middle and 0.691 (0.297- 1.610) for left thumb, as shown in table (2).

Table 2: Association between left hand finger print and both individual groups

Finger print	Control	Cases	P value	OR (95% CI)
Left little				
Arch	9 (36)	16(64)	0.105	2.144 (0.842- 5.459)
Whorl	3 (30)	7(70)	0.182	2.550 (0.620- 10.492)
Loop	38 (58.5)	27(41.5)	0.021	0.371 (0.158- 0.871)
Left ring				
Arch	5 (27.8)	13(72.2)	0.075	3.162 (1.032- 9.685)
Whorl	30 (83.3)	6(16.7)	0.000	0.091 (0.033- 0.253)
Loop	15 (32.6)	31(67.4)	0.001	3.807 (1.657- 8.747)
Left middle				
Arch	14 (63.6)	8(36.4)	0.148	0.490 (0.185- 1.300)
Whorl	4 (0.11)	32(88.9)	0.000	20.444 (6.322- 66.109)
Loop	32 (76.2)	10(23.8)	0.000	0.141 (0.057-0.347)
Left index				
Arch	12 (34.3)	23(65.7)	0.021	2.698 (1.148-6.341)
Whorl	17 (81)	4(19)	0.001	0.169 (0.052- 0.548)
Loop	21 (47.7)	23(52.3)	0.798	1.176 (0.534- 2.593)
Left thumb				
Arch	8 (36.4)	14(63.6)	0.391	0.691 (0.297- 1.610)
Whorl	16 (72.7)	6(27.3)	1.000	1.000 (0.299- 3.341)
Loop	23 (43.4)	30(56.6)	0.161	1.761 (0.796- 3.893)
Compound	3 (100)	0(0.0)	0.073	----------

Note: % taken by total row, *p value <0.05 (statistical significant), **Odd ratio (measure the risk)

In general, the whole and loop patterns of fingerprint were high in control group with 36.3% and 48.9% respectively than case group, while arch pattern was high in case group than control with 29.6%. The arch pattern high in middle digit of control group than case with 3.9%, while in case group the arch is high in little, ring, index and thumb digitals. The whorl pattern was high in case group in middle digit than others with 11.4%. while the loop patterns appear high in ring, index and thumb of case group than control one as seen in table 3 and figures 4 and 5.

Discussion

There are many people who suffer from some of the skin diseases; these diseases have a strong influence on the process of fingerprint recognition [19]. In the present study, it was observed that, the whorls patterns were higher in number in index fingers and the arch patterns were more in thumb fingers, while the loop pattern higher in number mainly in little and middle fingers respectively in both hands of leprosy group as compared to that of the control group.

Table 3: incidence of specific fingerprint patterns in both individual groups

Digital	Control			Cases		
	Arch	whorl	loop	Arch	whorl	loop
Little	11 (2.2)	44 (8.9))	45 (9.1)	30 (6)	37 (7.4)	33 (6.6)
Ring	10 (2.0)	58 (11.8)	32 (6.5)	33 (6.6)	6 (1.2)	61 (12.2)
Middle	19 (3.9)	10 (2.0)	69 (14)	18 (3.6)	57 (11.4)	25 (5)
Index	20 (4.1)	37(7.5)	43 (8.7)	38 (7.6)	8 (1.6)	54 (10.8)
Thumb	13 (2.6)	30 (6.1)	52 (10.6)	29 (5.8)	6 (1.2)	65 (13)
Total	73 (14.8)	179 (36.3)	241(48.9)	148 (29.6)	114 (22.8)	238 (47.6)

Note: Seven control cases had circular patterns (omitted in this table)

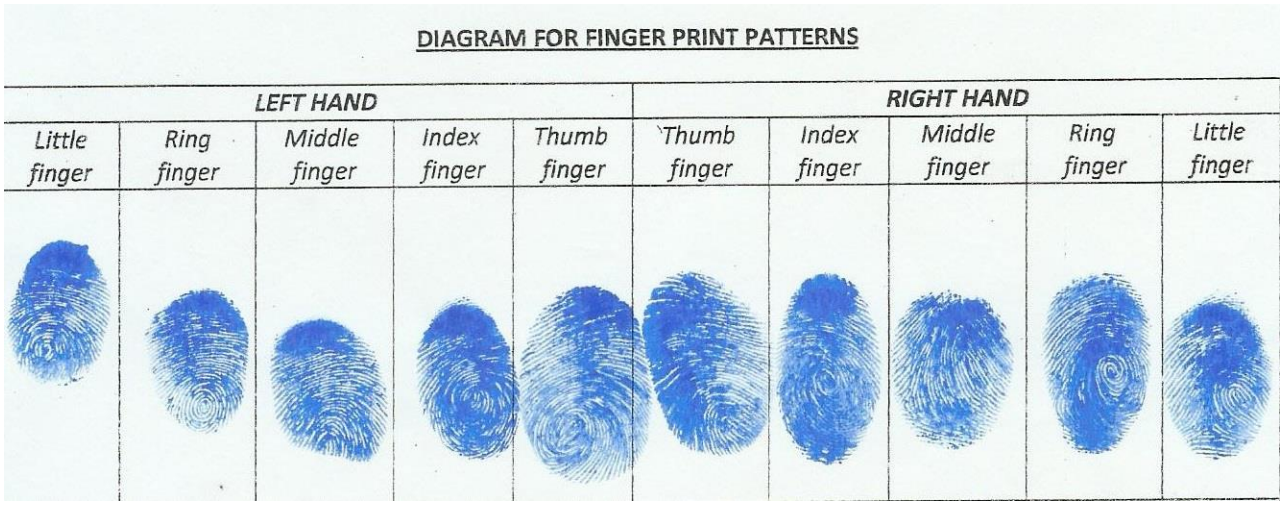

Figure 4. A sample of fingerprint patterns in leprosy

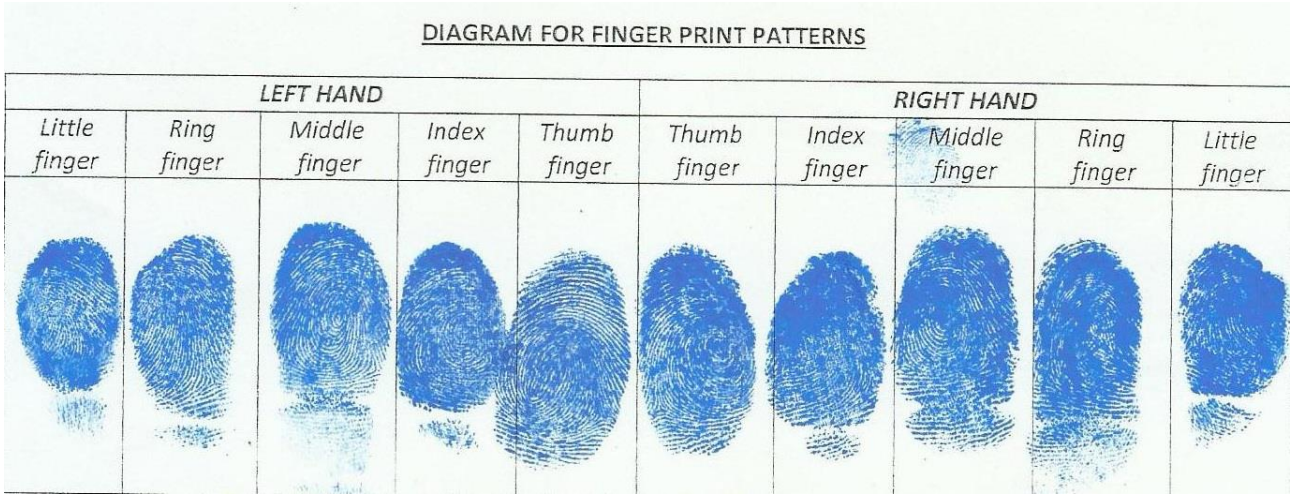

Figure 5. A sample of fingerprint patterns in leprosy

The authors observed a higher number of whorls found in the left ring finger in comparison to the right ring finger of leprosy group. On analysis of leprosy and control group, it has been found that the compound pattern appears only in control group mainly in right middle and right thumb fingers respectively.

In general, the study showed that loop patterns (47.6%) was increased in number follow by arch patterns (29.6%) with decrease in whorl patterns (22.8%) in leprosy group, while in control group it was found that higher numbers were loop (48.2%) followed by whorls (35.8%), with decrease in the number of arch (14.6%). This finding was in contradicts to those of other researchers such as Enna et al (1970) [15], Gupta et al (1986) [20], Natekar and Desouza (2007) who showed that the predominant fingerprint pattern was whorls (69.4%) and decrease in the loops (30.3%) in leprosy patients, whereas the control had decreased number of whorls (44.8%) and increased number of loops (54.7%) respectively [18].The number of arches both in the leprosy and control group were reduced in number and these differences were statistically insignificant. This finding also contradicts with the results of Kapoor and Verma (2011) who did not found any significant difference between fingerprint pattern of leprosy and control cases [16]. while the Ghei et al (1984) did not detect any significant changes in the pattern of whorls in leprosy patients [17].

This study concluded that it is evident from the results that loop patterns were affected in leprosy with decrease in whorl patterns in those patients. The dermatoglyphic features of the present study may be used as suggestive diagnostic tool to make a provisional diagnosis and to identify the persons who are at risk of leprosy. Similar studies can be conducted on large sample size of different population groups to generate more accurate and comprehensive data.

Acknowledgments

The authors are grateful to the subjects who have voluntarily participated and extended kind cooperation during this laborious and time-consuming data collection. Special thanks to Qadiri A. Hamdon for facilitating our work.

Author contributions:

Dr. Abdulla: Discussion and interpretation of results. Dr. Bahalah: Printing the leprosy fingerprints. Alnoban: Statistical analysis. Dr. AlQbaty: Data Collection and printing finger.

Conflict of interest

Forensic Medicine.

Funding

Authors own fund.

References

1. Fields R, Molina DK. A novel approach for fingerprinting mummified hands. J Forensic Sci. 2008; 53(4): 952-5. doi: 10.1111/j.1556-4029.2008.00768.x.
2. Trapecar M, Balazic J. Fingerprint recovery from human skin surfaces. Sci Justice. 2007; 47(3): 136-40. doi: 10.1016/j.scijus.2007.01.002.
3. Kaushal N. Fingerprints: Historical background and future trends. Int J Forensic Sci. 2010; 4(2): 30-4.
4. Mathew L, Hegde AM, Rai K. Dermatoglyphic peculiarities in children with oral clefts. J Indian Soc Pedod Prev Dent. 2005; 23(4): 179-82.
5. Bukelo MJ, Kanchan T, Unnikrishnan B, et al. Study of fingerprint patterns in children with acute lymphoblastic leukemia. Forensic Sci Med Pathol. 2011; 7(1): 21-5. doi: 10.1007/s12024-010-9190-9.
6. Bharat S, Jagdish C. Patel SV, et al. A study of palmar dermatoglyphic in leprosy in Bhavnagar District. Nat J Integ Res Med. 2011. 2(2): 46-9.
7. Araujo MG. Leprosy in Brazil. Rev Soc Bras Med Trop. 2003. 36(3): 373-82. https://dx.doi.org/10.1590/S0037-86822003000300010
8. Leprosy Fact Sheet N"101". World Health Organization. January 2014.
9. Suzuki K, Akama T, Kawashima A, et al. Current status of leprosy; epidemiology, basic sciences and clinical perspectives. J Dermatol. 2012; 39(2): 121-9. doi: 10.1111/j.1346-8138.2011.01370.x.
10. Hansen's disease (leprosy) transmission. 2015. https://www.cdc.gov/leprosy/transmission.
11. Eichelmann K, González González SE, Salas-Alanis JC, et al. Leprosy. An update: definition, pathogenesis, classification, diagnosis, and treatment. Actas Dermosifiliogr. 2013; 104(7): 554-63. doi: 10.1016/j.adengl.2012.03.028.
12. Schreuder PAM, Noto S, Richardus JH. Epidemiologic trends of leprosy for the 21st century. Clin Dermatol. 2015. 34(1): 24-31.
13. WHO. Global leprosy update, 2013; reducing disease burden. Wkly Epidemiol Rec. 2014; 89(36): 389-400.
14. Barret R. Self-mortification and the stigma of leprosy in Northern India Med Anthropol Q. 2005; 19(2): 216-30.
15. Enna CD, Elliott JP, Stockwell FE. An Evaluation of Dermatoglyphics in Leprosy. A pilot Study. Int J Lepr Other Mycobact Dis. 1970; 38(2): 177-86.
16. Kapoor TR, Verma RD. Study of dermatoglyphics in tuberculoid leprosy. Lepr India. 1982; 54(1): 142-8.

17. Ghei SK, Girdhar BK, Ramu G, et al. Dermatoglyphics in Leprosy (I. Finger Patterns). Indian J Lepr. 1984; 56(4): 855-60.
18. Natekar PE, DeSouza FM. Digital Dermatoglyphics in Leprosy. Anthropologist. 2007; 9(1): 63-6.
19. Drahansky M, Dolezel M, Urbanek J, et al. Influence of Skin Diseases on Fingerprint Recognition. J Biomed Biotechnol. 2012; 2012: 626148. doi: 10.1155/2012/626148.
20. Gupta CM, Tutakne MA, Tiwari VD. Study of fingerprint pattern in leprosy. Indian J Lepr. 1986; 58(1): 79-85.

Correspondence to Dr. Nagi A. Abdulla
E-mail: nagiali1966@hotmail.com
Received Oct. 10th 2016
Accepted Mar. 30th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

Levels of Tumor Necrosis Factor Alpha and Interleukin-17 in Fertile and Infertile Women with Endometriosis

Vean S.A. Al-Azawy *MSc*

Dept. of Basic Sciences, College of Dentistry, University of Baghdad, Iraq

Abstract

Background Endometriosis is described by the existence of endometrial tissue outside the uterine cavity. Infertility is one of clinical manifestation of endometriosis shown by the difference of fecundity.

Objective To compare the serum levels of TNF-α and IL-17 in fertile and infertile endometriosis patients.

Methods This study was conducted on 55 women patients with endometriosis (30 infertile women and 25 fertile women) and twenty apparently healthy controls. The technique used to reveal serum level of TNF-α and IL-17 was Enzyme-linked immune sorbent assay (ELISA).

Results This study revealed significant increase ($p<0.05$) in serum levels of TNF-α in infertile patients other than that fertile. On the other hand, there were no significant differences ($p>0.05$) between controls and each group of patients. Moreover, there were significant increase ($p<0.05$) in IL-17 levels in infertile patients than that fertile patients and controls, while there were no significant differences ($p>0.05$) between fertile patients and controls.

Conclusion The current results indicate that TNF-α and IL-17 might play a crucial role in endometriosis-related infertility.

Keywords Endometriosis, TNF-α, IL-17.

Citation Vean S.A. Al-Azawy. Levels of tumor necrosis factor alpha and interleukin-17 in fertile and infertile women with endometriosis. Iraqi JMS. 2017; Vol. 15(2): 159-164 . doi: 10.22578/IJMS.15.2.8

List of abbreviations: ELISA = Enzyme-linked immune sorbent assay, IL-17 = Interleukin-17, TNF-α= Tumor necrosis factor alpha

Introduction

Endometriosis is a prevalent benign chronic inflammatory gynecologic disorder, described as the proliferation and presence of functional endometrial glands (endometrial-like tissue) external of the normal location (uterine cavity) [1,2]. It is affecting about 6-10% of women of reproductive age. These women may be asymptomatic, but the majority will present with pelvic pain, infertility, or an adrenal mass. In fact, endometriosis has been reported to be as high as 35–50% in women presenting with infertility [3]. Endometriosis is a major cause of infertility due to inflammation-associated reductions in oocyte quality and endometrial receptivity to embryonic implantation. However, the connection between infertility and endometriosis is especially obvious for advanced levels of the disease [3]. Though its pathogenesis still unknown, there is evidence showing that environmental factors, immunological, endocrine, and genetic factors play an important role in the development of endometriosis and genesis [4]. Endometriosis is associated with several immunological alterations, which are identified in infertile

patients [5,6]. These alterations participate in the development and progression of endometriosis and infertility [7].

Moreover, endometriosis may be considered an autoimmune disorder due to its immune aberrations, including elevated local production of several proinflammatory cytokines as well as increased autoantibody production and revocation of local and systemic cell-mediated immunity [8]. Strong evidence proposed that endometriosis was correlated with a state of subclinical peritoneal inflammation, noteworthy by raised growth factors and inflammatory cytokines [9].

The role of cytokines in women with endometriosis has been reported by various researches [10]. Tumor necrosis factor-alpha (TNF-α) is known as a pluripotent mediator and angiogenic cytokine that condense the production of other cytokines, including IL-8 in diverse cells as well the production of cytokine in endometriotic tissue. TNFα may be regarded as a key cytokine that actuate many other cytokines in the peritoneal cavity of endometriosis patients [11].

IL-17 is a representative cytokine excreted from Th17 cells. IL-17 deed on a wide range of cell types, including those of the mesenchymal lineages, epithelial, endothelial, and hemopoietic [12]. It has been shown that IL-17 can stimulate the expression of intracellular adhesion molecule (ICAM)-1 and increased the proinflammatory responses induced by IL-1h and TNF-a. In addition, IL-17 has been implicated in several inflammatory disorders [13].

Therefore, the present study aimed to compare between serum of TNF-α and IL-17 levels in fertile and infertile endometriosis patients.

Methods

A total of 55 serum samples were collected from endometriosis patients (30 infertile women and 25 fertile women) aged between 21-43 years who were attended to Kamal Al-Samari Hospital and Baghdad Medical City Teaching Hospital from June 2014 to January 2015. Twenty fertile healthy controls women were enrolled in this study. The diagnosis was done by the gynecologist, which was based on laparoscopy. They were recently diagnosed and all of the patients without treatment and other chronic diseases. 3 ml of venous blood was withdrawn from every subject (patients and control) under aseptic technique and positioning in the plain test tube with no anticoagulant, left to clot at room temperature and then separated the serum by centrifugation at 3000 rpm for 15 minutes, divided into aliquots and kept at -20 °C until used for investigations.

Levels of serum TNF-α and IL-17 have been measured by using commercially available ELISA and accomplished as recommended in leaflet with kit (TNF-α and IL-17 Boster/USA).

Statistical analysis

Comparison of serum levels of TNF-α and IL-17level among groups were counted by student's t-test and ANOVA test. P-values of $P<0.05$ was deemed significant.

Results

The age of the two groups of patients and control was matched. Mean age of infertile patients was 27.5 ± 2.39 year, while a fertile patient was 27.9±1.98 year and for controls was a 26.3±1.35 year as shown in table (1).

This study observed that there were significant differences ($p<0.05$) in mean serum levels of TNF-α between infertile and fertile females' patients (24.54±4.60 vs. 39.77±7.64). Furthermore, there were no significant differences ($p>0.05$) between controls and each group of patients as shown in table (2).

Table (3) revealed the increased ($p<0.05$) in mean serum IL-17 levels in infertile patients (72.95 ± 16.84) than that fertile patients (35.51 ± 5.57) and controls (40.21 ± 6.32), whereas there were no significant differences ($p>0.05$) between fertile patients and controls.

Table 1. Age distribution of the studied groups

Age (years)	Patients		Healthy control n=20	*P* value (ANOVA)
	Infertile n=30	Fertile n=25		
Range	(21-40)	(22-43)	(20-39)	
Mean ± SE	27.5 ± 2.39	27.9 ± 1.98	26.3 ± 1.35	1.39 NS

NS: Non-significant; SE: Standard error

Table 2. Mean serum levels of TNF-α among studied groups

Marker	Infertile patients N=30 Mean ± SE	Fertile patients N=25 Mean ± SE	Healthy control N=20 Mean ± SE	*P (T-test)*
Serum TNF-α (pg/ml)	39.77 ± 7.64	24.54 ± 4.60	31.22 ± 5.12	Infertile vs Fertile 0.041* Infertile vs Control 1.271 *NS* Fertile vs Control 0.881 *NS*

*: Significant; SE: Standard error; NS: Non-significant

Table 3. Mean serum levels of IL-17 among studied groups

Marker	Infertile patients N=30 Mean ± SE	Fertile patients N=25 Mean ± SE	Healthy control N=20 Mean ± SE	*P (T-test)*
Serum IL-17 (pg/ml)	72.95 ± 16.84	35.51 ± 5.57	40.21 ± 6.32	Infertile vs Fertile 0.030* Infertile vs Control 0.049* Fertile vs Control 0.221 *NS*

*: Significant; SE: Standard error; NS: Non-significant

Discussion

It has been reported that about 25-50 % of infertile women possess endometriosis and that 30-50% of endometriosis women are infertile [14]. D'Hooghe and colleagues showed that the prevalence of endometriosis is significantly higher in infertile than fertile women, as well as the infertile women are more probably to have the disease in advance stage [15]. In spite of extensive research, no agreement has been reached and various mechanisms have been suggested to explain

the association between infertility and endometriosis. These mechanisms contain distorted pelvic anatomy, altered peritoneal function, ovulatory abnormalities and endocrine, and altered humeral and cell-mediated functions in the endometrium [16].

Systemic immune modification has also been characterized in endometriosis, with activation of peripheral blood monocytes, which secrete high levels of cytokines [17]. Many studies have involved TNF-α in the progression and pathogenesis of endometriosis as well as in infertility. TNF-α concentration have been shown to exhibit significant value as a qualitative diagnostic measure of women with endometriosis [18].

The current result found significant increase in mean serum levels of TNF-α among infertile patients as compared to fertile patients, and there are no significant differences between controls and each group of patients. Similarly, Malutan and colleagues found that significantly higher serum level of TNF-α in female with endometriosis compared to healthy controls [19].

In addition, Galo and colleagues reported the serum level of TNF-α in endometriosis group was significantly higher than that women without endometriosis group, and suggested that TNF-α serum levels are good marker for diagnosis of endometriosis as noninvasive methods [20].

In concern to the significant increase of TNF-α in infertile female as compared with fertile female, there were no other similar studies to compare with current study results.

Increased levels of cytokines in the serum and peritoneal fluid of endometriosis women may reflect increased synthesis of cytokines by peritoneal lymphocytes, macrophages, ectopic endometrial implants, or mesothelial cells of the peritoneum, all of which can produce cytokines [21,22].

Other important result in this work was significant increase in IL-17 levels in infertile patients than those fertile patients and controls, whereas there are no significant differences between fertile patients and controls, these result was in agreement with other local study conducted by Ali et al. 2016 [23] who showed that mean IL-17 was significantly elevated in patient with endometriosis as compared with healthy and concluded that serum level of IL-17 could be used as marker of susceptibility in endometriosis, and may play a major role in pathogenesis of this disease. Moreover, results reported by Ahn et al. showed the presence of IL-17 in plasma samples and ectopic tissue samples from women with endometriosis [24].

In contrast to others, Malutan et al. 2015, and one year before them, Beste et al. 2014 [19,25] revealed that IL-17 levels were not detected in peritoneal fluid and serum of endometriosis patients.

Furthermore, Zhang and colleagues indicated that the concentration of IL-17 was significantly higher in case of infertility that coexist and endometriosis this result confirms current result [26]. Endometriotic lesions themselves secrete pro-inflammatory cytokines and this inflammatory state, which is thought to reduced fertility by having a toxic effect on embryos, gametes and impairing tubal motility. This finding supports the hypothesis that elevated levels of cytokines may be implicated in the pathogenesis of endometriosis associated infertility [27].

In contrast to the present results, Andreoli and colleagues showed that IL-17 level was similar between infertile and fertile patients with endometriosis [28].

In conclusion, the current results showed that TNF-α and IL-17 might play an important role in endometriosis-associated infertility.

Acknowledgments

Author likes to express thanks to all patients were included in this study.

Conflict of interest

The author reports no conflicts of interest.

Funding

Self-funding.

References

1. Arellano Estrada C, Barcena de Arellano ML, Schneider A, et al. Neuroimmunmodulation in the pathogenesis of endometriosis. J Brain Behav Imm. 2013; 29: S2-9. https://doi.org/10.1016/j.bbi.2013.01.008.
2. Vergetaki A, Jeschke U, Vrekoussis T, et al. Differential expression of CRH, UCN, CRHR1 and CRHR2 in eutopic and ectopic endometrium of women with endometriosis. PloS One. 2013; 8(4): e62313. https://doi.org/10.1371/journal.pone.0062313.
3. Giudice LC, Kao LC. Endometriosis. Lancet. 2004; 364(9447): 1789-99. doi: 10.1016/S0140-6736(04)17403-5.
4. Kobayashi H, Yamada Y, Morioka S, et al. Mechanism of pain generation for endometriosis-associated pelvic pain. Arch Gynecol Obstet. 2014; 289(1): 13-21. doi: 10.1007/s00404-013-3049-8.
5. Hernandez Guerrero CA, Vadillo Ortega F, Tlapanco Barba R, et al. Changes in the systemic immunologic response in association with endometriosis using an animal model. Ginecol Obstet Mex. 2002; 70: 171-81.
6. Podgaec S, Abrao MS, Dias JA Jr, et al. Endometriosis: an inflammatory disease with a Th2 immune response component. Hum Reprod. 2007; 22(5): 1373-9. doi: 10.1093/humrep/del516.
7. Kyama CM, Debrock S, Mwenda JM, et al. Potential involvement of the immune system in the development of endometriosis. Reprod Biol Endocrinol. 2003; 1: 123. doi: 10.1186/1477-7827-1-123.
8. Ellis A, Bennett DL. Neuroinflammation and the generation of neuropathic pain. Br J Anaesth. 2013; 111(1): 26-37. doi: 10.1093/bja/aet128.
9. Seli E, Arici A. Endometriosis: interaction of immune and endocrine systems. Semin Reprod Med. 2003; 21(2): 135-44. doi: 10.1055/s-2003-41320.
10. Gazvani R, Templeton A. Peritoneal environment, cytokines and angiogenesis in the pathophysiology of endometriosis. Reproduction. 2002; 123(2): 217-26.
11. Sakamoto Y, Harada T, Horie S, et al. Tumor necrosis factor-alpha-induced interleukin-8 (IL-8) expression in endometriotic stromal cells, probably through nuclear factor-kappa B activation: gonadotropin-releasing hormone agonist treatment reduced IL-8 expression. J Clin Endocrinol Metab. 2003; 88(2): 730-5. doi: 10.1210/jc.2002-020666.
12. Bettelli E, Oukka M, Kuchroo KT. T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol. 2007; 8(4): 345-50. doi: 10.1038/ni0407-345.
13. Albanesi C, Cavani A, Girolomoni G. IL-17 is produced by nickel specific T lymphocytes and regulates ICAM-1 expression and chemokine production in human keratinocytes: synergistic or antagonist effects with IFN-gamma and TNF-alpha. J Immunol. 1999; 162(1): 494-502.
14. Missmer SA, Hankinson SE, Spiegelman D, et al. Incidence of laparoscopically confirmed endometriosis by demographic, anthropometric, and lifestyle factors. Am J Epidemiol. 2004; 160(8): 784-96. doi: 10.1093/aje/kwh275.
15. D'Hooghe TM, Debrock S, Hill JA, et al. Endometriosis and subfertility: is the relationship resolved? Semin Reprod Med. 2003; 21(2): 243-54. doi: 10.1055/s-2003-41330.
16. Gupta S, Goldberg JM, Aziz N, et al. Pathogenic mechanisms in endometriosis-associated infertility. Fertil Steril. 2008; 90(2): 247-57. doi: 10.1016/j.fertnstert.2008.02.093.
17. Carmona F, Chapron C, Martínez-Zamora MÁ, et al. Ovarian endometrioma but not deep infiltrating endometriosis is associated with increased serum levels of interleukin-8 and interleukin-6. J Reprod Immunol. 2012; 95(1-2): 80-6. doi: 10.1016/j.jri.2012.06.001.
18. Shakiba K, Falcone T. Tumour necrosis factor-alpha blockers: potential limitations in the management of advanced endometriosis? A case report. Hum Reprod. 2006; 21(9): 2417-20. doi: 10.1093/humrep/del179.
19. Malutan AM, Drugan T, Costin N, et al. Pro-inflammatory cytokines for evaluation of inflammatory status in endometriosis. Cent Eur J Immunol. 2015;40(1):96-102. doi: 10.5114/ceji.2015.50840.
20. Galo S, Zúbor P, Szunyogh N, et al. TNF-alpha serum levels in women with endometriosis: prospective clinical study. Ceska Gynekol. 2005; 70(4):286-90.
21. Tabibzadeh SS, Santhanan V, Sehgal PB, et al. Cytokine induced production of IFN-β2/IL-6 by freshly implanted human endometrial stromal cells: modulation by estradiol 17β. J Immunol. 1989; 142: 3134-9.
22. Betjes MG, Tuk CW, Struijik DG, et al. Interleukin-8 production by human peritoneal mesothelial cells in response to tumor necrosis factor-α, imterleukin-1 and medium conditioned by macrophages co-cultured with staphylococcus epidermidis. J Infect Dis. 1993; 168(5): 1202-10.
23. Ali AM, AL-Ghurabi BH, Nader MI. Serum levels of IL-17A in Iraqi female patients with endometriosis. World J Pharmaceut Sci. 2016; 4(3): ISSN (Online): 2321-3086.
24. Ahn SH, Edwards AK, Singh SS, et al. IL-17A contributes to the pathogenesis of endometriosis by triggering proinflammatory cytokines and angiogenic growth factors. J Immunol. 2015; 195(6): 2591-600. doi: 10.4049/jimmunol.1501138.
25. Beste MT, Pfäffle-Doyle N, Prentice EA, et al. Molecular network analysis of endometriosis reveals a novel role for c-Jun=regulated macrophage activation. Sci Transl Med. 2014 Feb 5;6(222):222ra16. doi: 10.1126/scitranslmed.3007988.
26. Zhang X, Xu H, Lin J, et al. Peritoneal fluid concentrations of interleukin-17 correlate with the severity of endometriosis and infertility of this disorder. BJOG. 2005; 112(8): 1153-5. doi: 10.1111/j.1471-0528.2005.00639.x

27. Piva M, Horowitz GM, Sharpe-Timms KL. Interleukin-6 differentially stimulates haptoglobin production by peritoneal and endometriotic cells in vitro: a model for endometrium– peritoneum interaction in endometriosis. J. Clin Endocrinl Metab. 2001; 86(6): 2553=61. doi: 10.1210/jcem.86.6.7613.

28. Andreoli CG, Genro VK, Souza CA, et al. T helper (Th)1, Th2, and Th17 interleukin pathways in infertile patients with minimal/mild endometriosis. Fertil Steril. 2011; 95(8): 2477-80. doi: 10.1016/j.fertnstert.2011.02.019.

E-mail: veanalazawy@yahoo.com
Received Feb. 5th 2017
Accepted Apr. 12th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

The Profile of Matrix Metalloproteinase-9 in Relation to Coiling Index of Human Umbilical Cord

Hiba A. Abdul Sattar[1] *MSc,* **Hayder J. Mubarak**[2] *PhD*

[1]Dept. of Medical Laboratory Techniques, Osol Aldeen University College, Baghdad, Iraq, [2]Dept. of Human Anatomy, College of Medicine, Al-Nahrain University, Baghdad, Iraq

Abstract

Background Fetal blood flow through the umbilical cord vessels associated with abnormal coiling of the cord can have serious deleterious effects on the health of the fetus and newborn. Matrix metallopeptidase-9 (MMP-9) is a class of enzymes that are involved in the degradation of the extracellular matrix collagen and other proteins.

Objective To investigate the profile of MMP-9 immunohistochemical reactivity in the umbilical cord with variable coiling indices.

Methods In this study, 60 umbilical cords with inclusion criteria (full term newborns with normal perinatal outcome whose mothers were normal) collected from labor rooms of Ibn Al-Balady Hospital in Baghdad. The cords were used for immunohistochemistry matrix metaloprotenase-9 study.

Results The results of Immunohistochemical study showed variability in mesenchymal tissue reactivity among the three groups of the umbilical cords in different regions (sub amniotic region, perivascular region and central region) of the umbilical cords. The results obtained were evaluated by using the Aprio image scope analysis software.

Conclusion The immunohistochemical reactivity could indicate that the MMP-9 localization has role in degradation of collagen and proteins to maintain a harmless perivascular pressure in association with hypercoiling and hypocoiling of the cord and that could preserve the normal vascular blood flow to a certain limit.

Keywords Umbilical cords, human, coiling index, matrix metalloprotienase-9, normal pregnancy, immunohitochemistry.

Citation Hiba A. Abdul Sattar, Hayder J. Mubarak. The profile of matrix metalloproteinase-9 in relation to coiling index of human umbilical cord. Iraqi JMS. 2017; Vol. 15(2): 165-174 . doi: 10.22578/IJMS.15.2.9

List of abbreviations: ECM = Extracellular matrix, MMPs = Matrix metalloproteinase

Introduction

Abnormalities of the umbilical cord can lead to a major fetal morbidity and mortality [(1)]. The umbilical cord is protected by amniotic fluid, Wharton's jelly, helical patterns, and coiling of vessels [(2)]. The umbilical vessels' coiling develops as early as 28 days after conception and is existent in about 95% of fetuses by 9 weeks of pregnancy [(3)].

Umbilical cord coiling index can be defined as number of coils per one centimeter of length of the cord. Normal index is one coil for a length of five centimeters. If the numbers of coils are more per centimeter it is called as hypercoiled and less than it is called as hypocoiled umbilical cord [(4)].

Most published studies define hypocoiled (undercoiled) or hypercoiled (overcoiled) umbilical cords as below the 10th and above the 90th percentiles, respectively [5,6].

Umbilical coiling may serve to improve cord hemodynamics, as arterial pulsations transmitted to the vein may assist pump blood back up the cord from the placental capillary bed. Both abnormally coiled cords (hypercoiled and hypocoiled cords) are associated with an increased risk of adverse perinatal outcome. Abnormal cord coiling in its two shapes has been reported to be associated more frequent with preeclampsia and gestational diabetes [2,5].

Elastin and collagen are the important component of extracellular matrix (ECM). It is known that activities of collagenolytic enzymes like matrix metalloprotenase-9 are an essential factor regulating the degradation of collagen [7]. Matrix metalloproteinase (MMPs) are able to break down all components of ECM. They are involved in many remodeling processes of the connective tissue [8,9].

MMPs constitute a family of enzymes with 25 members identified to date, which are all extracellular of two subgroups: some membrane-bound or type MT-MMPs which are anchored to the plasma membrane, six MMPs have been assigned to this subgroup, and predominantly pericellular or soluble MMPs (chiefly 1, 2, 3, 8 and 9) in that gelatinase A is (MMP-2) while gelatinase B is (MMP-9). MT1-, MT2-, MT3-, MT4-, MT5- and MT6-MMP (MMP-14, -15, -16, -17, -24 and -25 respectively) [10]. MMPs are calcium and zinc-binding endopeptidases i.e. requiring Ca^{2+} and Zn^{2+} for their enzymatic activity [11].

Matrix metallopeptidase-9 (MMP-9), also known as 92 kDa type IV collagenase, 92 kDa gelatinase or gelatinase B (GELB), is a matrixin, a class of enzymes that belong to the zinc-metalloproteinases family involved in the degradation of the extracellular matrix [12].

In this study, the profile of MMP-9 immunohistochemical reactivity in the umbilical cord with variable coiling indices will be investigated.

Methods

This study is a cross sectional study, done in the period between (February 2016 and October 2016) in the Department of Human Anatomy, College of Medicine, Al-Nahrain University.

One centimeter piece of twenty (normociled, hypocoiled and hypercoiled) umbilical cords was taken from a full term, healthy (not have hypertension, diabetes mellitus, any other gynaecological problems or major diseases, nonsmoker and normal vaginal delivered) women who admitted to the Obstetric Ward of Ibn Al-balady Hospital in Baghdad and transferred immediately to be fixed with 10% formalin and processed for paraffin blocks.

The total number of the cords used in this study was 60; these include 20 cords for each of the three coiling indices (namely; hypercoiled, hypocoiled and normocoiled cords).

Each of the umbilical cords collected was examined grossly for its length, coiling pattern (presence or absence of segmental variability in the coiling density) and the umbilical coiling index was calculated.

Measurement of the length done by tape measure with consideration of the umbilical stump of the cord that remains attached to the umbilicus of the baby.

An umbilical coiling index of less than (0.17) and more than (0.37) was accepted as being hypocoiled and hypercoiled respectively, between (0.17 and 0.37) was normocoiled [1].

Serial paraffin sections of 4-5 µm thickness were cut using the electrical microtome and set on positive charged slides (Fisher Scientific, USA) used for the immunohistochemical studies. In the present study, (Expose Mouse and Rabbit Specific HRP/DAB detection IHC Kit (ab80436) from (abcam, UK) was used. Slides; for three types of umbilical cords, immunohistochemical studies had been examined using a light microscope (Olympus BX41, Japan). Assessment of immunohistochemical staining was achieved by applying Aperio positive pixel count algorithms program (from Aperio Image Scope software v11.1.2.760 (Aperio Technologies Inc, USA),

which can be used to analyze digital slides. The Aperio positive pixel count algorithm can be used to quantify the amount of a specific stain present in a slide image. These inputs have been pre- configured for brown color quantification in the three intensity ranges (weak positive, positive, and strong positive).

For statistical analyses, we used the SPSS software program software, version 20. The data are expressed as mean and standard error of the mean (SEM). Analysis of variance (ANOVA) was used to examine the statistical significance differences between the mean percentages of the subamniotic, perivascular and central region (for normocoiled, hypercoiled and hypocoiled cords). Also, it was used for testing the statistical significance differences between the mean percentages of the subamniotic, perivascular and central region within the same type of umbilical coiling cords. P value ≤ 0.05 denoted a statistically significant difference.

Results

Analysis of variance (ANOVA) of the counted mean values (percentage of positive reactivity) of matrix metalloproteinase-9 antibody immuno-histochemical reactivity in the mesenchymal tissue of the umbilical cord obtained by the application of the Aperio positive pixel count algorithms program (Aperio Image Scope software) showed a significantly more intensity in hypercoiled and hypocoiled cords compared to the reactivity in normocoiled cords ($p<0.05$). This significant variability was considered when comparing the subamniotic, perivascular, and central regions of each of umbilical cords.The light microscopic examination of the immunohistochemical reactivity of the umbilical cords did not show accurate measurable different between the regions of different coiling umbilical cords (Figure 1).

The counting of the mean value of the number of positive pixels in the normocoiled cords was significantly higher in the subamniotic region compared to perivascular and central regions. Non-significant variability was obtained between the counted mean values of perivascular and central regions of the noromocoiled cords (Figure 2).

The counting of the mean value of the number of positive pixels in the hypocoiled cords showed significant variability between the subamniotic, perivasclar, and central regions. The highest intensity was shown in perivascular region, and the least was in the central region (Figure 3).

The counted mean value of the number of positive pixels in hypercoiled cords was significantly higher in the perivascular region compared to both subamniotic and central regions. Non-significant variability was shown between the values of subamniotic and central regions of these cords (Figure 4).

The mean values of MMP-9 antibody reactivity in the subamniotic region showed highest intensity in hypocoiled cords and the least in normocoiled cords (Figure 5).

The counted mean values of MMP-9 antibody reactivity in the mesenchyme of the perivascular region obtained showed highest intensity in hypercoiled cords and the least in normocolied cords (Figure 6).

The mean values of the reactivity in the mesenchyme of the central region showed highest intensity in hypercoiled cord and the least in normocoiled cords (Figure 7).

Discussion

The Dynamic of Adverse Effect of Hypercoiling of Umbilical Cord

It was concluded that hypercoiling results in mechanical compression and diminished the blood flow in umbilical vessels as a result of the forceful obstruction of the umbilical circulation. Hypoxia subsequently resultant is described as (cord accident), that represent unconventional behavior with an outcome similarly happening in cases of cord prolapsed or true knots [13,14].

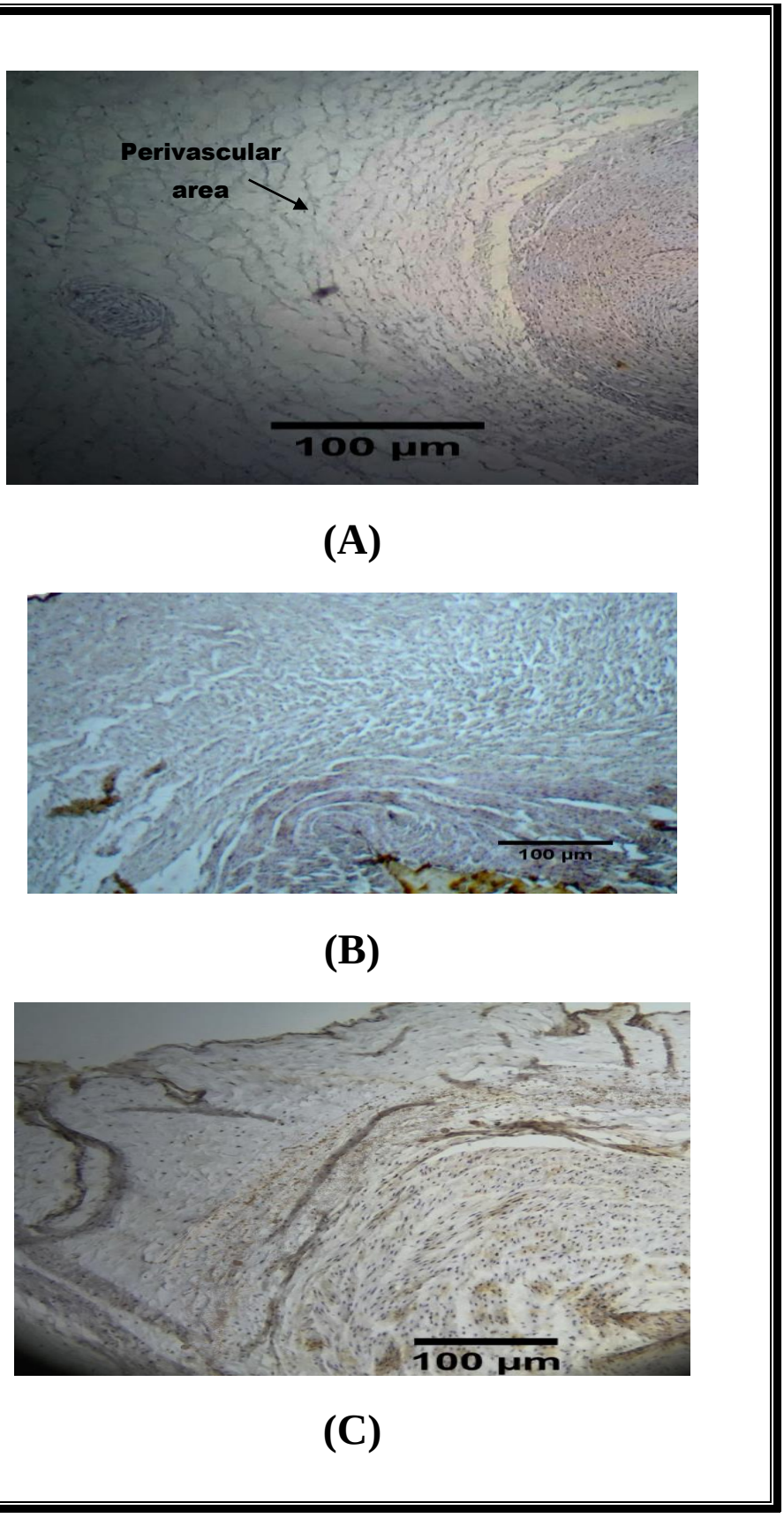

Figure 1. (A) Perivascular region of normocoiled cord, (B) Perivascular region of hypocoiled cord and (C) Perivascular region of Hypercoiled cord

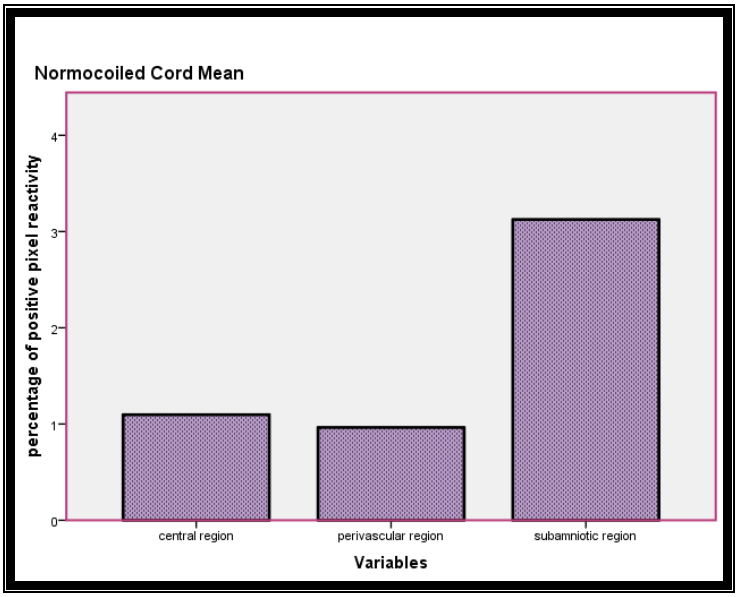

Figure 2. The mean value of the number of percentage positive pixels reactivity in the normocoiled between the mean values of perivascular, subamniotic and central regions

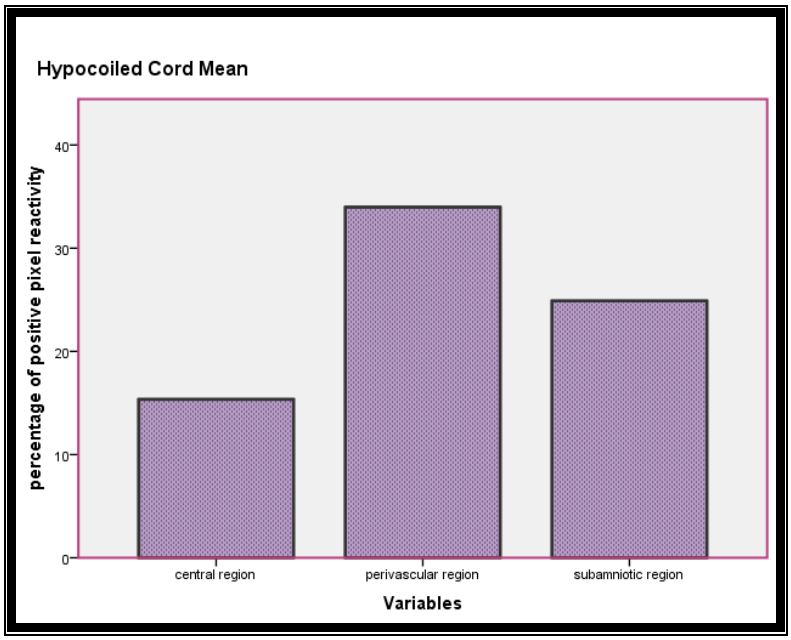

Figure 3. The mean value of the number of percentage positive pixel reactivity in the hypocoiled cords between the mean values of subamniotic, perivasclar, and central regions

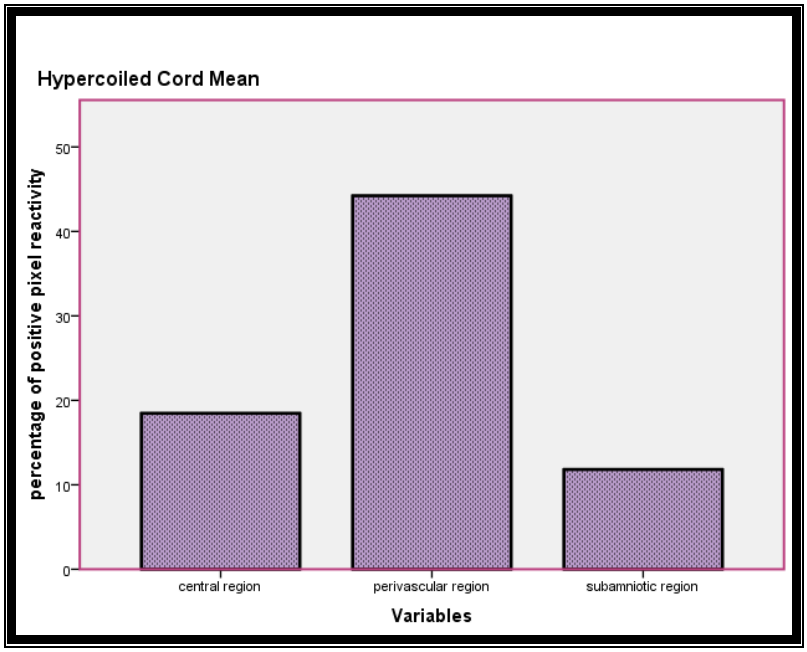

Figure 4. The mean value of the number of percentage positive pixel reactivity in the hypocoiled cords between the mean values of subamniotic, perivasclar, and central regions

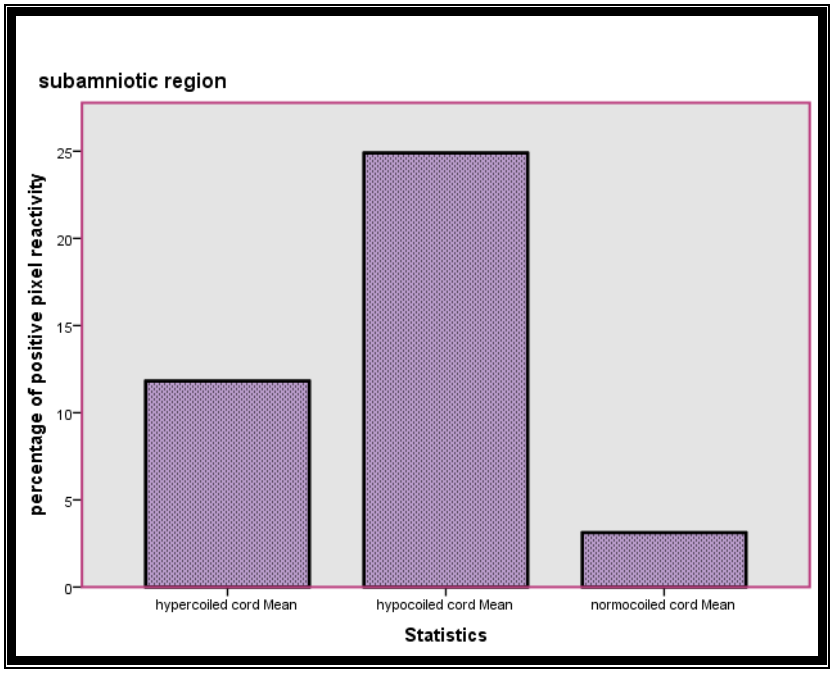

Figure 5. The mean values of percentage of positive pixel reactivity reactivity of the subamniotic region in hypocoiled, hypercoiled and normocoiled cords

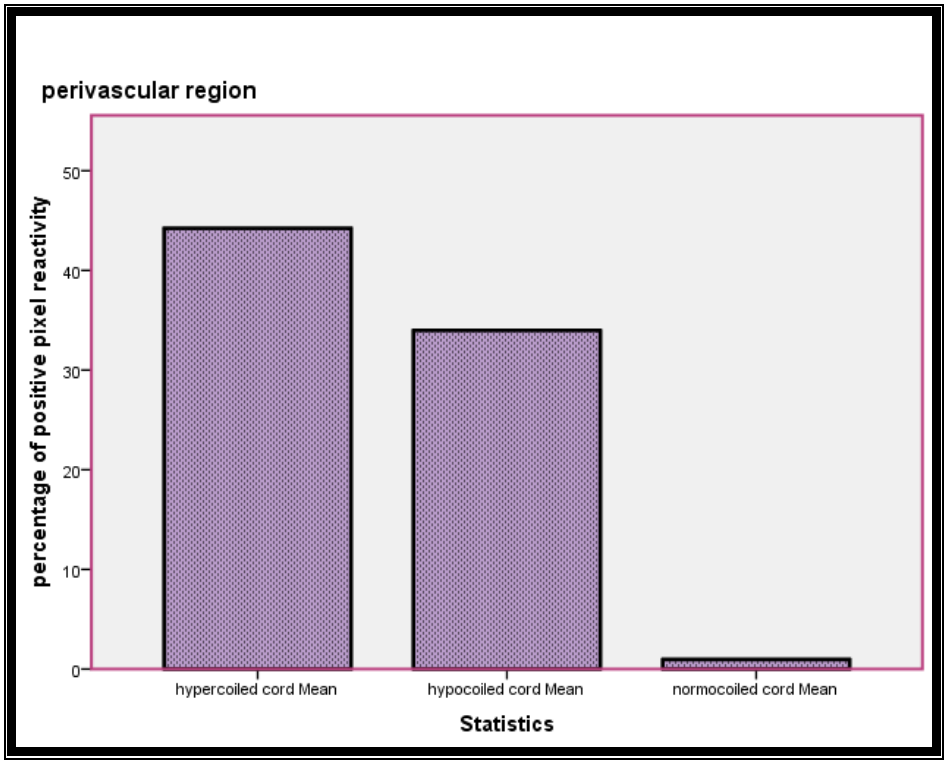

Figure 6. The mean values of percentage of positive pixel reactivity reactivity of the perivascular region in hypocoiled, hypercoiled and normocoiled cords

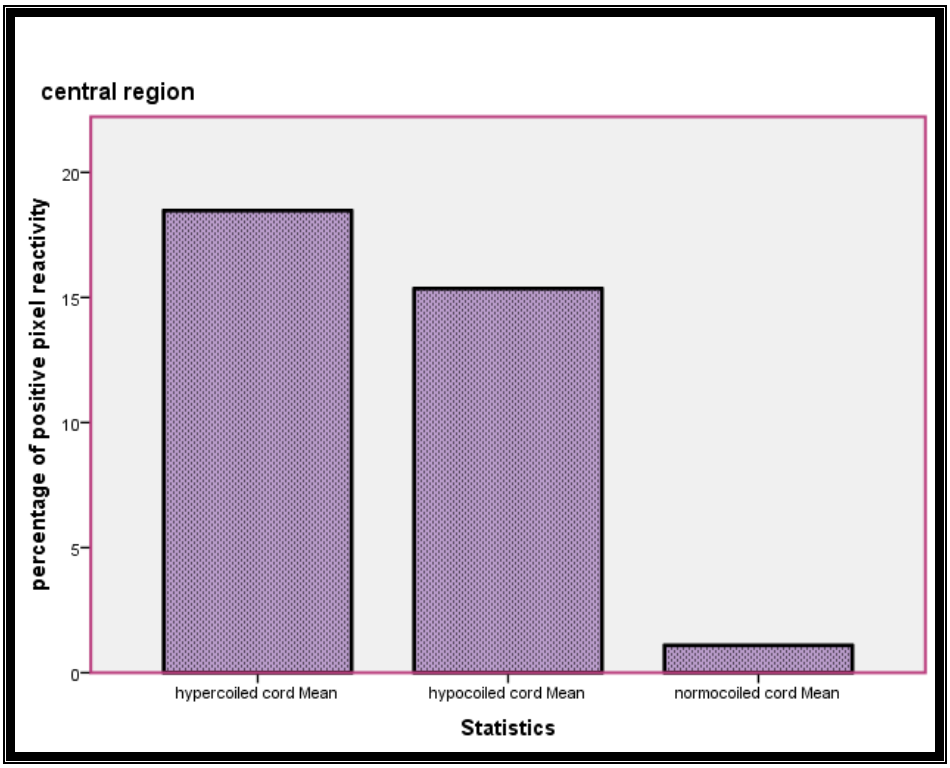

Figure 7. The mean values of percentage of positive pixel reactivity reactivity of the central region in hypocoiled, hypercoiled and normocoiled cords

In this study, normal fetal outcome with normal Apgar score was an inclusion criterion for the cord samples collected. The immune-histochemical matrix metalloproteinase-9 (MMP-9) reactivity demonstrated could be considered as an illumination of sequel of hypercoiling that is well tolerated by the fetus, the exaggeration of coiling could be associated with optimization of the sequel to the extent that disturb the umbilical circulation. This conclusion is supported by many reports documenting that (cord accident) including that of hypercoiling is dependent on the duration and degree of this circulatory obstruction, it may occur acutely or may be intermittent and causes chronic circulatory obstruction that may be associated with many intrapartum complications and adverse prenatal outcomes (as still stillbirth, intrauterine growth retardation, non-reassuring fetal heart tracing, low Apgar scores, and meconium staining) [15-18].

The Dynamic of Adverse Effect of Hypocoiling of Umbilical Cord:

It was concluded that the increased amount of hyaluronan in the umbilical cords of trisomy 21 fetuses would affect the morphology of the umbilical cord resulting in hypocoiling [19]. This conclusion was formulated depending on the fact that Wharton's jelly (a connective tissue responsible for the mechanical properties of umbilical cord) is composed of collagens, glycosaminoglycans, proteoglycans, and other microfilaments [20,21]. Hyaluronan is the major glycosaminoglycans in extracellular matrix of the umbilical cord; it inhibits angiogenesis and therefore has an influence on the growth of umbilical vessels and coiling formation resulting in hypocoiling [19].

The hypocoiled umbilical cord losses the advantage of coiling that make the cord as a semierectile organ which is more resistant to snarling torsion, stretch, and compression and consequently resulting in impair umbilical circulation [22,23].

The mesenchymal tissues in the hypocoiled cords are associated with much collagen accumulation; this massive collagenous buildup provided substantial mass effects that interfere with the normal coiling status.

The role of MMP-9 in the Mesenchyme of Abnormally Coiled Cords:

It is well known that the collagenolytic enzymes including matrix metalloprotenases MMPs are of vital importance in regulation of collagen degradation; MMPs degraded all components of the extracellular matrix and are involved in connective tissue remodeling [8,24]. Accordingly, the results of this study enlighten the cause-effect relationship of hypercoling and hypocoiling of the cord that could be associated with perinatal outcome. This relationship is affected by the counter effect of matrix metalloproteinase digestion of collagen and other ECM proteins resulting in reduction of the tissue masses around the umbilical vessels to relieve the stressful compressiom of umbilical circulation.

From the interpretation of discussing the dynamic of the adverse effect of hypercoiling and hypocoiling of the umbilical cord, it could be concluded that the forceful compression of the umbilical vessels circulation in hypocoiled and hypercoiled cord is produced by different mechanisms. During hypercoiling, mechanical compression plays the major role, while during hypocoiling, the increased extracellular matrix hayluronat in addition to accumulation of collagen and some other proteins in Wharton jelly produce massive increment of the connective tissues surrounding the umbilical vessels of hypocoiled cords resulting in their forceful compression.

The (MMP-9) immunohistochemical reactivity around the perivascular mesenchymal tissue was shown in this study to be significantly higher in hypercoiled and hypocoiled cords compared to normal cords. This result is supported by the establishment that MMP-9 is an enzyme synthesized by blood cells and blood vascular cells; it degradation ECM

components [25,26]. The MMP-9 breaks the ECM component around the umbilical vessels to relieve the compression of these vessels in both types of abnormal coiled cords. The limited effect of MMP-9 could secure satisfactory space around the umbilical vessels to a certain point beyond which obstruction of the umbilical circulation take place.

The increased MMP-9 immunohistochemical reactivity associated with abnormal cord coiling documented in this study indicates the high degradation of ECM components in the subamniotic mesenchyme. The increased MMP-9 activity reduces the subamnion mesenchyme to minimize the strain of the overlying amnion surrounding the cord. The marked increased subamnion MMP-9 reactivity in hypocoiled cords is a predictable outcome to minimize the massively increased mesenchyme resulting from the dual effect of increased hyalunate and collagen accumulation in hypocoiled cord.

Therefore, the perivascular immunohistochemical reactivity could indicate that the MMP-9 localization has a role degradation of the extra-tissue collagen and proteins to maintain a harmless perivascular pressure that preserve the normal vascular blood flow.

The results of this study do not contradict with the suggestion that reduced activity of collagen-degrading enzymes is a possible factor which enhances the accumulation of collagen and some other proteins in the pre-eclamptic umbilical cord tissues resulting in hypocoiling [27]. The pattern of MMP-9 immunohistochemical reactivity found in this study is a protective physiological response that preserves the healthy outcome of pregnancy resulting in a normal newborn with a normal Apgar score.

Acknowledgments

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

Authors contribution

Abdul Sattar: M.Sc. candidate performing the laboratory research work. Dr. Mobarak: The advisor of the M.Sc. Research performing the production and interpretation of the results.

Conflict of interest

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

Funding

The research working funding was provided by the authors.

References

1. Kashanian M, Akbarian A, Kouhpayehzadeh J. The umbilical coiling index and adverse perinatal outcome. Int J Obstetetric Gynecol. 2006; 95(1): 8-13. doi: 10.1016/j.ijgo.2006.05.029.
2. Chitra T, Sushanth YS, Raghavan S. Umbilical Coiling Index as a Marker of Perinatal Outcome An Analytical Study. Obstet Gynecol Int. 2012; 2012: 213689. doi: 10.1155/2012/213689.
3. de Laat MW, Franx A, Bots ML, et al. Umbilical Coiling Index in Normal and Complicated Pregnancies. Obstet. Gynecol., 2006; 107(5): 1049-55. doi: 10.1097/01.AOG.0000209197.84185.15.
4. Tahmasebi M. and Alighanbari R. Evaluation of umbilical cord thickness, cross-sectional area, and coiling index as predictors of pregnancy outcome. Indian J Radiol Imaging. 2011; 21(3): 195-8. doi: 10.4103/0971-3026.85367.
5. Ezimokhai M, Rizk DE, Thomas L. Abnormal vascular coiling of the umbilical cord in gestational diabetes mellitus. Arch Physiol Biochem. 2001; 109(3): 209-14. doi: 10.1076/apab.109.3.209.11593.
6. Ezimokhai M, Rizk DE, Thomas L. Maternal risk factors for abnormal vascular coiling of the umbilical cord. Am J Perinatol. 2000; 17(8): 441-6. doi: 10.1055/s-2000-13452.
7. Van Lint P, Libert C. chemokine and cytokine processing by matrix metalloproteinases and its effect on leukocyte migration and inflammation. J Leukoc Biol. 2007; 82(6): 1375-81. doi: 10.1189/jlb.0607338.
8. Woessner JF. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J. 1991; 5(8): 2145-54.
9. Galewska Z, Romanowicz L, Jaworski S. Matrix metalloproteinase-7 of the umbilical cord in preeclampsia. Arch Perinat Med. 2008; 14(2): 41-4.

10. Wang Y, Luo W, Reiser G. Trypsin and trypsin-like proteases in the brain: proteolysis and cellular functions. Cell Mol Life Sci. 2008; 65(2):237-52. doi: 10.1007/s00018-007-7288-3.
11. Mannello F, Tonti G, Papa S. Are matrix metalloproteinases the missing link. ISJ. 2005; 2(69): 69-74.
12. Nagase H, Woessner JF Jr. Matrix metalloproteinases. J Biol Chem. 1999; 274(31): 21491-4.
13. Ikechebelu J, Eleje G, Ofojebe C. True umbilical cord knot leading to fetal demise. Ann Med Health Sci Res. 2014; 4(Suppl 2): S155-8. doi: 10.4103/2141-9248.138044.
14. Baergen RN. Cord abnormalities, structural lesions, and cord accidents. Semin Diagn Pathol. 2007; 24(1): 23-32.
doi: http://dx.doi.org/10.1053/j.semdp.2007.02.004.
15. Collins JH. Umbilical Cord Accidents. BMC Pregnancy and Childbirth. 2012; 12(1): 1. doi: 10.1186/1471-2393-12-S1-A7.
16. Parast MM, Crum CP, Boyd TK. Placental histologic criteria for umbilical blood flow restriction in unexplained stillbirth. Hum Pathol. 2008; 39(6): 948-53. doi: 10.1016/j.humpath.2007.10.032.
17. Peng HQ, Levitin-Smith M, Rochelson B, et al. Umbilical cord stricture and overcoiling are common causes of fetal demise. Pediatr Dev Pathol. 2006; 9: 14-9. doi:10.2350/05-05-0051.1.
18. French AE, Gregg VH, Newberry Y, et al. Umbilical cord stricture: a cause of recurrent fetal death. Obstet Gynecol. 2005; 105: 1235-9. doi: 10.1097/01.AOG.0000159041.55845.
19. Phithakwatchara N, Phatihattakorn C. Specificity of hypocoiled umbilical cord in prediction of fetal trisomy 21 in the second trimester. Siriraj Med J. 2010; 62(4): 170-2.
20. Mauro AM, Buscemi M, Leone A, et al. Role of matrix metalloproteinases 2, 9 (MMP− 2, − 9), orphanin FQ and others peptides in vascular tone of human umbilical cordo at term. Dent Med Probl. 2008; 45(2): 111-20.
21. Sobolewski K, Bankowski E, Chyczewski L, et al. Collagen and glycosaminoglycans of Wharton's jelly. Biol Neonate. 1997; 71(1): 11-21. doi: 10.1097/01.AOG.0000159041.55845.
22. Wadhwa PD, Buss C, Entringer S, et al. Developmental origins of health and disease: brief history of the approach and current focus on epigenetic mechanisms. Sem Reproduct Med. 2009; 27(5): 358-68. doi: 10.1055/s-0029-1237424.
23. Bańkowski E, Pawlicka E, Jaworski S. Collagen biosynthesis in the wall of the umbilical cord artery and its alteration in EPH-gestosis. Pathophysiology. 1997; 4(1): 9-13. doi: http://dx.doi.org/10.1016/S0928-4680(96)00141-1.
24. Cox TR, Erler JT. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. Dis Models Mech. 2011; 4(2): 165-78. doi: 10.1242/dmm.004077.
25. Davis GE, Senger DR. Endothelial extracellular matrix biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circulation Res. 2005; 97(11): 1093-107. doi: 10.1161/01.RES.0000191547.64391.e3.
26. Daniels JT, Schultz GS, Blalock TD, et al. Mediation of transforming growth factor-β1-stimulated matrix contraction by fibroblasts: a role for connective tissue growth factor in contractile scarring. Am J Pathol. 2003; 163: 2043-52.
27. Romanowicz L, Galewska Z. Extracellular matrix remodeling of the umbilical cord in pre-eclampsia as a risk factor for fetal hypertension. J Pregnancy. 2011; 69(4): 212-8. doi: 10.1155/2011/542695.

Correspondence to Hiba A. Abdul Sattar
E-mail: hibaahmad455@gmail.com
Received Sep. 29th 2016
Accepted Feb. 12th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

The Role of Collagen Binding Assay in Classification of von Willebrand Disease

Suha K. Jabbar[1] *MSc*, **Subh S. Al-Mudallal**[1] *FICMS*, **Zeyad A. Shabeeb**[2] *PhD*, **Yusra G. Al-Obaidy**[2] *MSc*, **Hind S. Al-Mamoori**[1] *FICMS*

[1]Dept. of Pathology and Forensic Medicine, College of Medicine, Al-Nahrain University, Baghdad, Iraq, [3]Dept. of Immunology, National Center of Hematology, Baghdad, Iraq

Abstract

Background von Willebrand disease (VWD) is the most common, genetically, hereditary and clinically heterogeneous bleeding disorder caused by qualitative or quantitative defects in von Willebrand factor (VWF) that leads to imperfect VWF interaction between platelets and injured blood vessel wall which deteriorate primary hemostasis.

Objective To assess the role of collagen binding assay in the classification of VMD.

Methods A cross sectional study was conducted on 52 suspected known patients with VWD with no consideration to their age or gender who attended the National Center of Hematology. They were submitted to von Willebrand factor antigen (VWF:Ag), factor VIII (FVIII), ristocetin co factor assay (VWF:RCo), ristocetin induced platelet aggregation (RIPA), and collagen binding assay (VWF:CB) at the same center.

Results The patients mean age was 14.42 ±1.64 years, median 10 years, and range of 1-40 years. More than half of cases below 10 years with M:F ratio 1:1.26. Two out of 9 cases of subtype I was diagnosed as subtype II, 4 out of 7 cases of subtype II sub classified as subtype2M and the rest 3 cases sub classified as 2A, 11 out of 36 of subtype III diagnosed as 8 cases for subtype I, and 3 cases for subtype II.

Conclusion VWF:CB assay is an important and effective supplementary test, in addition to three test panel FVIII, VWF:Ag, VWF:RCo, and multimere analysis, for sub classification of VWD. VWF:CB assay has a role in reclassified VWD patients with highly variable clinical presentation and laboratory values. Type III VWD is most frequent diagnosed type among symptomatic patients due to high consanguinity rate which detected earlier with severe bleeding tendency.

Keywords Von Willebrand disease, collagen binding assay, VWF:RCo.

Citation Suha K. Jabbar, Subh S. Al-Mudallal, Zeyad A. Shabeeb, Yusra G. Al-Obaidy, Hind S. Al-Mamoori. The role of collagen binding assay in classification of von Willebrand disease. Iraqi JMS. 2017; Vol. 15(2): 175-180. doi: 10.22578/IJMS.15.2.10

List of abbreviation: FVIII = Factor VIII, RIPA = Ristocetin induced platelet aggregation, VWD = von Willebrand disease, VWF = von Willebrand factor, VWF:Ag = VWF antigen, VWF:CB = VWF collagen binding assay, VWF:RCo = VWF ristocetin co factor assay

Introduction

Von Willebrand disease (VWD) is the most common, genetically and clinically heterogeneous bleeding disorder caused by qualitative or quantitative defects in VWF that leads to imperfect von Willebrand factor (VWF) interaction between platelets and injured blood vessel wall which deteriorate primary hemostasis [1,2].

VWF is produced in vascular endothelium and megakaryocytes. It acts as an adhesive protein,

which binds to several ligands that are essential for the hemostatic process. VWF stimulate platelet adhesion to the sub endothelium to make platelet aggregation support and bind to factor VIII (FVIII) to avoid its premature degradation [3,4].

Incidence of VWD in all developing countries recognized subtype III VWD as the most common subtype then subtype I and II. About 60-70% cases of subtype III VWD are associated with consanguineous marriages of parents [5].

There are three main subtypes of VWD; subtype I and III exist with quantitative VWF deficiency while subtype II presents with qualitative VWF deficiency that subdivide into four variants 2A, 2B, 2M, and 2N. The determination of the exact subtype is important in treatment and prognosis [6].

The diagnosis of VWD depend on the history of mucocutaneous bleeding, a family history of bleeding, and a laboratory evaluation, which include VWF antigen (VWF:Ag), FVIII, ristocetin co factor assay (VWF:RCo), = VWF collagen binding assay (VWF:CB) and ristocetin induced platelet aggregation (RIPA) [7].

This study obejectives was to assess the role of collagen binding assay in the classification of VMD.

Methods

Patients

A cross sectional study was conducted on 52 patients who attended the National Center of Hematology collected over a period of 10 months from December 2015 till September 2016.

The VWF:Ag, FVIII, VWF:RCo, and RIPA results were done at the National Center of Hematology for 28 newly diagnosed cases, while 24 cases obtained from patients files with no consideration to their age and gender.

The FVIII, VWF:RCo, and RIPA results were obtained from the patient files at the National Center of Hematology that diagnosed VWD by depending on VWF:RCo/Ag ratio. When VWF:RCo/Ag ratio ≥ 0.6 diagnosed as subtype I and <0.6 diagnosed as subtype II, while subtype III exhibited markedly reduced FVIII and VWF:Ag, and absent VWF:RCo8. The VWF:CB assay and VWF Ag were done at the National Center of Hematology.

Materials

VWF:Ag and VWF:CB were assessed by Asserachrom kit (Diagnostica Stago/ France) via sandwich ELISA maneuver by Reader device, 3.5 ml of venous blood was collected from VWD patients in plastic tube containing 9:1 ratio of blood to 3.2% trisodium citrate anticoagulant. Pediatric volume of 2.5 ml in appropriate ratio provided that the blood to anticoagulant then put this sample in the centrifuge to separate the plasma and subject for the test [8].

Statistical analysis

The statistical analysis of this prospective study performed with the statistical package for social sciences (SPSS) 21.0 and Microsoft Excel 2013. Numerical data were described as mean and standard error. Analysis of variance (ANOVA) used for comparison among three groups. While, categorical data described as count and percentage, Chi-square test used to estimate the association between variables. The lower level of accepted statistical significant difference is below to 0.05.

Results

The study was performed on 52 patients with mean age of 14.42±1.64 years, median 10 years, and range of 1-40 years. More than half of cases below 10 years with M:F ratio 1:1.26. About 70% of cases were of type III VWD.

Forty five patients presented with 2nd degree consanguineous marriage of their parents. By adapting the results of FVIII, VWF:RCo, and RIPA obtained from the patients files at the National Center of Hematology, 9 cases were subtype I, 7 cases were subtype II, and 36 cases were subtype III (Table 1). After applying collagen binding assay and VWF:Ag results to those parameters, the classification of the patients as shown in (Table 2).

Table 1. VWF Ag, FVIII, VWF:RCo and PTT in VWD types at presentation

Classification at presentation	N	VWF:Ag (IU/dl)	FVIII (IU/dl)	VWF:RCo (IU/dl)	VWF:RCo/Ag (IU/dl)
Subtype I	9	26.40±4.87	51.43±11.48	25.21±3.89	2.20±1.29
Subtype II	7	21.89±4.50	44.33±7.32	6.47±0.97	0.32±0.05
Subtype III	36	6.75±1.26	16.93±5.08	0.00±0.00	0.00±0.00
P value		<0.001**	0.010*	<0.001**	<0.001**

*Statistically significant, ** High statistically significant (P<0.001)

Table 2 VWF Ag, FVIII, VWF:RCo and VWF:CB in VWD types in reviewed data

Reviewed data	N	VWF:Ag (IU/dl)	FVIII (IU/dl)	VWF:RCo (IU/dl)	VWF:RCo /Ag	VWF:CB (IU/dl)	VWF:Ag (IU/dl)	VWF:CB/ Ag
Subtype I	15	20.04 ±3.06	46.97 ±8.77	15.42 ±4.99	1.20 ±0.65	20.11 ±3.12	17.66 ±2.67	1.38 ±0.15
Subtype II	12	22.42 ±2.77	47.75 ±6.55	7.12 ±2.04	0.30 ±0.07	11.98 ±1.64	21.44 ±2.69	0.61 ±0.096
Subtype III	25	3.39 ±0.40	7.40 ±4.95	0.00 ±0.00	0.00 ±0.00	3.18 ±0.12	3.3 ±0.32	1.29 ±0.22
P value		<0.001**	<0.001**	<0.001**	0.055 NS	<0.001**	<0.001**	0.050 NS

NS: Not statistically significant (p>0.05), ** High statistically significant (P<0.001)

Some cases of VWD had change in their classification by using VWF:CB assay as well as to VWF:Ag, FVIII, and VWF:RCo. Two out of 9 cases of subtype I was diagnosed as subtype II, 4 out of 7 cases of subtype II sub classified as subtype2M and the rest 3 cases subclassified as 2A, 11 out of 36 of subtype III diagnosed as 8 cases for subtype I, and 3 cases for subtype II (Table 3 and 4).

Discussion

In the current study, the patient age ranged between 1-40 years and they were grouped according to 10 years interval, more than half of cases (55.77%) were ≤ 10 years of age, and the least percentage of cases were in the third decade of life. This due to that subtype III, which characterizes by the severe bleeding tendency due to markedly reduced of VWF and FVIII levels; (<10% for both) [9,10], which was the commonest subtype.

That result was similar to a study done by Sanders et al. [11], stating that subtype III VWD will be presented early in life.

Table 3. VWF:Ag, FVIII, PTT, VWF:RCo and VWF:CB, of VWD patients who had re-classification

Changed data	N	VWF:Ag7 (IU/dl)	FVIII (IU/dl)	VWF:RCo (IU/dl)	VWF:RCo/Ag	VWF:CB (IU/dl)	VWF:Ag (IU/dl)	VWF:CB /Ag	PTT (Sec)
Subtype I	2	29.45 ±5.65	42.50 ±17.5	20.35 ±2.65	0.68 ±0.03	12.46 ±4.74	29.45 ±5.65	0.41 ±0.09	33 ±6
Subtype III	11	15.86 ±2.39	38.59 ±9.70	0.00 ±0	0.00 ±0	12.02 ±1.16	13.64 ±1.71	1.06 ±0.17	36.55 ±1.15
P value		0.388 NS	0.119 NS	0.844 NS	0.424 NS	0.438 NS	0.439 NS	0.399 NS	0.061 NS

NS: Not statistically significant (p>0.05)

Table 4 VWF:Ag, FVIII, PTT, VWF:RCo and VWF:CB, of subtype II VWD patients who had changed their sub classification

Subtype II	N	VWF:Ag (IU/dl)	FVIII (IU/dl)	VWF:RCo (IU/dl)	VWF:RCo/Ag	VWF:CB (IU/dl)	VWF:Ag (IU/dl)	VWF:CB/Ag
2M	4	20.60 ±1.98	53.0 ±7.68	6.30 ±1.04	0.31 ±0.06	18.15 ±1.64	19.53 ±2.63	0.97 ±0.15
2A	3	22.98 ±9.60	38.7 ±11.70	6.53 ±1.58	0.34 ±0.07	7.10 ±1.19	23.10 ±9.51	0.40 ±0.12
2A or 2B	5	23.54 ±4.42	49.0 ±13.70	20.35 ±2.65	0.68 ±0.3	9.96 ±1.89	21.98 ±3.91	0.44 ±0.06
P value		0.003*	0.008*	0.232 NS	0.018*	0.138 NS	0.006*	0.029*

NS: Not statistically significant (p>0.05), * statistically significant

In agreement with the present study, an Iranian [12], Turkish [13], and Indian studies [14] found that subtype III was the commonest subtype whereas a Canadian [10] and European [9,11,15] studies found that subtype III was the latest frequent subtype. This controversy result due to high rate of consanguinity in Middle East and India compared to Western countries; which has an impact mainly on subtype III, since it is inherited as autosomal recessive trait [11,14] as well as subtype III VWD has severe symptoms and required medical consultation while subtype I is subclinical case.

After collagen binding assay and VWF:Ag were applied, there was change in the classification of VWD subtypes. Two out of 9 cases of subtype I was diagnosed as subtype II, 4 out of 7 cases of subtype II sub classified as subtype2M and the rest 3 cases sub classified as 2A, 11 out of 36 of subtype III diagnosed as 8 cases for subtype I, and 3 cases for subtype II.

These results were parallel to an American [16] and Australian [17] studies, which stated that a high background diagnostic error rate was identified in laboratories that performed the standard three test panel FVIII, VWF:Ag, and VWF:RCo; and after the application of collagen binding test, there was nearly 20% error rate for type I VWD misidentified as type II VWD, nearly 30% for type II VWD misidentified as type I VWD, and an error rate of 90% in type III misidentified as type I and type II VWD [17].

During current research, it was observed that most of changed cases presented with unexpected findings regarding age of presentation, history of bleeding tendency, and FVIII at presentation.

For example, young female presented with mild bleeding tendency at 12 years old, the result of her investigation as follow; VWF:Ag was 29, FVIII was 65, and VWF:RCo was absent that diagnosed as subtype III VWD. When applied collagen binding assay the diagnosis is sub classified to type I.

Second case, female patient presented with mild bleeding tendency at 9 years old, found that VWF:Ag was 31, FVIII was 10, and VWF:RCo was absent also diagnosed as subtype III. When applied collagen binding assay the diagnosis is sub classified to type II.

Third example, one year male infant presented with severe bleeding tendency, the VWF:Ag was 35, FVIII was 45, and VWF:RCo was 23 diagnosed as subtype I. When applied collagen binding assay the diagnosis is sub classified as type II.

There are multiple studies worldwide on VWD that considered VWF:CB assay as an effective test, in addition to the three test panel FVIII, VWF:Ag, and VWF:RCo, in classification of VWD [16,18,19].

Although VWF:RCo test display poor assay reproducibility, poor low VWF level sensitivity, high relative intraassay and interassay variability, and time consuming; however, it is

widely applied for the diagnosis of VWD and sub classification particularly in the VWF dysfunction of type 2 VWD such as types 2A , 2B, and 2M [20].

As conclusions; the collagen binding assay cannot replace the ristocetin co factor assay per se but collagen binding assay should be added to the test panel to reduce the classification errors, if the results of VWF:RCo values were highly variable among different center, collagen binding should replace the VWF:RCo in order to classify VWD.

Acknowledgments

Special thanks to all staff members of National Center of Hematology for their valuable help and advice.

Author contributions:

Dr. Jabbar collected the samples, organized the data, performed ELISA test and analyzed the results. Dr. Al-Mudallal helped in study design and supervising the work. Dr. Shabeeb helped in ELISA technique working. Dr. Al-Obaidy helped in the samples collection and performed VWF:RCo and RIPA tests. Dr. Al-Mamoori helped in progress of this study and manuscript organization and editing.

Conflict of interest

The authors have no conflicts of interest.

Funding

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

References

1. Ng C, Motto DG, Di Paola J. Diagnostic approach to von Willebrand disease. Blood J. 2015; 125(13): 2029-38. doi: 10.1182/blood-2014-08-528398.
2. De Jong A, and Eikenboom J. Developments in the diagnostic procedures for von Willebrand disease. J Thromb Haemost. 2016; 14(3): 449-60. doi: 10.1111/jth.13243.
3. De Meyer SF, Deckmyn H, Vanhoorelbeke K. von Willebrand factor to the rescue. Blood. 2009; 113(21): 5049-57. doi: 10.1182/blood-2008-10-165621.
4. Sadler JE, Mannucci PM, Mazurier C, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand Factor. J Thromb Haemost. 2006; 4(10): 2103-14. doi: 10.1111/j.1538-7836.2006.02146.x.
5. Ghosh K, Shetty S. Epidemiology, diagnosis, and management of von Willebrand disease in India. Semin Thromb Hemost. 2011; 37(5): 595-601. doi: 10.1055/s-0031-1281048.
6. National Heart, Lung and Blood Institute. The diagnosis, evaluation, and management of von Willebrand disease. NIC Publications, 2007. https://www.nhlbi.nih.gov/files/docs/guidelines/vwd.pdf. Accessed at 2016-7-21.
7. Lillicrap D, James P. von Willebrand disease : an introduction for the primary care physician. World Fed Hemoph. 2009; 47: 1-7.
8. Cleveland Clinic Laboratories. Collagen binding activity assay for von Willebrand disease. http://clevelandcliniclabs.com/wp-content/assets/pdfs/technical-briefs/collagen-87899.pdf. Accessed at 2016-5-3
9. Abshire TC, Federici AB, Alvárez MT, et al. Prophylaxis in severe forms of von Willebrand's disease: results from the von Willebrand Disease Prophylaxis Network (VWD PN). Haemophilia. 2013; 19(1): 76-81. doi: 10.1111/j.1365-2516.2012.02916.x.
10. James PD, Lillicrap D. von Willebrand disease: clinical and laboratory lessons learned from the large von Willebrand disease studies. Am J Hematol. 2012; 87(Suppl 1): S4-11. doi: 10.1002/ajh.23142.
11. Sanders YV, Fijnvandraat K, Boender J, et al. Bleeding spectrum in children with moderate or severe von Willebrand disease: Relevance of pediatric-specific bleeding. Am J Hematol. 2015; 90(12): 1142-8. doi: 10.1002/ajh.24195.
12. Lak M, Peyvandi F, Mannucci PM. Clinical manifestations and complications of childbirth and replacement therapy in 385 Iranian patients with type 3 von Willebrand disease. Br J Haematol. 2008; 111(4): 1236-9. doi: 10.1111/j.1365-2141.2000.02507.x.
13. Hampshire DJ, Abuzenadah AM, Cartwright A, et al. Identification and characterisation of mutations associated with von Willebrand disease in a Turkish patient cohort. Thromb Haemost. 2013; 110(2): 264-74. doi: 10.1160/TH13-02-0135.
14. Trasi S, Shetty S, Ghosh K, et al. Prevalence and spectrum of von Willebrand disease from western India. Indian J Med Res. 2005; 121(5): 653-8.
15. Sanders Y, Giezenaar M a, Laros-van Gorkom B, et al. von Willebrand disease and aging: an evolving phenotype. J Thromb Haemost. 2014; 12(7): 1066-75. doi: 10.1111/jth.12586.
16. Adcock D, Bethel M, Valcour A. Diagnosing von Willebrand Disease: A Large Reference Laboratory's Perspective. Semin Thromb Hemost. 2006; 32(5): 472-9. doi: 10.1055/s-2006-947860.
17. Favaloro E, Bonar R, Kershaw G, et al. Reducing errors in identification of von Willebrand disease: the experience of the royal college of pathologists of Australasia quality assurance program. Semin

Thromb Hemost. 2006; 32(5): 505-13. doi: 10.1055/s-2006-947865.

18. Favaloro E. Toward a new paradigm for the identification and functional characterization of von Willebrand disease. Semin Thromb Hemost. 2009; 35(1): 60-75. doi: 10.1055/s-0029-1214149.

19. Federici AB, Canciani MT, Forza I, et al. Ristocetin cofactor and collagen binding activities normalized to antigen levels for a rapid diagnosis of type 2 von Willebrand disease-single center comparison of four different assays. Thromb Haemost. 2000; 84(6): 1127-8.

20. Favaloro E. An update on the von Willebrand factor collagen binding assay: 21 Years of age and beyond adolescence but not yet a mature adult. Semin Thromb Hemost. 2007; 33(8): 727-44. doi: 10.1055/s-2007-1000364.

Correspondence to Dr. Suha K. Jabbar
E-mail: suhakreem2@gmail.com
Received Nov. 28th 2016
Accepted Mar. 22nd 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

Inter sphincteric Botulinum Toxin A Injection for the Management of Chronic Anal Fissure

Nashwan Q. Mahgoob[1] *FRCMS,* **Muhammed M. kamal**[2] *PhD, MRCS*

[1]Dept. of Surgery, College of Medicine, Mosul University, [2]Dept. of Surgery, Hawler Medical University

Abstract

Background Surgical sphincterotomy (SS) has been the most commonly used treatment for chronic anal fissure (CAF). Although effective, it is associated with gas and or fecal incontinence in (0-20%) inter-sphincteric Botulinum Toxin A (BTXA) injection is a non-surgical technique that may be used as an option under certain circumstances for treatment of such condition.

Objective To verify the effectiveness of the BTXA injection in relieving symptoms and healing of chronic anal fissures.

Methods Thirty patients with CAF were treated by BTXA 1 U/kg injected into the inter-sphincteric plane; as an outpatient procedure, patients were re-evaluated after 1 week, and then every 2 weeks until the fissure healed or surgery was required. The patients were followed up for one year by regular attachment through phone call or visit, to evaluate the effects of treatment. Results, complications and follow up were recorded.

Results In 25 patients (83%), the pain was disappeared after the first week; 20 patients (66%) presented with a complete healing of the fissure in a period ranging between 1 to 3 months. Gas incontinence was reported in 2 patients (6%) and solved spontaneously. No major complications were found, in 3 cases (10%) surgery was needed later on.

Conclusion Since it avoids the greater risk of incontinence associated with SS, and it can be done as outpatient procedure without admission or general anesthesia. We recommend the use of BTXA as the first therapeutic approach for patients with chronic anal fissure.

Keywords Botulinum Toxin A, anal fissure.

Citation Nashwan Q. Mahgoob, Muhammed M. kamal. Inter sphincteric botulinum toxin A injection for the management of chronic anal fissure. Iraqi JMS. 2017; Vol. 15(2): 181-187. doi: 10.22578/IJMS.15.2.11

List of abbreviations: BTXA = Botulenim toxin A, CAF = Chronic anal fissure, LIS = Lateral internal sphincterotomy, NIPC = National Initiative on Pain Control, SS = Surgical sphincterotomy

Introduction

An anal fissure is a common problem that causes substantial morbidity in people who are otherwise healthy. It is one of the most common proctological diseases, which manifests as pain and rectal bleeding on defecation [(1)]. Chronic anal fissure (CAF) is associated with persistent hypertonia and fibroses of the internal anal sphincter. Manometric evidence of sphincter spasm reflects elevated resting anal pressure [(2,3)], which is responsible for its chronicity, thus, conservative therapy alone rarely suffices. Surgical sphincterotomy (SS) is highly effective in providing good symptomatic relief and healing. However, in up to 16% of cases, it can be associated with significant complications, such as fecal incontinence [(4,5)], as well as, it is a relatively invasive procedure requiring hospital

admission and a general anesthesia. In order to reduce the risk of fecal incontinence, alternative non-surgical approaches have been described in the international literatures during the last three decades to decrease the spasm in the internal anal sphincter, thereby allowing the fissure to heal with less complications and morbidity [(6)]. Jost and Schimrigk reported for the first time the treatment of anal fissures with Botulinum Toxin A (BTXA) in 1993 [(7)]. Pharmacological treatments such as BTXA produce a reversible reduction in sphincter pressure and have become the first line of treatment for CAF due to their lack of permanent complications [(8-10)].

The objective of this study is to evaluate the effectiveness using BTXA technique in healing rate and to describe its side effects in the management of CAF.

Methods

The study included 30 patients who have presented to authors private clinic with CAF between August 2014 to September 2015; the diagnosis was on clinical bases. The procedure was discussed thoroughly with the patients regarding its effectiveness and side effects; a formal consent was signed by them.

Patients selection

The patients in whom the injection method was applied have to fulfill the following criteria:

1- Single posterior or anterior fissure.
2- No previous anal surgery.
3- No underling pathology for the fissure.
4- No other anal condition.
5- Patient acceptance for the treatment.

Procedure

The patients were injected with BTXA (Botox, Allergen100U). A 1ml insulin syringe with 26 G and 0.8 mm length needle was used for injection. The content of the vial was diluted in 1 ml isotonic saline and a dose of 1 U/kg (minimum of 50 U and maximum of 100 U, each 0.1 ml contain 10 U) was injected at 9 o'clock (lithotomy position) in the inter sphincteric plane while the patient laying on his/her left side (figure 1). The site of injection determined by putting the left index finger at the anus and the thumb at inter sphincteric plane, the needle introduced for its full length and the drug injected slowly by using the right hand. No sedation was used but local EMLA cream (lidocaine 2.5% and prilocaine 2.5%) was applied to the anal verge 10 minutes before the procedure. The patients were followed up after one week and then every 2 weeks until the fissure healed or surgery was required. The patients were followed up for one year by regular attachment through phone call or on need visit, to evaluate the effects of treatment. No special advices wear given to patients. The patients' records were reviewed for improvement, healing, occurrence of side effects, surgery, as well as recurrence of symptoms.

Results

The study composed of 30 patients (20 males, 10 females; mean age (SD) of 33.3 (8.3) years), the pain threshold was assisted by using Numeric Pain Rating Scale by The National Initiative on Pain Control (NIPC). From total of 30 patients, 25 patients (83%) were pain free after the first week of injection; 4 patients (14%) experienced less pain in comparison to pre-injection time but still have pain especially after defecation, one patient (3%) showed no improvement regarding the pain. Bleeding was the next main presentation, which was found in 22 patients; it was disappeared from 17 patients (77%) after 1 week and from other 3 patients (13%) within two weeks, but continues in 2 patients (9%) after one month of injection. Twenty patients (66%) presented a complete healing of the fissure in a period ranging between 1 to 3 months; other 6 patients (20%) had healing within 6 months; 4 patients failed to show healing 6 months after injection (13%) and 3 of them (10%) underwent surgery, while one patient failed to be followed. Gas incontinence was reported in 2 patients (6%) and solved spontaneously within 2 weeks. No major complications were found. All patients remained continent for stool after 1 year. From 26 patients who underwent complete healing

in the first 6 months, 2 patients (7%) had recurrence of the fissure within 6 months post BTXA injection the patients follow up is shown in table (1).

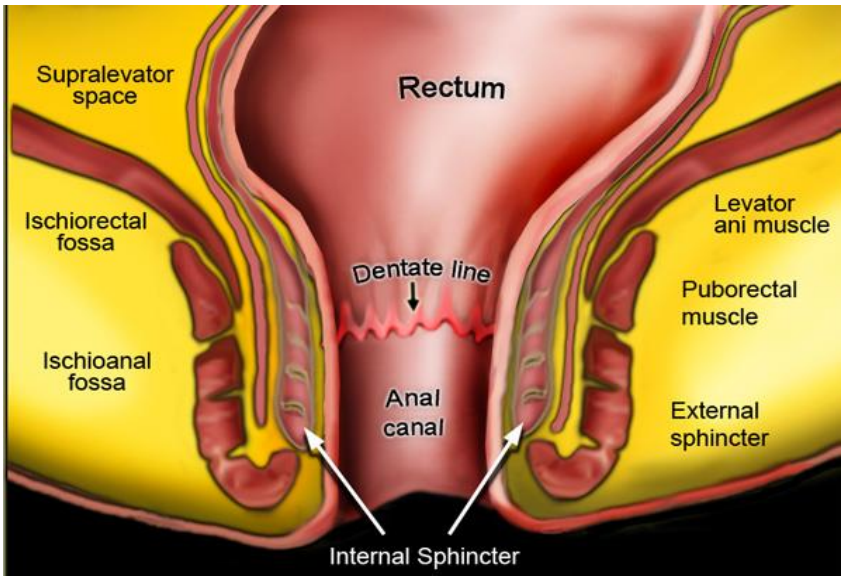

Figure 1. Site of Botulinum Toxin A injection. Picture by Henry Vandyke Carter [Public domain], via Wikimedia Commons

Table 1. Follow up of patients

Follow up	Number and percentage
Persistent post-injection pain	1 (3%) from 30 patients
Persistent post-injection bleeding	2 (9%) from 22 patients
Gas incontinence	2 (6%) from 30 patients
Fecal incontinence	0% from 30 patients
Failure of healing	4 (13%) from 30 patients
Recurrence of fissure	2 (7%) from 26 patients
Surgery needed	3 (10%) from 30 patients

Discussion

Anal fissure was first described by Recamier in 1829 who recommended stretching the anal sphincter to treat this condition [11]. Anal fissure affects all age groups, particularly young adults. Ninety percent of all fissures occur posteriorly and 10 percent anteriorly. Less than 1 percent of patients have both anterior and posterior fissures [12]. The etiopathogenesis of CAF is not well understood. There is increased intra-anal pressure at rest that might contribute to an ischemic state of the anal sphincter muscles. Indeed, the anodermal blood flow of the posterior midline has been shown to be reduced [13] and once wound happened by trauma, which is mostly caused by passing of hard stool, a devil's circle of spasm, pain, constipation and re-wounding develops [14,15]. The conservative treatment involves using appropriate nutrition, fluid intake, laxative and pain killer. Such treatment is easy, cheap, safe and remains the basis for all other treatments as well, but rarely suffices alone in CAF. Surgical sphincterotomy is the standard surgical management of CAF. However, it can be associated with significant

complications, such as fecal incontinence [5,6], which may occur many years after sphincter damage by anal surgery because sphincter tone decreases by age [16]. For that reason, incontinence after SS has probably been underestimated [17].

A Cochrane review [18], including three trials on 235 patients, showed that lateral internal sphincterotomy (LIS) compared to BTXA had superior healing rates (94% versus 67%). But BTXA caused no incontinence compared to LIS (0% versus 11%) in two trials including 191 patients [18]. Same results through a meta-analysis, Sajid *et al*. compared surgical vs chemical sphincterotomy using BTXA for the treatment of CAF found that SS had a significantly higher healing rate [P < 0.011] and a significantly lower recurrence rate [P < 0.0221] than BTXA. However, there was a higher complication rate [P < 0.0163] and a higher risk of transient fecal incontinence [P < 0.0001] in the sphincterotomy group than in the BTXA group [19].

The mechanism of action of BTXA is probably by reduction in squeeze pressure and resting pressure of the internal sphincter, these effects should be related to the fibrosis of the internal anal sphincter that is more prominent in the site of the fissure than other sites in the smooth muscle, BTXA blocks sympathetic and parasympathetic nerve function and myognic tone of the internal anal sphincter and the acetylcholine receptor at external sphincter (chemical sphincterotomy), (Acetylcholine is a neurotransmitter at somatic neuromuscular junctions, the parasympathetic nervous system and sympathetic preganglionic fibers (cholinergic fibers), eliminating the sphincter hypertonia resulting in increase in the local tissue perfusion and healing of the CAF [20,21]. Muscle paralysis occurs within hours and the effect remains for 3-4 months. This prolonged effect allows the fissure to heal. This effect is reversible because it is followed by axonal regeneration and formation of new nerve endings which avoids the risk of permanent injury to the sphincter [22,23]. Sphincter manometry after BTXA injection has demonstrated a lowering of resting internal pressure [24]. On the other hand, BTXA has a direct analgesic effect causing relief of pain before the healing of the fissure [25-27]. BTXA has also a good safety profile and tolerability [28].

BTXA is either injected directly into the internal anal sphincter muscle to promote anal sphincter relaxation and subsequent healing or into both the internal as well as the external sphincter muscles [29] since both sphincters play a role in the pathogeneses of CAF. Maria *et al*. demonstrated that a peri fissural injection in the posterior-median localization is less effective than an injection along the anterior midline. They observed an increased relaxation effect by the latter technique [30]. Huang *et al*. [31] by using three-dimensional trans-perineal sonographic evaluation of the anal sphincter complex and Starck *et al*. by using endosonography [32] showed that, the internal anal sphincter started about 6 mm from the anal verge, at the junction between subcutaneous and the superficial part of the external anal sphincter and ended at the ano-rectal junction and it possess the same thickness throughout its length (0.19 ± 0.09 to 0.21 ± 0.75 cm), while the external sphincter is thicker at its superficial part and more thicker at 3 and 9 o'clock (0.40 ± 0.10 cm and 0.39 ± 0.11 cm respectively). Tacking in consideration that BTXA shows a three-dimensional diffusion of about 2 cm, which is considered adequate to reach both the internal and external anal sphincter [33], we choose the inter sphincter plane for injection to cover both external and internal sphincter, and to have the injection at the thickest part of the sphincter complex.

Different dosage schedules have been used for BTXA injection in the treatment of CAF. Most investors use higher dosages as the response rate is higher and the relapse rate is lower. Up to 100 U BTXA have been used without any severe adverse effects [33]. The median lethal dose (LD50) of BTXA is estimated to be 3000 U for a 70-kg male [23]. We think that a dose of 1

U per/kg is safe and effective and reflect the slandered dose through all other references. The current recommendation of the American Society of Colon and Rectal Surgeons states that BTXA injection may be used for CAF that fail to respond to conservative measures [34] (evidence level II, recommendation B). However, there was no consensus on dosage, precise site of administration (internal anal sphincter, external anal sphincter, or intersphincteric space) and number of injections. There were large variations between studies in reported fissure healing and recurrence rates with BTXA) injection. This may be partly because of differences in the specific toxin brand used, injection site, unit dose, volume of solution injected, and most importantly, length of follow-up used to assess fissure outcomes.

Radwan *et al*. study showed that treatment with BTXA was effective in 89% of patients (from 38 patients) with chronic uncomplicated anal fissure. Two patients' experienced minor incontinence in the form of a fecal soiling which disappeared later [35]. Others reported transient incontinence for air between 6 to 10% and for stool in up to 5% of patients [36-39]. Recurrence rates vary between 10 and 55% [40]. In this study, gas incontinence was reported in 2 patients (6%) and solved spontaneously within 2 weeks. No major complications were found. All our patients remained continent for stool after 1 year.

Abscess formation is very rare, whereas this adverse effect is seen in about 13% of SS patients [41]; we did not face this complication in our series. In Cutan study, the healing rates (≤ 6 months) are 60-90% and partial responses are seen in many patients. However, non-responders are rare (< 5%) resulting mostly due to incorrect technique and/or diagnosis [29]. Menteş *et al*. [5] showed complete healing after single injection in 45 of the 61 patients (73.8 %) at the second month. After therapy with BTXA, higher recurrence rates are expected, because the sphincter tone is only temporarily reduced. However, we and others have shown that relapse rates after BTXA injection was very low [36-42]. However, in March 2013, the Medicines and Healthcare Products Regulatory Agency advised that all patients receiving any product containing BTXA should be warned of the signs and symptoms of toxin spread, such as muscle weakness and breathing difficulties. They should be advised to seek medical attention immediately if they experience breathing difficulties, choking, or any new or worsening swallowing difficulties, as such side effects may be life-threatening. We included this warning in our consent form.

Regarding the cost, its worthy to said that 100 U of BTX A (Botox, Allergen) in the pharmacy is about 200 $ only in our markets, the whole procedure cost about 400 $, in comparison with SS which cost about 800 $ as an average.

In conclusion BTXA injection for the management of CAF is a simple procedure, can be done in the outpatient clinic, avoids surgery and general anesthesia, cost effective and leads to healing of the fissure in an acceptable percentage, with its very low potential risk of incontinence. It can be regarded as the first-line treatment of choice for CAF. Surgery should be offered to patients who do not improve with BTXA. Patients presenting with CAF should be informed about the available treatment options as well as their risks.

The inter-sphincteric plane is preferable site for injection; more studies are needed to compare various sites of injections with each other. We think that a dose of 1 U per/kg is safe and effective and reflect the slandered dose through all other references.

Acknowledgments

Authors thank all patients that participated and cooperated with us in the study.

Authors' contribution

Both authors cooperate in the research and sampling, in addition to preparation of manuscript for publication.

Conflict of interest

Authors declare no conflict of interest.

Funding

There was no funding source for this research.

References

1. Lund NJ, Scholfield JH. Aetiology and treatment of anal fissure. Br J Surg. 1996; 83(10): 1335-44. http://dx.doi.org/10.1002/bjs.1800831006.
2. Gibbons CP, Read NW. Anal hypertonia in fissures: causes or effect? Br J Surg. 1986; 73(6): 443-5.
3. Oh C, Divino CM, Steinhagen RM. Anal fissure. 20-year experience. Dis Colon Rectum. 1995; 38(4): 378-82. http://dx.doi.org/10.1007/BF02054225.
4. Nyam DC, Pemberton JH. Long term results of lateral internal sphincterotomy for chronic anal fissure with particular reference to incidence of faecal incontinence. Dis Colon Rectum. 1999; 42(10): 1306-10.
5. Menteş BB, Irkörücü O, Akin M, *et al*. Comparison of botulinum toxin injection and lateral internal sphincterotomy for the treatment of chronic anal fissure. Dis Colon Rectum. 2003; 46(2): 232-7.
6. Steele SR, Madoff RD. Systematic review: the treatment of anal fissure. Aliment Pharmacol Ther. 2006; 24(2): 247-57. doi: 10.1111/j.1365-2036.2006.02990.x.
7. Jost WH, Schimrigk K. Use of botulinum toxin in anal fissure. Dis Colon Rectum. 1993; 36(10): 974.
8. Jost WH. Ten years' experience with botulinum toxin in anal fissure. Int J Colorectal Dis. 2002; 17(5): 287-97.
9. Brisinda G, Maria G, Bentivoglio AR, *et al*. A Comparison of Injections of Botulinum Toxin and Topical Nitroglycerin Ointment for the Treatment of Chronic Anal Fissure. N Engl J Med. 1999; 341(8): 65-9. doi: 10.1056/NEJM199908193410823.
10. Evans JE, Luck A, Hewett P. Glyceryl trinitrate vs. lateral sphincterotomy for chronic anal fissure: prospective, randomized trial. Dis Colon Rectum. 2001; 44(1): 93-7.
11. Kodner IJ, Fry RD, Fleshman JW, *et al*. Colon, rectum and anus. In: Schwartz SI, Fischer JE, Daly JM, *et al*., (eds). Principles of surgery. 7th ed. USA: Mac Grow-Hill; 1999. p. 1265-82.
12. Gui D, Rossi S, Runfola M, *et al*. Botulinum toxin in the therapy of gastrointestinal motility disorders. A review. Aliment Pharmacol Ther. 2003; 18(1): 1-16.
13. Utzig MJ, Kroesen AJ, Buhr HJ. Concepts in pathogenesis and treatment of chronic anal fissure: A review of the literature. Am J Gastroenterol. 2003; 98(5): 968-74. https://doi.org/10.1016/S0002-9270(03)00141-2.
14. Hörsch D, Kirsch JJ, Weihe E. Elevated density and plasticity of nerve fibers in anal fissures. Int J Colorectal Dis. 1998; 13(3): 134-40.
15. Wollina U, Konrad H. Botulinum toxin A in anal fissures: A modified technique. J Eur Acad Dermatol Venereol. 2002; 16(5): 469-71.
16. Lunniss PJ, Gladman MA, Hetzer FH, *et al*. Risk factors in acquired faecal incontinence. J R Soc Med 2004; 97(3): 111-6.
17. Casillas S, Hull TL, Zutshi M, *et al*. Incontinence after a lateral internal sphincterotomy: are we underestimating it? Dis Colon Rectum 2005; 48(6): 1193-9. doi: 10.1007/s10350-004-0914-3.
18. Nelson R. Non-surgical therapy for anal fissure Cochrane Database Syst Rev. 2012; (2):CD003431. doi: 10.1002/14651858.
19. Sajid MS, Vijaynagar B, Desai M, *et al*. Comparison of surgical vs chemical sphincterotomy using botulinum toxin for the treatment of chronic anal fissure: a meta-analysis. Colorect Dis. 2007; 10(6): 547-52. http://dx.doi.org/10.1111/j.1463-1318.2007.01388.x
20. Cook TA, Brading AF, Mortensen NJ. The pharmacology of the internal anal sphincter and new treatments of anorectal disorders. Aliment Pharmacol Ther. 2001; 15(7): 887-98. http://dx.doi.org/10.1046/j.1365-2036.2001.00995.x.
21. Jones OM, Moore JA, Brading AF, *et al*. Botulinum toxin injection inhibits myogenic tone and sympathetic nerve function in the porcine internal anal sphincter. Colorectal Dis. 2003; 5(6): 552-7. http://dx.doi.org/10.1046/j.1463-1318.2003.00484.x.
22. Jankovic J, Brin MF. Botulinum toxin: Historical perspective and potential new indications. Muscle Nerve Suppl. 1997; 6: S129-45.
23. Brin MF. Botulinum toxin: chemistry, pharmacology, toxicity, and immunology. Muscle Nerve Suppl. 1997; 6: S146-68.
24. Wollina U. Botulinum toxin: Non-cosmetic indications and possible mechanisms of action. J Cutan Aesthet Surg. 2008; 1(1): 3-6. http://dx.doi.org/10.4103/0974-2077.41148.
25. Radwan MM, Ramdan K, Abu-Azab I, *et al*. Botulinum toxin treatment for anal fissure. Afr Health Sci. 2007; 7(1): 14-7. doi: 10.5555/afhs.2007.7.1.14.
26. Kharkar S, Ambady P, Venkatesh Y, *et al*. intramuscular botulinum toxin in complex regional pain syndrome: case series and literature review. Pain Physician. 2011; 14(5): 419-24.
27. Runfola M, Di Mugno M, Balletta A, Magalini SC, Gui D. Antinociceptive effect of botulinum toxin: an added value to chemical sphincterotomy in anal fissure? Dis Colon Rectum. 2006; 49(7): 1078-9.
28. Naumann M, Jankovic J. Safety of botulinum toxin type A: a systematic review and meta-analysis. Curr Med Res Opin. 2004; 20(7): 981-90. DOI: 10.1185/030079904125003962.
29. Cutan J. Pharmacological Sphincterotomy for Chronic Anal Fissures by Botulinum Toxin A. Aesthet Surg. 2008; 1(2): 58-63. http://dx.doi.org/10.4103/0974-2077.44160.
30. Maria G, Brisidina G, Bentivoglio AR, *et al*. Influence of botulinum toxin site of injections on healing rate in patients with chronic anal fissure. Am J Surg. 2000; 179(1): 46-50. http://dx.doi.org/10.1016/S0002-9610(99)00255-X.

31. Huang WC, Yang SH, Yang JM. Three-dimensional transperineal sonographic characteristics of the anal sphincter complex in nulliparous women. Ultrasound Obstet Gynecol. 2007; 30(2): 210-20. doi: 10.1002/uog.4083.

32. Starck M, Bohe M, Fortling B, *et al*. Endosonography of the anal sphincter in women of different age and parity. Ultrasound Obstet Gynecol. 2005; 25(2): 169-76. doi: 10.1002/uog.1818.

33. Wollina U. Treatment of anal fissures with botulinum toxin. Zentralbl Chir. 2008 Apr;133(2):123-8. doi: 10.1055/s-2008-1004733.

34. Orsay C, Rakinic J, Perry WB, *et al*. Practice parameters for the management of anal fissures (revised). Dis Colon Rectum. 2004; 47(12): 2003-7. doi: 10.1007/s10350-004-0785-7.

35. Radwan MM, Ramdan K, Abu-Azab I, *et al*. Botulinum toxin treatment for anal fissure. Afr Health Sci. 2007; 7(1): 14-7. doi: 10.5555/afhs.2007.7.1.14.

36. Maria G, Cassetta E, Gui D, *et al*. A comparison of botulinum toxin and saline for the treatment of chronic anal fissure. N Engl J Med. 1998; 338(4): 217-20. doi: 10.1056/NEJM199801223380402.

37. Brisinda G, Maria G, Sganga G, *et al*. Effectiveness of higher doses of botulinum toxin to induce healing in patients with chronic anal fissures. Surgery. 2002; 131: 179-84. doi: 10.1067/msy.2002.119314.

38. Jost WH, Schrank B. Repeat botulin toxin injections in anal fissure in patients with relapse and after insufficient effect of first treatment. Dig Dis Sci. 1999; 44(8): 1588-9.

39. Arroyo A, Pérez F, Serrano P, *et al*. Long-term results of botulinum toxin for the treatment of chronic anal fissure: Prospective clinical and manometric study. Int J Colorectal Dis. 2005; 20(3): 267-71. doi:10.1007/s00384-004-0644-y.

40. Jost WH. One hundred cases of anal fissure treated with botulin toxin: early and long-term results. Dis Colon Rectum. 1997; 40(9): 1029-32.

41. Floyd ND, Kondylis L, Kondylis PD, *et al*. Chronic anal fissure: 1994 and a decade later - are we doing better? Am J Surg. 2006; 191(3): 344-8. doi: 10.1016/j.amjsurg.2005.10.035.

42. Gui D, Cassetta E, Asastasio G, *et al*. Botulinum toxin for chronic anal fissure. Lancet. 1994, 344: 1127-8.

Correspondence to Dr. Nashwan Q. Mahgoob
E-mail: nashwanmahgoob@yahoo.com

Received Aug. 11th 2016
Accepted Jan. 18th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

Gomco Clamp Circumcision in Neonates and Infants: A Preliminary Experience in Iraq

Ali E. Joda *FIBMS*

Dept. of Surgery, College of Medicine, Al-Mustansiriyah University, Dept. of Pediatric Surgery, Central Child Teaching Hospital, Baghdad, Iraq

Abstract

Background Circumcision and its complications are one of the most common cases that pediatric surgeons facing in their practice. There are many techniques that have been described to perform it either by the free hand operative methods or with the use of special devices.

Objective To evaluate the results of using Gomco clamp for circumcision as a preliminary experience in Iraq.

Methods A total of 120 babies were subjected to circumcision by this device during the period from April 2014 - November 2015; their age ranges from 7 days - 11 months and they were divided into 3 groups: neonates, infants 1-3 months, infants 4-11 month. By using three different sizes of the clamp, the procedure was performed under local anesthesia in the majority of cases in a form of subcutaneous ring block with aid of sedatives while general anesthesia was given to those cases of coincidental circumcision with other operations. The outcomes of the procedure were assessed after one-month period of follow up.

Results 12.5% of cases were neonates; the average duration of the procedure was 20 minutes. A total complication rate of 9.16% was recorded (11 cases out of 120. The lowest rate of complication (6.6%) was seen in neonates, while the group of infants older than 4 months had the highest complication rate (10%). The most frequent complication recorded was bleeding (36.3% of all complication rate) followed by excess skin, infection, meatitis, epidermal inclusion cyst, no other complications were recorded.

Conclusion Circumcision by Gomco clamp is a safe and simple method with good functional and cosmetic results if performed during neonatal period and early infancy. Choosing the suitable size of the clamp, adequate training and good postoperative care will minimize most of the complications.

Keywords Circumcision, Gomco clamp, neonate, infant, complications.

Citation Ali E. Joda. Gomco clamp circumcision in neonates and infants: a preliminary experience in Iraq. Iraqi JMS. 2017; Vol. 15(2): 188-197. doi: 10.22578/IJMS.15.2.12

List of abbreviations: None

Introduction

The word "circumcision" in Etymology means “cut around”, from circum = around; + caedō = cut (in Latin). The surgical removal of the prepuce, is the commonest and oldest procedure performed in males, it was dating back to 4000 BC where the ancient mummies were found to be circumcised as it has been mentioned in Egyptian papyri and wall carvings [(1)]. Perhaps it was initially performed in the ancient societies of the Middle East as a public health measure to prevent recurrent balanitis resulting from collection of sand under the foreskin [(2)]. About one out of three males are circumcised universally [(3)], it is practiced among Indian Muslims, in United States, Canada, various

countries of South East Asia and large parts of Africa [4,5] but the region of the Middle East constitutes the largest circumcised population due to their cultural and religious believes. Circumcision is a fundamental part of the traditions of many religions, particularly Judaism and Islam, in Judaism, male circumcision is considered a commandment from God whereas it is considered to be a Sunnah in Islam [6].

Indications

The most common reasons for performing circumcision are religious, ritualistic, and cultural reasons and it was not "medicalized." until the 19th century when it was justified by the relative medical indications [7] like urinary tract infections, prevention of HIV or other sexually transmitted infections, penile cancer, possible trauma to foreskin, and paraphimosis. While pathological phimosis and recurrent balanoposhtitis are considered to be an absolute indication. The only true contraindications for circumcision includes the presence of hypospadias or epispadias, as the prepuce may be needed for the creation of the neourethra.

Complications

Although circumcision can be regarded a relatively safe operation, it is not completely without complications and these are usually of low rate [3,8] when they done in hospitals or by the medical staff, while all of the community circumcisions that done by traditional circumciser have no recorded data of complication rates. In general, the rate of complication is dependent on age at circumcision, the technique or device used and the experience of the surgeon. Most of these complications are simple and easily treated [9] like mild bleeding, infection, pain, hematoma, simple glanular injury, urethral meatitis, meatal stenosis, urine retention, and other less common complications. Serious complications are often related to general anesthesia used, sepsis following severe infection, urethra-cutaneous fistula, ischemic necrosis of the glans and amputation [10] and even an extremely rare occurrence of circumcision-related deaths have been reported in some patients [11,12].

Technique of Gomco clamp circumcision

The device was invented by Hiram S. Yellen and Aaron Goldstein in 1934-1935 [1,13]. Gomco represents the Goldstein Medical Company, the original manufacturer of the clamp. It consists of 4-pieces (arm, plate, nut and stud) (figure 1). It has the advantages of glans protection, hemostasis and a platform for resecting the prepuce with no need of suturing [14]. In the United States, Gomco clamp has become a popular instrument used for performing non-ritual circumcision and preferred by both doctors and families [15] as it evident by its safety record in preventing injury and low risk of bleeding.

The objective of this study is to evaluate the results of Gomco clamp circumcision as a preliminary experience in our country, its advantages and disadvantages in neonates and infants.

Methods

This prospective study was done at the Department of Pediatric Surgery in the Central Child Teaching Hospital over the period from April 2014 – November 2015 during which, a total 120 babies were enrolled in the study, their ages ranging from 7 days – 11 months. The indications were parental decision, chronic urinary tract infection, recurrent balano-posthitis, phimosis, paraphimosis and coincidence with other surgery when circumcision advised by the doctor or requested by the family. For ethical consideration, the parents or guardians were asked for informed written consent to participate in the study. All of the babies underwent circumcision using Gomco clamp-by the same pediatric surgeon, a blood sample for a complete blood count, prothrombin time and partial thromboplastin time were routinely

done before the procedure. The exclusion criteria were:

- Re-circumcision.
- Age more than 1 year.
- Bleeding tendency.
- Congenital penile anomalies (hypospadias, epispadias, chordee, webbed penis, megalourethra).
- Microphallus.
- Infant obesity or baby with excessive suprapubic fat pad.
- Parent refusal.

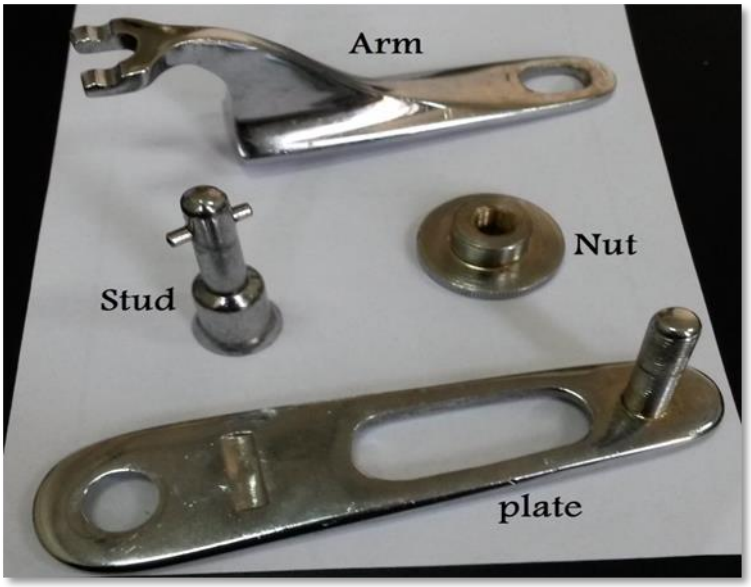

Figure 1. Parts of Gomco clamp

Three different sizes of Gomco clamp were used; one for neonatal age (Extra Small 1.1 cm) and the two others for older ages (1.3 cm and 1.45 cm), the procedure was performed in the operative theatre, under local anesthesia and sedatives while general anesthesia was used in cases of coincidental circumcision with other surgeries. The children were given oral chloral hydrate syrup 25 – 50 mg/kg 30 minutes before the procedure for sedation and paracetamol 125-250 mg suppositories for analgesia. after sterilization of the area with povidone iodine solution, local anesthesia was used in a form of subcutaneous ring block (SCRB) using 1% lidocaine without adrenalin through 25 G or 28 G syringe (insulin syringe), usually 1-1.5 ml of lidocaine was infiltrated around the penis 1 cm proximal to its base in band or ring form just in the subcutaneous tissue superficial to Buck's fascia.

The Gomco Technique [10,15,20]

The preputial opening is stretched with artery forceps (figure 2-A), the foreskin is grasped with two hemostats at 3 and 9 o'clock and separated completely from the glans by inserting a curved mosquito forceps between glans and foreskin at 12 o'clock down to the level of the corona and swept around the glans on both the right and left sides, avoiding the ventral frenulum (figure 2-B), the foreskin is then retracted proximally to check the glans for any hidden adhesions, then a hemostat is utilized to create a crush line on the dorsal aspect of the prepuce till the coronal sulcus in order to reduce bleeding (figure 2-C), the crushed skin is then cut with blunt scissors (figure 2-D) to make space for suitable size bell to be applied over the glans and under the foreskin (figure 2-E). After determining the amount of the shaft skin to be left below the corona, the edges of the dorsal slit are grasped

and the foreskin is pulled through the hole of the base plate and over the bell (figure 2-F), the clamp's arm is fitted, confirm the length of foreskin to be excised, the nut on the Gomco clamp is tightened and left in place for about five minutes so the prepuce is squeezed between the bell and the base plate to make it bloodless (figure 2-G), A scalpel is used to make a circumferential incision in the foreskin at the level of the base plate (figure 2-H). In such way, the clamp allows clotting and hemostasis to occur, the nut is then loosened, and clamp's base and bell are then removed from the penis. The penis now is inspected for any bleeding specially from the ventral frenulum region (figure 2-I). A dressing with Fucidin ointment gauze should be gently applied at the incision line, the baby observed for 2 hours in the ward then discharged home if no complications occurred.

Statistical analysis was done using SPSS-18 (statistical package for social sciences) software, and the results were expressed in mean ± SD and percentage.

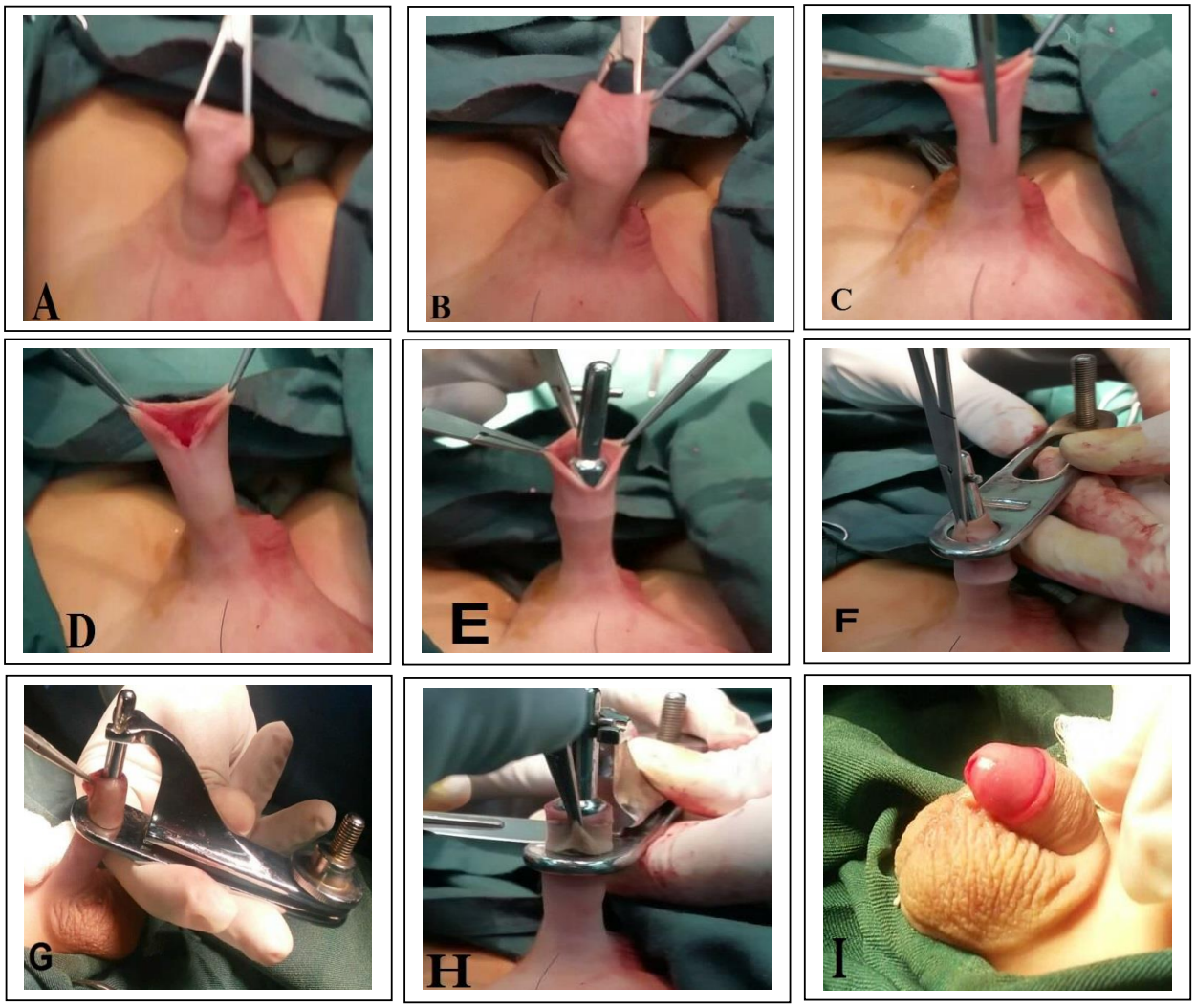

Figure 2. Stages of Gomco circumcision

Results

The cases were divided into 3 groups; neonates (15 cases) with mean age 15.6 days, infants 1-3 months (35 cases) with mean age 2.4 months and infants 4-11 months (70 cases) with mean age 6.5 months (Table 1).

The reasons of circumcision were as follow: according to the parent decision in 43 babies

(35.83%), coincidence with other surgeries (like inguinal herniotomy, orchidopexy, release of ankyloglossia, excision of external angular dermoid cyst) in 36 babies (30%) when circumcision advised by doctor or requested by the family, urinary tract infection in 20 babies (16.6%), recurrent balanoposthitis in 12 babies (10%), phimosis in 7 babies (5.83%), and paraphimosis in 2 babies (1.6%) (figure 3). The procedure was performed electively in all the cases except for the two cases of paraphimosis which were done as an emergency operation after failure of manual reduction.

Table 1. Age groups of circumcision

Age	No. of cases (%)	Mean±SD
Neonate	15 (12.5%)	15.6 days ± 3.9
1-3 months	35 (29.1%)	2.4 months ± 1.47
4-11 months	70 (58.3%)	6.5 months ± 1.65
Total	120 (100%)	

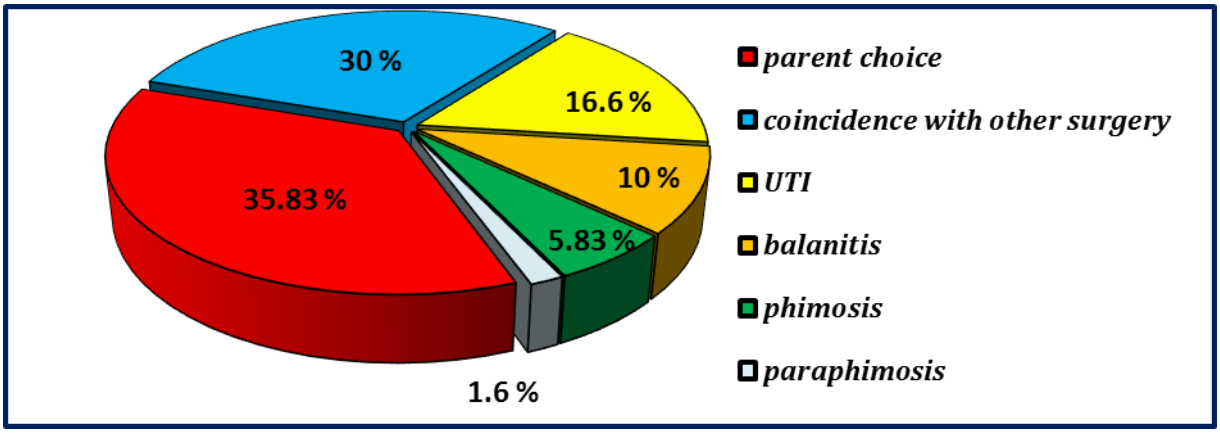

Figure 3. Reasons for circumcision

The procedure was done under local anesthesia in 70% of cases (84 babies) and general anesthesia was given for the remainder 36 babies (30% of cases) because it was coincidental with other surgeries. The average duration of the procedure was 20 minutes.

Complications were seen in 11 out of 120 cases with the total rate of complications was 9.16%. (Table 2). All of the complications were simple and no serious adverse results were noticed. The lowest rate of complication (6.6%) was seen in neonates, (9% of all complication), while the group of infants older than 4 months had the highest complication rate, 10% (63.6% of all complication), (figure 5).

The most frequent complication recorded was bleeding, (figure 4) which was seen in 4 cases all were developed when circumcision performed beyond neonatal age, in three of them the bleeding was stopped by suturing immediately following release of clamp and one case was treated by re-enforcing dressing after he was discharged to the ward. Excess skin was seen in 2 cases of infants, infection was developed in 2 cases one in neonate and the other at age of 3 months, epidermal inclusion cyst was developed in one infant of 6 months age, and two cases were developed meatitis treated by topical application of antibiotic. No other complications were recorded.

Table 2. Complications of Gomco circumcision in different age groups

	Complications	Cases	Neonates	1-3 m	4-11 m
Early	Bleeding	4	0	1	3
	Excess skin	2	0	0	2
	Glans injury	0	0	0	0
	Urethral injury	0	0	0	0
	Urine retention	0	0	0	0
	Edema	0	0	0	0
	Infection	2	1	1	0
Late	Epidermal inclusion cyst	1	0	0	1
	Preputial adhesion	0	0	0	0
	Buried penis	0	0	0	0
	Urethra-cutaneous fistula	0	0	0	0
	Meatitis	2	0	1	1
	Meatal stenosis	0	0	0	0
	Cosmetic dissatisfaction	0	0	0	0
	Total (%)	11 (9.16%)	1 (0.8%)	3 (2.5%)	7 (5.8%)

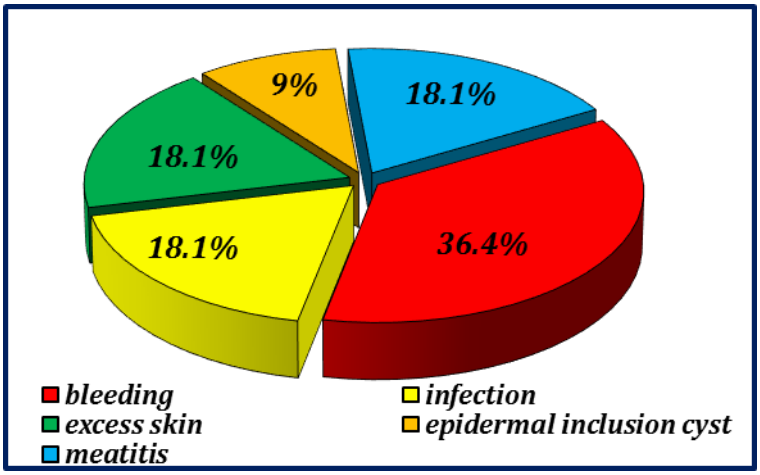

Figure 4. Types of complications

Discussion

A Gomco clamp is being used in different parts of the world like USA, Canada, South Africa, Turkey and Saudi Arabia [3], it was firstly started to be used in our country in 2014, so this study was implemented to present our preliminary experience with this device. We excluded the following groups of babies from the study in order not to affect the results:

- Cases of complicated previous circumcision that required re-circumcision.
- Infants older than one year due to unavailability of the suitable size clamp.
- Those infants with bleeding tendency
- When the family refused to subject their baby to this technique of circumcision.

The reported complication rates of circumcision depend on the type of study performed (retrospective or prospective), setting (medical or non-medical facility), operating personnel (traditional or medical practitioner), patient age (neonate, infant and

older child) and surgical technique or device used [3]. Our rate of complication was 9.16% which although higher than comparative studies (Table 3) but is accepted as an initial experience with this technique. This high rate may be attributed to the large percent of old infants included in the study compared with other studies.

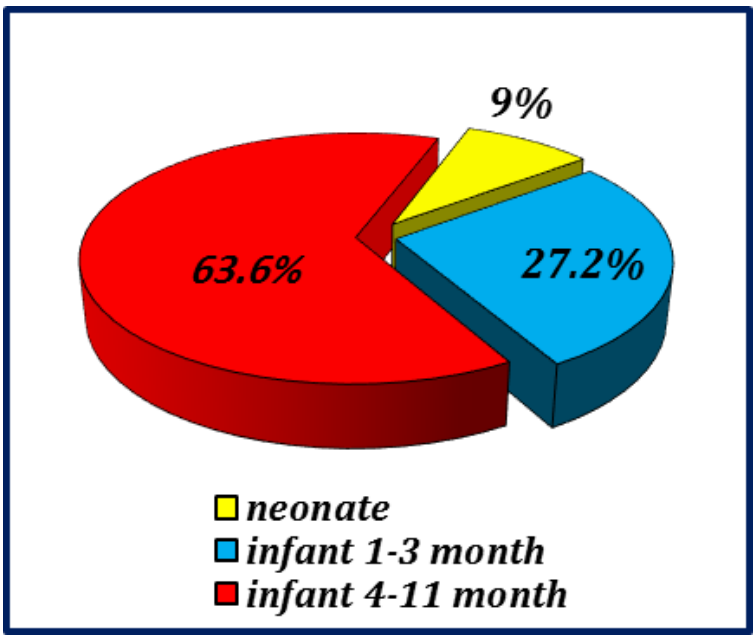

Figure 5. Complications in different age groups

Table 3. Comparative complication rate with other studies [3]

Study	Country	Year	No. of cases	Age	% of complication
Patel	Canada	1966	100	Neonates + infants	15 %
O'Brien	USA	1985	1951	Neonates	3.1 %
Amir	KSA	2000	1000	Neonates	1.6 %
Horowitz	USA	2001	130	Neonates + infants	7.4 %
Eroglu	Turkey	2002	214	Neonates	2.3 %
Perlmutter	USA	2007	51	Neonates	0.0 %
Banieghbal	South Africa	2009	583	Neonates	0.3 %
Current study	Iraq	2015	120	Neonates + infants	9.16 %

Most of complications from using Gomco clamp are resulting from technical error like not using suitable size of clamp (too large clamp result in removal of too much skin), the metal bell not covers the glans completely (result in glans injury), insufficient drawing up of skin will lead to an inadequate skin removal, insufficient tightening of the screw result in inadequate compression of the skin and subsequent bleeding, so it is better to keep the screw tightened for 5 minutes before its release to secure hemostatic compression.

Current data revealed that bleeding was the most frequent complication noticed accounting for 36.4% of all complications (figure 4). This is in agreement with other studies like Atikeler et al. [16], Burgu et al. [17]. It was noticed that bleeding rate in 3.3% of all Gomco procedures (four cases out of 120 procedures) compared to Feinberg et al. study who reported bleeding

in 4.47% of all Gomco procedures [18] (24 bleeds out of 537 procedures). All of these bleeding cases were recorded in babies beyond neonatal ages and they were simple and easily treated by re-enforcing dressing or suturing. Kurtoğlu and Baştuğ in their study [19] demonstrated a higher risk of bleeding during the "minipuberty" of infancy, which starts after four weeks of age extends to three months of age because of hormonally mediated increase in vascularity and size of penis and prepuce. This is also confirmed by Horowitz and Gershbein [20] who reported zero rate of bleeding in babies circumcised with a Gomco clamp in their first month of life compared to 30% bleeding complication rate requiring suturing or cauterization in those aged 3-8 months.

Infection was the second most frequent complication constituting 18% of all complications. Infection rate was 1.6% (2 cases out of 120), which goes with the systemic review and meta-analysis done by Ford et al. [21] who reported infection rate in the range of 0.3-1.85% of cases. Nevertheless, infection rate in Gomco clamp is still lower than those reported by other techniques, four out of 95 plastibell circumcisions (4.2%) had local sepsis requiring surgical toilet [22]. Bowa et al also reported 1.4% infection rate after plastibell circumcision compared to 1% after Gomco clamp due to the presence of foreign body in case of plastibell [23]. An increased infection rate is also expected to develop when electrocautery used for incision of circumcision as stated by NICE guidelines (National Institute for Health and Clinical Excellence) about surgical site infection [24]. Most of the reported infections are minor without significant consequences and can be prevented by proper patient preparation, wearing gloves, good local wound care including cleaning the penis after circumcision and application of antibiotic ointment with diaper changes [25].

The complication of excess skin was seen in 18% of our complication rate and this is quite common problem with many other techniques, in a retrospective review done in USA 2010, 40% of late complication following circumcision done by different techniques were inadequate circumcision (excess skin) [26]. This problem can be avoided by choosing a size of Gomco bell according to the glans diameter rather than the length of the penile shaft, because the too small bell will fail to protect the glans and causes insufficient foreskin to be pulled through the hole of the base plate resulting in excess foreskin.

Post-circumcision meatitis was seen in 18% of our complication rate. It may account for up to 26% of the late complication of circumcision in one study [26]. It has been postulated to be resulting from ammoniacal dermatitis to the meatus after the removal of the prepuce due to friction between the exposed meatus and diaper [25]. Weiss et al. in his systemic review reported this problem as a well-recognized late complication of circumcision performed during diaper age [3]. However, meatitis is a self-limiting problem and can be treated with topical antibiotics and keeping the area dry. While post-circumcision meatal stenosis may be due to chronic meatitis or resulting from ligation of the frenular artery [25] and can be treated by simple dilatation or meatatomy under general anesthesia.

Traumatic amputation of the glans is rare but a devastating complication seen with bone cutter, Mogen clamp or plastibell circumcision [27,28] because of inability to directly visualize the glans prior to incising the foreskin. Bone cutter circumcision technique is widely used in our country and partial glans amputation can occur if the operator inadvertently catches the glans in the cutter, this is quite contrast to Gomco clamp as this device incorporate a glans protective mechanism that prevents its inclusion and injury during circumcision (zero rate of glans injury in our data).

We got good results about shape of penis, stream of urine following circumcision as stated by the parents (zero rate of dissatisfaction). The cosmetic contentment following circumcision is highly dependent on

the socioeconomic status and educational level of the parents, so when circumcision is performed for religious reasons, the cosmetic result will be highly accepted from the parents [29]. Sexual satisfaction and penile sensitivity need to be assessed in adult life but in general male circumcision does not adversely affect sexual satisfaction or clinically significant function in men as reported by Kigozi et al. [30]. This study concluded that Gomco Clamp circumcision is a safe and simple method of circumcision with good functional and cosmetic results, having the advantages of straight circumcision scar, no associated injury to the underlying glans or urethra and less infection rate but carry its drawbacks of having several small parts that need to be sterilized to minimize the risk of infection, the procedure take longer time than other techniques like Mogen clamp or the traditional way with bone cutter. Most of the complications with this device can be prevented by choosing the suitable size of clamp, sufficient training and good postoperative nursing care. A higher complication rate is associated with using this device beyond the neonatal period.

Acknowledgments
None.

Conflict of interest
None.

Funding
Self-funding.

References
1. Totaro A, Volpe A, Racioppi M, et al. Circumcision: history, religion and law. Urologia. 2011; 78(1): 1-9. doi: 10.5301/RU.2011.6433.
2. Hutson JM. Circumcision: A surgeon's perspective. J Med Ethics. 2004; 30(3): 238-40. http://dx.doi.org/10.1136/jme.2002.001313.
3. Weiss HA, Larke N, Halperin D, et al. Complications of circumcision in male neonates, infants and children: a systematic review. BMC Urology 2010, 10(1): 2. doi: 10.1186/1471-2490-10-2.
4. World Health Organization, UNAIDS. Neonatal and child male circumcision: a global review. UNAIDS/10.07E – JC1672E; 2010. p. 8.
5. Drain PK, Halperin DT, Hughes JP, et al. Male circumcision, religion and infectious diseases: An ecologic analysis of 118 developing countries. BMC Infect Dis. 2006; 6(1): 172. doi: 10.1186/1471-2334-6-172.
6. Anwar MS, Munawar F, Anwar Q. Circumcision: a religious obligation or 'the cruellest of cuts'? Br J Gen Pract. 2010; 60(570): 59-61. doi: 10.3399/bjgp10X482194.
7. Padraig Malone, Henrik Steinberche. Medical aspect of male circumcision. BMJ. 2007; 335(7631): 1206-9. doi: 10.1136/bmj.39385.382708.AD.
8. Thorup J, Thorup SC, Ifaoui IB. Complication rate after circumcision in a paediatric surgical setting should not be neglected. Dan Med J. 2013; 60(8): A4681.
9. Muula AS, Prozesky HW, Mataya RH, et al. Prevalence of complications of male circumcision in Anglophone Africa: A systematic review. BMC Urol. 2007; 7(1): 4. doi: 10.1186/1471-2490-7-4.
10. Bhat NA, Raashid H, Rashid KA. Complication of circumcision. Saudi J Med Med Sci. 2014; 2(2): 86-9.
11. Meel BL. Traditional male circumcision-related fatalities in the Mthatha area of South Africa. Med Sci Law 2010; 50(4): 189-91. doi: 10.1258/msl.2010.010017.
12. Hedjazi A, Zarenezhad M, Hosseini SM, et al. Epidemiology of circumcision related mortality in Iran: A 10 year survey. N Am J Med Sci. 2012; 4(11): 608-10. doi: 10.4103/1947-2714.103338.
13. Wang J, Zhou Y, Xia S, et al. Safety and efficacy of a novel disposable circumcision device: A pilot randomized controlled clinical trial at 2 centers Med Sci Monit. 2014; 20: 454-62. doi: 10.12659/MSM.889722.
14. Bhat NA, Hamid R, Rashid KA. Bloodless, sutureless circumcision. Afr J Paediatr Surg. 2013; 10(3): 252-4.
15. Burns SD. GOMCO clamp circumcision. 2011. www.Naturalfamilyonline.com. [accessed at 18/11/2015].
16. Atikeler MK, Geçit I, Yüzgeç V et al. Complications of circumcision performed within and outside the hospital. Int Urol Nephrol. 2005; 37(1): 97-9. doi: 10.1007/s11255-004-6077-2.
17. Burgu B, Aydogdu O, Tangal S, et al. Circumcision: pros and cons. Indian J Urol. 2010; 26(1): 12-5. doi: 10.4103/0970-1591.60437.
18. Feinberg AN, Brust RA, Walker TA. Bleeding at Circumcision: Patient or Operator Issue? Clin Pediatr (Phila). 2010; 49(8): 760-3. doi: 10.1177/0009922810364658.
19. Kurtoğlu S, Baştuğ O. Mini puberty and its interpretation. Turk Pediatri Ars. 2014; 49(3): 186-91. doi: 10.5152/tpa.2014.2038.
20. Horowitz M, Gershbein AB. Gomco circumcision: when is it safe? J Pediatr Surg. 2001; 36(7): 1047-9. doi: 10.1053/jpsu.2001.24739.
21. Ford N, Chu K, Mills EJ. Safety of task shifting for male medical circumcision: a systematic review and meta-

analysis. AIDS. 2012; 26(5): 559-66. doi: 10.1097/QAD.0b013e32834f3264.
22. Lazarus J, Alexander A, Rode H. Circumcision complications associated with the Plastibell device. S Afr Med J. 2007; 97(3): 192-3.
23. Bowa K, Li MS, Mugisa B, et al. A controlled trial of three methods for neonatal circumcision in Lusaka, Zambia. J Acquir Immune Defic Syndr. 2013; 62(1): e1-6. doi: 10.1097/QAI.0b013e318275741b.
24. Surgical site infections: prevention and treatment. http://www.nice.org.uk/nicemedia/CG74NICEGuideline.pdf.2008. [accessed at 17/11/15]
25. Hutcheson JC. Male neonatal circumcision: indications, controversies and complications. Urol Clin North Am. 2004; 31(3): 461-7, viii. doi: 10.1016/j.ucl.2004.04.011.
26. Pieretti R V, Goldstein A M, Pieretti-Vanmarcke R. Late complications of newborn circumcision: a common and avoidable problem. Pediatr Surg Int. 2010 May;26(5):515-8. doi: 10.1007/s00383-010-2566-9.
27. Krill AJ, Palmer LS, Palmer JS. Complications of circumcision. Scient World J. 2011; 11, 2458-68. doi: 10.1100/2011/373829.
28. Bode CO, Ikhisemojie S, Ademuyiwa AO. Penile injuries from proximal migration of the Plastibell circumcision ring. J Pediatr Urol. 2010; 6(1): 23-7. doi: 10.1016/j.jpurol.2009.05.011.
29. Corduk N, Unlu G, Sarioglu-Buke A, et al. Knowledge, attitude and behaviour of boys and parents about circumcision. Acta Paediatr. 2013; 102(4): e169-73. doi: 10.1111/apa.12152.
30. Kigozi G, Watya S, Polis CB, et al. The effect of male circumcision on sexual satisfaction and function, results from a randomized trial of male circumcision for human immunodeficiency virus prevention, Rakai, Uganda. BJU Int. 2008; 102(6): 762-3. doi: 10.1111/j.1464-410X.2008.07995_3.x.

E-mail: ali.egab.joda@gmail.com
alieagle677@yahoo.com
Received Oct. 10th 2016
Accepted Feb. 21st 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

Histomorphometric and Endothelin-1 Immunohistochemical Study of Human Placental Villi Correlated with Umbilical Cord Coiling Index

Hayder J. Mubarak[1] *PhD*, **Sarah A. Ahmed**[2] *MSc*

[1]Dept. of Human Anatomy, College of Medicine, Al-Nahrain University, Baghdad, Iraq, [2]Dept. of Biology, College of Science, Baghdad University, Iraq

Abstract

Background Umbilical cord coiling index plays a role in predicting the pregnancy outcome and risk of low birth weight. Endothelin-1 binding sites exist on placental stem villi vessels and trophoblastic layer of the villi. Endothelin-1 is involved in regulation of the feto-placental circulation and specific trophoblastic functions.

Objective To investigate the profile of anti-endothelin-1 antibody expression in the human normal placental villi in relation to the coiling indices of the umbilical cords attached to these placentas.

Methods Normal human placentas were collected with inclusion criteria (full term newborns with normal perinatal outcome whose mothers were normal), and classified according to their umbilical cord coiling index into three groups: (N, H, and H), endothelin-1 marker used to investigate the localization of the endothelin-1 in the placental villi of each group.

Results There was a difference between the mean positivity percentage of endothelin-1 immunohistochemical reactivity in normocoiled group in comparison with hypercoiled group and hypocoiled group. There is a difference between the mean number of terminal villi in the three groups and in the perimeter of blood vessels.

Conclusion The pattern of endothelin-1 reactivity is associated with vasodilatation of the villous vascular bed to maximize the exchange function of the placenta as a physiological response to overcome the sequel of obstruction of umbilical vessels in both types of abnormal coiled cords during pregnancy.

Keywords Placental villi, umbilical cord, coiling index, normal pregnancy, endothelin-1, immunhistochemistry.

Citation Hayder J. Mubarak, Sarah A. Ahmed. Histomorphometric and endothelin-1 immunohistochemical study of human placental villi correlated with umbilical cord coiling index. Iraqi JMS. 2017; Vol. 15(2): 198-205. doi: 10.22578/IJMS.15.2.13

List of abbreviations: ET-1 = Endothelin-1, UCI = Umbilical cord coiling index

Introduction

The placenta is arguably the most important organ of the body, but paradoxically the most poorly understood [(1)].

Endothelin has three isoforms, endothelin-1 (ET-1), -2, and -3; endothelin-1 is the most potent and long-lasting vasoconstrictor known, being 100 times more potent than noradrenaline [(2)]. Mature ET-1 is a 21-amino acid peptide, and it is a main member of the endothelin peptide family. Endothelins has two receptors, ETA and ETB. ET-1 and -2 bind to ETA and ETB, while ET-3 only binds to ETB [(3)]. In the full-term human placenta, the immunoreactivity of ET-1 is local to endothelial cells of capillaries of placental microvilli, small-

and medium-sized arteries and veins in addition to placental syncytiotrophoblasts [4]. Several studies also confirmed a broad distribution of ET-1 expression in placentas throughout gestation in which, ET-1 expression is increased along with gestational age through the first trimester to full term [5]. Trophoblasts produce/release ET-1, human placenta also expresses ETA and ETB receptors. ETB is strongly expressed in placental trophoblasts and its expression is increased along with gestational age. In contrast, ETA is weakly expressed in placental trophoblasts [6]. Although the reason for the differential expression of ETA and ETB in placental trophoblasts is not clear, an in vitro binding assay has clearly shown that ET is capable of binding to the isolated trophoblast membranes. ET-1 is also involved in trophoblast invasion and differentiation of trophoblast cells isolated from first-trimester placentas [3].

The umbilical cord is the lifeline that attaches the placenta to the fetus [7]. The umbilical cord is not directly linked to the mother's circulatory system, instead it joins the placenta [8]. The attachment of the umbilical cord is generally eccentric, and to a less extent central, with a rare variety of being marginal. However, the umbilical cord, occasionally, is inserted into the chorionic membranes, to the outer surface placenta and this formal is known as velamentous cord insertion [9]. The coiling of the umbilical vessels grows as early as 28 days after conception and is present in about 95% of fetuses by 9 weeks of conception [10]. Umbilical cord coiling index (UCI) is well-defined as the total number of coils divided by the total length of the cord in centimeters [11]. The difference in coiling was described as an antenatal marker identifying fetus at risk. Majority of the studies on UCI had been done postnatally. Although UCI can be calculated antenatally by ultrasonography, limited data is available as to its accuracy [12]. The umbilical cords with abnormal coiling, were studied and showed that fetal death, fetal intolerance to labor, intrauterine growth retardation and choriomnionitis were associated with abnormal coiling [13]. In these abnormal cords, umbilical vein thrombosis, chorionic vessels thrombosis and umbilical cord structure was observed which can induce chronic (in the form of growth retardation) or acute (labor intolerance and fetal death) effects on fetal health [14].

This study was done to correlate the morphometric and the endothelin-1 immunohistochemical criteria of the placental tissues with the umbilical cord coiling index.

Methods

A sample of 30 normal human placentas were obtained from a full term, healthy (have no hypertension, diabetes mellitus, any other gynecological problem or major diseases), nonsmoker and normal vaginal delivered women admitted to the Obstetric Ward of Al-Bnouk Private Hospital. The gestational age was determined by ultrasound examination with the aid of history taken from those women prior to delivery to ensure a full-term pregnancy, which was measured as about 40±2 week, from the first day of the last menstrual period. The umbilical cords coiling indices of these placentas were examined. After being expelled, each of the placentas with their umbilical cords collected were examined grossly to ensure that they have an eccentric cord insertion with no abnormality or infraction, then the umbilical cord length and the umbilical coiling index were calculated. The placental samples were divided into three groups: (10 placentas with normocoiled coiling index, 10 placentas with hypercoilied coiling index, and 10 placentas with hypocoilied coiling index). Samples of placentas were selected from peripheral region (2 cm from placental margin near cord insertion). Placental samples were cut through the whole thickness of the placenta from fetal to maternal surfaces. Only the fetal sides of these sections were included in this study. Paraffin blocks were prepared for histological, and

immunohistochemical study [15]. Sections were cut at (5 µm) thickness serially.

The following variables were assessed for each section obtained from the regions of the three types of a sample.

1. The number of terminal villi per high power field.
2. The mean perimeter of the blood vessels in the terminal villi per high power field.

The 2nd variable was assessed by morphometric-image analysis system namely, Image J. After the random selection of a field has been done, the measurement of the perimeter of nine different sizes intravillous blood vessels; three large, three medium and three small vessels selected indiscriminately for each section, were obtained. So, each section will have 9 values for this parameter and later on the mean of these values will be obtained to be considered as the perimeter of blood vessels in terminal villi per power field which is calculated in µm. Immunohistochemical staining for Anti-ET-1 antibody was provided by USBiological with code no.15111257. It contains small molecule of synthetic endothelin-1 conjugated to bovine serum albumin, it is mouse monoclonal anti-human endothelin-1. It used with immunohistochemistry detection kit from Santa Cruz Biotechnology, Inc, evaluation of anti- ET-1 marker was done with aperio scope image analysis software v11.1.2.760, ten fields were randomly selected for each group (normocoiled, hypercoiled, and hypocoiled) and examined at power 100X to measure the percentage of the positive pixels. The percentage of the positive pixels count (number of positive excluded the weak positive pixels/number of total), is selected as it measures the anti- ET-1 in terminal villi tissue.

Statistical analysis of data was done by using Analysis of variance (ANOVA) test applied to look for statistical significance between the means of different groups for each variable. The statistical analysis of data done by using the SPSS software version 20 setup in a personal computer.

Results

ET-1 reactivity

There was a difference between the mean positivity percentage of ET-1 in normocoiled group in comparison with hypercoiled group and hypocoiled group. The highest positivity percentage was found in normocoiled group compared to hypercoiled group and hypocoiled group. Slightly higher value in hypocoiled group was observed in comparison to hypercoiled group. Table-1 shows the mean positivity percentage of ET-1 in terminal villi in the three groups. Table-2 shows that the difference between mean positivity percentage of ET-1 in terminal villi in the three groups was statistically significant (P value < 0.05).

The mean number of the terminal villi

Table 3 shows the mean number of terminal villi in the three groups. There is a difference between the mean number of terminal villi in the three groups. Table-4 shows that the difference between mean numbers of terminal villi in the three groups was statistically significant (P value < 0.05).

Table 1. The mean positivity percentage of ET-1 in termimal villi among the three groups

Groups	Mean	No.	Std. Deviation
Normocoiled	80.86	10	9.45
Hypercoiled	20.56	10	3.07
Hypocoiled	30.78	10	2.83

Table 2. ANOVA test between the mean positivity percentage of ET-1 in the terminal villi among the three groups

Groups	Mean positivity percentage of ET-1	p- value
Normocoiled	80.86	
Hypercoiled	20.56	0.000
Hypocoiled	30.78	

* Significance was accepted at p- value < 0.05

Table 3. The mean number of the terminal villi among the three groups

Groups	Mean	No.	Std. Deviation
Normocoiled	13.94	10	0.86
Hypercoiled	12.01	10	1.17
Hypocoiled	11.06	10	1.59

Table 4. ANOVA test between the mean number of the terminal villi among the three groups

Groups	Mean number of the terminal villi	p- value
Normocoiled	13.94	
Hypercoiled	12.01	0.041
Hypocoiled	11.06	

* Significance was accepted at p- value < 0.05

The mean blood vessels perimeter in terminal villi

Table 5 shows the mean perimeter of the blood vessels of terminal villi in the three groups. There is a difference between the mean perimeter of blood vessels in hypocoiled group in comparison with that of hypercoiled group and normocoiled group. The highest value of perimeter of blood vessels was found in hypocoiled group compared to that of hypercoiled group and normocoiled group, a slightly higher value in hypercoiled group was observed in comparison to normocoiled group. Table-6 shows that the difference between mean perimeter of blood vessels of the terminal villi in the three groups was statistically significant (P value < 0.05).

Discussion

Anti-endothelin-1 immunohistochemical of placental villi reactivity in response to umbilical cord coiling

The positive anti-endothelin-1 reactivity was seen in most of villous vascular endothelial cells of normocoiled group, this reactivity was weaker in these placental tissues of hypercoiled and hypocoiled groups.

Table 5. The mean perimeter of blood vessels (µm) of the terminal villi among the three groups

Groups	Mean	No.	Std. Deviation
Normocoiled	81.15	10	9.21
Hypercoiled	89.70	10	14.23
Hypocoiled	103.81	10	17.57

Table 6: ANOVA test between the mean number of the terminal villi among the three groups

Groups	Mean perimeter of blood vessels (μm) of the terminal villi	p- value
Normocoiled	81.15	
Hypercoiled	89.70	0.041
Hypocoiled	103.81	

* Significance was accepted at p- value < 0.05

ANOVA of the counted mean values of endothelin-1 antibody reactivity was evaluated collectively in groups of terminal villi using the Aperio Image Scope software. The counting of the mean value of the positivity percentage of normocoiled group was found to be significantly higher than that with hypercoiled and hypocoiled groups. Differential localization of endothelin (A) and (B) binding site in human placenta was investigated [16] on the bases that ET may have a role in the placenta by acting through its receptors to affect foetoplacental blood flow and other aspects of placental functions. It was concluded that specific high-density ET-1 binding sites were localized throughout the villous tree. Moderate to low density binding was found in the extravillous and villous trophoblast respectively. ET-A binding sites were found to be predominated in blood vessels in distal regions of villous tree. The proportion of ET-A/ET-B receptors varies between different regions of the placental vascular bed. ETB receptors have previously been considered only to occur on some vascular endothelial cells and may mediate vasodilatation [17]. ET-A receptors mediate the ET-induced arterial vasoconstriction [18]. It was established that hypercoling and hypocoiling of the cord could be associated with poor perinatal outcome. Both cord types were suggested to be less flexible or more prone to kinking and torsion, these criteria make them less tolerant to withstand the stress of pregnancy and labour resulting in the compression of these vessels [19,20]. Accordingly, the significant decrease of anti-endothelin-1 reactivity of the terminal villi investigated in the results of this study could be related to the need of eliminating the vasoconstriction effect of endothelin on the ET-A binding sites predominated in blood vessels in distal regions of villous tree. This reactivity is consequently associated with maximum vasodilatation of the villous vascular bed to maximize the exchange function of the placenta as a physiological response to overcome the sequel of obstruction of umbilical vessels in both types of abnormal coiled cords during pregnancy. This conclusion is supported by the reports that adaptations of fetal tissues (as the placenta) occur in response to failure of nutrients supply to overcome the deficit in fetal demands [21]. The results of this study agree with the finding of Jewsbury et al. (2007) [22] that vascular adaptation occurs with altered hemodynamic conditions arise due to variability of vasoconstrictor substances as a result of a pathophysiologic state. In support to this conclusion was the report that the increased ET associated with preeclampsia as supposed to be involved in impaired placental blood flow [23]. The relative density or proportion of vascular and villous ET receptor subtypes in placentae were similar in placentae obtained from pregnancies exhibiting preeclampsia compared with normal term controls [24].

Placental morphometry in relation to coiling index

The villous size, perimeter and vascularity were found be decreased in association with diseases decreasing the surface area available for gas exchange per villus [25]. Also, it was concluded that placental morphometric measures related to materno-fetal exchanges

as area and number of terminal villi and their respective villous vessels varied in associated with maternal diseases [26]. The morphometric evaluation of the placental tissues involved in this study was directed toward investigating the possible changes in parameters that could be considered as an accommodative response of placenta to improve the exchange function with hypercoiled and hypocoiled cord. The number of the terminal villi was found in this study to be significantly higher in normocoiled group compared to that of hypercoiled and hypocoiled groups. The terminal villi of the hypercoiled group showed statistically significant higher number compared to hypocoiled group. It was reported that placental nutrient transfer is regulated by blood flow and morphometric characteristics of the placenta [27]. This report is supportive to the morphometric results, the higher number of TV in normocoiled group found in this study is a normal morphometric criterion indicating normal placental transfer that is associated with normal blood flow in the placenta with normocoiled cords. The defected blood flow in the placenta with abnormal coiled cords is associated with defected placental transfer function, which is associated with the less number of placental villi. Hypocoiling was found to be associated with larger perimeter of villous vessels in the morphometric results of this study, statistically non-significant large perimeter of the villous blood vessels as found in hypercoiled group compared to normocoiled group. In conclusion, it seems that dilatation of the terminal villous blood vessels is a criterion associated with abnormal umbilical cord coiling. In support, it was hypothesized that abnormal placental morphology is an indication of deformed uteroplacental and foetoplacental vascular pathology that associated with negative effects on placenta and fetal development [28]. The results of this study illustrated that the consequences of altered umbilical cord coiling index on the placenta is an attribute for the remedial effect of the placenta in response to hypercoiling in form of both maintenance of the number of villi as possible and vasodilatation of villous blood vessels. The hypocoiled group showed vasodilation of the villous capillaries only, vasodilatation is more marked in hypocoiled group than hypercoiled group. It seems that vasodilation is the only mechanism to increase the uteroplacental blood flow in hypocoiled. Hypercoiling causes distress to fetoplacental blood flow in association with external compression, while hypocoiling was considered as a representation for an intrinsic abnormal development. The hypercoiling with external compression on the umbilical vessels reduces the blood flow to the placenta, a phenomenon that is not taking place in hypocoiled cord. This fact may be the attribute to the larger lumen of the placental villi vasculature found in this study, voluminous blood flow in the villous capillaries of the hypocoiled group (compared to hypercoiled group) is added to the vasodilation effect of diminished endothelin-1 receptors resulting in more capacious vascular lumen.

The conclusion interpreted in this study agree with the arguments that abnormal cord coiling (hypocoiling or hypercoiling) is not sufficient to cause adverse fetal outcomes, instead, placental changes could be involved. This conclusion is supported by the contribute of previous study [29].

Acknowledgments

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

Authors contributions

Ahmed: The M.Sc. candidate performing the laboratory research work and performing production of the results. Dr. Mubarak: The advisor of the M.Sc. research performing the interpretation of the results.

Conflict of interest

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

Funding

The research working funding was provided by the authors.

References

1. Burton G, Fowden A. The placenta: a multifaceted, transient organ. Philos Trans R Soc Lond B Biol Sci. 2015; 370(1663): 20140066. doi: 10.1098/rstb.2014.0066.
2. Alonso D, Radomski W. The nitric oxide-endothelin-1 connection. Heart Fail Rev. 2003; 8(1): 107-15.
3. Wang Y. Vascular biology of the placenta. In: Colloquium series on integrated systems physiology: from molecule to function. Morgan & Claypool Life Sciences; 2010. p. 1-98. http://dx.doi.org/10.4199/C00016ED1V01Y201008ISP009.
4. Bilban M, Barth S, Cervar M, et al. Differential regulation of endothelin secretion and endothelin receptor mRNA levels in JAR, JEG-3, and BeWo choriocarcinoma cell lines and in human trophoblasts, their nonmalignant counterpart. Arch Biochem Biophys. 2000; 382(2): 245-52. doi: 10.1006/abbi.2000.2016.
5. Barros J, Bairos V, Baptista M, et al. Immunocytochemical localization of endothelin-1 in human placenta from normal and preeclamptic pregnancies. Hypertens Pregnancy. 2001;20(1):125-37. doi: 10.1081/PRG-100104179.
6. Chakraborty C, Gleeson L, McKinnon T, et al. Regulation of human trophoblast migration and invasiveness. Can J Physiol Pharmacol. 2002; 80(2): 116-24. https://doi.org/10.1139/y02-016.
7. Mathew J. Timing of umbilical cord clamping in term and preterm deliveries and infant and maternal outcomes: a systematic review of randomized controlled trials. Indian pediatr. 2011; 48(2): 123-9.
8. Benirschke K, Burton G, Baergen R. Pathology of the human placenta. Berlin Heidelberg: Springer; 2012 p. 309-75.
9. Rocha J, Carvalho J, Costa F, Meireles I, et al. Velamentous cord Insertion in a singleton pregnancy: an obscure cause of emergency cesarean - a case report. Case Rep Obstet Gynecol. 2012; 2012: 308206. doi: 10.1155/2012/308206.
10. Chitra T, Sushanth Y, Raghavan S. Umbilical coiling index as a marker of perinatal outcome: an analytical study. Obstet Gynecol Int. 2012; 2012: 213689. doi: 10.1155/2012/213689.
11. Mittal A, Nanda S, Sen J. Antenatal umbilical coiling index as a predictor of perinatal outcome. Arch Gynecol Obstet. 2015; 291(4): 763-8. doi: 10.1007/s00404-014-3456-5.
12. Ezimokhai M, Rizk D, Thomas L. Abnormal vascular coiling of the umbilical cord in gestational diabetes mellitus. Arch Physiol Biochem. 2001; 109(3): 209-14. doi: 10.1076/apab.109.3.209.11593.
13. Diwakar RK, Naik MM, Jindal MM. Umbilical cord coiling: case report and review of literature. BJR Case Rep. 2017; 2: 20150152. http://dx.doi.org/10.1259/bjrcr.20150152.
14. Machin G, Ackerman J, Gilbert E. Abnormal umbilical cord coiling is associated with adverse perinatal outcomes. Pediatr Dev Pathol. 2000; 3(5): 462-71. doi: 10.1007/s100240010103.
15. Suvarna K, Suvarna K, Layton C, et al. Bancroft's Theory and practice of histological techniques. 7th ed. Churchill Livingstone; 2013. eBook ISBN: 9780702058172.
16. Min X, Kang W, Ya-Jun W, et al. Expression of endothelin-1 and endothelin receptors mRNA in placental villous from normal pregnancy induced hypertension. Med Life Sci. 2006; 8(1): 49-51.
17. Rauh A, Windischhofer W, Kovacevic A, et al. Endothelin (ET)-1 and ET-3 promote expression of c-fos and c-jun in human choriocarcinoma via ETB receptor-mediated Gi-and Gq-pathways and MAP kinase activation. Br J Pharmacol. 2008; 154(1): 13-24. doi: 10.1038/bjp.2008.92.
18. Mazzuca MQ, Khalil RA. Vascular endothelin receptor type B: structure, function and dysregulation in vascular disease. Biochem Pharmacol. 2012; 84(2): 147-62. doi: 10.1016/j.bcp.2012.03.020.
19. Ohno Y, Terauchi M, Tamakoshi K. Perinatal outcomes of abnormal umbilical coiling according to a modified umbilical coiling index. J Obstet Gynaecol Res. 2016; 42(11): 1457-63. doi: 10.1111/jog.13092.
20. Jessop F, Lees C, Pathak S, et al. Umbilical cord coiling: clinical outcomes in an unselected population and systematic review. Virchows Arch. 2014; 464(1): 105-12. doi: 10.1007/s00428-013-1513-2.
21. Godfrey RW, Dodson RE. Effect of supplemental nutrition around lambing on hair sheep ewes and lambs during the dry and wet seasons in the US Virgin Islands. J Anim Sci. 2003; 81(3): 587-93.
22. Jewsbury S., Baker P. and Wareing M. Relaxation of human placental arteries and veins by ATP-sensitive potassium channel openers. Eur J Clin Invest. 2007; 37(1): 65-72. doi: 10.1111/j.1365-2362.2007.01749.x
23. LaMarca B. Endothelial dysfunction; an important mediator in the Pathophysiology of Hypertension during Preeclampsia. Minerva Ginecol. 2012; 64(4): 309-20.
24. Dieber-Rotheneder M, Stern C, Desoye G, et al. Up-regulation of the endothelin receptor A in placental tissue from first trimester delayed miscarriages. J Soc Gynecol Investig. 2006 Sep;13(6):435-41. doi: 10.1016/j.jsgi.2006.05.006.
25. Chaikitgosiyakul S, Rijken MJ, Muehlenbachs A, et al. A morphometric and histological study of placental malaria shows significant changes to villous

architecture in both Plasmodium falciparum and Plasmodium vivax infection. Malar J. 2014; 13: 4. doi: 10.1186/1475-2875-13-4.

26. Calderon IM, Damasceno DC, Amorin RL, et al. Morphometric study of placental villi and vessels in women with mild hyperglycemia or gestational or overt Diabetes Res Clin Pract. 2007; 78(1): 65-71. DOI: 10.1016/j.diabres.2007.01.023.

27. Fowden AL, Ward JW, Wooding FP, et al. Programming placental nutrient transport capacity. J Physiol. 2006; 572(Pt 1): 5-15. doi: 10.1113/jphysiol.2005.104141.

28. Yampolsky M, Salafia CM, Shlakhter O, et al. Modeling the variability of shapes of a human placenta. Placenta. 2008; 29(9): 790-7. doi: 10.1016/j.placenta.2008.06.005.

29. Predanic M, Perni S, Chasen S, et al. Ultrasound evaluation of abnormal umbilical cord coiling in second trimester of gestation in association with adverse pregnancy outcome. Am J Obstet Gynecol. 2005; 193(2): 387-94. doi: 10.1016/j.ajog.2004.12.092.

Correspondence to Sarah A. Ahmed
E-mail: saabio248@yahoo.com
Received Nov. 14th 2016
Accepted Mar. 30th 2017

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2017; Vol. 15(2)

Immunohistochemical Expression of Aldehyde Dehydrogenase 1 (ALDH1) in Renal Cell Carcinomas

Ahmed M. Hassan[1] *MSc*, **Ban J. Qasim**[1] *PhD*, **Zaidoon A. Musa**[2] *FIBMS*

[1]Dept. of Pathology and Forensic Medicine, College of Medicine, Al-Nahrain University, Baghdad, Iraq, [2]Al-Imamein Al-Kadhimein Medical City, Baghdad, Iraq

Abstract

Background Renal cancer is the 13th most common malignancy globally. Cancer stem cells display progenitor cell properties such as self-renewal and ability to re-establish tumors that explain the tumor of origin. Aldehyde dehydrogenase 1 (ALDH1) has been known as a general marker of both normal stem cells and cancer stem cells.

Objective To evaluate the immunohistochemical (IHC) expression of cancer stem cell markers ALDH1 in renal cell carcinomas (RCCs).

Methods A study was designed that included 70 paraffin blocks of renal cell carcinoma tissues obtained from patients who underwent nephrectomy. The control group included 30 samples of normal renal tissue of autopsy cases from the Forensic Medicine Institute. From each two sections of 5 µm thickness were taken, one section was stained with Hematoxylin and Eosin (H&E) and the other section was immunohistochemically stained for ALDH1A1 (an isoform of ALDH1 that is expressed in the renal epithelium).

Results The difference in the IHC expression of ALDH1 is highly significant between RCC cases and control group ($p<0.001$). ALDH1 expression was increased in high tumor grade of RCC cases ($p= 0.007$) and also its high expression was noted in advanced tumor stage ($p= 0.002$), and with tumor size >4cm ($p=0.023$). RCC cases with renal vein invasion revealed high expression of ALDH1 ($p=0.003$), also ALDH1 expression increased in cases with perinephric fat invasion ($p=0.014$).

Conclusion ALDH1 showed higher expression in RCC tissues than normal renal tissues and it is associated with clinicopathological variables (tumor grade, tumor size, tumor stage, renal vein invasion, and perinephric fat invasion). This may reflect the role of ALDH1 in disease progression and poor prognosis of RCC.

Keywords Renal cell carcinoma, cancer stem cell marker, ALDH1, immunohistochemistry.

Citation Ahmed M. Hassan, Ban J. Qasim, Zaidoon A. Musa. Immunohistochemical expression of Aldehyde Dehydrogenase 1 (ALDH1) in renal cell carcinomas. Iraqi JMS. 2017; Vol. 15(2): 206-213. doi: 10.22578/IJMS.15.2.14

List of abbreviations: ALDH1 = Aldehyde dehydrogenase 1 CSCs = Cancer stem cells, IHC = Immunohistochemical, RCC = Renal cell carcinoma

Introduction

The National Cancer Institute has defined renal cell carcinoma (RCC) as the most common type of malignant tumors in the kidney, which arises from lining of the kidney renal tubules [(1)].

Renal cancer is the 13th most common malignancy globally [(2)]. Iraqi cancer registry recorded 386 cases in year 2010 with male to female ratio is about 1.4:1 [(3)]. The peak incidence exists in the sixth and seventh decades of life [(4,5)]. The histological

classification of RCC is very important, considering the significant effects of the subtypes in the prognosis and treatment of this tumor. The most common histological subtypes are clear cell renal cell carcinomas, papillary renal cell carcinomas, and chromophobe renal cell carcinomas. These three subtypes composed more than 90% of all RCCs [6].

Cancer stem cells (CSCs) display progenitor cell properties such as self-renewal, clonogenic ability and multipotency which are responsible for initiation and maintenance of cancer. Renal CSCs may have a significant role in tumor establishing, progression, and recurrence for their resistant to chemo and radio therapy [7].

Aldehyde dehydrogenase 1 (ALDH1) has been known as a general marker of both normal stem cells and CSCs. It was reported that ALDH1 expression is associated with nuclear grade of tumor cells in RCC. ADLH1 positive cells have greater ability in tumor development than ALDH1 negative cells and also are resistant to conventional therapies for RCC [8].

The objectives of this study was to evaluate the IHC expression of cancer stem cell markers ALDH1A1 in renal cell carcinomas.

Methods

A case control study was designed that included 70 paraffin blocks of RCC tissues obtained from patients who underwent nephrectomy, which were collected from Teaching Laboratory of Al-Imamein Al-kadhimein Medical City, Pathology Departments of Ghazi Al- Harreri Surgical Specialties Teaching Hospital and private laboratories for the period from January 2012 to April 2016. The control group included 30 samples of normal renal tissue of autopsy cases from the Forensic Medicine Institute with their relative consent for the period from December 2015 to February 2016. These specimens were processed and paraffin embedded in the Pathology Department in the Medical College of Al-Nahrain University. So, the total number of samples was 100 cases. The clinicopathological parameters were taken from patients' admission case sheets and pathology reports.

From each block, two sections of 5µm thickness were taken, one section was stained with Hematoxylin and Eosin for the histopathological diagnosis revision and the other section was deparafinized and dehydrated. Antigen target retrieval solution (DAKO, Denmark) (ready to use) (pH 6.0, 20 minutes in microwave) was used. ALDH1A1 (an isoform of ALDH1 that is expressed in the renal epithelium) was used in this study. Monoclonal ALDH1A1 rabbit antibodies, clone (EP1933Y) (Abcam, United Kingdom) (dilution 1:100) were incubated overnight. After that sections were treated with ab80436 – EXPOSE Mouse and Rabbit Specific Streptavidin and Di-amino-benzidine chromogen Detection immunohistochemical (IHC) Kit (Abcam, United Kingdom), and counterstained with hematoxylin. Technical negative control was obtained by omission of primary antibody.

Interpretation of ALDH1 IHC staining and quality control

Brown membranous and/or cytoplasmic staining pattern of epithelial cells even if staining was focal in tumor cell [9]. Positive control is the human liver tissue. Technical negative control was obtained by omission of primary antibody.

The results of IHC expressions of ALDH1 were scored semi-quantitative through assessing both staining intensity and percentage of stained cells (staining ratio) concerning the total number of cells and as following [10]:

Staining intensity

Score 0: negative staining intensity,
Score 1: weak staining intensity,
Score 2: moderate staining intensity,
Score 3: severe staining intensity.

Proportion of positive cells

Score 0: negative,
Score 1 positive in <25%,
Score 2 positive in 25-50%,

Score 3 positive in 51-75%,
Score 4 positive >75%.

Then the two scores were multiplied for each case, and the expressions were graded as:
Score 0: was negative,
Score 1-4: was low expression grade,
Score 5-12: was high expression grade

Statistical analysis

The statistical analysis of this case control study was performed with the statistical package for social sciences (SPSS) 21.0 and Microsoft Excel 2013. Numerical data were described as mean and standard error. Independent t-test was used for comparison between groups. While, categorical data were described as count and percentage, and Chi-square test was used to estimate the association between variables. The lower level of accepted statistical significant difference is below 0.05.

Results

According to ALDH1 grading score, 36 (51.43%) cases were considered high expression grade, and 34 (48.57%) cases were considered low expression grade. All control cases had shown low expression grade. The difference in the IHC expression of ALDH1 is highly significant between RCC cases and control group (p< 0.001) (Table 1).

ALDH1 expression displayed non-statistical significant association with age, sex, and histopathological types of RCC (p>0.05) (Table 2).

High expression grade of ALDH1 was recorded in 24 out of 47 cases of clear cell type (Figure 1), 5 out of 10 cases of papillary type (Figure 2), 3 out of 5 cases of sarcomatoid type (Figure 3), 2 out of 5 cases of chromophobe type, 1 out of 2 cases of granular type, and in the only one case of collecting duct RCC (Figure 4).

High expression grade of ALDH1 recorded high percentage in tumors with high nuclear grade and advanced stage, tumors that measure more than 4 cm, and in RCC cases that have renal vein invasion and/or perinephric fat invasion. So that expression of ALDH1 showed significant association with tumor grade, tumor size, tumor stage, renal vein invasion, perinephric fat invasion of RCC cases with p value (p=0.007), (p=0.023), (p=0.002), (p=0.003), (p=0.014) respectively (Table 2).

Discussion

In the present study, high expression of ALDH1 marker was recorded in 51.43% of RCC cases, which is higher than in normal tissue of control group that is parallel to Wang et al. [10] study in which, high expression was shown in 55.8% of RCC cases. A Turkish study done by Ozbek et al. [11] recorded higher expression of ALDH1 in cancerous tissue than in normal tissue.

Table 1. IHC expression of ALDH1 in RCC cases and control group according to expression grade

Score			Study groups		Total
			Control	RCC cases	
ALDH1 grade	Low (1-4)	Count	30	34	64
		%	100%	48.57%	64.00%
	High (5-12)	Count	0	36	36
		%	0.00%	51.43%	36.00%
Total		Count	30	70	100
		%	100%	100%	100%
p value			<0.001**		

** High statistically significant (P<0.001)

Table 2. ALDH1 expressions in RCC cases

Parameter		Total N=70	(ALDH1A1 expression) Low N=34	High N=36	P value
Sex	Female	26	11	15	0.644 NS
	Male	44	23	21	
Age group (years)	<50	23	11	12	0.999 NS
	≥50	47	23	24	
Tumor grade	Grade 2	50	30	20	0.007*
	Grade 3	14	2	12	
	Grade 4	6	2	4	
TNM staging of tumor	Stage-I	27	20	7	0.002*
	Stage-II	17	8	9	
	Stage-III	23	6	17	
	Stage-IV	3	0	3	
Histopathological types of RCC	Clear cell	47	23	24	0.928 NS
	Papillary	10	5	5	
	Chromophobe	5	3	2	
	Sarcomatoid	5	2	3	
	Granular RCC	2	1	1	
	Collecting duct	1	0	1	
Tumor size	≤ 4cm	13	10	3	0.023*
	> 4cm	57	24	33	
Renal vein invasion	Absent	62	34	28	0.003*
	Present	8	0	8	
Perinephric fat invasion	Absent	41	25	16	0.014*
	Present	29	9	20	

NS: non-statistical significance (p>0.05), *statistically significant

Furthermore, all apparently normal renal tissue samples in the present study showed low expression of ALDH1 marker, while in Wang et al. [10] study 79.3% of samples exhibited low expression while about 21.7% showed high expression.

This variance may be due to technical differences; such as the type of antibody, different methodology of IHC, and may be in the mode of selecting samples because the present study used autopsy samples, while Wang et al. study used biopsy samples from areas adjacent to non -tumor tissues that may lead to higher detection of enzymes and antibodies.

Ma et al. [12] revealed that ALDH1 enzymatic activity was recognized as a marker of both normal stem cells and CSCs. Furthermore, it was observed that ALDH1 distribution patterns in normal tissues were dissimilar, and were classified into three types: tissues with absent or limited expression, tissue with relatively weak expression, and tissue with high expression which is not used as s a CSC marker [13].

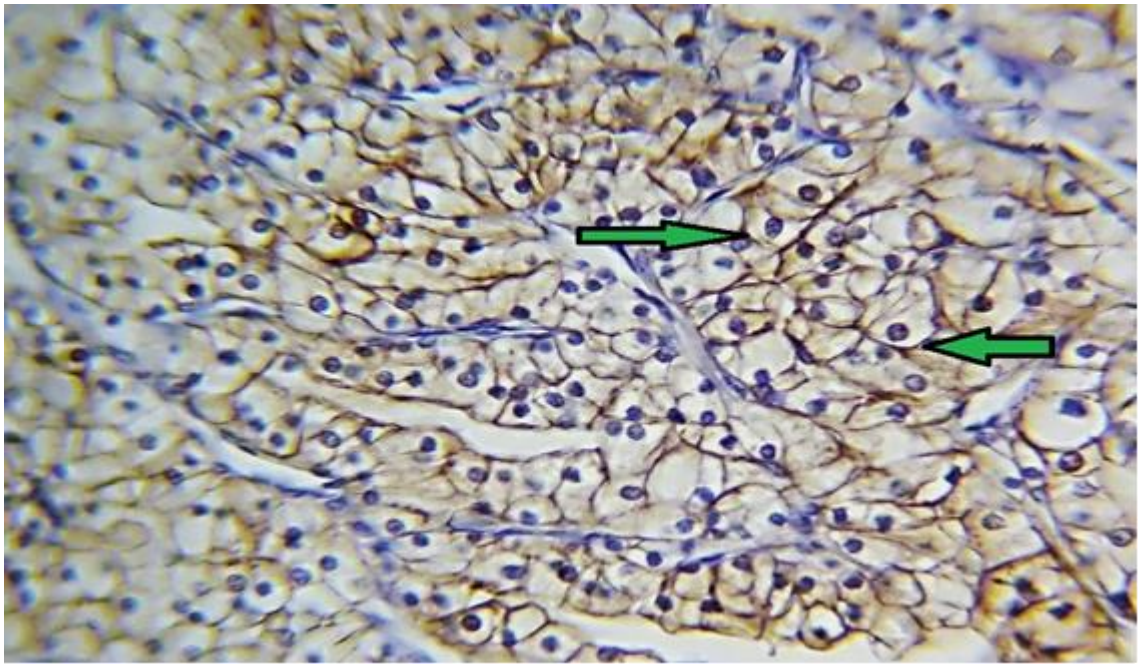

Figure 1. Clear cell RCC (grade 2, stage III) showing high IHC expression (score 6) of ALDH1A1 with brown membranous staining (arrows)(40X)

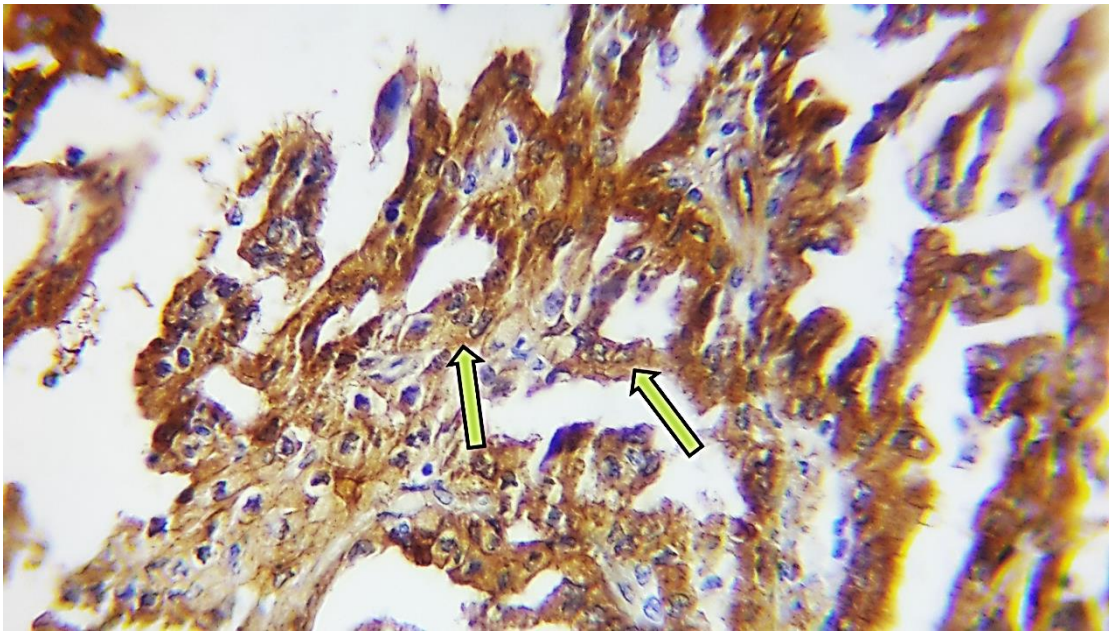

Figure 2. Papillary type RCC (grade 3, stage III) showing high IHC expression grade (score 6) of ALDH1A1 with brown membranous and cytoplasmic staining (arrow) (40X)

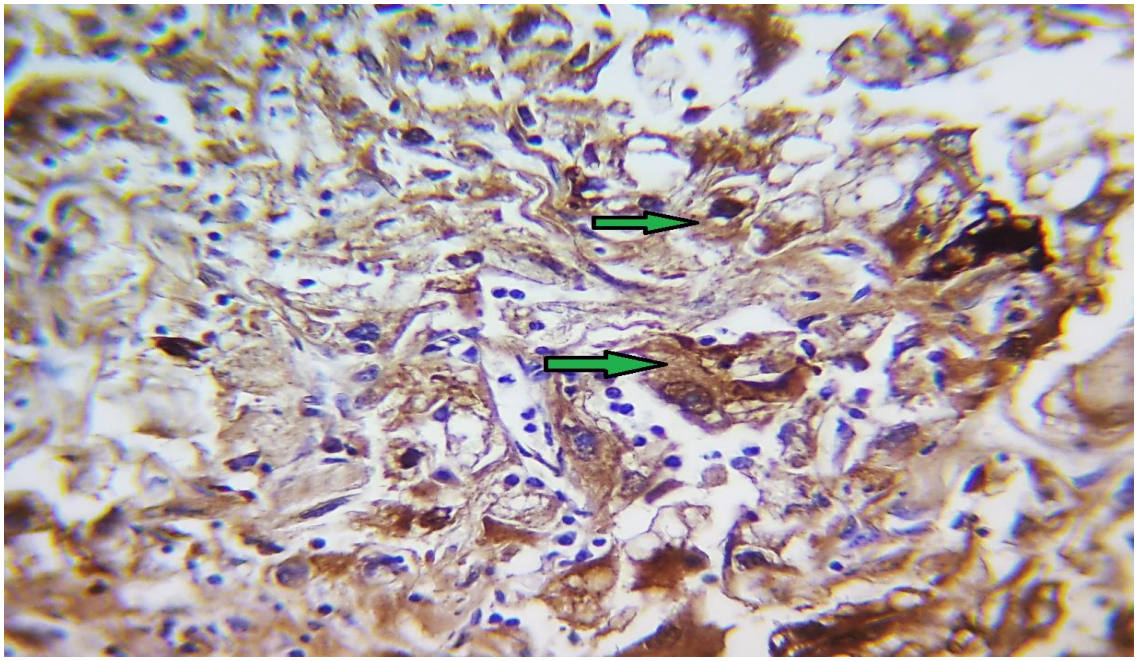

Figure 3. Sarcomatoid type RCC (grade 3, stage III) showing (score 12) ALDH1A1 IHC brown membranous and cytoplasmic staining (arrows) (40X)

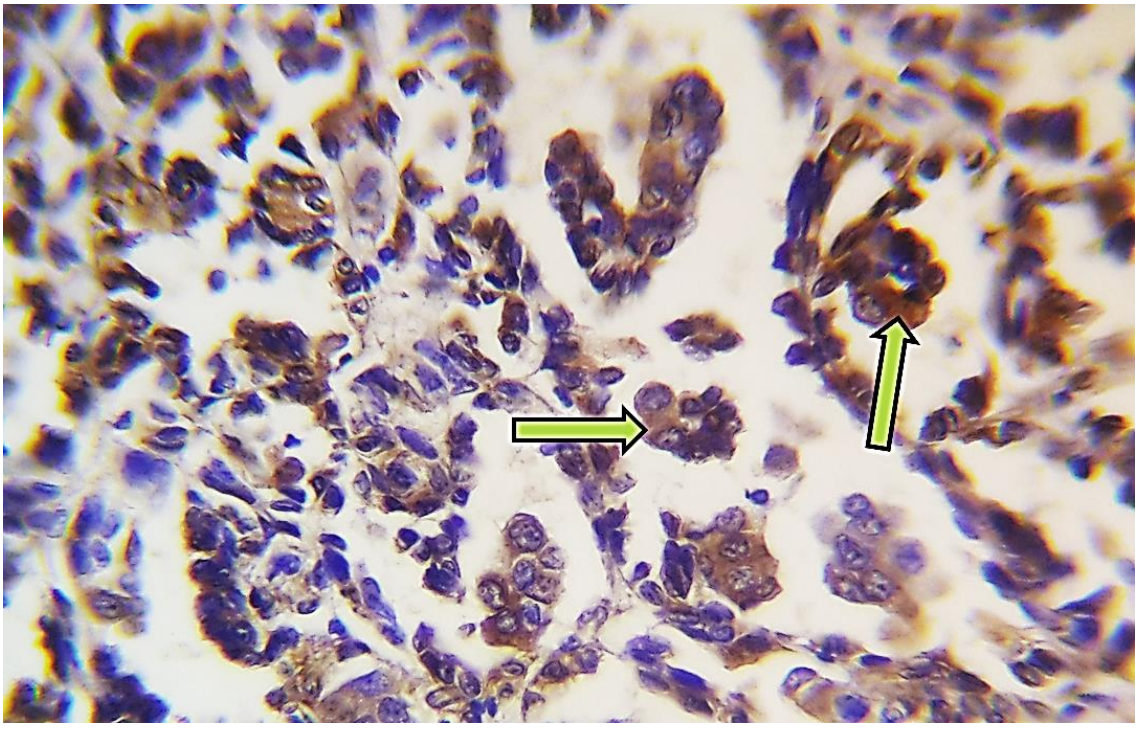

Figure 4. Collecting duct type RCC (grade2, stage III) showing high IHC expression grade (score 9) of ALDH1A1 with brown membranous and cytoplasmic staining (arrows) (40X).

ALDH1 has been recently introduced as a possibly reliable CSC marker and it has a role in RCC pathophysiology [9,11]. ALDH1 was expressed in non-cancerous and cancerous renal tissues and located in the cytoplasm and cytomembrane [10]. Some studies have reported that high expression of ALDH1 was associated with drug resistance due to cellular protection against cytotoxic drugs that exhibited poor prognosis [12].

The frequency of cases with high expression of ALDH1 in patient <50 years was 52% which is slightly higher than that of patient ≥50 years (51%). These findings are parallel to Wang et al. study [10].

The higher frequency of ALDH1 expression in young age group may be attributed to the intermediate tumor size (4-12 cm), which is associated with poor prognosis and rapid metastasis [14].

In present study, the low expression of ALDH1 was more frequently seen in stage I and II, while cases in stage III and IV were associated with higher frequency of high expression. The frequency of low expression of ALDH1 was increased in low grade (2), while high expression frequency was increased in high grade (3,4). There is a significant relation of ALDH1 expression with tumor stage and with tumor grade that is parallel to Ozbek et al. [11] and Wang et al. study. This association may reflect that high expression of ALDH1 is associated with poor prognosis cases [15] with high tumor grade and advanced stage [16,17]. Abourbih et al. [9] have found discordant results to the current study which may be due to different sample size or method of selection as well as the using of old sample from 1985 to 2006 in which the antigens may be presented at low levels to be detected efficiently by monoclonal antibody.

In the current work, expression of ALDH1 is increased when tumor size is more than 4cm in present study. There is significant relation between ALDH1 expression and tumor size that is parallel to Wang et al. [10] study.

In present study, high expression of ALDH1 was higher in cases with renal vein invasion that is parallel to Wang et al. [10] study.

Tumors with renal vein invasion has worse prognosis and become more aggressive [18,19] when show higher ALDH1 expression [10].

Current work showed that non-statistical significant relation between the ALDH1 expression and histopathological types of RCC.

The only one case (100%) of collecting duct type in the current research exhibited high ALDH1 expression may be due to this case presented with renal vein invasion. Furthermore, sarcomatoid type recorded high ALDH1 expression (60%) followed by clear, papillary, granular, then chromophobe. This result parallel to literature which stated that sarcomatoid type has worst prognosis followed by clear cell type, papillary and lastly chromophobe type [20].

In conclusion, ALDH1 showed higher expression in RCC tissues than normal renal tissues and it is associated with clinicopathological variables (tumor grade, tumor size, tumor stage, renal vein invasion, and perinephric fat invasion). This may reflect the role of ALDH1in IHC expression could act a necessary role in disease progression and poor prognosis of RCC.

Acknowledgments

Authors are deeply thankful to the staff of Laboratory of Pathology Department in the Medical College of Al-Nahrain University for their valuable support and advice, and also to all Doctors & staff of the Histopathology Department in the Teaching Laboratories in Al-Imamein Al-kadhimein Medical City.

Authors contributions

Dr. Hassan collected the cases, performed IHC test and analyzed the results. Dr. Qasim helped in study design and supervising the work. Dr. Musa participated collection of cases and revision of histopathological sections.

Conflict of interest

The authors have no conflicts of interest

Funding

This research was funded by College of Medicine/Al-Nahrain University

References

1. Matak D, Szymanski L, Szczylik C, et al. Biology of renal tumour cancer stem cells applied in medicine. Contemp Oncol (Pozn). 2015; 19(1A): A44-A51. doi: 10.5114/wo.2014.47128.
2. Qu Y, Chen H, Gu W, et al. Age-dependent association between sex and renal cell carcinoma mortality: a population-based analysis. Sci Rep. 2015; 5: 9160-6. doi:10.1038/srep09160.
3. Iraqi Cancer Registry (Ministry of Health).; 2010.
4. Ng CS, Wood CG, Silverman PM, et al. Renal cell carcinoma: diagnosis, staging, and surveillance. Am J Roentgenol. 2008; 191: 1220-32. doi: 10.2214/AJR.07.3568.
5. Ljungberg B, Bensalah K, Bex A, et al. Guidelines on Renal Cell Carcinoma. Eur Assoc Urol. 2014; 2: 1-70.
6. Muglia VF, Prando A. Renal cell carcinoma: histological classification and correlation with imaging findings. Radiol Bras. 2015; 48(3): 166-74. doi: 10.1590/0100-3984.2013.1927.
7. Bussolati B, Dekel B, Azzarone B, et al. Human renal cancer stem cells. Cancer Lett. 2013; 338(1): 141-6. doi: 10.1016/j.canlet.2012.05.007.
8. Marcato P, Dean CA, Giacomantonio CA, et al. Aldehyde dehydrogenase its role as a cancer stem cell marker comes down to the specific isoform. Cell Cycle. 2011; 10(9): 1378-84. doi: 10.4161/cc.10.9.15486.
9. Abourbih S, Sircar K, Tanguay S, et al. Aldehyde dehydrogenase 1 expression in primary and metastatic renal cell carcinoma: an immunohistochemistry study. World J Surg Oncol. 2013; 11: 298. doi: 10.1186/1477-7819-11-298.
10. Wang K, Chen X, Zhan Y, et al. Increased expression of ALDH1A1 protein is associated with poor prognosis in clear cell renal cell carcinoma. Med Oncol. 2013; 30: 574. doi: 10.1007/s12032-013-0574-z.
11. Ozbek E, Calik G, Otunctemur A, et al. Stem cell markers aldehyde dehydrogenase type 1 and nestin expressions in renal cell cancer. Arch Ital Urol Androl. 2012; 84(1): 7-11.
12. Ma I, Allan AL. The role of human aldehyde dehydrogenase in normal and cancer stem cells. Stem Cell Rev Reports. 2011; 7(2): 292-306. doi: 10.1007/s12015-010-9208-4.
13. Deng S, Yang X, Lassus H, et al. Distinct expression levels and patterns of stem cell marker, aldehyde dehydrogenase isoform 1 (ALDH1), in human epithelial cancers. PLoS One. 2010; 5: 1-11. doi: 10.1371/journal.pone.0010277.
14. Nguyen MM, Gill IS. Effect of renal cancer size on the prevalence of metastasis at diagnosis and mortality. J Urol. 2009; 181(3): 1020-7. doi: 10.1016/j.juro.2008.11.023.
15. Hampshire DJ, Abuzenadah AM, Cartwright A, et al. Identification and characterisation of mutations associated with von Willebrand disease in a Turkish patient cohort. Thromb Haemost. 2013; 110(2): 264-74. doi: 10.1160/TH13-02-0135.
16. Ueda K, Ogasawara S, Akiba J, et al. Aldehyde dehydrogenase 1 identifies cells with cancer stem cell-like properties in a human renal cell carcinoma cell line. PLoS One. 2013; 8: 1-10. doi: 10.1371/journal.pone.0075463.
17. Su Y, Qiu Q, Zhang X, et al. Aldehyde dehydrogenase 1 A1-positive cell population is enriched in tumor-initiating cells and associated with progression of bladder cancer. Cancer Epidemiol Biomarkers Prev. 2010; 19(2): 327-37. doi: 10.1158/1055-9965.EPI-09-0865.
18. Ball MW, Gorin MA, Harris KT, et al. Extent of renal vein invasion influences prognosis in patients with renal cell carcinoma. BJU Int. 2015; 118(1): 112-7. doi: 10.1111/bju.13349.
19. Kim HL, Zisman A, Han K-R, et al. Prognostic significance of venous thrombus in renal cell carcinoma. Are renal vein and inferior vena cava involvement different? J Urol. 2004; 171(2): 588-91. doi: 10.1097/01.ju.0000104672.37029.4b.
20. Amin MB, Amin MB, Tamboli P, et al. Prognostic impact of histologic subtyping of adult renal epithelial neoplasms: an experience of 405 cases. Am J Surg Pathol. 2002; 26(3): 281-91.

Correspondence to Dr. Ahmed M. Hassan
E-mail: drahmedmousa12@gmail.com

Received Nov. 28th 2016
Accepted Mar. 19th 2017

المجلد الخامس عشر، العدد الثاني، 1438 هـ، 2017م
DOI: 10.22578/IJMS.15.2.

المجلة العراقية للعلوم الطبية

عنوان المراسلات إلى المجلة العراقية للعلوم الطبية، صندوق بريد 70044 بغداد، العراق. تلفون (7717516090 964+).
رقم الإيداع في دار الكتب والوثائق ببغداد 709 لسنة 2000

Iraqi Journal of Medical Sciences

IRAQI JMS

المجلة العراقية للعلوم الطبية

Volume 15, Number 2, 2017
April-June
P- ISSN 1681-6579
E- ISSN 2224-4719

Contents