IRAQI JOURNAL OF MEDICAL SCIENCES

Iraqi Journal of Medical Sciences

Al-Nahrain College of Medicine Committee Members

Professor Adnan A. Anoze	Dean of the Medical College
Professor Hussam H. Ali	Vice Dean for Administrative Affairs
Assistant Prof. Abdul-Razak H. Ahmed	Vice Dean for Scientific and Students Affairs and Head of Microbiology Department
Professor Hashim M. Hashim	Head, Department of Medicine
Professor Alaa Gh. Hussien	Head, Department of Pathology and Forensic Medicine
Professor Farqad B. Hamdan	Head, Department of Physiology and Medical Physics
Assistant Prof. Samir M. Jasim	Head, Department of Chemistry and Biochemistry
Assistant Prof. Abd A. Muhsin	Head, Department of Surgery
Assistant Prof. Haider J. Mobarak	Head, Department of Human Anatomy
Assistant Prof. Lamia A.K. AL-Saady	Head, Department of Pediatric
Assistant Prof. Atheer J. Al-Saffar	Head, Department of Community
Assistant Prof. Liqaa R. Musa	Head, Department of Gynecology and Obstetrics
Lecturer Abdul-Kareem H. Abd	Head, Department of Pharmacology and Theraputices
Assistant Prof. Mohammed A. Kadhim	Representative of Teaching Staff Members

Scientific Advisory Board

Professor Akram Al-Mahdawi	(Iraq)
Professor Akram J. Abood	(UAE)
Professor Ameera Shubbar	(Iraq)
Professor Anam R. AL-Salihi	(Iraq)
Professor Basim Yamout	(Lebanon)
Professor Dhiaa J. Al-Timimi	(Iraq)
Professor Faiq A. Bakir	(Qatar)
Professor Faiq H. Mohammed	(Jordan)
Profesor Farooq H. Al-Jawad	(Iraq)
Professor Hikmat A.R. Hatam	(Iraq)
Professor Husam Hasson	(Iraq)
Professor Lilyan W. Sarsam	(Iraq)
Professor Mahmood H. Hamash	(Jordan)
Professor Saad Sh. Mansour	(UAE)
Professor Sami E. Matlob	(Iraq)
Professor Sawsan S. Al-Haidari	(Iraq)
Professor Shawqi Ghazala	(Iraq)
Professor Usama S. Al-Nasiri	(Iraq)
Professor Walid W. Al-Rawi	(Iraq)
Professor Yarub I. Khattab	(Iraq)

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. They include forensic medicine, history of medicine, medical ethics, and religious aspects of medicine, and other selected topics.

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 - Depart. Of Training & Improving skill - Research & Educational facilities, the approval has to be stated separetly in the method section.

C. Publication fees are 50,000 Iraqi dinars and extra fees will be taken for extended paper (6000 dinars for each additional page(more than six pages)and up to 24000 dinars only).

- Manuscripts submitted to IJMS are subject to editorial evaluation and revision by three referees.
- 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 double spaced on size A4 (29.5x21 cm) paper with wide margins. 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 IJMS.
- The 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.

Abstract: manuscript should include an abstract of not more than 150 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 Arabic and English. As far as possible, be selected from the National Library of Medicine Medical Subject Headings.
- The Arabic abstract should follow the United Medical Dictionary (Council of Arab Ministers of Health/WHO/ Arab Medical Union/ALESCO, 3rd edition.
- Manuscript format: It should be divided into the following parts: introduction, materials and 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. 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 6.
 Halliwell B, Gutteridge JMC. Oxygen toxicity, Oxygen radicals, transition metals and disease. Biochem J. 1984; 219: 1-14.
2. Books: Mann JI, Pyorala K, and Teuscher A. Diabetes in epidemiological perspective. London: Churchill Livingstone. 1983.
3. Chapter in book: Phillips SJ, and Whisnant JP. Hypertension and strock. In: Laragh JH, and Brenner BM. editors. Hypertension: Pathophysiology, diagnosis, and management. 2nd ed. NewYork: Raven Press; 1995. p. 465-78.

- **Tables**: Each table should be typed on a separate page double-spaced, including all headings, number all tables with English 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. Figure number, name of senior author, and title of the work should be written lightly on the back with red pencil. 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.

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.

- All corresponding to be addressed to the Chief Editor on the address below:

Iraqi Journal of Medical Sciences
College of Medicine,
Al-Nahrain University,
P.O. Box 14222,
Tel. 5231521,
Al-Kadhiymia,
Baghdad,
IRAQ.

Iraqi Journal of Medical Sciences

A Medical Journal Encompassing All Medical Specializations
Issued Quarterly

CONTENTS

. .

. . .

. . .   ′

. . .   ‒

. . .

.   . . .

‒

.   ‒

.   ‒

.

–		
2000	1	1
2001	2	1
2001	3	1
2002	4	1
2002	1	2
2003	3	2
2003	Suppl. 1–3	2
2004	1–3	3
2005	1–2	4
2006	1	5
2007	2	5
2007	3	5
2008	1–3	6
2009	1–4	7
2010	1–4	8

.

:

. . .

:   .

.

;   .

.

:   .

.   (ISSN)

.

;   .

:

)   .

.

.

)

.(

.

.(Impact score)

.

.(

:   .

.   .

Does Post-laparoscopic Cholecystectomy Intra-abdominal Drain Reduces Postoperative Shoulder Pain?

Hassan A. Hassan[1] *FRCS*, **Anees K. Nile**[1] *FIBMS*, **Ahmed H. Ismael**[2] *PhD*

[1]Dept. of General Surgery, [2]Dept. of Pharmacology and Therapeutics, College of Medicine, Al-Nahrain University.

Abstract

Background In the context of the much-heralded advantages of laparoscopic surgery, it can be easy to overlook post-laparoscopy pain as a serious problem, yet as many as 80% of patients will require opioid analgesia. It is generally accepted that pain after laparoscopy is multifactorial, and the surgeon is in a unique position to influence many of the putative causes by relatively minor changes in technique.

Objective To determine whether a drain placed in the peritoneal cavity during laparoscopy is both clinical and cost-effective method of reducing postoperative shoulder pain.

Methods One hundred female patients were having laparoscopic cholecystectomy were divided into two groups, a control group (50 patients) where no intra-peritoneal drain was inserted and second group (50 patients) in which the patients had intra-peritoneal gas drain sited in the subhepatic area. Patients' age, weight, height, operative time, total amount of CO_2 and amount of analgesia used were recorded for each patient in both groups. Shoulder pain was assessed using visual analogue score (VAS) from 1-5 scale at 4, 8, 24 & 48 hours postoperatively, where as abdominal pain was assessed at 48 hour post-operatively. pH of the abdominal fluid was assessed in the second group of patients by using pH meter 48 hours postoperatively.

Results Shoulder pain may occur in many of patients of the control group more frequent than those of the second group, where as postoperative abdominal pain was found to be greater in patients with subhepatic drain after laparoscopic cholecystectomy.

Conclusion Low-cost drain decreased the frequency of shoulder pain and reduced the need for analgesia, but increases the abdominal pain; however it is less cost-effective than simple oral analgesia after laparoscopy.

Keywords laparoscopic cholecystectomy, intra-abdominal drain, shoulder pain

Introduction

Laparoscopic procedures, compared to laparotomies, are associated with lower morbidity, shorter hospitalizations, smaller incisions, earlier return to normal activity, and less postoperative pain [1-4].

Carbon dioxide has been the favored gas used to create pneumo-peritonium because of its high solubility in the blood and the fact that it does not support combustion. Although the physiologic problems resulting from carbon dioxide are well documented, they are becoming of more concern in long extensive laparoscopic procedures in elderly and debilitated patients [5].

Release of gas at the end of the operation is inefficient regardless of the method used [6]. After laparoscopy, CO_2 gas remains within the peritoneal cavity for a few days [7], commonly causing pain at this time [7,8] particularly soon after the start of activity and ambulation [9].

The pain is thought to be due to peritoneal irritation by carbonic acid and to the creation of space between the liver and the diaphragm, leading to loss of suction support of the heavy liver.

Several studies have shown benefits from preoperative methods for reducing abdominal and shoulder pain after laparoscopic cholecystectomy, but the problem is under investigation [10-13].

Methods

A prospective randomized controlled study was carried out in Al-Kadhmyia Teaching Hospital, Baghdad, Iraq from April 2009 until January 2010 involving 100 female patients, of age ranging from 20 to 55 years (mean =37.5), all of them were having laparoscopic cholecystectomy. They were divided into two groups, first group (involving 50 patients) was considered to be the control group, where no intra-peritoneal drain was inserted, and second group (50 patients) was the group in which the patients had intra-peritoneal gas drain sited in the sub-hepatic area. Cases with real indications for drain post-laparoscopic cholecyst-ectomy (suspected bile or blood leak) were excluded from the study. Cases involved in the study were uncomplicated and have no other associated diseases (diabetes mellitus, hypertension and ischemic heart diseases) and were approved about the study.

Patients age, weight, height, operative time, total amount of CO_2 used were recorded for each patient in both groups. Shoulder pain was assessed using visual analogue score (VAS) from 1-5 scale at 4, 8, 24 and 48 hours postoperatively, where as abdominal pain was assessed at 48 hour postoperatively.

Drains were removed and patients of the two groups were discharged after 48 hour post-operatively. pH of the abdominal fluid was assessed in the second group of patients by using pH meter. The type and amount of analgesia needed in the two groups was recorded.

Statistical analysis

All data were collected and analyzed by using SPSS. Statistical analysis was performed using Chi-squared test to compare discrete variables and two tailed paired Student's t-test to compare continuous variables between groups. $P < 0.05$ was considered statistically significant for all tests.

Results

The study involved 100 female patients having laparoscopic cholecystectomy; they were of age ranging from 20-55 years, as seen in Table 1 and Figure 1.

The patients of both groups of the study were of weight ranging from 69.7 to 84.38 kg, and of height from 161.7 to 163.1 cm, and consequently of body mass index (BMI) ranging from 25.6 to 26.5 as seen in Table 1 and Figures 2,3,4.

Volume of CO_2 used in both groups of the study was shown in Table 1 and Figure 5.

Operative time in both groups of the study was recorded and shown in Table 1 and Figure 6.

pH of the abdominal fluid was assessed in the second group of patients and shown in Table 1.

The type and amount of analgesia needed in the two groups was recorded and shown in Table 2.

Shoulder pain was assessed at 4, 8, 24 and 48 hours postoperatively, where as abdominal pain was assessed at 48 hour post-operatively as shown in Tables 2, 3, and 4 also seen in Figure 7.

Table 1: distribution of age, weight, height, BMI, CO$_2$ amount and operative time in both groups of the study and pH values in patients of group 2 only

	Study groups	N	Mean	Std. Deviation	Std. Error Mean	Sig. (2-tailed)
Age	Without drain	50	32.32	7.386	1.044	0.212
	With drain	50	30.58	6.443	0.911	
Weight	Without drain	50	84.38	99.760	14.108	0.304
	With drain	50	69.70	12.193	1.724	
Height	Without drain	50	161.7	5.832	1.323	0.521
	With drain	50	163.1	6.324	1.285	
BMI	Without drain	50	25.6	2.443	0.943	0.426
	With drain	50	26.5	3.754	0.954	
CO$_2$ amount	Without drain	50	42.12	7.441	1.052	0.894
	With drain	50	41.92	7.586	1.073	
Operation time	Without drain	50	28.00	8.981	1.270	0.926
	With drain	50	28.14	5.668	0.802	
pH	With drain	50	6.8	1.546	0.654	

Table 2: Analgesia needed abdominal pain score and shoulder pain score at 4, 8, 24 and 48 hours post operatively in patients of both groups of the study

	Pearson Chi-Square Tests	Study groups
Analgesia needed	Chi-square	0.332
	df	2
	Sig.	0.847
Abdominal pain score	Chi-square	23.522
	df	3
	Sig.	0.000
Shoulder pain score 4 hrs	Chi-square	34.213
	df	4
	Sig.	0.000
Shoulder pain score 8 hrs	Chi-square	37.690
	df	3
	Sig.	0.000
Shoulder pain score 24 hrs	Chi-square	40.527
	df	3
	Sig.	0.000
Shoulder pain score 48 hrs	Chi-square	0.062
	df	2
	Sig.	0.970

Table 3: Shoulder pain score in both groups of the study at 4, 8, 24, 48 hours post-operatively

Shoulder pain score	Study groups															
	Without drain								With drain							
	4hrs		8hrs		24hrs		48hrs		4hrs		8hrs		24hrs		48hrs	
	Count	Percentage	Count	Percentage	Count	Percentage	Count	Percentage	Count	Percentage	Count	Percentage	Count	Percentage	Count	Percentage
1	8	16%	8	16%	7	14%	35	70%	30	61%	31	62%	33	66%	36	72%
2	13	27%	13	26%	14	28%	11	22%	15	31%	17	34%	15	30%	10	20%
3	24	49%	25	50%	25	50%	4	8%	3	6%	2	4%	2	4%	4	8%
4	4	8%	4	8%	4	8%	0	0%	1	2%	0	0%	0	0%	0	0%
Total	49	100%	50	100%	50	100%	50	100%	49	100%	50	100%	50	100%	50	100%

Table 4: Chi-square test of shoulder pain score of both groups of the study

Pearson Chi-Square Tests			
		Without drain	With drain
Shoulder pain score	Chi-square	59.298	6.526
	df	9	9
	Sig.	0.000	0.686
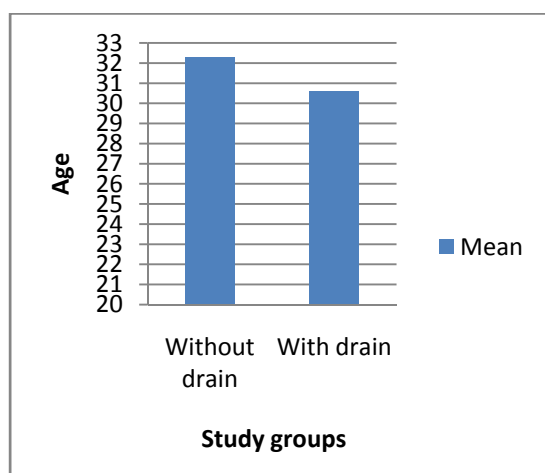
Figure 1: Age distribution in both groups of the study
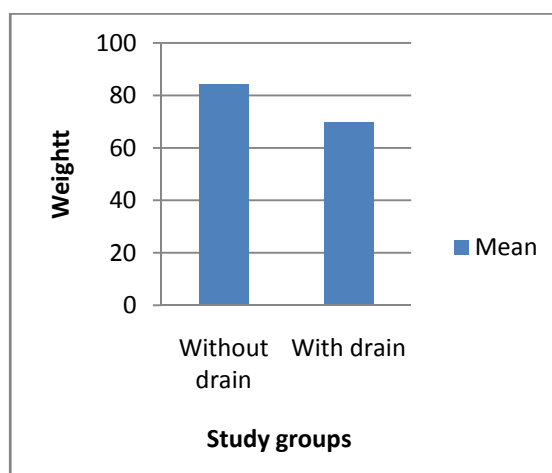
Figure 2: Weight distribution in both groups of the study
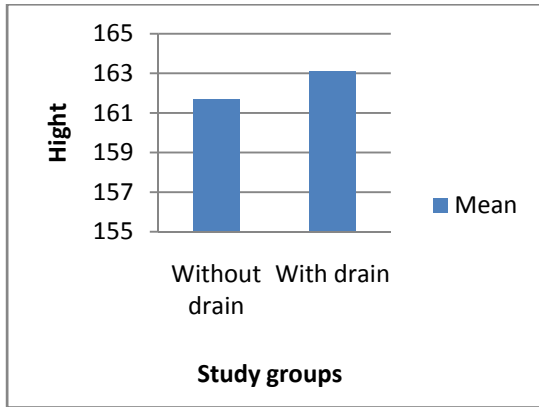
Figure 3: Height distribution in both groups of the study

Figure 4: BMI of patients in both groups of the study

Figure 5: Amount of CO_2 needed in patients of both groups of the study

Figure 6: Operative time in patients of both groups of the study

Figure 7: VAS score of shoulder pain in both groups of the study at 4, 8, 24, 48 hours post-operatively

Discussion

This prospective randomized study was carried out on 100 female patients; they were uncomplicated and have no other associated diseases. They were divided into two groups, control group (50 patients) where no intra-peritoneal drain was inserted and second group (50 patients) in which the patients had intra-peritoneal gas drain sited in the sub-hepatic area.

Patients of two groups of the study were well matched for age, weight, height, BMI, volume of CO_2 needed, operative time and amount of analgesia needed, as shown in Figures 1, 2, 3, 4, 5 and 6 and seen in Tables 1and 2, where

the standard error mean difference is not significant. The fact that the above parameters are not significant is also mentioned by other studies [1,4,6,10,12].

Shoulder pain was assessed in this study by using visual analogue score (VAS) from 1-5 scale at 4, 8, 24 & 48 hours postoperatively as shown in Tables 2, 3, and 4 also seen in Figure 7.

Pain after laparoscopy may be transient or persist for at least three days [3,5]. Shoulder pain may occur in as many as 63 % [1], or as few as 35% of patients [8]. Prolonged presence of shoulder tip pain suggests excitation of phrenic nerve [3,5,8]. This pain can be reduced by aspiration of gas under diaphragm by the use of gas drain [2]. Low-pressure CO_2 pneumoperitoneum reduces the number of patients complaining of shoulder-tip pain and the intensity of the pain after laparoscopic cholecystectomy [4,7]. The addition of intraperitoneal normal saline infusion to low-pressure CO_2 pneumoperitoneum seems to reduce the intensity but not the frequency of shoulder-tip pain after laparoscopic cholecystectomy [11,13].

Suxamethonium used during anesthesia may cause pain across the shoulder but its avoidance is not associated with a reduction of pain score [7,9,13].

Abdominal pain was assessed 48 hours postoperatively by using visual analogue score (VAS) as in Table 2. Postoperative abdominal pain was found to be greater in patients with subhepatic drain after laparoscopic cholecystectomy, as suggested by other studies [7,11].

Drain use after elective laparoscopic cholecystectomy increases wound infection rates and delays hospital discharge. There is no evidence to support the use of drain after laparoscopic cholecystectomy as some papers stated higher wound infection ratio in drain group in comparison to non drain group [9,11,13].

The routine use of a drain in elective laparoscopic cholecystectomy has nothing to offer, in contrast, it is associated with increased pain. It would be reasonable to leave a drain if there is a worry about an unsolved or potential bile leak [4,6,11]. Subdiaphragmatic drain offers only minor, if any, benefit on postoperative pain, nausea and vomiting after laparoscopic cholecystectomy, and this effect is probably clinically irrelevant [1,2,5,13].

Shoulder pain is significantly lower in the second group probably due to aspiration of dissolved CO_2 in the subhepatic area and less formation of carbonic acid which irritant to diaphragm and subsequent less shoulder pain and this was confirmed by measuring pH of aspirated fluid through intra-abdominal drain which was mostly towards the acidic side [5,8]. On the other hand, shoulder pain is more in the first group (control group), the presence of intra-abdominal drain leads to mild to moderate discomfort (pain) which needs some sort of analgesia which is nearly equal to that used in the absence of drain [4,6,11].

From the above we can conclude that a low-cost drain decreased the frequency of shoulder pain and reduced the need for analgesia, however it is less cost-effective than simple oral analgesia after laparoscopy. Removal of as much intraperitoneal gas as possible before incision closure, in conjunction with postoperative analgesics, analgesics, remains the best practice for reducing postoperative pain.

References

1. Alexander JI, and Hull MGR. Abdominal pain after laparoscopy: the value of a gas drain. *Br J Obstet Gynaecol*, 1987; 94: 267.

2. Al-Mulhim A, Al-Ali A, Albar A, Bahnassy AA, Abdelhadi M, Wosornu L, et al. Increased rate of cholecystectomy after introduction of laparoscopic cholecystectomy in Saudi Arabia. *World J Surg*, 1999; 23: 458-462.

3. Chamberlain G. The recovery of gases insufflated at laparoscopy. *Br J Obstet Gynaecol*, 1984; 91: 367.

4. Dobbs FF, Kumar V, Alexander JI, and Hull MGR. Pain after laparoscopy related to posture and ring versus clip sterilization. *Br J Obstet Gynaecol*, 1987; 94: 262.

5. Duchene P. Analgesie apres coelioscopie: interet du serum physiologique intraperitoneal. *Ann Fr Anesth Reanim*, 1994; 13: 435.

6. Grace PA, Quereshi A, Coleman J, Keane R, McEntee G, and Broe P. Reduced postoperative hospitalization after laparoscopic chole-cystectomy. *Br J Surg*, 1991; 78: 160-162.

7. Narchi P, Benhamou D, and Fernandez H. Intraperitoneal local anesthetic for shoulder pain after day-case laparoscopy. *Lancet*, 1991; 338: 1569.

8. Radke OC, DEAA, Phelps P, Cakmakkaya OS, and Apfe CC. A simple clinical maneuver to reduce laparoscopy induced shoulder pain. *Obstet Gynecol*, 2008; 111(5): 1155-1160.

9. Ortega AE, Hunter JG, Peters JH, Swanstrom LL, and Schirmer B: A prospective, randomized comparison of laparoscopic appendectomy with open appendectomy. Laparoscopic Appendectomy Study Group. *Am J Surg*, 1995; 169: 208-212.

10. Perry CP, Trombello R. Effect of fluid instillation on postlaparoscopy pain. *J Reprod Med*, 1993; 38: 768.

11. Riedel HH, Semm, K. Das post-pelvis kopische (laparoskopische) Schmerz-syndrom (The post-laparoscopic pain syndrome). *Geburtshilfe Frauenheilkd*, 1980; 40: 635.

12. Sharp JR, Pierson WP, and Brady CE. Comparison of CO_2- and N_2O-induced discomfort during peritoneoscopy under local anaesthesia. *Gastroenterology*, 1982; 82: 453

13. Valla JS, Limonne B, Valla V, Montupet P, Daoud N, Grinda A, et al. Laparoscopic appendectomy in children: report of 465 cases. *Surg Laparosc Endosc*, 1991; 1: 166–72.

Correspondence to: Dr. Anees K. Nile,
E-mail: aneesnile74@yahoo.com

Selenium Level in Lichen Planus and in Psoriasis and Its Relation to Chronicity and Severity of Both Diseases

Nadheer A. Matloob *FICMS; CABD*

Dept. Medicine, Dermatology Section, College of Medicine, Al-Nahrain University.

Abstract

Background   Lichen planus characterized by its violaceous color most commonly seen on the flexor surfaces of the upper extremities, the genitalia and the mucous membranes. Psoriasis is a common, chronic, relapsing, inflammatory skin disorder with a strong genetic basis. Plaque type of psoriasis is the most common. Selenium is a component of the enzyme glutathione peroxidase, and is important in protection against damage by peroxides and free radicals.

Objectives   To measure selenium level in Iraqi patients with lichen planus and in patients with psoriasis and its relation to the chronicity and the severity of both diseases.

Methods   One hundred twenty patients were included in this study, 68 males and 52 females, with ages between 18-54 years. Full history and examination, including dermatological examination, were done for all patients. The patients were divided into three groups. The first group includes lichen planus patients, the second group includes psoriasis patients and the third group was a control group study. Serum selenium level was measured for all patients by spectrophotometer.

Results   Serum selenium level was decreased in 20 (50%) patients of the first group, in 32 (80%) patients of the second group and 14 (35%) of the third group. The results were of high statistical significance when compared between groups 2 & 3 but it was insignificant when compared between groups 1 & 3. Selenium level was decreased in 12 of the first group who had the disease for two years and above while it was decreased in 18 of the second group who had psoriasis for two years and above, selenium level was decreased in patients with severe and diffuse variants of both lichen planus and psoriasis.

Conclusion   Serum selenium level was decreased in both lichen planus and psoriasis but it was more significant in psoriasis and this decrease was related to both chronicity and severity.

Keywords   Selenium, Lichen planus, Psoriasis, Chronicity, Severity.

Introduction

Lichen planus (LP) is a fairly common skin disorder presented with a pruritic, papular eruption characterized by its violaceous color, polygonal shape and sometimes, fine scales.

Lichen planus is most commonly found on the flexor surfaces of the upper extremities, the genitalia and the mucous membranes especially the oral cavity [1,2]. Lichen planus is most likely an immunologically mediated reaction of unknown origin. No significant geographical variation in frequency was noted. No racial predispositions have been noted. No significant differences in incidence are noted between male and female patients, but in women, lichen planus may present as desquamative inflammatory vaginitis [3].

More than two thirds of patients are aged 30-60 years; however, lichen planus can occur at any age [4]. Many clinical variants of the disease are present (according to the morphology or the areas of involvement) [1,2]. Psoriasis is a common, chronic, relapsing, inflammatory skin disorder with a strong genetic basis. Plaque type of psoriasis is the most common, although several other distinctive clinical variants of psoriasis are recognized (Guttate Psoriasis; Pustular Psoriasis; Psoriatic Arthritis; flexural Psoriasis; Erythrodermic Psoriasis ... etc)[5,6]. Plaque psoriasis is most typically characterized by circular-to-oval red plaques distributed over the extensor body surfaces and the scalp. The plaques usually exhibit scaling as a result of epidermal hyperproliferation and dermal inflammation. The extent and duration of plaque psoriasis is highly variable from patient to patient. Acute flares or relapses of plaque psoriasis may also evolve into more severe disease, such as pustular or erythrodermic psoriasis [1,7]. Psoriasis affects adult males and females equally. Plaque psoriasis first appears during two peak age ranges. The first peak occurs in persons aged 16-22 years, and the second occurs in persons above 50 years [8].

Selenium (Se) is one of the trace elements, which include in addition, iron, copper, iodine, chromium and zinc. All of them are required for physiological functions in amounts less than 100 mg daily [9]. Normal serum concentration of selenium is 80-130 µg/l [10]; the concentration of selenium in blood is highly responsive to changes in the selenium level in the diet over a wide range [10]. Supplemental selenium has restored cell proliferation defects associated with aging mice by increasing the number of high affinity IL-2 receptors and improved T-cell response to phytohaemoagglutination and significant progressive increase in delayed type hypersensitivity in hemodialysis patients [11]. Selenium is a component of the enzyme glutathione peroxidase, and is important, together with vitamin E, in protection against damage by peroxides and free radicals (it is important for the integrity of the immune system in human body). Selenium acts as anti toxic element, can binds cadmium, mercury and other metals, it mitigates their toxic effect, and even the toxic level in tissues remain unchanged. On the other hand selenium may be toxic when ingested water containing a high amounts of the metal [12,13]. Some studies indicate that selenium can be useful in the treatment of acne and it also helps in treatment and prevention of dandruff and some other skin disorders [14]. Many studies showed the relation between selenium level and psoriasis, but no known study was done to show the relation between selenium level and lichen planus and so this study was performed to measure selenium level in Iraqi patients with lichen planus and in patients with psoriasis and its relation to the chronicity and the severity of both diseases.

Methods
One hundred twenty patients were included in this study, 68 (56.7%) of them were males and 52 (43.3%) were females, with ages between 18-54 years and a mean age of 35.7±10.8 years. The study was conducted from July 2007 till the end of December 2009 in the Department of Dermatology of Al-Kadhymia Teaching Hospital in Baghdad.

Three groups were present in this study and each one contained 40 patients, the first one included patients with lichen planus, the second included patients with psoriasis and the third was a control group. The diagnosis of both diseases was done depending on the clinical bases. Full history was obtained from each patient including age, occupation,

duration of the disease (regarding both LP and psoriasis), history of treatments (for both diseases), also full examination, including dermatological examination, were done for all patients by the same dermatologist. Some patients needed full biochemical investigations according to the clinical variants of the two dermatoses and some of them needed skin biopsy to settle the diagnosis.

Methods of determination of serum selenium:

A (5 ml) sample of blood were taken from all study subjects and allowed to clot then centrifuged at 3000 rpm for 5 minutes. The clear serum was transferred to a plastic tube by disposable syringe and tapped by a plastic stopper, then stored a deep frozen at -20 °C before analysis (all glassware and bottles used for the isolation of serum and for analysis were previously soaked in diluted nitric acid (10%) and rinsed thoroughly with de-ionized water, this procedure was followed in order to exclude the possibility of contamination with trace elements). Serum was aliquoted into a vessel-tube for mineralization with 5 ml of $HNO_3/HCLO_4$ (4:1 v/v). The temperature of this mixture was slowly increased to 175 °C until fumes of $HCLO_4$ appeared. The mixture was then heated according to the following (temperature/time) scheme: 175 °C/60 min, 200 °C/60 min and finally 250 °C for 60 min. The mixture was then left to cool down to room temperature. HCL 6 N (10 ml) was added and heated to 170 °C for 30 min to reduce the Se (VI) to Se (IV). After cooling to room temperature, Se concentration was determined using the hydride generation atomic absorption spectrophotometry (Atomic absorption spectrophotometer Shimadzu, AA-680). Sodium bromohydride solution (3 g $NaBH_4$, 1 g NaOH in 100 ml of

mili-Q water) was used as a reducing agent. Samples were diluted (1:4) with de-ionized water and measured directly at 196 nm. A standard curve was made from dilutions solution of 1 mg/ml [15].

Statistical analysis

Continuous variables were expressed as mean and standard deviation. Categorical variables were expressed as percentages. Descriptive characteristics of patients were compared using χ2 tests with Yate's correction for continuity. All database management and statistical analyses were performed with SPSS software (10th version). The level of significance was set at (*p*-value < 0.05). All probability values were two-sided [16].

Results

Duration of LP in group 1 patients was between one month & 5 years while the duration of psoriasis in group 2 patients was between two weeks & 15 years.

Selenium level shown to be decreased in 20 (50%) patients of the 1st group (patients with LP), in 32 (80%) patients of the 2nd group (patients with psoriasis) and 14 (35%) of the 3rd group (control), (selenium level was between 40 and 70 µg/l in those patients and was 80 µg/l and more in patients with normal selenium levels) and the results were of high statistical significance when compare between groups 2 and 3 but it was insignificant when compare between groups 1 and 3 (Table 2 and Figure 1).

Selenium level was decreased in 12 of the 1st GP Patients who had LP for two years and above, while it decreased in 18 of the 2nd GP Patients who had psoriasis for two years and above, also selenium level decreased in patients with severe and diffuse variants of both LP and psoriasis (eruptive, ulcerative and diffuse lichen planus as well as

erythrodermic psoriasis, generalized pustular psoriasis (GPP), psoriatic arthritis and diffuse plaque psoriasis) (Tables 3, 4 and 5).

Table 1: The age and the sex of the patients

Age (Years)	L.P	M	F	Psoriasis	M	F	Control	M	F	Total	%
10 - < 20	4	2	2	5	3	2	3	2	1	12	10
20 - < 30	6	4	2	7	4	3	10	5	5	23	19.2
30 - < 40	14	8	6	13	8	5	11	6	5	38	31.7
40 - < 50	10	5	5	9	5	4	11	6	5	30	25
50 - < 60	6	3	3	6	4	2	5	3	2	17	14.1
Total	40	22	18	40	24	16	40	22	18	120	100
Mean age	36.7 ± 10.4			35.7 ± 10.9			35.4 ± 10.8			35.7 ± 10.8	

Table 2: The decrease in selenium level in relation to the three groups

Group	Decrease in selenium level (40-70mcg/l)	%	Normal selenium level (≥ 80mcg/l)	%	p-value	Chi-square
1st group	20	50	20	50	0.2581	1.279
2nd group	32	80	8	20	0.0001*	14.783
3rd group	14	35	26	65	-	-

* Values considered statistically significant when P - value < 0.05 (Compare both the 1st and the 2nd group with the 3rd group).
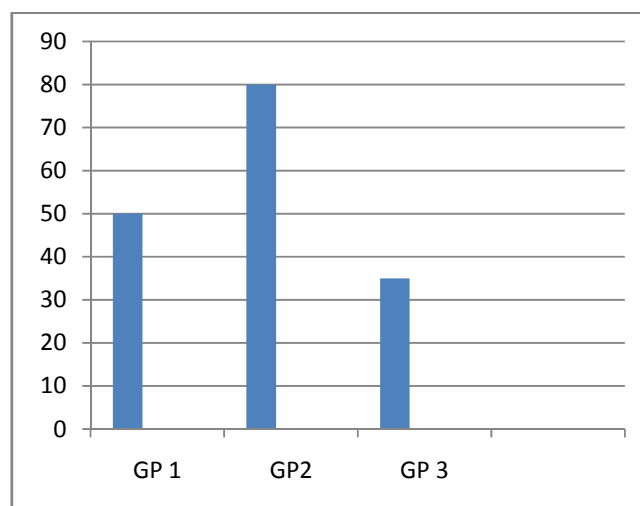

Figure 1: The decrease in selenium level in relation to the three groups

Table 3: The decrease in selenium level in relation to the duration of both diseases

Group	< 1 Year No.	1 − 2 Years No.	> 2 years No.
Lichen planus	2	6	12
Psoriasis	4	10	18

Table 4: The decrease in selenium level in relation to the severity (variant) of Lichen planus

Clinical Variant	Decreased level	Normal level	Total
Classical	14	12	26
Actinic	1	5	6
Eruptive	3	1	4
Ulcerative	2	-	2
Hypertrophic	-	1	1
Atrophic	-	1	1

Table 5: The decrease in selenium level in relation to the severity (variant) of Psoriasis

Clinical Variant	Decreased level patients	Normal level patients	Total patients
Plaque Diffuse	18	8	26
plaque	5	-	5
Erythrodermic	3	-	3
Psoriatic arthritis	3	-	3
GPP	3	-	3

Discussion

Selenium is considered as one of the important trace elements that had important functions in human including its relation to the immune system and its action as an antioxidant and antitoxic material [17]. Selenium have relation to many common skin diseases like psoriasis, eczema, dandruff ….etc [14] (especially those dermatoses with immune aspects in their pathogenesis) and to the best of our knowledge, this study is the first in Iraq regarding the relation between selenium and psoriasis as well as its duration and severity; it was also the first study in Iraq and even in the literature regarding the relation between selenium and lichen planus as well as its duration and severity.

Generally, this study clearly showed that there is an inverse relationship between the level of selenium and the duration of both LP and psoriasis and the explanation of this reduction in selenium level is that selenium itself has a fundamental role in the regulation of the immune system and any decrease in the level of selenium will cause a change in the immune system, and this will affect LP and psoriasis which both had an immune etiology, [13] and this study also showed that patients with lichen planus had a 50% decrease in selenium level but this result was statistically insignificant in comparison with the control group (35%) and this may be due to the relatively small number of LP patients or due to the increase in the deficiency in selenium in Iraqi people in general due to many years of sanctions and blockade, however, the more the chronic and the more diffuse and sever LP was, the more the decrease in selenium level was seen. This recent study also showed that selenium level

had a significant inverse relation to psoriasis as well as to its duration, variants and severity and this point is in agree with Serwin et al study [18] which showed the same findings, however, this old study showed a relation of selenium level to psoriasis of more than three years duration and this differs from this study which showed that selenium level was decreased even in psoriatic patients who had less than one year history of the disease and this difference may be due to the differences in the etiopathogenic or the provoking factors of psoriasis from country to country and also (as mentioned before)due to the fact that Iraqi people may already had deficiency in selenium because of the years of sanctions and blockade [18]. This study was also in agreement with Hinks et al study [19] which showed a significant inverse relationship between selenium level and psoriasis, however, this recent study differs from Donadini et. al. study [20] that showed the absence of relation between selenium level and psoriasis and this difference may be due to (and as mentioned before) the differences in the etiopathogenic or the provoking factors from country to country as well as the differences in the number of the patients and the clinical variants of the disease in the study. The relation between the clinical variants of psoriasis and selenium level that was mentioned in this study was not shown in the literature, however, many studies abroad claimed that psoriasis may be improved by the use of selenium supplements [21,22] but no such studies were found regarding lichen planus.

Selenium level was decreased in both LP and psoriasis but it was more significant in psoriasis and this decrease was related to both chronicity and severity.

Recommendation
Other studies with a higher number of patients and with more clinical variants of both LP and psoriasis are needed to get more accurate results about the relation between the two diseases and selenium, a future studies about the effects of selenium supplements on both LP and psoriasis and studies about other trace elements in relation to both disease as well as other skin diseases are needed.

Acknowledgment

References
1. Champion RH. Psoriasis. In Rook, Wilkinson, Ebling Textbook of Dermatology. Vol 3, 6th ed. Malden, Mass: Blackwell Science; 2004; p. 1899-1916.
2. Fitzpatrick T, Eisen A, and Wolff K. Psoriasis. In: Dermatology in General Medicine. Vol 1, 4th ed. New York, NY: McGraw-Hill; 1993; p. 1134-1144.
3. Murphy R, and Edwards L. Desquamative inflammatory vaginitis: what is it? *J Reprod Med*, Feb 2008; 53(2): 124-128.
4. Balasubramaniam P, Ogboli M, and Moss C. Lichen planus in children: review of 26 cases. *Clin Exp Dermatol*, 2008; 33(4): 457-459.
5. Schon MP, and Boehncke WH. Psoriasis. *N Engl J Med*, 2005; 352(18): 1899-1912.
6. Christophers E, and Mrowietz U. Psoriasis. In: Freedberg IM, Eisen AZ (eds). Fitzpatrick's Dermatology in General Medicine. Vol 1. New York, NY: McGraw-Hill; 1999; p. 495-521.
7. Nickoloff BJ, Bonish BK, Marble DJ, Schriedel KA, DiPietro LA, Gordon KB, and Lingen WV. Lessons learned from psoriatic plaques concerning mechanisms of tissue repair, remodeling, and inflammation. *J Investig Dermatol Symp Proc*, 2006; 11(1): 16-29.
8. Camp RDR. Psoriasis. In: Champion RH, Burton JL, Burns DA, Breathnach S, eds. Textbook of Dermatology. Vol2. Oxford, England: Blackwell Science; 1998; p. 1589-1649.
9. Mayes PA, Digestion N, and Absorption. In: Murray R, and Grinner D. Harpers Biochemistry 1st ed.

Appleton and Lange. 1996; p. 625-635.

10. Underwood EJ. Trace elements in human and animal. Nutrition 9th ed., Academic press. 1977; p. 106-120.

11. Marshall WJ, and Bangert SK. Clinical nutrition. In: Clinical Chemistry, 6th ed. London, New York, 2008; p. 371-382.

12. Falchuk KH. Disturbance in trace elements. In: Harrison's principles and practice of internal medicine 14th ed. New York; McGraw-Hill, 1998; p. 489-92.

13. Ananda D, Prasad S. Trace elements in human health and disease. 2nd ed., New York, London, Academic press, 1976; p. 117-119.

14. http://www.healthvitaminsguide.com. Retrieved in 2008.

15. Safaralizadeh R , Kardar GA , Pourpak Z, Moin M, Zare A, and Teimourian S. Serum concentration of Selenium in healthy individuals living in Tehran, Nutrition, 2005; 4: 32.

16. Duncan RC, Knapp RG, Miller MC (Eds). Introductory biostatistics for the health sciences. Wiley & Sons Inc., 1977; p. 79-85.

17. Rayman MP. The importance of selenium to human health. *The lancet*, 2000; 356: 233-241.

18. Serwin AB, Wasowicz W, Gromadzinska J, and Chodynicka B. Selenium status in psoriasis and its relations to the duration and severity of the disease. *Nutrition*, 2003; 19(4): 301-304.

19. Hinks L J, Young S, and Clayton B. Trace element status in eczema and psoriasis. *Clin Exp Dermatol*, 1987; 12(2): 93-97.

20. Donadini A, Fiora C, Regazzini C, Perini D, and Minoia C. Selenium plasma levels in psoriasis. *Clin Exp Dermatol*, 1992; 17(3): 214-216.

21. http://www.vitamin-update.com/health-problems-detail.cfm/id/30.html. Retrieved in 2008.

22. Serwin AB, Chodynicka B, Wasowicz W, and Gromadzinska J. Selenium nutritional status and the course of psoriasis. Polski Merkuriusz Lekarski, May 1999; 6(35): 263-265.

Correspondence to: Dr. Nadheer A. Matloob,
E-mail: ndnav36@yahoo.com

Evaluation of Cytomorphological Changes in Urine Samples of Uremic Patients Undergoing Regular Hemodialysis

Arif S. Malik *FICMS*

Dept. Medicine, College of Medicine, Al-Nahrain University.

Abstract

Background   Dialysis is one of the common strategies of renal replacement therapy for patients with chronic renal failure; however it harbors significant cellular changes in various body fluids.

Objective   To evaluate the cellular changes in urine samples of patients undergoing dialysis.

Methods   Seventy-two fresh midstream, spontaneously voided urine samples, they were included in the study. Early morning samples were excluded, Duration of dialysis was taken into consideration (short term and long term dialysis). Samples were centrifuged at 3000 round per minute for 15 minutes, the supernatants were decanted and the sediments were examined cytomorphologically.

Results   The gross appearance of all urine samples was neither purulent nor hemorrhagic. Microscopically there was an excessive shedding of urothelial cells in urine samples of patients undergoing dialysis compared with samples of the control group which showed evidence of normal shedding. There were no significant cytological atypia or malignancy in all urine samples. The excessive exfoliation in the absence of significant inflammation, hemorrhagic, or cytological atypia was compared with control group.

Conclusions   The study revealed that some cytological changes do occur in the urothilial cells in patients undergoing dialysis; these changes need further attention to disclose their real causes.

Key words   chronic renal failure, hemodialysis, cytomorphology, epithelial exfoliation

Introduction

Chronic renal failure remains a major health problem. Dialysis (hemo- and peritoneal) is regarded one of the most common strategy of renal replacement therapy and the main sole for saving the life [1]. Urine cytology has an acceptable sensitivity and specificity. It is easy, cheap, quick, readily accepted by the patients, and can be repeated many times without the need for preparations of the patients for the test [2,3]. The most important accomplished cytology of urinary tract is the diagnosis of clinically suspected cases of carcinoma particularly carcinoma in situ [4].

Routine screening is performed for the detection and diagnosis of tumors and precancerous state of urinary tract, the incidence of false positive and false negative result is 5% and 15% respectively [5,6].

Various types of cells may appear during cytomorphological study of the urine samples including physiological exfoliation, transitional, sequamouse, columnar, traumatic exfoliation, red blood cells and cast [7].

Dialysis (mainly peritoneal of any duration) can induce significantly atypical changes in mesothelial cells [8].

The abnormal cells can be benign cytological findings, precancerous or neoplastic and dysplastic changes [9-12].

Dialyses harbor some cellular changes in various body fluids [13-15].

The aim of the study was to analyze the cytomorphplogical changes of exfoliated urothilial cells in urine samples of the patients undergoing dialysis and to determine the relation-ship between the degree and type of cellular changes and the duration of dialysis.

Methods

From April 2005 to August 2005, 72 urine samples were collected from patients with end stage renal failure undergoing dialysis at Al-Kadhimya Teaching Hospital.

Patients with indwelling catheters, previous history of passing renal stone, chronic irritants (I mean like exposure to the chemical materials and drugs). Malignant and benign tumors of urinary tract and those with significant hematuria (16 cases) were excluded from the study.

A spontaneously passed, freshly voided, midstream urine samples (15 ml) in three disposable tubes (5 ml each) were collected from each patient and send for cytological examination. The presence of red blood cells (RBC) was regarded significant when the number of RBC was more than 3-4 in female and 2-3 in male. The number of exfoliated cells was considered low if it was ranged from 0-1, moderate from 2-6 and high if the number was more than 6 cells per HPF.

The patients were divided into two groups; the first group was those with short term dialysis (duration of dialysis of less than one year), the second group were those with long term dialysis (duration of dialysis of more than one year). The mean duration of dialysis was 14.6 months. Of all 68% underwent hemodialysis six hours every week, the remaining (32%) had nine hours per week. Gambro AK96 machines were used. A third group of 20 healthy subjects were included as a control group.

Descriptive statistical analysis studies were applied for each group including mean, median, mode and standard deviation. Chi-square tests were applied for comparison, p value < 0.005 was regarded significant.

Results:

Seventy two patients on dialysis were subjected to urine cytomorphological examination, 41 were males and 31 were females, they were divided into two groups according to the duration of dialysis

Group I: included patients undergoing dialysis for less than, or equal to one year (n= 33).

Group II: included patients on dialysis for more than one year (n= 39).

Group III: Included control persons (n=20) of matching age and sex.

The age range was 20-65 years with a mean of 49.06 years. The distribution of age groups in the three groups is shown in Table 1. The sex distribution is in Table 2.

The most probable causes of end stage renal disease based on clinical ground are shown in Table 3.

Table 4 shows that the urine color was deep orange in 91.7% in groups I and II, clear yellow color in 65% of the control group.

The exfoliation of urothilial cells in each group was shown in Table 4, there was high exfoliation (> 6 cells/HPF) in groups I and II in comparison to the control group who showed low exfoliation (0-1 cells/HPF).

Both single and cluster cells were found in the same smear in 80.6% in urine samples of group I and II while the arrangements of exfoliated urothilial cells in urine samples of control group were arranged in single pattern (Table 4).

The frequencies of red blood cells (acute and chronic) in urine samples of each group studied are shown in Table 5.

The atypical or malignant cytological features were not found in all groups.

Table 1: Age distribution of the study groups

Age Group(year)	Study Groups					
	Group I	(%)	Group II	(%)	Group III	(%)
20-25	3	(9.1)	6	(15.4)	4	(20)
30-39	5	(15.2)	4	(10.3)	14	(70)
40-49	4	(12.1)	8	(20.5)	2	(10)
50-59	6	(18.2)	8	(20.5)		
60 and above	15	(45.5)	13	(33,3)		
Total	33	(100)	39	(100)	20	(100)
Chi-square test	value		df		*p* value	
	85.873		8		0.000 (highly significant)	

Table 2: The sex distribution of the study groups

Study Groups	SEX		Total (%)
	Male (%)	Female (%)	
Group I	17 (51.5)	16 (48.5)	33 (100)
Group II	24 (61.5)	15 (38.5)	39 (100)
Group III	10 (50)	10 (50)	20 (100)
Total	51(55.4)	41 (44.6)	92 (100)

Chi-sequare test *p* value > 0.005 (not significant)

Table 3: The urine color of the study groups (macroscopical appearance)

Urine color	Group I (%)	Group II (%)	Group III (%)	Total (%)
Deep orange	29 (87.9)	37 (94.4)	2 (10.0)	68 (73.9)
Straw color	3 (9.1)	1 (2.6)	5 (25.0)	9 (9.8)
Yellow	1 (3.0)	1 (2.6)	13 (65.0)	15 (16.3)
Total	33 (100)	39 (100)	20 (100)	92 (100)
Pearson Chi- square test	value	df	*p* value	
	58.239	4	000	

Table 4: The exfoliation of urothilial cells and their patterns of arrangement in the urine sample smears of both dialyzing and control groups

No. of exfoliated urothilial cells	Group I (%)	Group II (%)	Group III (%)	Total (%)
Low (0-1 cells/HPF)	-	-	20 (100)	20 (21.7)
Moderate (2-6 cells/HPF)	7 (21.2)	5 (12.8)	-	12 (13.0)
High (> 6 cells/HPF)	26 (78.8)	34 (87.2)	-	60 (65.2)
Urothilial cells arrangement				
Single	6 (18.2)	6 (15.4)	20 (100)	32 (34.8)
Cluster	2 (6.1)	-	-	2 (2.2)
Both	25 (75.8)	33 (84.6)	-	58 (63.0)
Total	33 (100)	39 (100)	20 (100)	92 (100)

Table 5: The type (inflammatory or red cells) and the distribution of cells in both dialyzing and control groups

Red blood cells in urine samples	Group I (%)	Group II (%)	Group III (%)	Total (%)
Significant	6 (18.2)	8 (20.5)	-	14 (15.2)
Non	27 (81.8)	31 (79.5)	20 (100)	78 (84.8)
Total	33 (100)	39 (100)	20 (100)	92 (100)
Inflammatory cells in the urine samples				
Lymphocytic	3 (9.1)	1 (26)	-	4 (4.3)
Lymphocytic and neutrophilic	1 (3.0)	1 (26)	-	2 (2.2)
Non	29 (87,9)	37 (94.9)	20 (100)	86 (93.5)
Total	33 (!00)	39 (100)	20 (100)	92 (100)

Discussion

For the most practicing nephrologists and pathologists, the term urinary tract cytology brings to mind almost immediately the diagnosis of urinary tract neoplasm. Obviously many non neoplastic disorders may also be reflected in the urine cytology specimens [17]. Thus the technique of the cytological diagnosis of urinary tract malignancy has been around for well over 150 years and widely published for at least 65 years [18].

Under normal circumstances mid stream freshly voided urine contains relatively scattered urothilial cells and few cells of other types, including polymorph nuclear leukocytes, red cells and macrophages [19], however, urinary cytology preparations are usually not ordered by the clinicians unless having a clinical suggestion of urinary tract disease, an abnormal urine analysis, or both [20]. Thus the sparsely cellular "normal urinary cytology preparation is unusual in

day to day practice, that's why taking the control group of patients from wide age range to confirm the previous knowledge. The current results are nearly similar findings in the series conducted by Yarub et al and Kutaibah et al [17,18].

The number of urothilial cells and nonepithilial cells in a given freshly voided urine sample may vary widely depending not only on the how long the disease process goes on, but also on the manner by which the specimens were collected. Excluded in this study all the specimens which were obtained by catheterization, irrigation, ordinary brushing techniques, which normally yields a cellular smear. In addition to that the specimen preparation may also have some effect on the cell yield and the individual cell characteristics and that is why using an ordinary centrifugation (not cytocentrifuge or membrane filter technique) to overcome excessive normal yield of the urothilial cells [21]. The method of fixation, slide preparation (pre-albumin coated slides method used to prevent cell loss), and staining (routine alcohol fixed papanicolaou stain were used to exclude counting of keratinized sequamous cells especially in female patients which relatively resemble superficial urothilial cells. All these might interfere or change the diagnostic yield in the interpretation of data.

Despite the above mentioned facts, the diagnostic yield is relatively more accurately correlate with the number and volume of the urine sample [22,23].

In comparison with control group, the urine samples of uremic patients undergoing dialysis showed excessive shedding of urothilial cells in the form of clusters. The number of samples (small samples) and the mean duration of hemodialysis (the mean duration of hemodialysis in the current study less than 15 months) and the presence of comorbid disease (anemia and

malnutrition) which are highly prevalent among our patients might affect the results. In conclusion, there was excessive shedding of urothilial cells in the urine samples of patients undergoing dialysis (short and long term) compared with control group.

There was a significant cell cluster arrangement of exfoliated urothilial cells in the urine samples of both groups (short and long term dialysis) compared with control group which were mainly single cells.

There were no significant cellular cytomorphological changes of both groups (short and long term dialysis) and no cell atypia and / or malignant changes in the urine samples of both groups (short term and long term dialysis)

I recommend that every cyto-pathologist should be aware of these changes and more advanced study or studies should done to know the exact pathophysiology and cellular molecular biology in predialysis and in patients undergoing different forms of dialysis.

References

1. Rubin NT. Treatment of irreversible renal failure in, Arend, Armitage, Clemmons, Drazen, Griggs, Larusso, Cecil Text book Of Medicine 23rd edition, Chapter 133 Saunders 2007; 936-47.

2. Mariappan P, Smith G, Lamb AD, Grigor KM, Tolley DA. Pattern of recurrence changes in noninvasive bladder tumors observed during 2 decades. *J Urol*, 2007; 177: 867-70.

3. Joste N. Overview of the cytology laboratory: specimen processing through diagnosis. *Obstet Gynecol Clin North Am*, 2008; 35: 549-53.

4. Siegel R, and Ward E. Cancer statistics, 2009. *CA Cancer J Clin*, 2009; 41; 89-92.

5. Rathert P. Urinary cytology in bladder cancer. *Urol A*, 2003; 42: 980-991.

6. Dacron M. Urinary tract infection in adult leukocytoria. *Rev-Prat*, 2003; 15: 1137-42.

7. Bhuiyan J. Performance characteristics of multiple urinary tumor markers and sample collection technique in the detection of transitional cell carcinoma of the bladder. *Clin Chim Acta*, 2003; 13: 69-77.

8. Nabi G. How important is urinary cytology in the diagnosis of urological malignancy. *Eur Urol*, 2003; 15: 632-6.

9. Paiton E. Improved detection of urothilial carcinoma with fluorescence immune-cytochemistery and urinary cytology. *Lab Invest*, 2003; 11: 845-52.

10. Al-Rawi K, Salem MS, and Ibrahim I. Exfoliative cytology in Iraqi school children infested with Schistosoma hematobium. *J Faculty Medicine Baghdad*, 1988; 30(1): 53-7.

11. Al-Saleem T, Alash NI, and Taofik L. Bladder cancer in Iraq, the histopathological subtypes and their relationships to schistomiasis. *Ann Saudi Med*, 1990; 10: 158-63.

12. Tatomirovic Z. Importance of cytological examination of urine in the diagnosis of renal transplant function disorders. *Vojnosanit Pregl*, 2003; 60(3): 299-304.

13. Riech O. How reliable is conventional urine cytology in post transplant patients? *Urol Int*, 2003; 8: 176-7.

14. Kern WH. Comprehensive histopathology 2nd ed., Marlac Bibbo, WB Saunders Company. Philadelphia Pennsylvania 1997; p.p. 445-75.

15. Dapier E. Value of urine cytological test for detection and follow up of bladder tumor. *Prog Urol*, 2003; 13: 222-6.

16. Hsusc SC. Extra pulmonary tuberculosis infection manifested as peritoneal fluid eosinophilia in a continuous ambulatory peritoneal dialysis patient. *Nephrol Dial Transplant*, 2000; 15(2); 284-5.

17. Yarub IK. Urine cytology in patients with long standing dialysis. *Al-Kindy Coll Med J*, 2004; 15: 8-12.

18. Khutaiba K, Alash NI, and Luay T. Urine cytological examination in clinical practice. *J Faculty Med Baghdad*, 1988; 30(4): 377-88.

19. Caldorella A. Thin layer cytology in urinary diagnosis. *Pathologica*, 2003; 11: 37-41.

20. Lorenzo-Gomez MF. The role of tumor markers in urologic consultation for screening. Diagnosis and follow up of bladder cancer. *Acta Urol Esp*, 2003; 27: 110-16.

21. Nasser H, Austin RM, and Cox JT. Use of thin preparation monolayer technique and cytospin preparation ion urine cytology, a comparative analysis. *Diagno Cytopath*, 2003; 28: 115-8.

22. Gogus C, and Chapel P. Value of selective upper tract cytology for recognition of upper tract tumors after treatment of superficial bladder cancer. *Int J Urol*, 2003; 10(5): 243-8.

23. Danot SM, Khoury C, Lavey DD, and Lamb AD. Fluid intake and of the tumor recurrence in patients with superficial bladder cancer. *J Urol*, 2003; 170(5): 1777-80.

Correspondence to: Dr. Arif S. Malik,
E- mail: dr_arif31@yahoo.com

Circulating-Peripheral Blood Naturally Occurring CD4+CD25+ Regulatory T Cells and CD4+ T Cells in Chronic Rheumatic Heart Disease

Nidhal A.M. Mohammed[1] *PhD*, **Zaman I.L. Al-Kaabi[2]** *MSc*.

[1]Dept. of Microbiology, College of Medicine, Al-Nahrain University, [2]Dept of Medical Microbiology, College of Dentistry, Babylon University

Abstract

Background   The development of autoimmune disease involves a breakdown in the mechanisms that control T cell tolerance to self antigens, these mechanisms are many and complex, and they integrate as immunoregulation. Among the cells that might be responsible for this regulation is a specific type of T cells which has the ability to downregulate the differentiation of helper cells or antigen specific effector cells. The main subset of these suppressor T cells is the naturally occurring CD4+CD25+ regulatory T cells (n Tregs) which are the most important and they derived as a functionally mature population from the thymus.

Objective   The purpose of this study was to determine the correlation between the numbers of circulating CD4+CD25+ regulatory T cells (nTregs) and CD4+ T cells in chronic rheumatic heart disease patients.

Methods   Peripheral blood samples were taken from 48 Iraqi patients with chronic rheumatic heart disease (CRHD). Lymphocytes were isolated from the peripheral blood, nTregs and CD4+ T cells; also, cell numbers were detected by using immunofluorescence technique.

Results   In general, nTregs were found in lower numbers in the peripheral blood of CRHD patients in different study groups than in healthy control group, whereas, CD4+ T cells were found in higher numbers in some of patients than controls. Also, our results revealed that there was a significant negative correlation between naturally occurring CD4+CD25+ regulatory T cells and CD4+ T cells in all study groups.

Conclusions   Our finding confirmed that there is a significant correlation between circulating nTregs and CD4+ T cells in chronic rheumatic heart disease.

Key words   CD4+CD25+ regulatory T cells, CD4+ T cells, chronic rheumatic heart disease.

Introduction

An organism's immune system is designed to maintain a balance between responsiveness to infectious agents and maintenance of self-tolerance. An unresponsive immune system may result in pathogen invasion; in contrast, a hypersensitive immune reaction may result in damaging inflammatory responses [1]. Thus, the development of autoimmune disease involves a breakdown in the mechanisms that control T cell tolerance to self-antigens [2]; these mechanisms are many and complex, and they collectively known as immuno-regulation.

Among the cells that might be responsible for this regulation are a specific type of T cells which has the ability to downregulate the differentiation of helper cells or antigen-specific effector cells [3]. The main subset of these suppressor T cells are the

naturally occurring CD4+CD25+ regulatory T cells (nTregs) which are the most important and they are derived as a functionally mature population from the thymus.

Other regulatory T cells are the type 1 regulatory (Tr1) cells and Th3 cells [4,5]. A better understanding of the mechanisms underlying the induction and functions of T regulatory cells in controlling the immune system is critical in view of a future cellular therapy to modulate immune-mediated pathologies. nTreg cells were first defined in 1995 by Sakaguchi and colleagues, who showed that the passive transfer of T cells lacking in the nTregs subset into a thymic nude mice resulted in the spontaneous development of various T cell-mediated autoimmune diseases. These cells appear to be capable of suppressing a wide variety of immune cells, consisting of those from both the innate and adaptive immune systems [6].

Rheumatic heart disease (RHD) is an autoimmune most severe sequel of group A streptococcal upper respiratory tract infection complicated by rheumatic fever (RF) [7]. It describes a group of acute (short-term) and chronic (long-term) heart disorders and many of its features in the chronic stage are a result of fibrosis occurring during the healing of the acute lesion [8]. Adaptive immune responses are characterized by the capacity to recognize and remember pathogen-specific antigens. When a cognate antigen is encountered, lymphocytes become activated, undergo clonal proliferation and acquire effector functions that enable the activated cells to eliminate the intruder. However, in the acute phase of streptococcal pharingitis, streptococcal antigens (especially M protein) act as the promoter of T cell activation. It is assumed that the streptococcal antigens are initially taken up and processed by antigen-presenting cells, predominantly macrophages, which

present the antigens in the context of MHC II molecules to CD4-positive T cells [9]. CD4+ T cells are the major effectors of heart tissue lesions, and *Streptococcus*-primed T cells are able to recognize heart proteins by molecular mimicry. These T cells show a degenerate pattern of antigen recognition (streptococcal antigens and autoantigens) [10].

Naturally occurring CD4+CD25+ regulatory T cells, which comprise approximately 5-10% of peripheral CD4+ T cells, are a central component of active immune suppression [11] and populate the periphery as long-lived cells to control autoimmunity and regulate ongoing immune responses [12,13]. Here, in this study we try to determine the numbers of both nTregs and CD4+ T cells in the peripheral blood of chronic rheumatic heart disease patients to highlights the correlation between them.

Methods

This study was conducted from October 2006 to September 2007. Blood samples were taken from 48 patients with chronic rheumatic heart disease in Ibn Al-Bitar Hospital for Cardiac Surgery, Baghdad – Iraq. All patients were divided according to the positive or negative history of rheumatic fever (PHORF and NHORF), PHORF patients were subdivided according to the frequency of rheumatic fever, and according to the period of medication treatment into single attack under continuous medication (SA^{UCM}), single attack without continuous medication (SA^{WCM}), high risk under continuous medication (HR^{UCM}), and high risk without continuous medication (HR^{WCM}). Lymphocytes isolation was performed by using Ficoll method [14], nTregs and CD4+ T cells, also cell numbers were detected by using immunofluorescence staining technique [15] in the presence of Mouse anti- Human CD4 (FITC), CD25 (PE) Proteins, Dual Color (USBiological , USA)

and immuno-fluorescence microscopic slides (Biomerieux, USA). Fluorescence microscope was used with 40 X-magnification lenses at 490 nm to examine the slides immediately or 1-3 days later as a maximal duration. Detection of positive cells by observing a dot-like apple green fluorescent colored light (FITC) and red fluorescent colored light (PE) on the surface of nTregs. Whereas, CD4+ T cells were detected by observing only a dot-like apple green fluorescent colored light (FITC) on the surface of positive cells.

Statistical Analysis

The percentage of both nTregs and CD4+ T cells was measured by counting the number of positive cells/field in 5 to 10 microscopic fields to the total lymphocyte count as follows:

The percentage of positive cells = *the number of positive cells / the number of total cells X 100*.

The correlation coefficient (*r*) was calculated as a quantitative descriptive to the association between the mean percentage of CD4+ T cells and CD4+CD25+ nTreg cells among different study groups.

All statistical analysis was performed with the SPSS 10.01 statistical package for social sciences and also Excel 2003. A *p* value of less than 0.05 ($p < 0.05$) was considered significant.

Results

The percent of naturally occurring CD4+CD25+ cells was evaluated by immunofluorescence staining technique and the results shown in (Table 1 & Figure 1) displayed the difference in the mean percentage among all groups under study. High risk groups (HR^{UCM} and HR^{WCM}) displayed lower CD4+CD25+ regulatory T cells expression than single attack groups. In HR^{WCM} patients, lower percent of nTregs was recorded (1.45%) when compared with SA^{WCM} group (1.56%) and this difference was also found between groups of continuous medication (HR^{UCM} and SA^{UCM}) (3.75% and 4.12% respectively). These results refer to the difference between groups of continuous medical care which displayed higher numbers of nTregs than patients of intermittent medical therapy.
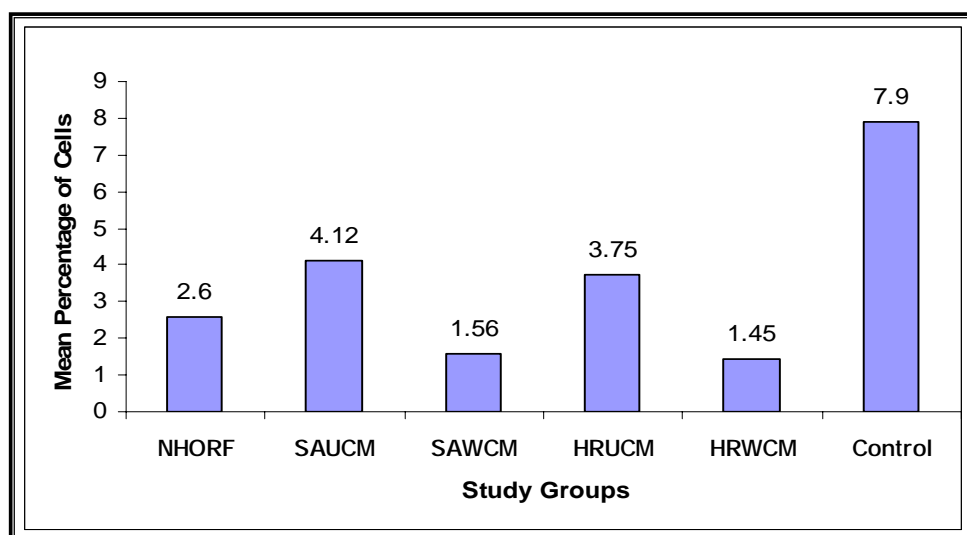

Figure1: Mean percentage of peripheral blood nTreg cells among different study groups

Negative history (NH) group had high mean percentage (2.6%) than both HR^{WCM} and

SA^{WCM} groups. In general, CD4+ CD25+ nTregs were found in lower numbers in the

peripheral blood of CRHD patients in different study groups than in healthy control group (7.9%).

Table 1: Comparison in the mean percentage of peripheral blood CD4+CD25+ regulatory T cells and CD4+ T cells among different study population groups

Group type		Patients No.	Patients (%)	Lymph No.	CD4+ & nTregs	No.	Mean percent ± SD*	Min.*	Max.*
NHORF		14	20.59	43.79	CD4+	23.43	53.16±4.710	46.42	59.57
					nTregs	0.59	2.6±0.718	1.42	4.61
PHORF	SA	5	7.35	38.8	CD4+	19.4	49.75±3.707	43.33	52.38
					nTregs	0.8	4.12±0.389	3.64	4.62
		18	26.47	48.11	CD4+	27.28	56.11±5.706	45.45	65.22
					nTregs	0.41	1.56±0.398	1	2
	HR	4	5.88	37.25	CD4+	19.5	51.72±5.536	44.82	57.5
					nTregs	0.7	3.75±0.954	2.6	4.62
		7	10.29	57.86	CD4+	38.14	65.89±4.298	63.33	68.33
					nTregs	0.54	1.45±0.539	1	2.1
Control		20	29.41	31.25	CD4+	15.55	49.82±2.721	45.83	55.55
					nTregs	1.24	7.9±1.303	5.71	10

*SD = Standard Deviation,*Min. = Minimum, *Max. = Maximum.

In this study, approximately one-half of the circulating apparently healthy human peripheral blood lymphcytes express CD4 (49.82%), and we showed that CD4+ T cells were found in higher numbers in the peripheral blood of some CRHD patients than in controls (Figure 2), and, there was a difference in the mean percentage of CD4+ T cells expression among different study groups (see table 1). From the total lymphocyte count, CD4+ T cells represents (65.89%) in HR^{WCM} group which was higher than that in SA^{WCM} patients (56.11%). Lower numbers were recorded in groups under continuous medication (UCM); HR^{UCM} and SA^{UCM} (51.72 and 49.75% respectively) when compared with patients without continuous medication (WCM). CD4 expression in patients with negative history was 53.16%, which is considered higher than that in SA^{WCM} patients but lower than HR^{WCM} group. Some of CRHD patients had normal CD4+ T cells numbers and we found that the total lymphocyte count and CD4+ T cells percentage in patient number (7-negative history), (3, 4-SA^{UCM}), (1, 4, 6-SA^{WCM}), and (1, 4-HR^{UCM}) were (28 and 46.42%), (30, 43.33%; 21, 52.38%), (22, 45.45%; 37, 54.05%; 32, 62.5%), and (29, 44.82%; 36, 50%) respectively.
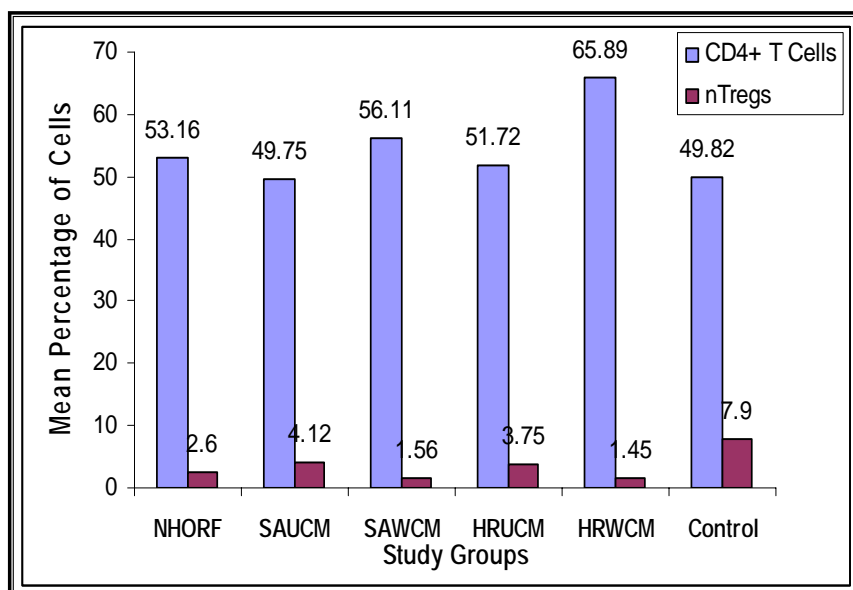
Figure 2: Mean percentage of peripheral blood CD4+CD25+ nTregs and CD4+ T cells among different study groups

According to the results above, spearman's correlation test revealed a significant negative correlation between naturally occurring CD4+CD25+ regulatory T cells and CD4+ T cells in all study groups. The groups SA^{UCM}, SA^{WCM}, and HR^{UCM} exhibited high significant difference in the mean percentage of these cells ($p < 0.05$). Also

there was a highly significant negative correlation between nTregs and CD4+ T cells ($p < 0.01$) in both negative history group and patients of HRWCM (Table 2). Figure 3 shows immunofluorescence staining (dual color) of CD4+CD25+ nTreg and CD4+ T cells in the peripheral blood of CRHD patients.

Table 2: The difference in the mean percentage of nTregs and CD4+ T cells expression among different study groups

Group type		Patients		Correlation Coefficient (r =)	P value
		No.	(%)		
NHORF		14	20.59	- 0.831	*P < 0.01 ***
PHORF	**SA^UCM**	5	7.35	- 0.921	*P < 0.05 **
	SA^WCM	18	26.47	- 0.483	*P < 0.05 **
	HR^UCM	4	5.88	- 0.973	*P < 0.05 **
	HR^WCM	7	10.29	- 0.892	*P < 0.01 ***

*Significant,   **Highly significant.
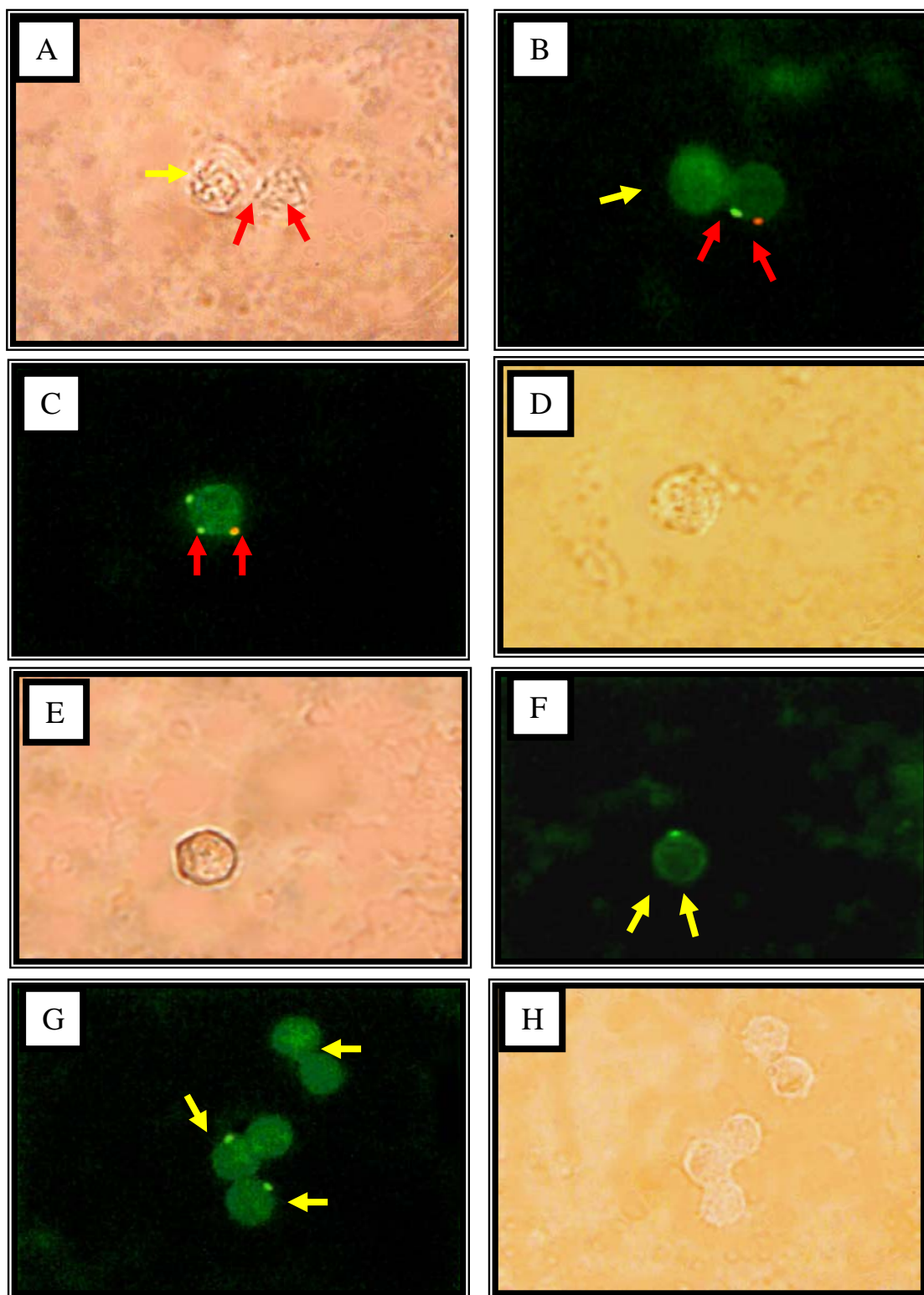
Figure 3: Immunofluoresence staining for circulating human CD4+CD25+ nTregs and CD4+ T cells in chronic rheumatic heart disease patients using mouse anti-human CD4-(FITC), CD25 (PE) proteins, dual color. A & B show CD4+ CD25+ nTregs (red arrows) and CD4+ T cells (yellow arrows). C & D indicate CD4+ CD25+ nTreg cells. E, F, G, & H show CD4+ T cells. Pictures in black color are under UV light of the immunofluoresent microscope, whereas, light pictures are under the light microscope. Microscopic magnification power: X1000.

Discussion

CD4+CD25+ nTregs represent a population of T cells that are highly specialized for suppression of immune responses and are known to play a critical role in the maintenance of peripheral self-tolerance. Approximately one-half of the circulating human peripheral blood lymphocytes express CD4, and of these roughly 10% express the IL-2 growth factor receptor α-chain, CD25, and only those which express high levels of CD25 exhibit suppressive activity in vitro,[16] but, why nTregs cannot eradicate the massive autoimmune response in vivo?

In the present work, circulating CD4+CD25+ regulatory T cells appear in lower numbers in patients with chronic rheumatic carditis than normal persons suggesting an important role for these regulatory T cells in controlling a post-infectious autoimmune disease. Our results (Table 1) show that HR^{WCM} had displayed low prevalence of nTreg cells (1.45%) than SA^{WCM}, negative history and groups of medical care, whereas, patients of SA^{UCM} group had recorded high CD4+CD25+ nTreg cell numbers (4.12%) when compared with HR^{UCM} and patients without continuous medication. Continuous inflammatory state during acute and chronic rheumatic carditis in addition to recurrent attacks of acute rheumatic fever due to exposure to a group A *Streptococcus pyogenes* antigens may lead to reduce frequency of the CD4+CD25+ nTreg cells in the peripheral blood of patients which may occur as a result of an active recruitment of regulatory T cells from circulation to the site of inflammation as a strategy of the immune system to fight an ongoing inflammation. This is consistent with the findings in animal models of chronic inflammatory colitis [17]. A previous study showed that patients with chromosome 22q11.2 deletion syndrome with developmental thymic hypoplasia had markedly fewer CD4+ CD25+ nTreg cells in infancy. That study suggested that patients with chromosome 22q11.2 deletion syndrome had a relatively pure quantitative defect in T-cell production and inclusively CD4+CD25+ nTreg cells which found in fewer numbers in infants, in addition to that, the study suggested that regulation of nTreg-cell production early in life, in humans, is directly related to thymic capacity, and this phenomenon could play a role in the predisposition to autoimmune disease in patients with chromosome 22q11.2 deletion syndrome [18] which may be related with the lower numbers of nTregs in the present CRHD patients if this syndrome is considered one of the causes of autoimmunity in our study.

Nevertheless, we found some of patients had nTregs numbers near to the normal values as in patients with continuous medication, patients number 1, 2, and 3-SA^{UCM} who had 4.17, 4.34, and 4.62 % , and patients number 1, and 4-HR^{UCM} who had 4.62, and 4.44% respectively, and also patient number 7-negative history who had 4.61% of nTregs.

Therefore, many factors may interfere with nTregs function to make them inactive and abrogate their suppressive function. One of these factors believed to play an important role in inhibiting the functional activity of nTreg cells is the tumor necrosis factor alpha. Also, toll-like receptors (TLRs) are primary sensors of both innate and adaptive immune systems and play a pivotal role in the response against structurally conserved components of pathogens. Many researchers found that toll-like receptor-2 signaling in T cells had distinct effects on effectors and nTreg cells. In addition to that, they showed that bacterial lipoprotein (BLP), together with anti-CD3 antibody [T cell receptor (TCR) activation], induced proliferation of both CD4+CD25+ nTregs and CD4+CD25-

(effector) T cells in the absence of antigen-presenting cells. The expanded nTregs showed a transient loss of suppressive activity and suppression of the induction of Foxp3 mRNA in Tregs at the first 8-15 hours after T cell receptor activation [19]. One explanation for the infection-dependent induction of TLR2 might be related to the presence of Gram-positive *Streptococcus pyogenes* [20], therefore, the presence of streptococcal M protein which is known to bind with TLR-2 may be play an important role in loss of naturally occurring CD4+CD25+ nTreg cells their suppressive function.

According to our results, there was no correlation between disease severity and the low prevalence of CD4+CD25+ nTreg cells when compared with patient's clinical and physical parameters because the severity of disease may return to more than one factor that affect the disease activity, among them, number of acute rheumatic fever attacks, duration of disease, treatment, host susceptibility to suppressed autoimmunity, and the host susceptibility to the formation of fibrosis after tissue damage. Peripheral blood CD4+ T cells appeared in high numbers in CRHD patients when compared with controls. For several factors that make patients of HR^{WCM} group more exposed to group A streptococci infection and recurrent attacks of acute rheumatic fever, HR^{WCM} group displayed the highest percentage of CD4+ T cells (65.89%) than all groups under study which confirm the continuous inflammatory state even in the chronic stage of RHD. No significant difference was recorded in the mean percentage of CD4+ T cells among negative history, SA^{WCM}, HR^{WCM}, and also in SA^{UCM}, and HR^{UCM} groups when compared with others. The main cause of increasing the number of CD4+ T cells in some of patients with continuous medical therapy [SA^{UCM}-patient number 1: 51.06% , 2: 50% , 4: 52.38% and

5: 52%] and [HR^{UCM}-patient number 2: 57.5 %, 3: 54.54%, and 4: 50 %] may be referred to the bacterial resistance for penicillin. Long-term management is known to involve regular penicillin prophylaxis in high-risk patients, to prevent further episodes of rheumatic fever [21]. *Streptococcus pyogenes* has been shown to be resistant to penicillin, but because penicillin is inexpensive and available in most countries, it remains the drug of choice for treating group A streptococcal infections [22,23]. Also, other study found that streptococci are becoming increasingly resistant to penicillin and other β-lactams, owing to a decreased β-lactams affinity of their membrane-bound penicillin binding proteins [24] .Thus, more attacks of acute rheumatic fever due to penicillin resistant *Streptococcus pyogenes* A bacteria will affect the heart and lead to increase in the inflammatory response in which CD4+ T cells play the major role. Although CD4+ T cells displayed high numbers in some of patients with medical care, but this study found that other patients had lower values nearest to the normal range [SA^{UCM}-patient number 3: 43.33 %], and [HR^{UCM}-patient number 1: 44.82%], and this result may confirm the immunosuppressive role of naturally occurring CD4+CD25+ nTreg cells (which was found in high numbers in these medical care groups) against autoreactive CD4+ T cells.

In this regard, there was a significant correlation between CD4+CD25+ nTreg cells and CD4+ T cells expression in SA^{UCM}, SA^{WCM}, HR^{UCM} groups (p< 0.05), and highly significant correlation was recorded in the negative history and HR^{WCM} patients (p< 0.01). These results reveal the important role of CD4+CD25+ nTreg cells in autoreactive CD4+ T cells suppression leading to reverse the autoimmune reaction against the heart. At the same time these results strongly explain the role of CD4+ T cells in increasing the severity of

disease which is considered the main enhancer of acute and chronic rheumatic heart damage.

CD4+CD25+ regulatory T cells which has a critical role in immune suppression and reversing the autoimmunity were found in very lower numbers, and this may explain to a certain degree why the autoimmune-inflammatory process still active in rheumatic heart disease.

Acknowledgement

The authors would like to thank all the staff in Microbiology and Pathology Departments / College of Medicine / Al-Nahrain University, Ibn Al-Bitar Hospital for Cardiac Surgery, Al-Kadhimya Teaching Hospital, and Laboratory of Health Centre for their assistance in this study.

References

1. Lan R, Ansari A, Lian Z, and Gershwin M. Regulatory T cells: Development, function and role in autoimmunity. *Autoimmunity Reviews*, 2005; 4: 351-363.
2. Crispin J, Vargas M, and Alcocer-Varela J. Immunoregulatory T cells in autoimmunity. *Autoimmunity Rev*, 2004; 3: 45-51.
3. Bach J F. Regulatory T cells under scrutiny. *Nature Rev Immunol*, 2003; 3: 189-198.
4. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S., et al. Immunologic tolerance maintained by CD4+CD25+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev*, 2001; 182: 18-32.
5. Berthelot JM and Maugars Y. Role for suppressor T cells in the pathogenesis of autoimmune diseases (including rheumatoid arthritis). *Facts and hypotheses. Jt Bone Spine,* 2004; 71(5): 374-380.
6. Fehervari Z and Sakaguchi S. CD4+ Tregs and immune control. *J Clin Invest*, 2004; 114(9): 1209-1217.
7. Hernandez-Pacheco G, Flores-Dominguez C, and Rodriguez-Perez J. Tumor necrosis factor-alpha promoter polymorphisms in Mexican patients with rheumatic heart disease. *J Autoimmun*, 2003; 21: 59-63.
8. Julian D, Cowan J, and McLenachan J. Cardiology. 7th ed. London, WB Saunders Company Ltd., WBC, Bridgend, Mid Glamorgan. 1998.
9. Bronze MS and Dale JB. The re-emergence of serious groups A streptococcal infections and acute rheumatic fever. *American Journal of Medical Science,* 1996; 311(1): 41-54.
10. Guilherme L, Fae K, Oshiro S, Tanaka AC, Pomerantzeff PM, and Kalil J. How S. pyogenes-primed peripheral T cells trigger heart valve lesions. *Ann NY Acad Sci,* 2005; 1051: 132-140.
11. Shevach EM. Regulatory T cells in autoimmunity. *Annu Rev Immunol*, 2000; 18: 423-449.
12. Lerman MA, Larkin J 3rd, Cozzo C, Jordan MS, and Caton AJ. CD4+CD25+ regulatory T-cell repertoire formation in response to varying expression of a neo-self-antigen. *J Immunol*, 2004; 173: 236-244.
13. Hsieh CS, Liang Y, Tyznik AJ, Self SG, Liggitt D, and Rudensky AY. Recognition of the peripheral self by naturally arising CD4+CD25+ T-cell receptors. *Immunity*, 2004; 21: 267-277.
14. Boyum A. Isolation of Mononuclear Cells and Granulocytes from Human Blood. Scand. *J. Clin. Lab. Invest*, 1968; 21 (97): 77-89.
15. Wigzell H, and Anderson B. Isolation of Lymphoid Cells Active Surface Receptors Sites. *Annu. Rev. Microbiol*, 1971; 25: 291-299.
16. Baecher-Allan C, Brown J, Freeman G, and Hafler D. CD4+CD25 high Regulatory cells in human peripheral blood. *The Journal of Immunology*, 2001; 167: 1245-1253.
17. Mottet C, Uhlig H, and Liew F. Cutting edge: Cure of colitis by CD4+CD25+ regulatory T cells. *J Immunol*, 2003; 170: 3939.
18. Sullivan K, McDonald-McGinn D, and Zackai E. CD4+CD25+ T-Cell Production in Healthy Humans and in Patients with Thymic Hypoplasia. Clinical and diagnostic laboratory immunology. *American Society for Microbiology*, 2002; 9(5): 1129-1131.
19. Liu W, Putnam AL, Xu-yu Z, Szot GL, Lee MR, Zhu S, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ Tregs. *J Exp Med*, 2006; 203: 1701-1711.
20. Heine H, and Lien E. Toll-like receptors and their function in innate and adaptive immunity. *Int Arch Allergy Immunol*, 2003; 130: 180-192.
21. Bayer AS, Bolger AF, Taubert KA, Wilson W, Steckelberg J, Karchmer AW, et al. Diagnosis and management of infective endocarditis and its complications. *Circulation*, 1998; 98: 2936-2948.
22. Warrington RJ, Lee KR, and McPhillips S. The value of skin testing for penicillin allergy in patient population: Analysis of the subsequent

patient management. *Allergy Asthma Procedings*, 2000; 21(5): 297-299.

23. Forrest DM, Schellenberg RR, Thien VV, King S, et al. Introduction of practice guidelines for penicillin skin testing improves the appropriateness of antibiotic therapy. *Clinical Infectious Diseases*, 2001; 32(12): 1685-1690.

24. Moreillon P, and Que Y. Infective endocarditis. *Lancet*, 2004; 363: 139-149.

The Effect of Body Mass Index and Waist Circumference on Prostate Specific Antigen in Patients with Benign Prostatic Hyperplasia (BPH)

Saad D. Farhan *FICMS*

Dept. of Urology, College of Medicine, Baghdad University.

Abstract

Background   Obesity may be associated with lower prostate specific antigen(PSA) values, If true, this would result in fewer obese men having an elevated PSA, fewer biopsies performed, and fewer cancers detected , consequently cancers may be missed or not detected until at a more advanced stage.

Objective   We examined the influences of age, body mass index (BMI) and waist circumference (WC) on PSA before and after adjusting for prostate volume. We also examined associations among age, body mass index, waist circumference and prostate volume (PV).

Methods   We analyzed 125 Iraqi men aged 40 to 84 years old who attained the urological outpatient clinic for BPH evaluation during 2009. Curent health status information including prostate related problems, medical interview, basic physical examination and anthropometric measurements including height, weight, BMI and waist circumference were taken for all patients. Blood tests including PSA concentration were performed after overnight fast. A radiologist performed transrectal prostate ultrasound. PSA measurements preceded routine digital examination and transrectal prostate ultrasound.

Results   The median serum PSA was significantly lower among obese subjects compared to normal BMI subjects. BMI showed a statistically significant moderately strong negative linear correlation (r = -0.5) with serum PSA. Waist circumference showed a similar pattern with a statistically significant linear correlation with serum PSA (r = -0.43); the median serum PSA was significantly lower among subjects with highest waist circumference compared to subjects in the lowest quartile of waist circumference. The median PSA was significantly higher among subjects with large prostate size compared to those with lowest quartile prostate size. The anthropometric measures were tested for association with PSA density, to adjust for the effect of prostate size on serum PSA.

Conclusion   The current data suggest that the PSA cut-points used to recommend biopsy need to be adjusted for the degree of obesity.

Keywords   body mass index, waist circumference, prostate specific antigen.

Introduction

Many investigators evaluated the relationship between body habitus, body mass index (BMI), and obesity and lower urinary tract symptoms/benign prostate hyperplasia (LUTS/BPH). There are plausible biologic considerations: adipose tissue is the main source of aromatization of testosterone to estrogen, and men with lower BMI have higher serum testosterone levels [1]. Several caveats must be mentioned:

digital rectal examination (DRE) is less likely to yield a diagnosis of BPH and prostate enlargement in very obese patients because of anatomic obstacles and patients with high BMI may be biased against surgical interventions [1,2].

Recent studies [3-8] suggest obesity may be associated with lower prostate specific antigen (PSA) values, If true, this would result in fewer obese men having an elevated PSA, fewer biopsies performed, and fewer cancers detected, consequently cancers may be missed or not detected until at a more advanced stage.

In contrast, in a study of 68 men, the average prostate weight increased both with age and with increasing obesity together with an increase in serum estradiol levels [9].

Despite its wide spread use, the PSA test is limited by low specificity and reduce sensitivity beyond a specific cutoff value [10,11]. Another problem is that PSA is affected by many non cancer related factors, Understanding those factors and how they interrelate would increase test usefulness, PSA is known to increase with age and prostate volume, and investigators have suggested that age specific PSA and density might provide more accurate clinical assessments than a single reference range applied to men of all ages [12].

Waist circumference (WC), which represents central obesity, is an important clinical parameter that has greater impact than BMI on metabolic disease incidence and its related mortality [13].

To clarify the influence of obesity, defined by BMI or WC, on serum PSA, We examined the magnitude of the association among BMI, WC, prostate volume (PV), and PSA.

Methods

Eligible subjects were 125 men of 40 to 84 years old who attained the urological outpatient clinic for BPH evaluation during 2009. Current health status information including prostate related problems, medical interview and basic physical examination, and anthropometric measurements including height, weight, BMI, and WC were done for all patients.

BMI was calculated as weight in kilograms divided by the square of the height in meters (Kg/m^2) [12].

WC was measured at the part of the trunk located midway between the lower costal margin (bottom of lower rib) and the iliac crest (top of pelvic bone) while the person is standing, with feet about 25-30 cm apart (10-12 in). The measurer should stand beside the individual and fit the tape snugly, without compressing any underlying soft tissues. The circumference should be measured to the nearest 0.5 cm (1/4 in), at the end of a normal expiration.

Blood tests including PSA concentration were performed after subjects fasted overnight; the test was done in the same Lab. A radiologist performed transrectal prostate ultrasound and PSA measurements precede routine digital examination and transrectal prostate ultrasound. PSA density (adjusted PSA for prostate volume) was calculated as PSA divided by Prostate volume.

We exclude from the study men who had a history of prostate cancer, prostate surgery or prostatitis, and who were taking anti androgenic medication such as finasteride. We then exclude men whose serum PSA concentration was greater than 6.5 ng/ml because of the increase the probability of prostate related disease or data error [14]. Patients with PSA density more than 0.12 and those with abnormal DRE were excluded because of increased risk of malignancy, and those with neurological history for lower urinary tract like diabetes mellitus [14].

Results

The results presented in this study were based on the analysis of 125 males with symptoms of BPH. Their age ranged between 40 and 84 years with a mean of 62.7±8.9(SD) years. About one half of the samples were in the 6th decade of life, while those under 50 years of age constituted only 7.2% of the sample. About quarter of the sample were of normal BMI, while obese subjects constituted 39.2% of the sample (Table 1).

The median serum PSA showed week significant changes with age, however no obvious or statistically significant linear trend (r= -0.12, *p*= 0.2) was elucidated.

The median serum PSA was significantly lower among obese subjects (1.5) compared to normal BMI subjects (3.8). BMI showed a statistically significant moderately strong negative linear correlation (r= -0.5) with serum PSA.

Waist circumference showed a similar pattern with a statistically significant linear correlation with serum PSA (r= -0.43). Serum PSA was significantly lower among subjects with highest waist circumference (1.6) compared to subjects in the lowest quartile of waist circumference [3].

The median PSA was significantly higher among subjects with largest prostate size [4] compared to those with lowest quartile prostate size [2]. The prostate size showed a statistically significant weak positive linear correlation (r= -0.24) with serum PSA (Table 2).

The anthropometric measures were tested for association with PSA density, to adjust for the effect of prostate size on serum PSA.

The median PSA density was significantly lower among obese subjects (0.024) compared to normal BMI subjects (0.051) and lowest quartile of waist circumference (0.05), both BMI and WC showed a similar pattern with statistically significant negative linear correlation with PSA density (r= -0.47) (r= -0.51) (Table 3).

A multiple linear regression model was used to study the net and independent effect of each anthropometric measure on serum PSA after adjusting for age and prostate size. Age had no important or statistically significant association with serum PSA after adjusting for other explanatory variables included in the model.

Prostate size and the anthropometric had a significant association with serum PSA. All these explanatory variables were of almost equal importance in predicting the magnitude of serum PSA.

For each one cm^3 increase in prostate size the serum PSA is expected to increase by mean of 0.017 after adjusting for the remaining explanatory variables included in the model.

For each one Kg/m^2 increase in BMI the serum PSA is expected to decrease by a mean of 0.102 after adjusting for the remaining explanatory variables included in the model.

For each one cm increase in waist circumference the serum PSA is expected to decrease by a mean of 0.041 after adjusting for the remaining explanatory variables included in the model (Table 4).

Table 1: Frequency distribution of the study sample by age and Body mass index.

Age group (years)	No (%)
<50	9(7.2%)
50-59	30(24%)
60-69	59(47.2%)
70+	27(21.6%)
BMI (Kg/m^2)-Categories [15,16]	
Normal (< 25)	30(24%)
Overweight (25-29.9)	46(36.8%)
Obese (30+)	49(39.2%)
Total	125(100%)

Table 2: The median serum PSA by age and selected anthropometric measures.

	PSA (prostate specific antigen)				
	Range	Median	Interquartile range	No.	p
Age group (years)					0.016
<50	(1.8-5.6)	2	(2-3.3)	9	
50-59	(0.1-6)	1.6	(1.5-2.1)	30	
60-69	(0.2-10)	3	(1.6-4)	59	
70+	(0.1-10)	2.2	(1.5-4.8)	27	
r= -0.12 p= 0.2[NS]					
BMI (Kg/m^2)-Categories					<0.001
Normal (< 25)	(1-10)	3.8	(2-5)	30	
Overweight (25-29.9)	(0.1-10)	3	(2-4.2)	46	
Obese (30+)	(0.1-8)	1.5	(1-2.1)	49	
r= -0.47 p < 0.001					
Waist circumference (cm)-Quartiles					< 0.001
First (lowest) quartile (≤ 86.4)	(1-10)	3	(2-5)	40	
Second quartile (86.5-94.0)	(1.2-5.6)	3.2	(1.7-4.8)	23	
Third quartile (94.1-109.2)	(0.1-7)	1.8	(1-3.4)	43	
Fourth (Highest) quartile (109.3+)	(0.1-3)	1.6	(1-2.5)	19	
r= -0.51 p < 0.001					
Prostate size (cm^2)-Quartiles					0.01
First (lowest) quartile (≤ 44)	(0.1-8)	2	(1.5-2.5)	32	
Second quartile (45-70)	(0.1-10)	2	(1.5-3)	35	
Third quartile (71-92)	(0.1-10)	2	(1.5-3)	29	
Fourth (Highest) quartile (93+)	(0.2-6)	4	(2.3-5)	29	
r= 0.24 p < 0.001					

Table 3: The median PSA density by age and selected anthropometric measures.

		PSA density				
		Range	Median	Interquartile range	No.	P
1.	Age group (years)					0.13[NS]
	<50	(0.022-0.071)	0.05	(0.034-0.063)	9	
	50-59	(0.002-0.182)	0.032	(0.015-0.05)	30	
	60-69	(0.001-0.182)	0.033	(0.024-0.05)	59	
	70+	(0.001-0.148)	0.05	(0.026-0.06)	27	
	r= -0.03 p= 0.71[NS]					
2.	BMI (Kg/m^2)-Categories					< 0.001
	Normal (< 25)	(0.011-0.133)	0.051	(0.029-0.063)	30	
	Overweight (25-29.9)	(0.002-0.182)	0.045	(0.033-0.059)	46	
	Obese (30+)	(0.001-0.182)	0.024	(0.015-0.041)	49	
	r= -0.47 p < 0.001					
3.	Waist circumference (cm)-Quartiles					< 0.001
	First (lowest) quartile (≤ 86.4)	(0.011-0.182)	0.05	(0.034-0.106)	40	
	Second quartile (86.5-94.0)	(0.015-0.148)	0.04	(0.03-0.05)	23	
	Third quartile (94.1-109.2)	(0.001-0.152)	0.029	(0.015-0.05)	43	
	Fourth (Highest) quartile (109.3+)	(0.001-0.06)	0.024	(0.011-0.035)	19	
	r= -0.51 p < 0.001					

Table 4: Multiple Linear regression model with serum PSA (prostate specific antigen) as the dependent (outcome) variable and age, prostate size in addition to selected anthropometric measures as the independent (explanatory) variables

	Unstandardized Regression coefficient	Standardized Regression coefficient	Sig.
(Constant)	-7.6		0.10[NS]
Age (years)	0.026	0.114	0.17[NS]
Waist circumference (cm)	-0.041	-0.288	0.002
Prostate size (cm^2)	0.017	0.297	< 0.001
BMI (Kg/m^2)	-0.102	-0.278	0.002

R^2=0.34
p (model) < 0.001

Discussion

When we evaluate obese men with prostate enlargement, it may be important to consider that the obesity my lower base line PSA and obese men with early prostate cancer disease are increased risk for having PSA lower than the screening cutoff value [10,11,17].

Thus, it is important to examine the influence of obesity and its related factors including PV on PSA in the general screening population.

In the present study BMI and WC were negatively associated with PSA and adjusted PSA for prostate volume (PSA density). Therefore, we concluded that PSA is

negatively associated with obesity (measured by BMI or WC), positively associated with PV and weakly associated with age.

Our study demonstrated that although obese men generally have higher PV than non obese men, they have a lower PSA, but the reason for that is not well known. Previous investigators have suggested that the inverse link between obesity and PSA levels is explained either by endocrine disturbances associated with abdominal obesity, Obesity leads to greater aromatization of testosterone and may associated with lower PSA production [17-19].

The volume dilution theory appears to more closely predict the inverse association between prostate-specific antigen (PSA) levels and obesity than the hormone disturbance theory. A more recent suggestion is that lower PSA are largely due to haemodilution by a large plasma volume in obese men [20-23].

These data demonstrate that PSA concentrations in prostate-cancer-free men inversely correlate with BMI, due to a rise in plasma volume with increasing BMI. Studies confirmed that Higher BMI correlated with higher plasma volumes. Partially adjusted mean PSA levels, on the other hand, decreased with increasing BMI. This relationship between BMI and PSA did not exist when investigators controlled for plasma volume. These data demonstrate that PSA concentrations in prostate-cancer-free men inversely correlate with BMI, due to a rise in plasma volume with increasing BMI [20-23].

In one theory, fat mass, but not lean mass and abdominal fat will be inversely associated with PSA levels, while both lean and fat mass, independent of body fat distribution, will be inversely associated with PSA levels in the second theory [17-19].

In men undergoing radical prostatectomy, higher BMI was associated with higher plasma volume; haemodilution may therefore be responsible for the lower serum PSA concentrations among obese men with prostate cancer [24-26].

To investigate further, Be-Long Cho, from Seoul National University Hospital, and colleagues studied 3593 Korean men aged 30-79 years who received regular check-ups at a health examination center and for whom prostate volume data were available; Obesity had a negative association with prostate specific antigen regardless of prostate volume, and a positive association with prostate volume. Age was not associated with prostate specific antigen after prostate volume adjustment. Obese men, especially those with a small prostate volume, may have lower baseline prostate specific antigen and, thus, be at higher risk for having prostate cancer undetected in a prostate specific antigen screening test [12].

The current data suggest that the PSA cut-points used to recommend biopsy need to be adjusted for the degree of obesity [10,12,27]. Further studies to correlate these results in relation to different races, geographic distribution, and specific age group relation may be needed.

References

1. Kristal AR, Chi C, Tangen CM, Goodman PJ, Etzioni R, and Thompson IM. Association of demographic and life style characteristics with prostate specific antigen (PSA) concentration and rate of PSA increase. *Cancer*, 2006; 106: 320.
2. Riehmann M, Rhodes PR, Cook TD, Grose GS, Bruskewitz RC. Analysis of variation in prostate-specific antigen values. *Urology*, 1993; 42: 390-397.
3. Culp S, and Porter M. The effect of obesity and lower serum prostate-specific antigen levels on prostate-cancer screening results in American men. *BJU Int*, 2009; 104(10): 1457-1461.
4. Ando R, Nagaya T, Hashimoto Y, Suzuki S, and Itoh Y. Inverse relationship between obesity and serum prostate specific antigen level in healthy Japanese

men: a hospital-based cross sectional survey, 2004-2006. *Urology*, 2008; 72: 561.

5. Werny DM, Thompson T, Saraiya M, and Freedman D. Obesity is negatively associated with prostate-specific antigen in U.S. men, 2001-2004. *Cancer Epidemiol Biomarkers Prev*, 2007; 16: 70-76.

6. Fowke JH, Signorello LB, Chang SS, Matthews CE, Buchowski MS, Cookson MS, et al. Effects of obesity and height on prostate-specific antigen (PSA) and percentage of free PSA levels among African-American and Caucasian men. *Cancer*, 2006; 107(10): 2361-2367.

7. Randle A, Richards C, Neugut AI. Body composition of abdominal fat distribution and prostate specific antigen test results. *Cancer Epidemiol*, 2009; 18(1): 331-336.

8. Ando R, Nagaya T, Hashimoto Y, Suzuki S, Itoh Y, Umemoto Y, et al. Inverse relationship between obesity and serum prostate-specific antigen level in healthy Japanese men: a hospital-based cross-sectional survey, 2004-2006. *Urology*, 2008; 72(3): 561-565.

9. Chia SE, Lau WK, Chin CM, Tan J, Ho SH, Lee J, et al. Effect of ageing and body mass index on prostate-specific antigen levels among Chinese men in Singapore from a community-based study. *BJU Int*, 2009; 103(11): 1487-1491.

10. Schröder FH, Roobol MJ. Defining the optimal prostate-specific antigen threshold for the diagnosis of prostate cancer. *Curr Opin Urol*, 2009; 19 (3): 227-231.

11. Catalona WJ, Ramos CG, Carvalhal GF, and Yan Y. Lowering PSA cutoffs to enhance detection of curable prostate cancer. *Urology*, 2000; 55: 791-795.

12. Parka JH, Chob BL, Kwon HT, Lee CM, Han HJ. Effect of body mass index and waist circumference on prostate specific antigen and prostate volume in a generally healthy korean population. *J Urol*; 2010; 182(1): 106-111.

13. Janssen I, Katzmarzyk P, and Ross R. Waist circumference and not body mass index explains obesity-related health risk. *Am J Clin Nutr*, 2004; 79: 379-384.

14. Joseph C, Presti JR, Christopher J, Kane MD, Shinohara K, and Carroll PR. Neoplasm of the prostate gland. Smith General Urology, Chapter 22, pp. 360-361.

15. Davies PS, Lanham DA, Stead MA, Tsang K. Body mass index and the prediction of percentage body fat in Australian and Chinese women. *Ann Hum Biol*, 2001; 28: 467-470.

16. Gallagher D, Visser M, Sepulveda D, Pierson RN, Harris T, and Heymsfield SP. How useful is BMI for comparison of body fatness across age, sex, and ethnic groups. *Am J Epidemiol*, 1996; 143: 228-239.

17. Beebe-Dimmer JL, Faerber GJ, Morgenstern H, Werny D, Wojno K, Halstead-Nussloch B, et al. Body composition and serum prostate-specific antigen: review and findings from Flint Men's Health Study. *Urology*, 2008; 71(4): 554-560.

18. Svartberg J, von Muhlen D, Sundsfjord J, and Jorde R. Waist circumference and testosterone levels in community dwelling men. The Tromso study. *Eur J Epidemiol*, 2004; 19: 657-663.

19. Baillargeon J, Pollock BH, and Kristal AR. The association of body mass index and prostate-specific antigen in a population-based study. *Cancer*, 2005; 103: 1092-1095.

20. Porter MP, Stanford JL. Obesity and the risk of prostate cancer. *Prostate*, 2005; 62: 316-321.

21. Bañez LL, Hamilton RJ, Partin AW, Vollmer RT, Sun L, and Rodriguez C. Obesity-related plasma hemodilution and PSA concentration among men with prostate cancer. *Eur Urol*, 2008; 54(1): 232-233.

22. Ohwaki K, Endo F, Muraishi O, Hiramatsu S, and Yano E. Relationship between Prostate-specific Antigen and Hematocrit: Does Hemodilution Lead to Lower PSA Concentrations in Men with a Higher Body Mass Index? *Urology*, 2010 Mar; 75(3): 648-52.

23. Lee SH, Kim JC, Lee JY, Kim JH, Oh CY, Lee SW et al. Effect of obesity on lower urinary tract symptoms in Korean benign prostate hyperplasia patients. *Asian J Androl*, 2009; 11: 663-668.

24. Price MM, Hamilton RJ, Robertson CN, Butts MC, and Freedland SJ. Body mass index, prostate-specific antigen, and digital rectal examination findings among participants in a prostate cancer screening clinic. *Urology*, 2008; 71(5): 787-791.

25. Potosky AL, Feuer EJ and Levin DL. Impact of screening on incidence and mortality of prostate cancer in the united states. *Epidemiol Rev*, 2001; 23: 181.

26. Partin AW, Piantadosi S, and Subong EN. Clearance rate of serum-free and total PSA following radical retropubic prostatectomy. *Prostate*, 1996; 7 Suppl: 35-39.

27. Catalona WJ, Hudson MA, and Scardino PT. Selection of optimal prostate specific antigen cutoffs for early detection of prostate cancer: Receiver operating characteristic curves. *J Urol*, 1994; 152: 2037-2042.

Correspondence to: Dr. Saad D. Farhan,
E-mail: Saadurology@yahoo.com
Received: 18th Feb. 2010, Accepted: 3rd Mar. 2010

Association of Dyslipidemia and Obesity After Menopause

Bushra H.B. Zillo[1] *PhD*, **Hedef D. El-Yassin**[2] *PhD*, **Hala A. Al-Moayed**[3] CABOG,
Ghassan A. Al-Shamma[1] *PhD*.

[1]Dept. of Chemistry and Biochemistry, [3]Dept. of Obstetrics & Gynecology, College of Medicine, Al-Nahrain University. [2]Dept. of Physiological Chemistry, College of Medicine, Baghdad University.

Abstract

Background   Aging and hormonal changes in menopause are factors which may play the most important role in the development of all events of this stage of women's life. Of importance in this respect is obesity and related events as insulin resistance, oxidative stress, dyslipidemia and consequent increase in the risk of cardiovascular disease.

Objective   To evaluate the association of dyslipidemia with the development of obesity and related events as lipid peroxidation after menopause, and to correlate the different parameters with each other.

Methods   Thirty seven premenopausal women aged (33.95±7.9 years) and 41 postmenopausal women aged (59.9±7.2 years) were involved in this study. All were normal and without a previous history of any disease or illness. Blood specimens were collected after 12 hour fast for measurement of serum lipids (total cholesterol TC, Triglycerides TG, and high density lipoprotein cholesterol HDL-C) by enzymatic spectrophotometric methods. Low density lipoprotein cholesterol (LDL-C) was estimated by calculation. The athrogenic index (AI) indicates the ratio of LDL-C to HDL-C, while sex hormones (estradiol, E_2, follicle stimulating hormone, FSH and luteinizing hormone, LH) and oxidized LDL (ox-LDL) were measured by ELISA methods. All participants were subjected to anthropometric measurements including body mass Index (BMI) and waist circumference (WC).

Results   Marked significant increase in BMI and WC in the postmenopausal women as compared with the premenopausal women and was associated with a significant low E_2, high serum TG, TC, LDL-C, AI and ox-LDL with low HDL-C.

Conclusion   Changes in serum levels of sex hormones at menopause may be the trigger for the development of post-menopausal obesity, dyslipidemia and elevated Ox-LDL, and that at a given age all these factors should be considered for evaluation of body fat distribution and related health risks.

Key Words   menopause, obesity, dyslipidemia, sex hormones, ox-LDL

Introduction

The main circulating estrogen during the premenopausal age is 17β- Estradiol. Levels of this hormone are controlled by the developing follicle and resultant corpus luteum. Essentially all estradiol in post-menopausal women is derived from peripheral conversion from estrone [1].

This is due to estrone production, resulting largely from peripheral aromatization of androstendione. This aromatase activity increases with aging by two to four folds; this is further amplified by increased adiposity that typically accompanies the aging process [2].

Menopause was found to associate changes in body weight or distribution of body fat.

An apple shaped body or male type distribution of adipose tissue, is associated with high risk of coronary heart disease (CHD) than pear-shaped body or female-type distribution of body fat. This factor may be quantified by using the waist circumference (WC) [3].

Insulin resistance, which associates obesity, increases the activity of hormone sensitive lipase in adipose tissue resulting in increased level of circulating fatty acids. These fatty acids are carried to the liver to be converted to TG and cholesterol. The major components of the dyslipidemia that may associate obesity are increased TG and modified small dense LDL particles with decreased HDL and impairment of LDL-receptor (LDL-R) activity which contributes to delayed TG rich lipoprotein clearance [4]. These modified LDL are mostly taken up by macrophage scavenger receptors, rather than the normal LDL-R pathway, thus inducing athero-sclerosis and increasing the risk of cardiovascular disease [4,5].

The present study was designed to study the link between obesity, dyslipidemia and ox-LDL with the changes in sex hormones in the postmenopausal period.

Methods
Subjects
The study was carried out during the period from August 2008 till January 2009. It included 37 pre-menopausal women with age range of 18-43 years and 41 postmenopausal women with age range of 47-73 years. All women were attending AL-Kadhimya Teaching Hospital. They were, all, healthy with no previous illness or taking any drug which may interfere with any of the tests in this study.

All women of the study were subjected to anthropometric measurements including BMI and WC, and both groups of the study were subdivided according to BMI into 3 subgroups: normal (< 25 Kg/m^2), overweight (25-29.9 Kg/m^2) and obese (> 30 Kg/m^2).

Methods
Ten mls of blood were collected into a plain tube in the morning after 12 hour fast. The serum obtained after centrifugation of blood at 3200 rpm for 10 min. was separated and divided into small aliquots for measurement of serum E2, FSH, LH and Ox-LDL by ELISA technique. Serum lipids (TG, TC, and HDL-C) were determined by enzymatic spectrophotometric methods (Kits from BioMereux, France). The LDL-C was calculated according to Friedwald formula [6] and the atherogenic index (AI) is the ratio of LDL-C to HDL-C.

Results
In addition to the higher age in the postmenopausal women and the significant reduction in E2 and elevation in FSH and LH, there was also a significant rise in TG, TC and LDL-C with a significant reduction in HDL-C and a significantly higher atherogenic index, AI (Table 1).

Table 1: Biochemical parameter values in the pre- and post menopausal women

Parameters	Pre-menopausal	Post-menopausal
Age (years)	33.95±7.9	59.9±7.2
BMI (Kg/m²)	26.66±4.86	28.58±4.78
WC (cm)	80.6±10.0	89.53±12.00*
TC (mg/dl)	178.3±21.3	207.5±34.4*
TG (mg/dl)	126.3±39.3	140.9±55.8
HDL-c (mg/dl)	53.3±6.0	47.02±6.9*
LDL-c (mg/dl)	99.0±24.7	132.12±34.65*
AI	1.9±0.7	3.00±1.14*
LH (ρg/ml)	6.6±2.3	41.58±11.64*
FSH (ρg/ml)	7.2±2.2	55.39±12.54*
E_2 (ρg/ml)	190.7±48.1	56.46±15.48*
OX-LDL (U/l)	57.8±22.3	79.85±35.29*

*p 0.05

In table 2 the comparison between different BMI subgroups of the pre-menopausal group showed a significant increase in BMI, WC, TG and ox-LDL ($P < 0.01$) with significant decrease in HDL-C in the obese subgroup as compared to the normal or overweight women, but no significant changes in the other parameters (TC, LDL-C and sex hormones) could be seen.

Table 2: The biochemical parameter values in the BMI subgroups of the pre-menopausal women

Parameters	Premenopausal > 25 (kg/m²)	Premenopausal 25-29.9 (kg/m²)	Premenopausal > 30 (kg/m²)
Number	13	13	11
Age (years)	30.23±8.39	35.23±6.7	36.82±7.6
BMI (kg/m²)	21.67±1.7	26.72±1.53	32.5±2.96*
WC (cm)	70.62±4.65	81.38±5.41	91.27±6.81*
TC (mg/dl)	170.92±21.35	180.15±19.26	184.73±22.67
TG (mg/dl)	103.46±23.81	117.69±29.47	163.45±39.24*
HDL-c (mg/dl)	56.23±4.4	53.54±5.16	49.45±6.73*
LDL-c (mg/dl)	93.92±25.0	100.77±23.42	102.82±26.95
AI	1.7±0.59	1.93±0.6	2.16±.084
OX-LDL(U/l)	44.61±17.55	57.82±14.29	73.91±25.71*
LH (ρg/ml)	7.02±2.44	6.33±2.26	5.12±2.22
FSH (ρg/ml)	8.33±1.99	6.78±2.22	6.44±2.1
E_2 (ρg/ml)	209±30.56	192.31±51.2	166.54±54.15

$p < 0.01$ by ANOVA test

The comparison (by ANOVA test) between different BMI subgroups of the postmenopausal group showed a gradual significant increase in age, BMI and WC. In addition, the obese women of this group had a significant elevation in serum TC, LDL-C, AI and ox-LDL-C ($p < 0.01$) with a significant reduction in HDL-C ($p < 0.01$).

There was no significant difference in TG, and serum sex hormones (Table 3).

Table 3: The biochemical parameters in the BMI subgroups of the post-menopausal women

Parameters	Premenopausal > 25 (kg/m²)	Premenopausal 25-29.9 (kg/m²)	Premenopausal > 30 (kg/m²)
Number	12	14	15
Age (years)	55.0±3.84	62.07±7.53	61.8±7.42*
BMI (kg/m²)	23.18±1.37	27.74±1.67	33.55±3.05*
WC (cm)	75.5±3.53	90.29±7.18	100.07±8.17*
TC (mg/dl)	186.5±20.95	206.71±37.87	225.0±31.45*
TG (mg/dl)	111.17±55.91	151.64±44.28	154.53±59.46
HDL-c (mg/dl)	51.75±5.91	46.36±5.96	43.87±6.67*
LDL-c (mg/dl)	113.17±21.52	126.5±36.14	152.53±32.7*
AI	2.24±0.61	2.98±1.13	3.63±1.15*
OX-LDL(U/l)	61.38±16.4	71.09±24.71	102.81±42.96*
LH (ρg/ml)	37.98±11.21	40.11±12.21	45.85±10.83
FSH (ρg/ml)	48.34±12.44	56.66±12.63	59.83±10.69
E_2 (ρg/ml)	61.63±12.99	57.61±16.6	51.25±15.59

* $p < 0.01$ by ANOVA test.

In table (4) the comparison between the obese pre- and post-menopausal women shows, in addition to higher age, FSH and LH, a significantly higher WC, TC, LDLC, AI and Ox-LDL (P <) with a significant reduction in HDL-C and E2 ($p < 0.01$)

The estradiol E2 was significantly negatively correlated with obesity (BMI) in the pre-menopausal women only (figure 1). While WC was positively correlated with each of serum TG and Ox- LDL (Figures 2 and 3).

Table 4: comparison between the obese subgroups in the pre- and post-menopausal women.

| Parameters	Pre BMI>30 Mean ±SD	Post BMI>30 Mean ±SD
Number	11	15
Age (years)	36.82±7.6	61.8±7.42*
BMI (kg/m²)	32.5±2.96	33.55±3.05
WC (cm)	91.27±6.81	100.07±8.17*
TC (mg/dl)	184.73±22.67	225.0±31.45*
TG (mg/dl)	163.45±39.24	154.53±59.46
HDL-c (mg/dl)	49.45±6.73	43.87±6.67*
LDL-c (mg/dl)	102.82±26.95	152.53±32.7*
AI	2.16±.084	3.63±1.15*
OX-LDL(U/l)	73.91±25.71	102.81±42.96*
LH (ρg/ml)	5.12±2.22	45.85±10.83*
FSH (ρg/ml)	6.44±2.21	59.83±10.69*
E_2 (ρg/ml)	166.54±54.15	51.25±15.59*

* $p < 0.01$
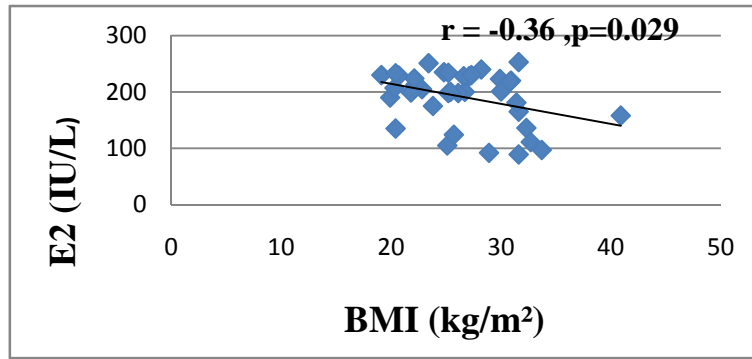
Figure 1: Correlation between body mass index (BMI) and serum estradiol (E2) in the pre-menopausal group.
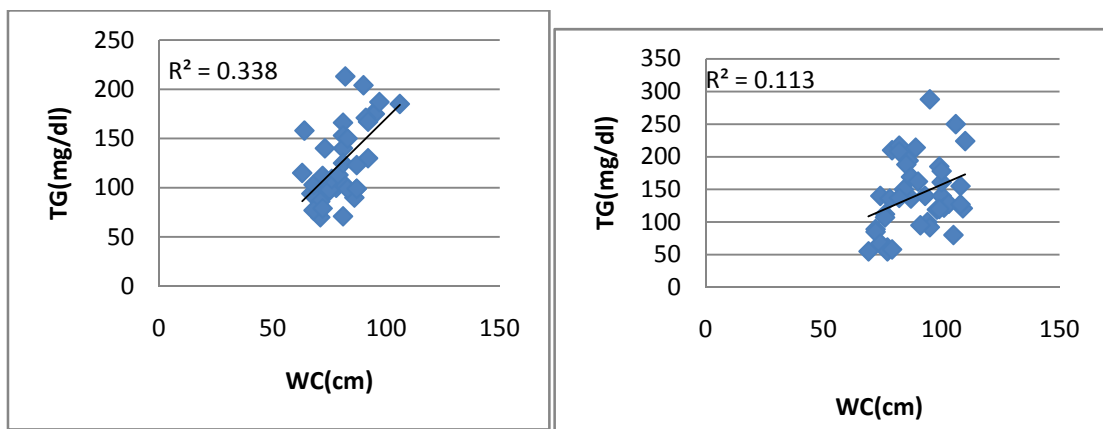
A- n: 37 , r = 0.58 , P < 0.01   B- n : 41 , r = 0.33 , P < 0.01

Figure 2: Correlation between the waist circumference (WC) and serum triglycerides (TG) in the pre-menopausal (A) and the post-menopausal (B) groups
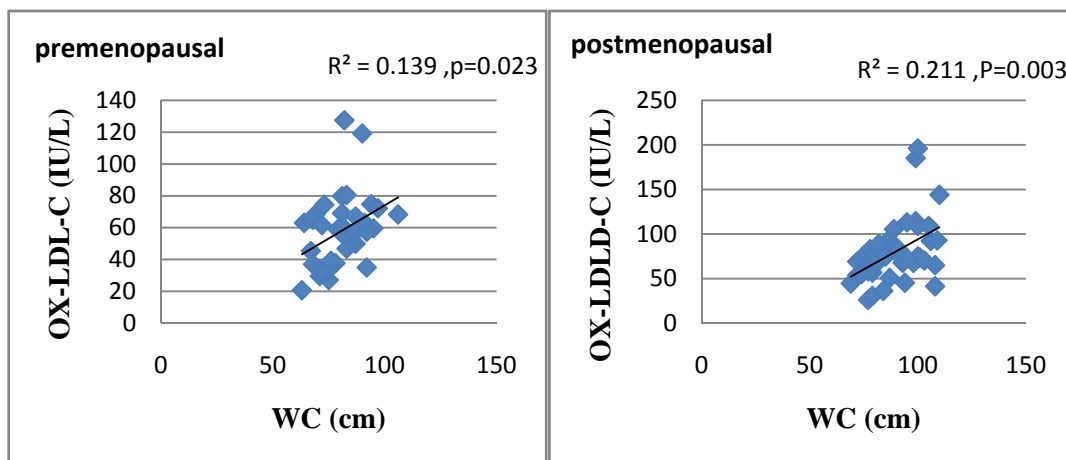

Figure3: Correlation between the waist cirumference (WC) and the Oxidized LDL (Ox- LDL) in the premenopausal and postmenopausal groups

Discussion

The results of the present study that compare between the pre-menopausal and the post-menopausal women (Table 1) reveals clearly that changes in sex hormones is the most important factor which differentiates between these two stages. However studying the relation of these hormones to other measured parameters showed that only E2 was significantly negatively associated changes in BMI in the pre-menopausal women only (Table 1 and Figure 1). This could be explained by the fact that E2 is scarce in the post-menopausal stage and may have no, or, negligible physiological effect; Moreover changes in serum lipids, and oxidative stress status observed in this study may be due to the increase in body weight (high BMI or WC) which may be triggered by the drastic reduction in E2.

Aging, on the other hand, appeared to have an effect on serum lipid concentrations presented by significant increase in TC, LDLC and TG with a significant reduction in HDLC and consequent increase in AI. This is evident in the different BMI sub- groups of the postmenopausal women who showed significant age difference (Table 3), while no such difference could be noticed in the BMI subgroups of the pre-menopausal women of the present study who showed no significant age difference (Table 2). However it should be kept in mind that age difference was associated with changes in sex hormones and accordingly it is probable that changes in serum lipids were due to the combined effects of age and sex hormones.

A recent study showed that fat mass and WC were higher in postmenopausal women compared with the premenopausal women and that weight gain during aging would occur predominantly in the abdominal region [7]. Furthermore, it is known that after menopause WC and visceral adipose tissue accumulation increases beyond the effect of aging. This predisposition processes are more likely to be associated with the time since the menopause than with biological age [8].

So it could be proposed that reduced E2 effect in the post-menopausal women of the present study had resulted in a significant increase in the body weight, presented by high BMI, or high WC, which was positively associated with dyslipidemia and increased Ox-LDL (Table 1 and Figure 2).

In the post-menopausal women aging has been associated with increased concentration of TC, TG, VLDL-C, and LDL-C, and decreased concentration of HDL-C, all of which contributed to a more atherogenic lipid profile (Tables 1 and 4). The cardio-protective effect of estrogen has long been related to its beneficial effect on cholesterol metabolism and deposition, contributing to inhibition of athero-sclerotic plague formation in the arterial walls [9]. Estrogen was reported to lower LDL-C by up regulating LDL receptors in the liver and enhancing LDL catabolism [10]. This could be attributed to the reducing action of estrogen on the activity of adipose tissue lipoprotein lipase which results in less rapid hydrolysis of the circulating triglyceride rich (chylomicrons and VLDL particles), and has been reported to decrease hepatic TG lipase activity [11]. In addition, estrogen is believed to enhance hepatic B/E receptors mediated lipoprotein uptake, and it appears to promote the hepatic synthesis and secretion of apo-A-I. Also Estrogen is thought to enhance the production of larger, less dense and presumably less atherogenic LDL particles [12]. This may explain the presence of higher Ox-LDL concentration in the obese post-menopausal women than their counterpart of the pre-menopausal group (Table 4) with the presence of a positive correlation between OX-LDL levels and obesity measures such as BMI and WC (Figure 3).

The present finding agrees with a previous report, considering BMI as one of the strongest predictors for circulating levels of Ox-LDL and demonstrating the effect of leptin on the generation of reactive species in the endothelial cells with a consequent LDL oxidation [13]

The mechanisms by which abdominal adiposity per se could induce increased oxidative stress are not clear. The oxidative stress could be induced by low grade of inflammation mainly characterized by high concentration of leptin, interlukin-6 (IL-6) and C-reactive protein [14]. Also several authors suggested that low degree of inflammation in obese person is caused by a high secretion of pro-inflammatory cytokines such as TNF-α [15]. This induces the production of IL6 which leads to low grade inflammatory state that leads to excessive production of free radicals and increased lipid peroxidation [16]. Estrogen, on the other hand, was considered a powerful antioxidant, which prevents lipid peroxidation and changes in lipid profile, as observed in the pre-menopausal women [9].

In conclusion these findings suggest that, for a given age, WC, BMI and menopausal status need to be considered when predicting abdominal adipose tissue distribution and related health.

References

1. Gardener DG, Shoback D. Menopause: Greenspan's basic and clinical endocrinology. 8th edition, 2007; Mc-Graw - Hill, 14: p. 537-543.
2. Scott JR, Gibbs RS, Karlan BY, and Haney AF. Menopause: Danforth's obstetrics and gynecology 9th edition, Lippincott Williams & Wilkins, 2003; 41: p. 721-728.
3. Herber D, Ashley JM, Leaf DA, and Barnard RJ. Reduction of serum estradiol in postmenopausal women given free access to low-fat high carbohydrate diet. *Nutrition J*, 1991; 7: 137.
4. Ruotolo G, Howard BV, Robbins DC. 2003. Dyslipidemia of obesity. www-endotext.org/obesity10/obesityframe10.htm.
5. Holvoet P, Deikeyzer D and Jarobs D. Oxidized LDL and metabolic syndrome. *Future lipidology J*, 2008; 6(3): 637-49.
6. Friedewald WT, Levy RL, and Fredrickson DS. Estimation of the concentration of low density lipoprotein cholesterol in plasma without use preparative ultracentrifuge. *Clin Chem*, 1972; 18: 499- 502.
7. Deibert P, König D, Vitolins MZ, Landmann U, Frey I, Zahradnik HP, et al. Effects of weight loss intervention on anthropometric measures and metabolic factors in pre- versus postmenopausal women. *Nutrition J*, 2007; 6: 31-46.
8. Tremollieres FA, Pouilles JM, and Ribot CA. Relative influence of age and menopause on total and regional body composition changes in postmenopausal women. *Am J Obstet Gynecol*, 1996; 175: 1594-1600.
9. Abbey M, Owen AA, Suzakawa M, Roach P, and Nestel PJ. Effects of menopause and hormone replacement therapy on plasma lipids, lipoproteins and LDL-receptor activity. *Maturities*, 1999; 33: 259-269.
10. Walsh BW, Schiff I, Rosner B, Greenberg L, Ravikar V, and Sacks FM. Effects of Post-menopausal estrogen replacement therapy on the concentrations and metabolism of plasma lipoproteins. *New England J Med*, 1991; 325: 1196-1204.
11. Vaidya D. Sex hormones and circulating lipoprotein levels. *Future Lipidol*, 2008; 3(6): 603-606.
12. Plouffe LJ, Ravnikar VA, Seperoff L, and Watts NB. Lipid metabolism and menopause: Comprehensive management of menopause. Springer Company, 1993; 20: p. 215-222.
13. Porreca E, Febbo CD, Moretta V, and Angelini A. Circulating leptin is associated with oxidized LDL in postmenopausal women. *Atherosclerosis*, 2004; 175: 39-43.
14. Hulthe J, Wikstrand J, and Fagerberg B. Relationship between C-reactive protein and intima- media thickness in carotid and formal arteries and to antibodies against oxidized LDL. *Clin Sci (Lond)*, 2001; 100: 371-378.
15. Mohamed-Ali V, Goodrick S, and Rawesh A. Subcutaneous adipose tissue release interlukin-6 but not tumor necrotic factor-alpha. *J Clin Endocrinol Metab*, 1997; 82: 4196-200.
16. Davi G, Guagnano G, Ciabattoni G, Basili S, Falco A, Marrinopiccoli M, et al. Platelet activation in obese women: role of inflammation and oxidant stress. *JAMA*, 2002; 288: 2008-2014.

Correspondence to: Dr. Ghassan A. Al-Shamma,
E-mail: ghassan.1971@yahoo.com

A Comparative Study of Fructose, Zinc and Copper Levels in Seminal Plasma in Fertile and Infertile Men

Nawal K. Hussain[1] *PhD*, **Shahlaa S. Rzoqi** [2] *MSc*, **Ali W. Numan[3]** *MSc*, **Dina T. Ali[1]**

[1]Institute of Embryo Research and Infertility Treatment, AL- Nahrain University, [2]Medical Technical Institute/Presidency of the Foundation of Technical Education/Erbil University, [3]Dept. of Chemistry, College of Science, Baghdad University

Abstract

Background   Human semen contains high concentrations of fructose, zinc (Zn) and copper (Cu) in bound and ionic forms for Zn and Cu. The presence of abnormal levels of fructose and those trace elements may affect spermatogenesis with regard to production, maturation, motility and fertilizing capacity of the spermatozoa.

Objective   To evaluate the levels of fructose, Zn and Cu in seminal plasma in different groups of male infertility and to correlate their concentrations with various sperm parameters.

Methods   The concentrations of fructose, Zn and Cu were measured in 114 semen samples from normozoospermic, oligozoospermic, astheno-zoospermic, and azoospermic men using the electrothermal-atomic absorption spectrometry for Zn and Cu determination. The concentration of fructose in seminal plasma was determined with a spectrophotometric method, using the resorcinol method.

Results   Results of the present study showed that there was an inverse relationship between fructose levels and sperm count. The mean value of seminal plasma fructose concentrations was significantly increased ($p ≤ 0.001$) in the three groups of infertile male subjects (azoospermia, asthenozoospermia and oligozoospermia) than in fertile males. The mean value of seminal plasma Zn concentrations was significantly decreased ($p ≤ 0.001$) in the three groups of infertile male subjects (azoospermia, asthenozoospermia and oligozoospermia) than in fertile males. A good correlation in a positive direction was noted between the sperm count and seminal plasma Zn concentration. There was significant decrease in seminal plasma Cu concentration between asthenozoospermia and control groups ($p ≤ 0.05$) and insignificant increase in oligozoospermic patients.

Conclusions   On the basis of the observations of the present study, seminal fructose, zinc and copper may contribute to fertility through their effects on various semen parameters.

Key words   Male infertility, fructose, zinc, copper.

Introduction

Infertility has often been defined as failure to achieve pregnancy within one year of unprotected intercourse. Infertility has multiple causes and consequences depending on the gender, sexual history, life style of society and cultural background of people it affects [1]. Infertility affects about 8-12% of the world's population and in about half of cases men are either the single cause of or contribute the couple's infertility [2].

Fructose concentration, because it is considered a measure of seminal vesicle function, has been studied in great detail. Studies indicate that there is a wide variation in fructose concentration [3], and

this concentration can be a function of a number of factors, including time since collection and the age of the donor [4]. Fructose is an important source of energy for the sperm, and, hence, measurements of fructose concentration in whole semen can change over time as a result of fructolysis, the primary source of lactic acid in semen [5,6]. Fructose is also likely involved in protein complexes, particularly in coagulated semen [7].

In fact trace elements calcium, magnesium, copper, selenium, and zinc play very vital role in affecting various parameters of semen. Among trace elements increasing evidence of a direct relationship of zinc was found with seminal parameters [8]. Zinc (Zn) in seminal plasma stabilizes the cell membrane and nuclear chromatin of spermatozoa [9]. It may also have an antibacterial function and protect the testis against the degenerative changes [10]. It may play a regulatory role in the process of capacitation and acrosome reaction [11].

The total zinc content in semen from mammals is high, and zinc has been found to be critical to **s**permatogenesis. Deficiency of zinc is associated with hypogonadism and insufficient development of secondary sex characteristics in humans [12], and can cause atrophy of the somniferous tubules in the rat and hence failure in spermatogenesis [13].

Zinc is excreted from the prostate as a low-molecular weight complex with citrate. After ejaculation, 50% is redistributed and bound to medium- and high-molecular weight compounds from the seminal vesicles [14].

Testicular Zn is critical for normal spermatogenesis and for sperm physiology; it preserves genomic integrity in the sperm and stabilizes attachment of sperm head to tail [13].

Copper is an important element for numerous metalloenzymes and metallo-proteins that are involved in energy or antioxidant metabolism. However, in its ionic form and at high level, this trace element rapidly becomes toxic to a variety of cells, including spermatozoa [15]. The present study was designed to evaluate seminal plasma levels of zinc, copper and fructose to correlate their concentrations with various sperm parameters among fertile and infertile male subjects.

Method

This study was carried out at the Institute of Embryo Research and Infertility Treatment, AL-Nahrain University, Baghdad, during the period from Sep. 2008 to Mar. 2009. Eighty six primary infertile male subjects, who had regular unprotected intercourse for at least one year without conception with their partners, aged (25-40) years were included in this study. Patients had no infections, traumatic abnormalities which could be implicated in the development of infertility. At first clinic attendance, a detailed background history and physical examination were done on both husband and wife.

Semen specimens from all infertile patients were collected into sterile polystyrene jars after an abstinence period of 3 to 5 days. Macroscopic and microscopic examination of semen was performed according to WHO recommendations [16]. A portion of each semen sample was examined for sperm count, motility and morphologic features. Infertile male patients were then divided into the following three groups count /motility and/or morphology, WHO criteria, 1992 [17].

Group I: Azoospermic (sperm concentration = zero, n=28),

Group II: Oligozoospermic (sperm concentration < 20 ×10 /ml, n= 30), and

Group III: Asthenozoosperic (sperm motility < 50%, n=28).

Twenty eight fertile males whose partners had conceived within one year and having sperm concentration more than 20 million/ml with motility and morphology more than 50% were selected from general population and taken as normospermic control group.

After liquefaction, the seminal plasma was collected after centrifugation at 3000 rpm for 15-20 minutes. The supernatant seminal plasma was transferred in fresh tubes and stored at -20 °C until assay. Seminal Zn and Cu measurements were performed by the electrothermal-atomic absorption spectrometry (AAS) method. The concentration of fructose in seminal plasma was determined with a spectrophotometric method, using the resorsinol method.

Statistical analysis

Descriptive statistics were represented as mean and SE. Statistical differences were analyzed using Independent sample-test when we had 2 groups, but ANOVA was used when we had more than 2 groups. P-values were considered statistically significant ($p < 0.05$). Pearson correlation was used to assess the relationship between studied variables.

Results

Table 1 summarizes the mean (±SEM) value on seminal plasma levels of Fructose, Cu and Zn in the three groups of infertile male subjects (azoospermia, oligozoospermia and asthenozoospermia) and in fertile control group.

The mean (±SEM) value of seminal plasma fructose concentrations was significantly increased in the three groups of infertile male subjects (azoospermia, asthenozoospermia and oligozoospermia) than in fertile males ($p < 0.001$, Table 1). There were no significant differences in seminal plasma Cu concentration between the azoospermia and the oligozoospermia groups of infertile male subjects and in fertile control group (Table 1), but there was significant decrease in seminal plasma Cu concentration between astheno-zoospermia group and control as shown in Table 2. The mean (±SEM) value of seminal plasma Zn concentrations was significantly decreased in the three groups of infertile male subjects (azoospermia, asthenozoospermia and oligozoospermia) than in fertile male (Table 1).

The results of Table 3 appeared positive and significant ($p < 0.001$) correlation between sperm concentration and total progressive motility. Meanwhile, negative weak and non significant ($p > 0.05$) correlations were assessed between sperm concentration and each of fructose and cupper. However, positive weak and non significant ($p > 0.05$) correlations were noticed between total progressive sperm and each of fructose and cupper. On the other hand, zinc appeared positive weak and non significant ($p > 0.05$) correlations with both sperm concentration and total progressive sperm. In contrast, zinc presented negative weak and non significant ($p > 0.05$) correlations with each of fructose and cupper.

Table 1: Fructose, Cu and Zn Concentration in Seminal Plasma in Three Groups of Infertile Males and Fertile Control Group

		N	Mean± Std. Error	Sig.
Sperm concentration	Control	28	73.393±5.692	≤ 0.001**
	Asthenozoospermia	28	34.964±1.597	
	Oligozoospermia	30	11.833±1.349	
Total progressive	Control	28	87.179±12.135	≤ 0.001**
	Asthenozoospermia	28	10.786±1.147	
	Oligozoospermia	30	5.667±1.015	
Fructose	Control	28	210.643±13.651	≤ 0.001**
	Azoospermia	28	392.500±17.170	
	Asthenozoospermia	28	266.536±17.096	
	Oligozoospermia	30	261.133±21.260	
Cu	Control	28	0.047±0.006	> 0.05^NS
	Azoospermia	28	0.044±0.010	
	Asthenozoospermia	28	0.025±0.004	
	Oligozoospermia	30	0.051±0.008	
Zn	Control	28	157.593±11.785	≤ 0.05*
	Azoospermia	28	105.893±6.664	
	Asthenozoospermia	28	132.250±11.590	
	Oligozoospermia	30	125.367±10.370	

The values are expressed as Mean (±SEM).
NS = no statistical significance $p > 0.05$.
* = statistical significance $p < 0.05$.
** = highly statistical significance $p < 0.001$.

Table 2: Cu and Zn Concentration in Seminal Plasma in Asthenozoospermia Group of Infertile Males and Fertile Control Group

	Study groups	Mean± Std. Error	Sig.
Sperm concentration	Control	73.393±5.692	≤0.001**
	Asthenozoospermia	34.964±1.597	
Total progressive	Control	87.179±12.135	≤0.001**
	Asthenozoospermia	10.786±1.147	
Fructose	Control	210.643±13.651	≤0.05*
	Asthenozoospermia	266.536±17.096	
Cu	Control	0.047±0.006	≤0.05*
	Asthenozoospermia	0.025±0.004	
Zn	Control	157.593±11.785	>0.05^NS
	Asthenozoospermia	132.250±11.590	

The values are expressed as Mean (±SEM).
NS = no statistical significance $p > 0.05$.
* = statistcal significance $p ≤ 0.05*$.
** = highly statistical significance $p ≤ 0.001**$.

Table 3: Pearson Correlation coefficient among studied variables

		Sperm concentration	Total progressive	Fructose	Cu	Zn
Sperm concentration	r	1	0.508	-0.047	-0.237	0.108
	p	0.000	≤ 0.001**	> 0.05[NS]	> 0.05[NS]	> 0.05[NS]
Total progressive	r		1	0.072	0.008	0.073
	p		0.000	> 0.05[NS]	> 0.05[NS]	> 0.05[NS]
Fructose	r			1	0.056	-0.131
	p			0.000	> 0.05[NS]	> 0.05[NS]
Cu	r				1	-0.181
	p				0.000	> 0.05[NS]
Zn	r					1
	p					0.000

NS= no statistical significance *p* >0.05.

* = statistical significance *p* ≤ 0.05*.

** = highly statistical significance *p* ≤ 0.001**.

Discussion

The normal function of seminal vesicle is essential for sustaining fertility. Decreased function of seminal vesicle affects the semen coagulation, sperm motility, stability of sperm chromatin, and semen immunoprotection. One of the most important markers for the seminal vesicular function is the concentration of fructose in seminal plasma [18].

The results of the present study showed that there was an inverse relationship between fructose levels and sperm concentration. Similar results have been reported by Manivannan et al [19]. However this finding was conflicting with others [20,21]. The lowest values of seminal fructose presented may be due to the increase of the process of fructolysis. Furthermore, the decrease of fructose concentration was significantly positively correlated with motile sperm concentration [18].

It appears that the abnormal concentrations of this substance are related to disturbances in the secretory activity of the seminal vesicles [22].

In this study, there was a significant low level of seminal plasma zinc levels in oligozoospermic and azoospermic males. Similar results have been reported by Hasan et al [23]. Our results are also incompatible with several studies [24]. A good correlation in a positive direction was noted between the sperm count and seminal plasma zinc concentration. This element has been shown to be highly important for conception, successful implantation and pregnancy outcome [25, 26]. Zinc is present at high concentrations in the seminal fluid and there is evidence that it may act *in vivo* as a scavenger of excessive O^{-2} production by defective spermatozoa and/or leukocytes in semen after ejaculation [27]. There is evidence that zinc plays a vital role in the physiology of spermatozoa and spermatogenesis. Specifically, Bedwal et al reported that shrinkage of seminiferous tubules Zinc is an essential nutritional component. A potential benefit of zinc supplementation for immuno-logical competence is currently widely discussed.

Zinc is present at high concentrations in the seminal fluid, and may play a multifaceted role in sperm functional properties. It has been suggested as being an important anti-inflammatory factor, and also to be involved in sperm oxidative metabolism [29]. A clinical study demonstrated that adult males experimentally deprived of zinc showed a disturbance of testosterone

synthesis in the Leydig cell. Since zinc plays an important role in 5α reductase enzyme that is necessary for the conversion of testosterone into biologically active form 5α dihydro-testosterone [23]. The authors concluded that adequate seminal concentration of the Zn is required for normal sperm function. It has been demonstrated that Zn in human semen is derived from the prostate [12]. Zn appears to be a potent scavenger of excessive superoxide anions produced by defective spermatozoa and/or leukocytes in human semen after ejaculation [30].

The results of this study also showed that Cu concentration was significantly decreased in seminal plasma of asthenozoospermic patients while insignificantly increased in oligospermic. This result is compatible with that observed by others [15]. Copper is an essential trace element that plays an important role in several enzymes such as cytochrome oxidase, ferroxidase, superoxide dismutase and spermin oxidase. Human spermatozoa are particularly susceptible to peroxidative damage because they contain high concentrations of polyunsaturated fatty acids and also possess a significant ability to generate a reactive oxygen species (ROS), mainly superoxide anion and hydrogen peroxide. Superoxide dismutase (Cu-metalloenzyme) protects human spermatozoa from this peroxidative damage [15]. Liang Lu et al 2009 [31] suggested that Cu^{2+} can affect male reproductive function through T-type Ca^{2+} channels. *Invitro* studies, Roblero et al [32] have demonstrated the effect of Cu^{2+} on the motility viability, acrosom reaction and fertilizing capacity of human spermatozoa.

On the basis of the present observations and those of others seminal fructose, zinc and copper may contribute to fertility through their effects on various semen parameters. Adequate seminal plasma concentration of fructose, Zn and Cu are required for normal sperm function and that high toxic concentrations of Zn and Cu in seminal plasma are apparently related to defective motility of sperm in infertile males. It seems that the estimation of seminal fructose, Zn and Cu may help in the investigation and treatment of infertile males.

References

1. Kerry LW. Defining infertility. *Family Health International,* 2003; 23: 4-8.
2. Barbara B. Men contribute to and suffer from Infertility. *Family Health International*, 2003; 23: 17-20.
3. Nun S, Musacchio I, and Epstein JA. Variations in seminal plasma constituents from fertile, subfertile, and vasectomized azoospermic men. *Fertil Steril,* 1972; 23: 357-360.
4. Kothari LK, Gupta AS, Chaturvedi KC, and Paliwal OL. Seminal fructose and acid phosphatase in vasectomised men. *Int J Fertil*, 1977; 22: 60-62.
5. King TE, and Mann T. Sorbitol metabolism in spermatozoa. *Proc R Soc Lond B Biol Sci,* 1959; 151: 226 -243.
6. Mann T, and Lutwak-Mann C. Male reproductive function and semen: Themes and trends in physiology, biochemistry and investigative andrology. New York, Springer-Verlag, 1981; p. 269-326.
7. Owen DH and Katza DF. Review of the physical and chemical properties of human semen and the formulation of a semen stimulant. *Journal of Andrology*, 2005; 26: 459-469.
8. Stanwell SRS, Thompson A, and Haines R. A comparative study of zinc, copper, cadmium and lead levels in fertile and infertile men. *Fertil steril*, 1983; 40: 670-677.
9. Kvist U. Sperm nuclear chromatin decondensation ability. An in vitro study on ejaculated human spermatozoa. *Acta Physiol Scand suppl*, 1980; 486: 1-24.
10. Batra N, Nehru B, and Bansal MP. Reproductive potential of male Portan rats exposed to various levels of lead with regard to zinc status. *Br J Nutr*, 2004; 91: 387-391.
11. Riffo M, Leiva S, and Astudillo J. Effect of zinc on human sperm motility and acrosome reaction. *Int J Androl*, 1992; 15: 229-237.
12. Prasad AS. Discovery of human zinc deficiency and studies in an experimental human model. *Am J Clin Nutr,* 1991; 53:403–412.
13. Millar MJ, Fischer MI, Elcoate PV, Mawson CA. The effect of dietary zinc deficiency on the

reproductive system of the male rat. *Can J Biochem Physiol*, 1958, 36: 557-569.

14. Mandal A, and Bhattacharyya AK. Biochemical composition of washed human seminal coagulum in comparison to sperm-free semen from the same donors. *J Reprod Fertil*, 1990; 88: 113-118.

15. Wong WY, Flic G, Groenen PMW, Swinkels DW, Thomas CMG, Copius-Peereboom JHJ, Merkus HMWM and Steegrers-Theunissen RPM. The impact of calcium, magnesium, zinc and copper in blood and seminal plasma on semen parameters in men. *Reproductive Toxicology*, 2001; 15: 131-136.

16. WHO World Health Organization laboratory manual for the examination of human semen and sperm-cervical mucus interaction. Cambridge University Press, Cambridge, UK; 1999.

17. World Health Organization. WHO Laboratory Manual for the Examination of Human Semen and Semen–cervical Mucus Interaction, 3rd ed. Cambridge University Press, Cambridge, UK; 1992.

18. Lu JC, Chen F, Xu HR, Yu-Feng Huang YF, and Lu NQ. Standardization and quality control for determination of fructose in seminal plasma. *Journal of Andrology*, 2006. 29: 1-25.

19. Manivannan B, Bhande SS, Panneerdoss S, Sriram S, and Lohiya NK. Safety evaluation of long-term vas occlusion with styrene maleic anhydride and its non-invasive reversal on accessory reproductive organs in langurs. *Asian J Androl*, 2005; 7: 195-204.

20. Videla E, Blanco AM, Gall ME, and Fernandes-Collazo E. Human seminal biochemistry: Fructose, ascorbic acid, citric acid, acid phosphatase and their relationship with sperm count. *Andrologia*, 1981; 13:212.

21. Sheriff DS. Setting standards of male fertility in semen analysis in 1500 patients - a report. *Andrologia*, 1983; 15: 687.

22. Andrad-Rocha FT. Seminal fructose levels in male infertility: Relationship with sperm characteristic. *International Urology and Nephrology*, 1999; 31(1): 107-111.

23. Hasan A, Masood A, Mukhtiar B, and Moazzam A. Relationship of zinc concentrations in blood and seminal plasma with various semen parameters in infertile subjects. *Pak J Med Sci*, 2007; 23(1): 111-114.

24. Wong WY, Merkus HM, Thomas CM, Menkveld R, Zielhuis GA, and Steegers- TRP. Effects of folic acid and zinc sulphate on male factor subfertility: A double-blind, randomized, placebo-controlled trial. *Fertile Steril*, 2002; 77: 491-498.

25. Prasad AS. Zinc: an overview. *Nutrition*, 1995; 11: 93-99.

26. Stephenson JL, and Brackett BG. Influences of zinc on fertilization and development of bovine oocytes in vitro. *Zygote*, 1999; 7: 195-201.

27. Mohammad EH, Alixides FA, and Mohammed FH. Reactive oxygen species and antioxidant in seminal plasma and their Impact on male fertility. *International Journal of Fertility and Sterility*, 2009; 3: 87-110.

28. Bedwal RS, and Bahuguna A. Zinc, copper and selenium in reproduction. *Experientia*, 1994; 50: 626-640.

29. Rohr G, Zwick EM, Knopf D, Batschulat K, Armbruster FP, Strowitzki TH and Eggert-Kruse W. Are zinc levels associated with semen quality? Abstracts of the 17th Annual Meeting of the ESHRE, Lausanne, Switzerland 2001.

30. Irvine DS. Glutathion as a treatment for male infertility. *Rev Reprod*, 1966; 1: 6-12.

31. Lu L, Changsong W, Gao X, Xu P, Wang J, Wang Q, Cheng J and Xiao H. Effects of Copper on T-Type Ca^{2+} Channels in mouse spermatogenic cells. *J Membrane Biology*, 2009; 227: 87-94.

32. Roblero L, Guadarrama A, Lopez T, and Zegers, Hochschild F. Effect of copper ion on the motility viability, acrosom reaction and fertilizing capacity of human spermatozoa in vitro. *Reprod Fertil Dev*, 1996; 8: 871-874.

Serum Cytokine Production in Patients with Cutaneous Leishmaniasis Before and After Treatment

Israa K. Al-Aubaidi *PhD.*

Dept. of Biology, Ibn Al-Haitham College of Education, Baghdad University

Abstract

Background Cutaneous leishmaniasis (CL) is caused by a protozoan from the genus *Leishmania* that infect macrophages of many mammals including humans, their infection induces both humoral and cellular immune responses, but the balance of their expression varies with the type of the disease.

Objective The aim of the present study is to understand the effect of antimonial compounds on some serum cytokines levels that include (IFN-γ, TNF-α, TGF-β, IL-1β, IL-6, IL-8) before, during and after treatment from CL infection.

Methods Eighty people were included in the present study, 60 patients with CL lesions and 20 healthy individuals (control). Patients were diagnosed on the basis of clinical and parasitological criteria. All patients treated with pentostam by intralesional injection. Serum (IFN-γ, TNF-α, TGF-β, IL-1β, IL-6, IL-8) levels were determined by ELISA using a quantitative sandwich enzyme immunoassay technique.

Results Serum levels of (TNF-α, IL-1β, IL-6, and IL-8) were significantly higher in patients group than healthy subjects ($p < 0.05$). INF-γ and TGF-β levels were decreased significantly during infection with CL. During therapy with pentostam, cytokines levels (IFN-γ, TNF-α, TGF-β, IL-1β, IL-6, IL-8) were significantly increased ($p < 0.05$). All cytokines levels returned to the normal values after three months of healing from CL lesions.

Conclusions Cytokines plays an important role in the resolution of CL infection. Pentavalent antimonials compounds may have immuno-stimulating effects which may be responsible for its antimicrobial activities.

Key words Cutaneous leishmaniasis, cytokines, treatment, pentostam.

Introduction

Human leishmaniasis includes a spectrum of diseases with variable severity ranging from cutaneous to visceral diseases, all of them caused by protozoan parasites of the genus *Leishmania* [1,2]. The cutaneous forms are the commonest (1.0 to 1.5 million cases each year), representing 50-75% of all new cases all over the world [2,3]. In Iraq *L. major* and *L. tropica* are the causes of cutaneous leishmaniasis [4].

Leishmania species are intra-cellular parasites invading monocytes, macrophages, and langerhans cell [5]. Their infection in man induces both humoral and cellular immune responses, but the balance of their expression varies with the type of the disease [6]. A variety of inflammatory mediators are produced by monocytes/macrophages during the course of infection [7], the importance of cytokines during leishmanial infection comes from the demonstration (on experimental murine leishmaniasis) of the existence of two distinct CD4+ Th1 and Th2 subsets [8].

Sodium stibogluconate (pentostam) remain the drugs of choice in the treatment of all forms of *Leishmania* infection, several studies have been demonstrated that there is an important immunological component in response to antimonial therapy [9-11].

The objective of the present study was to determine the serum cytokines levels of IFN-γ, TNF-α, TGF-β, IL-1β, IL-6, IL-8 before, during therapy and after healing in Iraqi patients with CL lesions.

Methods

Patients: This study was conducted at Baghdad Teaching Hospital in Baghdad, during the period from January 2008 to March 2009. Eighty people, 60 patients with CL lesion patients and 20 apparently healthy individuals (control), were included. Their age ranged from 5-40 years.

The clinical diagnosis was confirmed by laboratory demonstration of the parasite in the lesions by direct smears. Lesions were cleaned with ethanol, and punctured at the margins with a sterile lancet. Exudate materials was smeared, dried in air and fixed by methanol. The smears were stained with Giemsa's stain and examined by light microscope. Microscopic diagnosis was made when amastigotes were identified in the smears. In order to confirm the diagnosis, the material was also cultured on Novy Mac – Neal –Nicolle (NNN) medium for up to three weeks to detect the leishmanial promastigotes. Antimonial treatment with (pentostam) was given only through the lesions by intralesional injection, 1-3 ml of this drug was injected, and 1-2 doses were given weekly for two months. The patients were checked weekly for healing or recurrence.

Determination of cytokines: The blood samples were collected after the diagnostic procedure from healthy subjects, CL patients, during therapy and after three months when they healed from their infection. Ten ml of venous blood was withdrawn from each individual, and allowed it to clot for 30 minutes at 37 °C. The tubes were then centrifuged for 10 minutes at 4 °C and 2500 rpm. The serum was collected and stored at -20 °C until the time of the serological test. Serum IL-1β, IL-6, IL-8 and TNF- α levels were determined by ELISA using a quantitative sandwich enzyme immunoassay technique (EASIA kits for IL-β, IL-6, IL-8 by Bio source, Europe) and ELISA kits (Mabtech AB, Sweden) for IFN-γ, TNF-α, TGF-β. All tests were carried out by vigorously following manufacturer instructions. Serum cytokine levels were calculated by interpolating the standard absorbance readings of the test samples calculated from samples of known concentrations supplied with the kits and assayed in parallel. The data were processed using the SPSS PC statistical program (Statistical Package for Social Sciences, PC version 10.0). Initially, mean cytokine levels were compared between groups using Student t-test.

Results

The serum levels of IFN-γ, TNF-α, TGF-β, IL-1β, IL-6 and IL-8 in patients responsive and refractory to antimonial therapy (pentostam) as well as normal controls were determined by ELISA before and after treatment were shown in table 1. The results of the pretreatment determinations of the cytokines levels (TNF-α, IL-1β, IL-6 IL-8) in CL patients were higher significantly ($p < 0.05$) than those in normal controls (Table 2). Serum cytokine levels in patients during treatment are listed in table 1 also. CL patients who were responsive to therapy presented with elevated levels of these cytokines ($p < 0.05$) and they were significantly higher than in control group (Table 2). Low levels of IFN-γ and TGF-β were determined in serum of patients group when compared to control group (healthy), but during therapy these concentrations were increased significantly

($p < 0.05$) when compared to patient group (before treatment). After three months all patients were healing from the infection due to successful therapy, serum IFN-γ, TGF-β, TNF- α, IL-1β, IL-6 and IL-8 levels were returned to the normal values (Table 1) and did not show significant differences ($p > 0.05$) between control group (healthy) and healing group (Table 2).

Table 3 shows the comparative significance (p-value) for the repeated measurements at different periods of contrasts (before, during and post healing).

Table 1: Concentrations of cytokines in serum of different study groups

Cytokine	*Concentrations of cytokines (pg/ml)			
	Control group	Patients groups		
		Before treatment	During treatment	Post healing
IFN-γ	102.7 ± 11.46	77.5 ± 15.95	183.4 ± 26.55	103.3 ± 12
TNF-α	14.12 ± 2.359	28.25 ± 3.61	63.5 ± 7.5	14.75 ± 2.25
TGF-β	44 ± 7.17	30.4 ± 4.15	37.8 ± 1.48	43.2 ± 7.29
IL-1β	4.01 ± 0.89	9.31 ± 1.38	14.12 ± 2.23	3.87 ± 2.16
IL-6	5.47± 1.66	12.85 ± 1.28	48.36 ± 6.82	5.08 ± 0.70
IL-8	16.38 ± 1.61	32.22 ± 2.16	333.6 ± 69.97	15.51 ± 1.10

* All cytokine concentrations were expressed as means ± standard deviation

Table 2: Comparisons Significant between Control and treated groups in its' different periods of treatments (Before, during and post healing)

Parameters	Control X Before		Control X During		Control X Healing	
	t-value	P-value	t-value	P-value	t-value	P-value
IFN-γ	4.057	0.001	-8.825	0.000	-0.114	0.910
TNF-α	-9.257	0.000	-19.57	0.000	-0.542	0.596
TGF-β	3.666	0.006	1.892	0.126	0.175	0.866
IL-1β	-8.051	0.000	-12.71	0.000	0.004	0.997
IL-6	-9.936	0.000	-19.18	0.000	0.683	0.504
IL-8	-15.87	0.000	-14.33	0.000	1.204	0.000

p > 0.05 Non significant

Table 3: Comparison between Significant (p-value) for the repeated measurements by different periods of contrasts (Before, during and post healing)

(i)	(j)	IFN-γ	TNF-α	TGF-β	IL-1β	IL-6	IL-8
Before	During	0.000	0.000	0.025	0.001	0.000	0.000
	Healing	0.006	0.000	0.003	0.002	0.000	0.000
During	Healing	0.000	0.001	0.175	0.000	0.000	0.000

P > 0.05 Non significant

Discussion

Chemotherapeutic cure of leishmaniasis is largely dependent upon the development of an effective immune response that activates macrophages to produce toxic nitrogen and oxygen intermediates to kill the amastigotes. This process is suppressed

by the infection itself which down regulates the requisite signaling between macrophage and T cells [8].

Gamma interferon (IFN-γ) secreted by Th1 cells is the most potent macrophage-activating cytokine, leading to the host resistance to infection with *Leishmania* parasite [12]. A marked decrease in the production of IFN-γ was observed in patients group (Table 1). The deficient production of IFN-γ after exposure to *Leishmania* antigens is one of the commonly reported factors which associated with increase expression of CD4+ T cells. The disease susceptibility is associated with the inability to produce macrophage-stimulating profile including IFN-γ, IL-2, and IL-12 [13]. Coutinho et al. [14] detected a decrease in the level of *Leishmania* in supernatants from *Leishmania* stimulated cell cultures 2255±653 pg/ml before antimonial therapy and 3005±900 pg/ml at the end of the treatment. A significant increase in the level of IFN-γ was detected in the serum of patients during treatment with pentostam when compared to its level before treatment, this explain that a successful drug therapy were restored T-cell proliferation and IL-2, IFN-γ production in response to *Leishmania* antigen [15]. Furthermore, these findings confirmed the role of IFN-γ in the healing of the lesion due to involvment of CD4+ T cells in the healing process and elevated IFN-γ production at the end of the treatment in human [16]. Also, IFN-γ could be used as immunopotentiator for augmenting the capacity of macrophages to eliminate *Leishmania* infection [17].

Transforming growth factor (TGF-β) is a multipotential cytokine with diverse effects on immune cells, including the down-regulation of certain macrophages and the blockade of IFN-γ induced macrophages activation [18,19]. In this study the concentrations of TGF-β in patients and treated groups decreased significantly as compared to controls (30.4±4.15 pg/ml and 37.8±1.48 respectively). Li et al [20] showed that using anti-TGF-β treatment promotes rapid healing of murine leishmaniasis through enhancing *in vivo* nitric oxide (NO) production by activated macrophages.

In this study, TNF-α and IL-1 levels were found to be significantly higher for CL patients than for control group, and during therapy their concentrations were significantly elevated also.

T cells mediate activation of macrophages to produce NO, resulting in killing or control *L. major* parasites and the secretion of TNF-α by macrophages is sufficient to mediate production of NO and killing of *L. major* parasites [21]. Melby et al. [22] found significant increase in the expression of IL-1β, TNF-α, IL-10 and TGF-β in late lesions compared with that in early lesions, these finding were in agreement with our study.

IL-1 is primarily produced by cells of the mononuclear phagocytic lineage but is also produced by endothelial cells, keratinocytes, synovial cells, astrocytes, osteoblasts, neutrophils, glial cells, and numerous other cells. IL-1 production may be stimulated by a variety of agents, including endotoxins and other cytokines, microorganisms, and antigens. IL-1 is also cytotoxic to cancerous and virus – infected cells [8]. Sodhi et al. [23] demonstrated that IL-1 levels were significantly increased when *L. donovani* infected animals were treated with antimonium salts 14 days post infection; their findings appear to support our study. It is thought that TNF-α and IL-1 levels increase as a part of host defense strategies, and induction of the cytokines by antimonial therapy might be dependent on macrophage activation.

T cells, monocytes and fibroblast produces IL-6, which is a major cytokine involved in T and B cell regulation and also in some aspects of the inflammatory response [24]. Serum IL-6 levels were significantly

elevated in CL patient group compared to values seen for the controls. This cytokine was increased during antimonial therapy in patient group. Several *in vitro* studies demonstrated that some herb powders, such as Echinacea, activate macrophage to produce TNF-α, IL-β, and IL-6 as well as oxidative burst and killing of *Leishmania* parasite [25].

IL-8 is a chemokine produced by macrophages and other cell types such as epithelial cells and endothelial cells. It is a proinflammatory cytokines that chemoattract and activates blood cells, beside its central role in inflammation; other biological functions of IL-8 include T cell chemotaxis, angiogensis, and hematopoiesis [26,27]. IL-8 concentrations were significantly higher ($p < 0.05$) in CL patients before treatment (32.22±2.16) than in the control subjects (16.38±1.61) and this increment was still significantly higher during treatment (333.6±69.97). Lejon et al. [28] detected a significant elevation of IL-6 and IL-8 levels in patients of the late stage *Trypanosoma gambiense*. TNF-α stimulate release of IL-8 which may in turn play an important role in the inflammation reaction. The chemokines IL-8 essential to bring the more neutrophils at the site of infection, also other proinflammatory cytokines might induce production of IL-8 to a reactive oxygen species, which caused a direct intracellular killing to *Leishmania* parasite during treatment with antimonial salts [8,29].

References

1. Ahmed S, Clomenares M, Soong L, Goldsmith-Pestaana K, Muntermann L, Molina R. et al. Intradermal infection model for pathogenesis and vaccine studies for murine visceral leishmaniasis. *Infect Immun*, 2003; 71(1): 401-410.
2. Blum J, Desjeux P, Schwartz E, Beck B and Hatz C. Treatment of cutaneous leishmaniasis among travellers. *J Antimicrob Chemother*, 2004; 53(2): 158-166.
3. Tripathi P, Singh V, and Naik S. Immune response to *Leishmania*: Paradox rather than paradigm. *FEMS Immunol Med Microbiol*, 2007; 51(2): 229-242.
4. Gonzalez J. Baghdad boil festers as new enemy of G. I.s. New York Daily. 2003; News. http://www.commondreams.org.
5. Moll H, and Berberich C. Dendritic cell-based vaccination strategies: Induction of protective immunity against leishmaniasis. *Immunobiol*, 2001; 204: 659-666.
6. Launois P, Louis J A, and Milon G. The fate and persistence of *Leishmania major* in mice of different geneyic backgrounds: An example of exploitation of the immune system by intracellular parasites. *Parasitol*, 1997; 115(Suppl.): 25-32.
7. De-Souza-Neto SM, Carneiro CM, Vieira LQ, and Afanso LCC: *Leishmania braziliensis* partial control of experimental infection by interleukine-12 p40 deficient mice. *Mem Inst Oswaldo Cruz*, 2004; 99(3): 289-294.
8. A Wasthi A, Mathur RK and Saha B. Immune response to *Leishmania* infection. *Indian J Med Res*, 2004; 119: 238-258.
9. Beebe AM, Mauze S, Schork NJ, and Coffman RL: Serial backcross mapping of multiple loci associated with resistance to *Leishmania major* in mice. *Immunology*, 1997; 6: 551-557.
10. Croft SL, and Yardley V. Chemotherapy of leishmaniasis. *Curr Pharm Des*, 2002; 8: 319-42.
11. Vidyashankar C, and Agrawal R. Leishmaniasis. *eMed J*, 2002; 3(7): 1-2.
12. Scott P. INF-γ modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *J Immunol*, 1991; 147: 3149-3155.
13. Gama MEA, Costa JML, Pereira JCR, Gomes CMC, and Corbett CEP. Serum cytokine profile in the subclinical form of visceral leishmaniasis. *Braz J Med Biol Res*, 2004; 37: 129-139.
14. Coutinho SG, Da-Cruz AM, Bertho AL, Santiago MA, and De-Luca P. Immunologic patterns associated with cure in human American cutaneous leishmaniasis. *Braz J Biol Res*, 1999; 31: 139-142.
15. Anam K, Afrin F, Banerjee D, Pramanik N, Guha SK, Goswami RP, et al. Differential decline in *Leishmania* membrane antigen-specific immunoglobuline G (IgG), IgM, IgE and IgG subclass antibodies in Indian Kala- azar patients after chemotherapy. *Infect Immun*, 1999; 67(12): 6663-6669.
16. da Cruz AM, Conceicao-Silva F, Bertho AL, and Coutiho S. *Leishmania*-reactive CD4+ and CD8+ T cells associated with cure of human cutaneous

leishmaniasis. *Infect Immun*, 1994; 62: 2614-2618.

17. Das L, Datta N, Bandyopadhyay S, and Das PK. Successful therapy of lethal murine visceral leishmaniasis with cystatin involves up-regulation of nitric oxide. *J Immunol*, 2001; 166: 4020-4028.

18. Meymandi S, Dabiri S, Meymandi M, Mirshekari TR, Abdirad A, Nikpour H, et al. Immunopathology of autoleukocytic therapy of cutaneouse leishmaniasis. *Arch Iran Med*, 2005; 8(3): 229-233.

19. Barral-Netto M, Barral A, and Brownell CE. Transformin growth factor-beta in leishmanial infection a parasite escape mechanism. *Science*, 1992; 257: 548-555.

20. Li J, Hunter CA, and Farrell JP. Anti- TGF-beta treatment promotes rapid healing of *Leishmania major* infection in mice by enhancing *in vivo* nitric oxide production. *J Immunol*, 1999; 162: 974-979.

21. Belosevic M, Davis CE, Meltzer MS, and Nacy CA: Regulation of activated macrophages antimicrobial activities. Identification of lymphokines that cooperate with INF-γ for induction of resistance to infection. *J Immunol*, 1988; 141: 890-896.

22. Melby PC, Andrade-Narvaez FJ, Darnell BJ, Valencia-Pacheco G, Tryon VV, and Palomo-Cetina A. Increased expression of pro-inflammatory cytokine in chronic lesions of human cutaneous leishmaniasis. *Infect Immun*, 1994; 62: 837-842.

23. Sodhi S, Ganguly NK & Malla N, and Mahajan RC. Effect of sodium stibogluconate on the status of interleukine-1 production in normal and *Leishmania donovani* infected BALB/c mice. *Indian J Med Res*, 1990; 91: 344-348.

24. Blackwill F. Cytokines and cytokines receptors. In: Roitt I, Brastoff J, and Male D, (Eds.). Immunology, 6th ed., Spain, Mosby, 2002; p. 119-127.

25. Luetting B, Steinmuller C, Gifford GE, Wagner H, and Lohmann-Mathes ML. Marophage activation by the polysaccharide arabinogalactan isolated from plant cultures of *Echinaceca purpurea. J Natl Cancer Inst*, 1989; 1: 669-675.

26. Barach-Ben A, Michiel DF, and Oppenheim JJ. Signals and receptors involved recruitment of inflammatory cells. *J Biol Chem*, 1995; 270: 11703-11706.

27. Khabar KSA, Al-Zoghaibi F, Al-Ahdal M, Murayama T, Dhalla M, Mukadia N, et al. The α chemokine, Interleukine-8, inhibits the antiviral action of interferon α. *J Exp Med*, 1997; 186 (7): 1077-1085.

28. Lejon V, Lardon J, Kenis G, Pinoges L, Legros D, Bisser SN, et al. Interleukine (IL)-6, IL-8 and IL-10 in serum and CSF of *Trypanosoma brucei gambiense* sleeping patients before and after treatment. *Trans R Soc Trop Med Hyg*, 2002; 96(3): 329-333.

29. Hirota K, Akahoshi T, Eudo H, Kondo H, and Kashiwazaki S. Production of interleukine 8 by cultured synovial cells in responses to interleukine 1 and tumor necrosis factor. *Rheumatol Int*, 1992; 12: 13-16.

Correspondence to: Dr. Israa K. Al-Aubaidi,
Tel.: + 964 7703124491
Received: 27th July 2009, Accepted: 26th Sept. 2010.

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

Elham A. Alaswad[1] *PhD*, **Nidhal AM. Mohammed[2]** *PhD*, **Khudair J. Al-Rawak[3]** *MBChB MRT*

[1]Dept. of Medical Biology, [2]Dept. of Medical Microbiology, College of Medicine, Al-Nahrain University, [3]Dept. of Surgery, College of Medicine, Baghdad University

Abstract

Background	The Epstein -Barr virus (EBV) is the first human virus implicated in the carcinogenesis. EBV contributes to the carcinogenesis like Hodgkin's Lymphoma (HL) and Non Hodgkin's Lymphoma (NHL).
Objective	Quantitative Competitive Polymerase Chain Reaction (QC-PCR) and ELISA was used to quantitate the EBV DNA load in blood samples of HL and NHL patients pre and post therapy.
Methods	EBV DNA extracted from blood samples of 18 HL and NHL patients pre and post therapy, 9 apparently healthy controls used to quantify the EBV DNA load. Quantitative Competitive Polymerase Chain Reaction (QC-PCR) and ELISA were used to quantify EBV DNA load. Wild EBV DNA (WT) obtained by Transformation of Escherichia coli MM 294 with Wild type (WT) DNA plasmid *pGEMBamHI-K*.
Results	EBV DNA load in controls was found to be 7-1.99 × 10^3 in HL and NHL patients, while in patients it's ranged from zero to 1.936×10^9 copy numbers per ml of blood. High EBV load with the range of 10715(1.071×104) to 1936421960 (1.936×109) above cut-off value was detected in 66.7% of HL and 5861(5.86×103)-50118(5.01×104) copies/ml blood in 44.5 % of NHL patients pretherapy. After chemotherapy, 60% of HL patients and 100% of HL patients with high EBV load showed significant response. Low viral load was found in 44.45% of patients. Only 55% of lymphoma patients with high EBV load, after chemotherapy 16.6% of them continue to have high EBV DNA load compared to the control group, 38.3% of the patients showed response to chemotherapy when their viral load decreased below cut off value. While 11.1 % continue to have high DNA load. One patient (5.5%) showed an elevated EBV load after completion of chemotherapy.
Conclusions	EBV DNA load estimated by Quantitative Competitive Polymerase Chain Reaction considered as valuable promising tumor biomarker in the diagnosis and monitoring of EBV related HL and NHL patients.
Key words	Quantitative Competitive Polymerase Chain Reaction (QC-PCR), Epstein-Barr virus (EBV), Viral DNA load, Hodgkin's (HL) and non Hodgkin's Lymphoma (NHL) Patients.

Introduction

The Epstein Barr virus (EBV) is a lymphotropic virus that infects and immortalizes B-lymphocytes, which maintained their viral genome in a non-

replicating latent form EBV is mainly B-cell tropic but capable of infecting T-cells and epithelial cells. EBV is the first human virus implicated in the pathogenesis of lymphoid and epithelial malignancies which reach 80% in developing countries; these malignancies including Burkitt′s lymphoma (BL), undifferentiated nasopharyngeal carcinoma (UNFC), HL, NHL, post-transplant lympho-proliferative disease (PTLD), some T-cell lymphoma and more recently certain cancers of stomach and smooth muscles. Hodgkin's lymphoma is uncommon malignant tumor of the lymphatic system where approximately 40% of HL were shown to contain clonal EBV [2]. NHL are associated with EBV, like nasal T / natural killer (NK) cell lymphoma and angioimmunoblastic lymphadenopathy [3]. Due to EBV associated malignancies, recent advances in PCR technology called Quantitative Competitive PCR (QC-PCR) permit precise measurement of EBV DNA level in clinical samples called EBV viral load. QC-PCR is used to quantify PCR products; it's a method to quantitatively measure DNA amount, and number of its copies in the sample [4]. Epstein -Barr virus (EBV) viral load assays able to distinguish low-level infection in carriers from higher levels associated EBV diseased patients. The patients affected by EBV often have high levels of EBV DNA in their body fluids like blood, plasma or serum and this is used as specific marker for EBV carcinogenesis. The QC-PCR co-amplify EBV DNA and a spiked or endogenous control sequence called Internal standard (IS), the relative amount of EBV and control product was measured in EBV related disease for early diagnosis and for monitoring the efficiency of therapy [5]. QC-PCR used to quantitate, EBV-DNA in plasma of all EBERs-positive AIDS lymphoma patients; they concluded that QC-PCR is very promising in

diagnosis and management of EBV related lymphoma [6].

Aim of the study: Utilizing EBV DNA load as molecular biomarker to predict the prognosis and to check response to chemotherapy in HL and NHL patients.

Methods
Patients and samples preparation: Peripheral blood samples, were taken before and 3-4 months after chemotherapy from Eighteen HL and NHL patients at Baghdad Medical City Teaching Hospital. Sampling extended from Feb 2005 to Nov 2005. Nine apparently healthy individual were enrolled in this study as control group.

Viral DNA extraction: Fresh whole blood from healthy group and patients were diluted 10 times in NASBA lysis buffer contains 5M guanidine thiocyanate, 0.75% Triton X-100, 1 M Tris -HCL, stored at −20 °C until DNA extracted by silica based extraction method as described previously by Boom et al, 1990.

EBV DNA: EBV DNA obtained by Transformation of Escherichia coli MM 294 with Wild type (WT) DNA plasmid *pGEMBamHI-K* and according to those reports by Kushner, 1978.

Plasmid DNA used: WT DNA *pGEMBamHI-K* plasmid which has the prototype EBV B95-8 EBNA1 sequence, used as positive control and *PQPCR8* plasmid DNA as in Gene bank was used as Internal Standard (IS) which compete viral DNA in QC-PCR, both obtained from Dr. Stevens, S.J.C, University Hospital Vrije, the Netherlands.

Plasmid DNA extraction: Plasmid DNA extracted from *E. coli* cells as described by Pospiech and Neuman, 1995, number of WT and IS DNA copies was determined.

Primers and Probes: The nucleotide sequences and localization of primers are listed in Table 1.

Table 1: Primers and Probes used for QC-PCR [*]

Primers or probe	Sequence (5´ – 3´)	Localization (EBV B95-8 genome)
QP1	GCCGGTGTGTTCGTATATGG	109462-109482
QP2	bio-CAAAACCTCAGCAAATATATGAG	109652-109675
WT probe	dig-TCTCCCCTTTGGAATGGCCCCTG	109563-109563
IS probe	dig-CTATATGCCTGCTTCCTCCTCCGGCG	

* Stevens et al, 1999 [10]

Amplification reactions were carried out at a reaction volume of 50 µl containing PCR reaction buffer (50 Mm kCl, 1.5 Mm MgCl$_2$, 10 Mm Tris (pH 8.2), 200 µM (each) deoxynucleoside triphosphate, and 1U of Taq DNA polymerase (Roch, USA). 25 pmole of Primer QP1. A 25 pmole of antisense primer QP2, 5 µl of DNA elute was added and amplified as follows: Denaturation at 95 °C for 4 min. and subjected to 40 cycles, each cycle consist of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 70 dC for 1 min. Samples maintained at 72 dC for 3 min [10].

Qualitative EBNA-1 PCR:
Standard dilution curve of the wild type (WT) DNA of known copy number was constructed by making serial dilution of WT DNA (106, 105, 104, 103, 102, 101, and 100). Each one of these dilutions was amplified separately. The PCR cocktail preparation performed in a 50 µl volume reaction, Primers and probes are listed in table 1 [10].
The PCR product of each dilution was quantified by ELISA detection method and optical density (O.D) was read at 405 nm.
Standard curve was blotted between O.D. of each PCR product of each dilution and the number of WT copies present in each dilution (Figure 3).

Prescreening method for patients and healthy control samples:
DNA eluted from blood samples of each patient before treatment and controls DNA

was amplified as mentioned previously. By comparison of the results obtained to the standard dilution curve, the number of copies of EBV present in each sample was estimated.

Quantitative competitive PCR assay:
Five µl elute of DNA extracted from blood samples of each patient before and after treatment was amplified with IS DNA copies (104, 103, and 102) separately. Primers and probes used as in Table 1.

Quantification of PCR products by enzyme immunoassay (EIA):
EIA with a modified procedure Jacobs, 1996 used Density. Five µl of biotinylated PCR products were added to 50 µl of 1 x hybridization buffer, denatured by 0.2 M NaOH. A 50 pmol/ml WT Digoxigenine (DIG) labeled oligonucleotide probe was added to one of the wells then IS DIG labeled oligonucleotide probe was added to the other well. Antidigoxigenine – conjugated antibodies (75 mU/ml hybridization buffer) added to all wells followed by 100 µl of 2, 2-Azino-di (3-ethyle benzthioazoline sulphonate 6) diammonium salt (ABTS) substrate (Roch. USA). The color intensity was measured at 405 nm and the runs included positive control PCR product, where as distilled water (D.W) was used as negative control [11].

Results

Prescreening method for DNA extracted from patients:

The extracted DNA which obtained from whole blood samples was found to have the optical density $O.D_{260}$. (1.6-1.8). The size of the DNA fragments separated compared to the DNA Marker was found to be 5148bp (Figure 1).
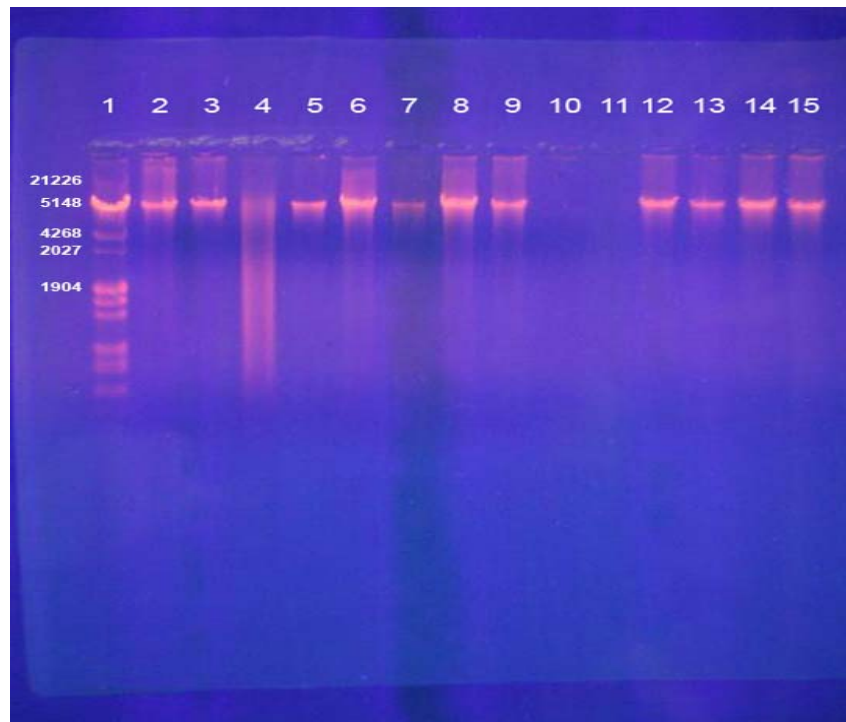

Figure 1: Ethidium bromide stained Agarose gel electrophoresis for the DNA extracted from the blood of the lymphoma patients, shows DNA fragments extracted from blood samples.
Lane (1): lambda DNA/ECOR 1+HindIII Marker 3.
Lane (2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14, and 15): DNA extracted from patient blood samples.
Lane (11): negative control.

Transformation:

Plasmid WT DNA *pGEM Bam HIK* (WT) was extracted from transformed E. coli MM294 by salting method compared to DNA Marker. The size of the prepared plasmid DNA was determined by comparison of their relative prepared position to that of the DNA ladder appeared in lane 8 (Figure 2).

Plasmid quantification:

WT plasmid DNA concentration:

The concentration of WT plasmid DNA was 635ng/µl and the number of copies of WT plasmid DNA was 0.075×10^{12} copies/µl.
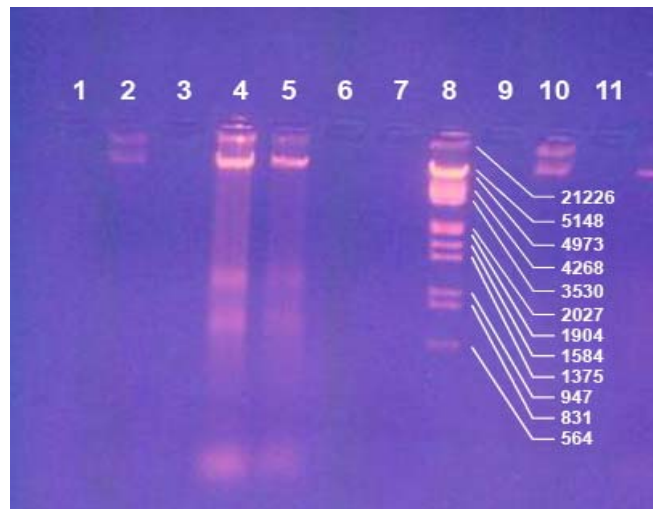
Figure 2: 0.5% agarose gel electrophoresis stained with ethidium bromide showing the Wild type (WT) DNA extracted from plasmid pGEMBam HIK extracted Lane 2, 10: WT DNA plasmid pGEMBam *HIK* purchased from Dr. Stevens S.J.C.
Lane 4: WT DNA plasmid pGEMBam *HIK* prepared high concentration.
Lane 5: WT DNA plasmid pGEMBam *HIk* prepared diluted.
Lane 2, 10: WT DNA plasmid pGEMBam *HIK* purchased from Dr. Stevens S.J.C.
Lane 7: negative control.
Lane 8: lambda DNA/*ECOR 1+Hind III*.

Standard dilution curve o f WT DNA was constructed by plotting optical density Product of each WT dilution against the number of WT copies present in each dilution (figure 3). It was observed that patient's samples have qualitatively EBV DNA copy numbers range from 102.4 to 10^6 DNA copy/µl.
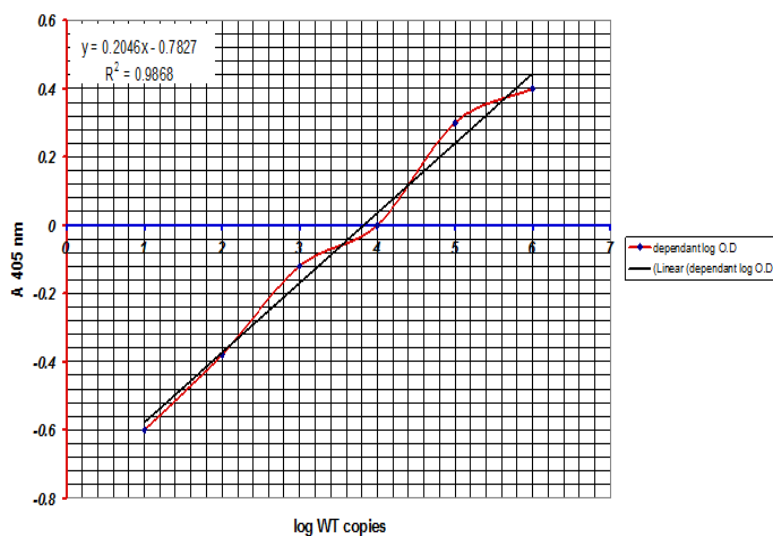

Figure 3: Standard curve of Wild type (WT) EBV DNA

Figure 4 reveals the Analytical sensitivity and amplification equivalence of QC-PCR for both WT and IS plasmid DNA. Five µl (10^1-10^5) /µl of each of WT and IS plasmid DNA copies were amplified separately. The amplified PCR products were analyzed using 1.5% gel electrophoresis and then visualized by ethidium bromide stain where 10^1 copies could be detected for both WT DNA and IS DNA (figure 4).
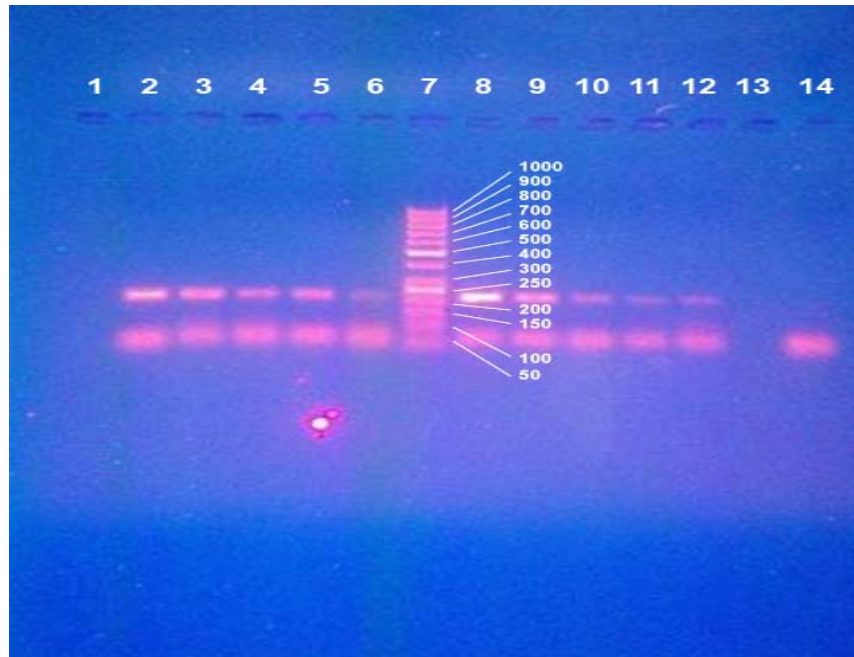

Figure 4: Ethidium bromide gel electrophoresis showing the analytical sensitivity of EBNA-1 QC - PCR for WT and IS plasmid DNA target
Lane 2, 3,4,5,6 having 10^5-10^1 copies of the WT PCR products
Lanes 8, 9, 10, 11, and 12 having 10^5-10^1 copies of the IS PCR products
Lane13 negative control (D.W)
Lane 14 positive control

As shown in figure 5, 10 fold serial dilution of 10^1-10^5 copies of WT DNA were spiked with increasing amounts of IS 10^1-10^5 DNA copies in separate reaction. Equivalent amplification and true competition was observed between two DNA template (WT and IS). This was confirmed by the production of equal signals when similar amounts (10^3) of WT and IS DNA were present in the reaction mixture.
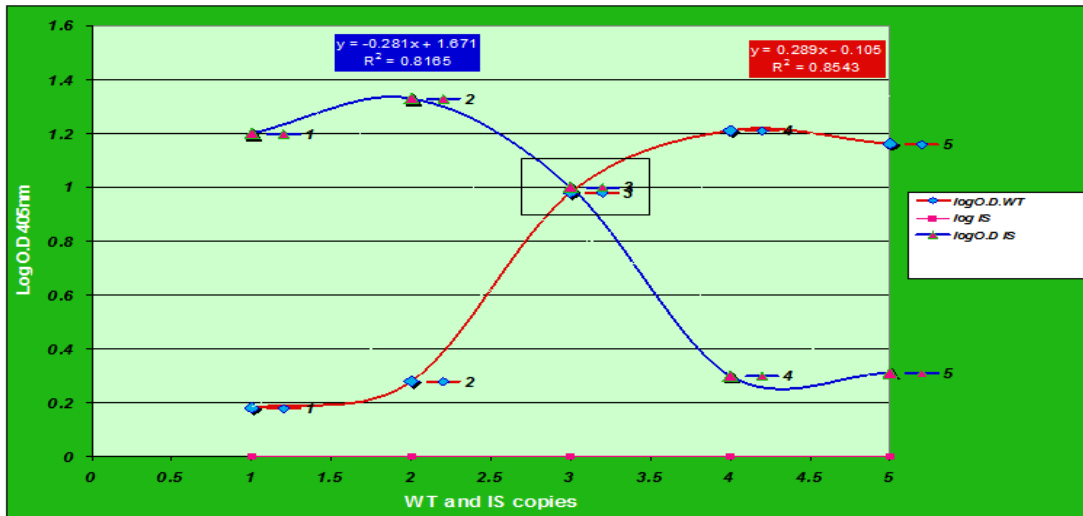
Figure 5: Serial 10 fold dilution of 10^1-10^5 WT copies were spiked with increasing amount of IS copies (10^5-10^1) in separate reaction. PCR products were detected by ELISA detection system.

The validation of accuracy of QC-PCR was reflected in Figure 6, where WT DNA estimated from linear regression curve was 822.2 which are nearly equal WT DNA copies added in the mixture amplified and this reflect the validation of accuracy of test since we have already added 1000 copies of WT DNA to the amplified reaction mixture.
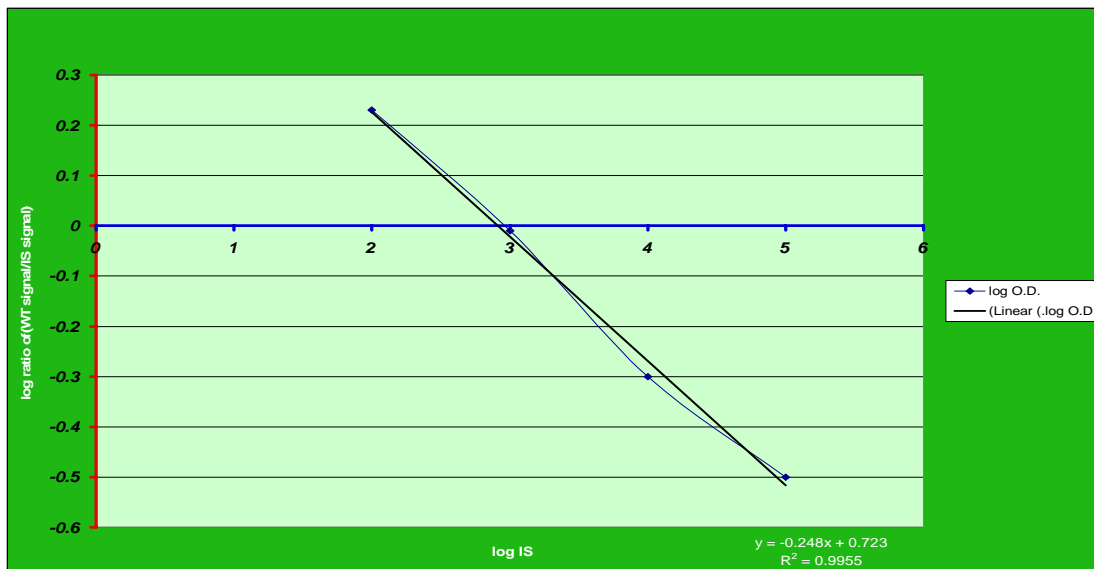

Figure 6: Quantification of WT EBV DNA .1000 copies of WT DNA were spiked with 10^2, 10^3, 10^4, 10^5 copies of IS in four separate reaction

Quantitative Competitive PCR assay:

The EBV DNA load in whole blood samples of patients and control was quantified through the estimation of linear regression curve between the logarithmic ratio of WT signal/IS signal produced by ELISA using WT and IS probe against the logarithmic values of IS copies added. The quantification of each sample estimated from the linear equation Y = ax + b pre and post therapy. The EBV load in those samples ranged from $0–1.936 \times 10^9$, while the EBV viral load in healthy controls were ranged from $7–1.9 \times 10^3$ there is significant difference between the viral load of patient and controls as shown in table 2 where P is 0.011 .

Cut off value: Healthy controls screened by QC-PCR, it was found that cut off value have EBV copy number ≤1.9× 103 EBV DNA copies/ml.

Follow up samples were available in 18 patients with active HL and NHL. Figure 7 showing the distribution of viral load values in patients from 0-1936421960 (1.936×109) EBV DNA copies/ml of blood and in controls from 7 to 1990 (1.99×103) EBV DNA copies/ml of blood .

Table 2 shows the EBV load in blood of patients at time of diagnosis and after completion of chemotherapy where 66.7% of HL Patients have viral load above cut off value while 44.5% of those patients with NHL have high viral load above cut off value. Regarding HL AND NHL patients response After completion of chemotherapy in table 3, the viral load declined in the group I, where 38.3% have high viral load above cut off value shows decline below cut off value while group II still have viral load above cut off value and elevated viral load above cut off value was detected in Group III.
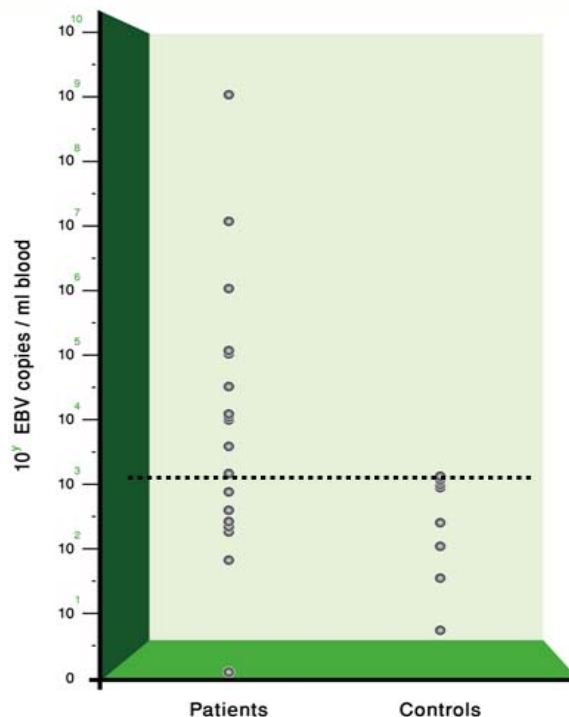

Figure 7: Distribution of EBV load in peripheral whole blood of lymphoma patients and controls.
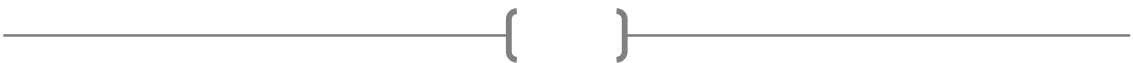
Table 2: Epstein Barr virus load in lymphoma patients as determined by EBNA-1 QC-PCR

Patient lymphoma type and No.	EBV viral load (copies/ml) pre therapy	EBV viral load above or below cut off value pre therapy	EBV viral load below cut off value percentage pre therapy	EBV viral load above cut off value percentage pre therapy	EBV viral load (copies/ml) post therapy	EBV viral load above or below cut off value post therapy	EBV viral load below cutoff value percentage post therapy	EBV viral load above cut off value percentage post therapy
HL 1	121060	Above	3(33.3%)	6 (66.7%)	5010	Above	6(66.7%)	3(33.4%)
HL 3	1778280	Above			501187	Above		
HL 6	340	Below			160	Below		
HL 7	16982436	Above			1280	Below		
HL 8	327	Below			1595	Below		
HL 9	840	Below			zero	Below		
HL 11	10715	Above			630960	above		
HL 17	109650	Above			10	Below		
HL 18	1936421960	Above			32	Below		
NHL 2	5861	Above	5(55.5 %)	4 (44.5%)	400	Below	9 (100.0%)	0(0%)
NHL 4	1860	Below			1000	Below		
NHL 5	80	Below			85	Below		
NHL 10	600	Below			zero	Below		
NHL 12	11220	above			45	Below		
NHL 13	50118	above			300	Below		
NHL 14	275	Below			130	Below		
NHL 15	Zero	Below			94	Below		
NHL 16	13500	Above			50	Below		

Table 3: Patients groups according to their viral load and their response to chemotherapy

Groups	Number (%)	EBV DNA load pre therapy in copies/ml	EBV DNA load post therapy in copies/ml	Response
I (High viral load)	7 (38.3%)	5861-1936421960 (Range)	10-1280 (Range)	Low viral load (Below cut off value)
П (High viral load	2 (11.1%)	121060-1778280	5010-501187	High viral load (Above cut off value)
Ш (High viral load	1 (5.5%)	10715	630960	Elevated viral load (Above cut off value)
IV (Low viral load	8 (44%)	0-1860	0-1595	Low viral load (Below cut off value)

Table 4: Quantitative Competitive Polymerase reaction (QC-PCR) Comparison of frequency between pre and post therapy

	Frequency of high viral load (Percentage)	Frequency of low viral load (Percentage)	Significance	
Pre therapy	10 (55.55%)	8 (44.45%)	Pre therapy *Vs* post therapy	0.036 *
Post therapy	3 (16.66%)*	15 (83.34%)	Pre therapy *Vs* control	0.011*
Control	0 (0%)	9 (100%)	Post therapy *Vs* control	0.202

Mann-Whitney Test: * Pre therapy *Vs* post therapy ($p < 0.05$)

* Pre therapy *Vs* control ($p < 0.05$)

The significance of QC-PCR assay results were highlighted and summarized in Table 4.

Discussion

The Epstein-Barr virus is a herpes virus which establishes a life-long persistent infection in over 90% of human adult population worldwide based upon its association with a variety of lymphoid and epithelial malignancy, EBV considered as group 1 carcinogen by the international agency for researches on cancer and it has a precise roles in development of virus associated human malignancies [12]. Patients viral load are included within the range of healthy controls.

In this study highly advanced molecular method was used to provide rapid and highly productive amplification of specific DNA sequence for achievement of an accurate and highly reproducible EBNA-1 QC-PCR which is important in diagnostic laboratories for diagnosis and monitoring of diseases [13]. The EBV load in patient blood samples ranged from 0– 1.936×10^9 while the EBV viral load in healthy controls were ranged from 7– 1.9×10^3 (Figure 7).

Table 2 shows that 66.7 % of HL patients and 44.5% of patients with NHL having EBV load above cut off value at time of diagnosis and before chemotherapy after starting of chemotherapy the EBV DNA load decreased below the cutoff value by 66.7% in HL

patients and by 100 % in NHL patients indicating good response to chemotherapy. These results come in agreement with Gandi et al, 2004 who stated that half of those patients having HL will respond to therapy and can be cured with conventional modality treatment also others found that after treatment no EBV genome were found in plasma of 6 HD patients which are stable with complete remission of the disease [15].

In group I HL patients with high EBV load at time of diagnosis with a range of 10715 (1.071×10^4) to 1936421960 (1.936×10^9) copies/ml blood, 50% of them showing response to chemotherapy with decline of viral load below cutoff value or to undetectable values, these results are in agreement with Gandi et al., 2006 who concluded that plasma EBV DNA have excellent sensitivity and could be used as a biomarker for EBV associated HL when he reported the presence of detectable viral load in 50% of EBV positive HL patients prior to therapy and after therapy EBV was undetectable unless in one patient under study.

In group I NHL the four patients who have a high EBV load above cut off value 5861 (5.86×10^3) -50118 (5.01×10^4) copies/ml blood

at time of diagnosis, they show 100% response and their viral load lowered below cutoff value, these results are in agreement with Josting et al, 2002 who observed continuously low or undetectable level of EBV in serum samples of NPC patients who reported that EBV DNA load is a valuable tool for monitoring of NPC patients against tumor recurrence.

Our results obtained confirm reports that patients with HL have an excellent prognosis with modern chemotherapy even if the disease is far advanced at diagnosis [18,19].

In studies assaying QC-PCR in lymphoma AIDS patients, our results come in agreement with others who reported that the viral load in 17 EBERS-positive lymphoma patients ranging from 34-1,500.000 copies per ml, this viral load fall rapidly upon initiation of lymphoma therapy and remaining undetectable except in two patients with persistent tumor [20].

In group II (Table 3) QC-PCR shows that Patients viral load decrease after chemotherapy but still above cutoff value, similar results obtained by Fan et al. 2004 who work with EBV viral load of lymphoma in AIDS patients , they found that the viral load usually falls upon initiation of chemotherapy except in two patients with persistent tumor. It is reported that when analyzing the EBV status in the peripheral blood of pediatric patients with HD where no EBV DNA was detected in plasma of HD with complete remission while 2 of 5 HD patients will relapse and are positive for EBV DNA [15].

In group Ш (Table 3) who have high viral load above the cutoff value and increase in the EBV load after chemotherapy. The presence of high viral load at diagnosis time is in agreement with Gallagher et al. 1999 who reported that EBV DNA was detected in 91% of serum samples from patients with EBV positive HL. The increase in the EBV load after completion of treatment are in

consistent with Drouet et al. 1999, that this viral DNA was probably a consequence of viremia which is related to increased viral replication in a non neoplastic compartment such as oropharynx which explain the increase in the viral load during the period of treatment.

In the field of EBV associated NPC, the same finding of persistence in the viral load after chemotherapy was observed, workers in this field explained that this persistence of viral load following primary therapy was predictive of relapse [23,24,25,16].

Other worker with EBV viral load of lymphoma in AIDS patients, found that the viral load usually falls upon initiation of chemotherapy except in two patients with persistent tumor [26], others analyze the EBV status in the peripheral blood of pediatric patients with HD where no EBV DNA was detected in plasma of HD with complete remission while 2 of 5 HD patients relapse and were positive for EBV DNA [15].

For group IV of patients with initial viral load at the time of diagnosis below cutoff value, some of them are EBV negative, after the completion of the chemotherapy they showed slightly increase in the viral load but still below the cutoff value. The absence of elevated EBV DNA load in those patients who constitute 33.3% in HL and 55.5% in NHL as shown in Table 2 and falls in group IV which constitute 44 .45% in table 4, This absence of elevation of EBV load in these groups of patients might be explained by the fact that those patients developed an EBV-negative lymphoma and these results could be debated by that some patients have lymphoma not due to oncogenic potential of EBV this is consistent with [10]. These results are also consistent with studies done [10,21], they reported that EBV DNA was detected in 23% of EBV negative HL patients. Others

found EBV DNA in 24% of EBV negative lymphoma [24].

The molecular nature of EBV DNA in these tumors elucidated by Fan and Gullay 2001, they found that circulating EBV DNA exists as short fragments of less than 200bp. This implies that the increase of EBV DNA is due to tumor release of EBV DNA fragments instead of virion reactivation and these fragments are naked molecules not protected by viral protein coat [27], They found a relationship between circulating EBV DNA and apoptosis that DNA is fragmented by caspase-activated DNAase and resulting in DNA fragments with length in multiple of nucleosomal DNA.

It's concluded that EBV could be considered as a target in the effective diagnosis of EBV associated tumors. EBV DNA load could be a promising marker for the patients who express EBV load above the cutoff value. The QC-PCR assay allow accurate quantification of EBV load and show promise as a tool to assist in diagnosis and management of EBV related lymphoma patient, it is potentially useful in the diagnosis and follow up as well as in the assessment of the efficiencies of chemotherapeutic regimens consequently, in these cancers EBV DNA may be considered as a real tumor biomarker.

Acknowledgments

References
1. Kieff E. Epstein-Barr virus and its replication. In: Field's virology by: Fields BN, Knipe DM, Howley PM, et al. 3rd ed. Philadelphia, Lippin Cott. Raven publisher 1996; p. 2343-2380.

2. Thompson MP, and R Kurzrock. Epstein-Barr virus and cancer. *Clin Cancer Res*, 2004; 10: 803-821.

3. Zimmermann K, and Mannhalter JW. Technical aspects of quantitative competitive PCR. *Biotechniques*, 1996; 21: 268-279.

4. Gustafsson A, Levitsky V, Zou JZ, Frisan, T, Dalianis, T, Ljungman, P, et al. Epstein-Barr virus (EBV) load in bone marrow transplant recipients at risk to develop posttransplant lymphoproliferative disease: prophylactic infusion of EBV-specific cytotoxic T cells. *Blood*, 2000; 95: 807-814.

5. Fan H, Kim SC, Chima CO, Israel BF, Lawless KM, Eagan PA, et al. Epstein Barr viral load as marker for lymphoma in AIDS patients. *J Med Virol*, 2004; 75: 59-69.

6. Boom R, Sol CJA, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol*, 1990; 28: 495-503.

7. Kushner SR. An improved method for transformation of Escherichia coli with Col E1 derived plasmids. In Boyer HW, and Nicosia S. Genetic Engineering, eds., New York NY, Elsevier/North–Holland Biomedical Press, 1978; p. 17-23.

8. Pospiech A, and Neumann B. A versatile quick-prep of genomic DNA from Gram-positive bacteria. *Trends Genet*, 1995; 11, 217-218.

9. Stevens SJC, Vervoort HJ, Van der Brule AJC, Meenhorst PL, Meijer CLJM, Middeldor JM. Monitoring Epstein-Barr virus DNA load in peripheral blood by quantitative competitive PCR. *J Clin Microbiol*, 1999; 37: 2852-2857.

10. Jacobs MV, van den Brule AJ, Snijders PJ, Helmerhorst TJ, Meijer CJ, Walboomers JM. A non radioactive PCR enzyme -immunoassay enables a rapid identification of HPV16 and 18 in cervical smears after GP5+/6+PCR. *J Med Virol*, 1996; 49(3): 223-229.

11. Niedobitek G, Meru N, and Delecluse H J. Epstein-Barr virus infection and human malignancies. *Int J Experimental Pathol*, 2001; 82: 149-170.

12. Zentilin L, and Giacco M. Competitive PCR for precise nucleic acid quantification. *Nature Protocols*, 2007; 2: 2092-2103.

13. Gandi MK, Tellam JI, and Khanna R. Epstein Barr virus associated Hodgkin's lymphoma. *Br J Haematol*, 2004; 125: 267-281.

14. Wagner HJ, Schlager F, Claviez A, Buesky P. Detection of Epstein-Barr virus DNA in peripheral blood of pediatric patients with Hodgkin's disease by real-time polymerase chain reaction. *Eur J Cancer*, 2001; 37: 1853-1857.

15. Gandhi MK, Lambley E, Burrows J, Dua U, Elliott S, Shaw PJ, et al. Plasma Epstein-Barr virus (EBV) DNA

is a biomarker for EBV-positive Hodgkin's lymphoma. *Clin Cancer Res*, 2006; 12: 460-464.

16. Lo YM, Chan LY, Lo KW, Leung SF, Zhang J, Chan AT, et al. Quantitative analysis of cell frees Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. *Cancer Res*, 1999; 59: 1188-1191.

17. Josting A, Franklin J, May M, Koch P, Beykirch MK, Heinz J, et al. New prognostic score based on treatment outcome of patients with relapsed Hodgkin Lymphoma registered in the data base of the German Hodgkins Lymphoma Study Group. *J Clin Oncol*, 2002; 20(1): 221-230.

18. Schmitz N, and Sureda A. The role of allogenic stem-cell transplantation in Hodgkin's disease. *Eu J Haematol*, 2005; 75: 146-149.

19. Fan H, Kim SC, Chima CO, Israel BF, Lawless KM, Eagan PA, Elmore S, et al. Epstein Barr viral load as a marker for lymphoma in AIDS patients. *J Med Virol*, 2004; 75(1): 59-69.

20. Gallagher A, Armstrong AA, MacKenzie J, Shield L, Khan G, Lake A, et al. Detection of Epstein Barr virus (EBV) genomes in the serum of patients with EBV-associated Hodgkin's disease. *Int J Cancer*, 1999; 84 (4): 942-948.

21. Drouet E, Brousset P, Fares F, Icart J, Verniol C, Meggetto F, et al. High Epstein-Barr virus serum load and elevated titers of anti ZEBRA antibodies in patients with EBV-harboring tumor cells of Hodgkins disease. *J Med Virol*, 1999; 57(4): 383-389.

22. Chan KC, Zhang J, Chan AT, Lei KI, Leung SF, Chan LY, et al. Molecular characterization of circulatory

EBV DNA in the plasma of nasopharyngeal carcinoma and lymphoma patients. *Cancer Res*, 2003; 63(9): 2028-2032.

23. Au WY, Pang A, Choy C, Chim CS, Kwong YL. Quantification of circulating Epstein-Barr Virus (EBV) DNA in the diagnosis and monitoring of natural killer cell and EBV positive lymphoma in immunocompetent patients. *Blood*, 2004; 104(1): 243-249.

24. Lin JC, Wang WY, Chen KY, Wei YH, Liang WM, Jan JS, et al. Quantification of plasma Epstein-Barr virus DNA in patients with advanced nasopharyngeal carcinoma. *N Eng J*, 2004; 350: 2461-2470.

25. Fan H, and Gullay ML. Epstein-Barr viral load measurement as a marker of EBV related disease. *Mol Diagnostic*, 2001; 6: 279-288.

26. Chan KC, Zhang J, Chan AT, Lei KI, Leung SF, Chan LY, et al. Molecular characterization of circulatory EBV DNA in the plasma of nasopharyngeal carcinoma and lymphoma patients. *Cancer Res*, 2003; 63(9): 2028-2032.

27. Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, et al. DNA fragments in the blood plasma of cancer patients: quantification and evidence of their origin from apoptotic and necrotic cells. *Cancer Res*, 2001; 61(4): 1659-1665.

IgE Level and Eosinophil Count in Relation with Type of Feeding in Children with Atopic Dermatitis

Ahmed H. Alanee[1] *CABP*, **Nawal B. Mahdi**[1] *CABP*, **Talal Sabbar**[2] *MBChB*,
Ehsan Mahmood[3] *FICMS*

[1]Dept. of Pediatrics, College of Medicine, Tikrit University, [2]Tikrit Teaching Hospital, [3]Dept. of Pathology, College of Medicine, Tikrit University

Abstract

Background   Atopic dermatitis (AD) is a chronic inflammatory disease of the skin that occurs in persons of all ages but is more common in children.

Objective   To determine the effect of breast feeding on atopic dermatitis.

Methods   A descriptive study carried on pediatric patients visiting asthma and allergy center and pediatrics out patient in Tikrit Teaching Hospital from March 2007 to August 2007. This study included 100 patients with AD; 58 cases (58%) were males and 42 cases (42%) were females, they all underwent full history, clinical examination and blood sampling for total WBC count, eosinophil cells count, and total serum IgE.

Results   Statistical analysis was done by using (ANOVA, Chi-square test). There was significant association between severity of AD according to scorad index (S.I) and type of infant feeding, the mean severity score (S.S) for the breast fed (B.F) children was 21.48±4.96, while the mean S.S for the formula fed (F.F) children was 35.70±9.79, *p* value ≤ 0.05. There was a significant association between the hematological parameters of disease activity (total serum IgE level, eosinophil count) and type of infants feeding; the mean total serum IgE level for the B.F children was 211±178.7 IU/ml, was lower than mean total serum IgE level for F.F children (638.2±355.8 IU/ml). The mean eosinophil cells count for the B.F children (355.7±193.1) cell/μl was lower than the mean eosinophil cells count for the F.F children (654.8±236.3) cell/μl.

Conclusions   There is significant association between the type of feeding and the level of hematological parameters (IgE and eosinophil count), and that breast feeding is protective against development of severe AD.

Key words   atopic dermatitis, breast feeding, IgE, eosinophil

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease that occurs with a peak onset in infancy and the majority of cases presenting in the first few years of life [1]. Atopic dermatitis is also known as atopic eczema. Exclusive breast feeding seems to have a preventive effect on the early development of allergic disease up to 2 years of age [2]. It is widely believed that B.F should be recommended for primary prevention of allergic disease. Exclusive B.F beyond four months of age reduces the development of atopic disease in early life [3,4]. Hanifin and Rajka (1980) proposed major and minor criteria based on their clinical experience. To diagnose atopic dermatitis we must have 3 or more of these major features plus three or more of minor features [5]. Elevated IgE levels are found in up to 80 % of affected patients, IgE

levels are also elevated in patients with other atopic diseases [6]. IgE antibodies mediate the immediate hypersensitivity reactions [7].

Serum IgE level are elevated above 200 IU/ml in 80-90% of patient with AD. Patient with very active disease may have IgE levels greater than 1000 IU/ml. However, 20% of patients with AD have normal or below normal levels of IgE, suggest that IgE elevations are a coincident feature of disordered cell regulation rather than pathogenic factor [8].Increased number of eosinophils in blood is frequently present in a variety of allergic conditions especially in atopic disorder. Eosinophilia is generally defined as the presence of more than 450 eosinophils/µl of blood. Seasonal increases in the number of circulating eosinophils may be observed in sensitized patients after exposure to allergens such as tree, grass, and weed pollens [9]. The clinical severity of AD was assessed by the Scorad index [10]:

The aim of this study was to clarify the association between breast feeding and severity of atopic dermatitis, total serum IgE level and eosinophil count.

Methods

A descriptive study was carried on pediatric patients visiting Asthma and Allergy Center & pediatric outpatient in Tikrit Teaching Hospital from March 2007 to August 2007.

Study population:

This study included 100 patients with atopic dermatitis (58 males & 42 females). Their ages were less than 18 year. All the patients met the diagnostic criteria for atopic dermatitis, as defined by Hanifin and Rajka [5]. None of these patients had received antihistamines, systemic or topical corticosteroids during the period of 3 weeks before clinical evaluation.

Data collection:

Each patient was assessed by full history and physical examination.

Early feeding method, the milk feeding history during the first 6 month of life was obtained for each child and classified as follow:

A. Breast feeding (children who had been totally breast fed since birth and never received cow's milk).

B. Bottle feeding (children who had been totally bottle fed since birth and never received breast milk).

 C. Mixed feeding (children who had been breast fed but had received some cow's milk before 6 months of age).

Each patient was generally examined for chest, face, color, eyes, nose, neck, hands and feet. Local examination included the skin lesions site, size, types; xerosis, erythema, edema, papulation, oozing, crusting, excoriation and lichenifecation.

The severity of atopic dermatitis was measured by using the Scorad index. The child must have 3 or more major features plus 3 or more minor features [5]. The diagnosis of atopic dermatitis depends on history and clinical examination and supported by differential white blood cell (WBC) cells count, eosinophil cell count and total serum IgE level.

Investigations:

The laboratory tests included:

1. Eosinophil count; eosinophil cells count of more than 450 cell/µl was considered pathological.

2. Total serum IgE which was determined by enzyme linked immuno-sorbant assay kit (Biomagreb) was determined in 32 sera of AD patients. Serum values of more than 200 IU/ml were considered high.

Statistical analysis:

The results were given as mean ± SD. Values and data were statistically analyzed using SPSS version (4). The difference between the study groups were tested by using (ANOVA, Chi- square). $P < 0.05$ was considered as statistically significant.

Results

Family history was positive in 84 cases (84%) while 16 cases (16%) had negative family history.

The mean S.S for the B.F patients was 21.48±4.96, for the mixed type of feeding was 33.34±12.92 while for the formula fed patients it was 35.7±9.7 as shown in Table 1.

Table 1: The mean severity score according to feeding history

Feeding history	Severity score		
	No.	Mean	SD
B.F	42	21.48	4.96
Mixed	16	33.34	12.9
Formula	42	35.70	9.79
Total	100	29.35	11.0

p-value = 0.000 (significant), B.F (breast feeding)

Table 2 reveals that most of the patients whose total serum IgE levels below 200 IU/ml (7 cases, 87.5%) were B.F, while most of the patients whose total serum IgE was above 200 IU/ml were formula fed patients (13 cases, 54.2%, p-value = 0.003). The mean IgE levels for the breast fed patients .002.

were significantly decreased as compared to mixed fed group & formula fed patients, the mean IgE level for the breast fed patients was 211±178.7 IU/ml, for the mixed fed was 472.3±22.4 IU/ml and for the formula fed patients was 638.2±355.8 IU/ml as shown in Figure 1, p-value = 0 .002.

Table 2: Patient distribution according to the total serum IgE level) and feeding history

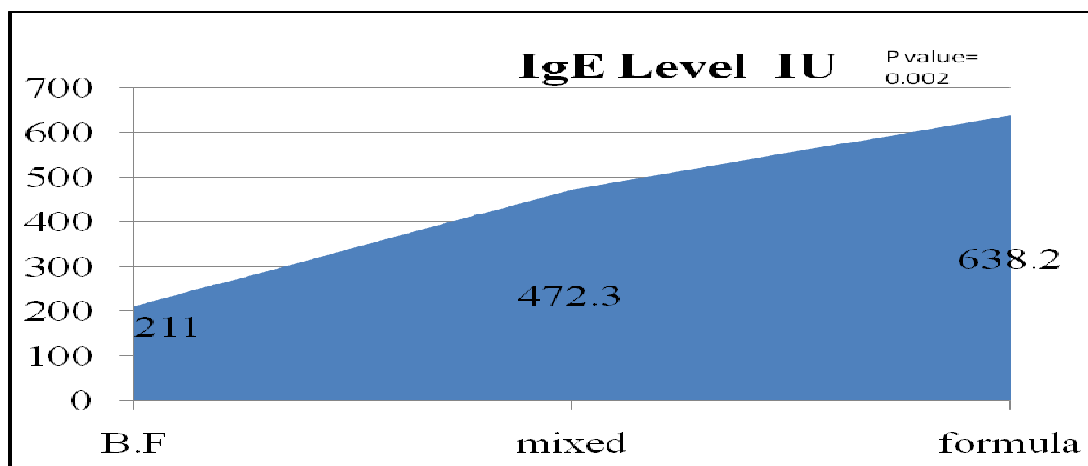| Feeding history	IgE count IU		Total
	<200	>200	
B.F	7(87.5%)	5 (20.8%)	12 (37.5%)
Mixed	0(0%)	6 (25%)	6 (18.8%)
Formula	1(12.5%)	13 (54.2%)	14 (43.8%)
Total	8(100%)	24 (100%)	32 (100%)

p-value = 0.003 (significant)

Figure 1: Mean IgE level according to feeding history
B.F (breast feeding), p-value = 0.002

Table 3 shows that most of the patients who had eosinophil count below 450 cell/µl were B.F (26 cases out of 34, 76.5%) and most of the patients that had eosinophil count above 450 cell /µl were formula fed (35 out of 66 cases, 53%, *p*-value = 0.000).

Table 3: The patient's distribution according to Eosinophil cells count and feeding history

| Feeding history | Eosinophil cells count | | | | Total | |
| | < 450 | | > 450 | | | |
	No.	Percentage	No.	Percentage	No.	Percentage
B.F	26	76.5 %	16	24.2 %	42	42 %
Mixed	1	2.9 %	15	22.7 %	16	16 %
Formula	7	20.6 %	35	53.0 %	42	42 %
Total	34	100 %	66	100 %	100	100 %

p-value = 0.000 (significant)

The mean eosinophils count for the B.F patients was 355.7±193.1 cell/µl, for the mixed fed patients was 688.1±217.8 cell/µl, while for the formula fed patient were 654.8±236.3 cell/µl ($p < 0.001$) as shown in table 4.

Table 4: The Mean eosinophil cells count according to feeding history

| Feeding history | Eosinophil cells count | | |
	No.	Mean	SD
B.F	42	355.7	193.1
Mixed	16	688.1	217.8
Formula	42	654.8	236.3
Total	100	534.5	263.2

p-value < 0.001   No. (number)   B.F (breast feeding)

Discussion

Atopic dermatitis is a distressing inflammatory skin disease affecting large number of children worldwide, with its variable clinical presentations and course, constitutes a syndrome made up of an identifiable group of signs and symptoms that represents the dermatological manifestation of the atopic diathesis [11].

The total number of the cases included in the study was 100 cases; most of the patients were males (58%). This goes with Adriana et al that showed 41.3% of AD patients were females and 58.7% were males [12]. It is well known fact that both sexes are affected, but in adults the disease is more common in females, while in children, atopic dermatitis is more common in males [13].

Male gender, and family history of atopy, was associated with increased risk of AD in the first 6 months of life. These findings suggest that the genetic and perinatal influences are important for this difference in both genders [14]. There is no clear reason for this difference; in that it is more common in males in childhood.

Family history was positive in 84 cases (84%) while, 16 cases (16%) had negative family history. This agrees with what was found by Blumenthal who showed that most of the cases with AD had positive family history [15]. This may be due to the fact that AD is an inherited disease that runs in families but there is no clear way of inheritance and this explains why clinically normal parents may have affected children,

which excludes simple dominant inheritance. On the other hand, in other families both parents may be affected but the children are normal excluding a simple recessive trait [16].

Regarding the mean severity score according to feeding history, the mean S.S for the B.F patients were (21.49±4.97), the mean S.S for the mixed fed patients were (33.34±12.92) while the mean S.S for the formula fed patients were (35.70+ 9.79). These results were consistent with another study by Host et al (1999) which showed that B.F has the ability to modifying the diseases severity [17]. Typically B.F can decrease the severity of AD, but cannot prevent its occurrence. It is widely recommended for the first 4-6 months. Human colostrums/milk facilitates maturation of the gut and provides passive protection against infectious agents and antigens [17]. Saarinen et al, 1979 reported also that the intensity of the manifestations of atopy were softened in children who were on B.F for the first 6 months compared with children who were not ,or who were B.F but for shorter periods of time (up to 2 months) [18].

Exclusive B.F is a protective factor for development of AD if compared with conventional cow's milk formula [19] .This protective effect may be related to the fact that allergic conditions in children are often related to food sensitivity, and B.F helps prevent this problem through a variety of mechanisms. Exclusive breast-feeding for 6 months means to avoid feeding the baby any food known as allergen and as precipitation factor of allergic diseases. Breast milk being rich in immunoglobulin A (IgA) can help to protect the gastrointestinal tract by binding foreign protein which has a potential to be allergenic and inhibit its absorption. Nutritional contents of breast milk will stimulate the maturation of gastrointestinal tract, so that it is ready to receive the antigens, maintain normal flora of gastrointestinal tract, and maintain the immunemodulatory factors [20].

Most of the patients whose total serum IgE levels were below 200 IU/ml were B.F, 7 cases (87.5%), while most of the patients who had total serum IgE above 200 IU/ml were formula fed, (54.2%). This goes with another study conducted by Businco et al. in 2005 who reported that the B.F patients had a total IgE level less than that of patients who were bottle fed [22]. This is due to the same reason mentioned above in that B.F is a protective factor against having an allergic disease while cow milk and cow milk protein are an aggravating factor for allergic disorder with subsequent increase in IgE level.

The results of IgE of the present study go with that found by Businco et al. (1983) who observed that the children fed breast milk until the age of 6 months presented significantly lower levels of IgE compared with children fed cow's milk. This may be due to that B.F avoiding the child from early sensitization to cow's milk protein and IgE over production [21].

Most of the study patients who had eosinophil counts below 450 cell /μl were B.F, 26 cases (76.5%) while most of the study patients who had eosinophil count above 450 cell /μl were formula fed (53%).

The eosinophils count for the present patients are in agreement with the fact that milk protein allergy can induce eiosinophilia [9]. Infants suffering from severe AD reveal a low serum albumin level, and electrolyte disturbances, and have significantly higher number of eosinophils and eosinophilic nuclear lobes, platelets, and total serum IgE level [22]. The clinical activity of the disease as recorded by the scorad index can be used as an indicator of the hematological abnormalities as well as to some extent as a prognostic indicator [23].

Conclusions Breast feeding can decrease the severity of the atopic dermatitis & decrease the peripheral blood eosinophil count and total serum IgE level.

References

1. Diepgen TL. Is the prevalence of atopic dermatitis increasing? In: Williams HC, Epidemiology of Atopic Eczema. Cambridge United Kingdom, 2000; p. 96-109.
2. Kull I, Wickman M, Lilja G, Nordvall SL, Pershagen G. Breast feeding and allergic diseases in infants. *Arch Dis Childhood*, 2002; 87(6): 478-481.
3. Leicht S and Hanggi M. Atopic dermatitis: how to incorporate advances in management. *Postgrad Med J*, 2001; 6: 119-127.
4. Reza G, and Zahra M. Effect of breast-feeding on the development of atopic dermatitis. *Iranian J Aller Asthma and Immunol*, 2005; 4(3): 129-132.
5. Hanifin J, and Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol*, 1980; 92: 44-47.
6. Leung D. Atopic dermatitis: the skin as a window into the pathogenesis of chronic allergic disease. *J Allergy Clin Immunol*, 1995; 96: 312-319.
7. Golds by Antibodies: Structure and Function. Kuby Immunology 5th ed. May 2002; p. 4, 76.
8. Thomas PH. Atopic dermatitis. Clinical dermatology: A color guide to diagnosis and therapy. 3rd ed., Mosby, 1996; p. 100-121.
9. Atkins D and Donald YML. Diagnosis of Allergic Disease. In: Richard E, Robert M, and Hal BJ. Nelson Text Book of Pediatrics, 17th ed. WB Saunders, 2003; p. 748-751.
10. Gdalevich M, and Mimouni D. Breast-feeding and the onset of atopic dermatitis in childhood. *Am Acad Dermatol*, 2001; 45: 520-527.
11. Beltrani VS, and Boguneiwicz M. Atopic Dermatitis. *Dermatol Online J*, 2003; 9: 1.
12. Leite AAC, Leite RMS, Borges W, Burns DA. Can breast-feeding modify atopy severity manifestation during childhood? Anais Brasileiros de Dermatologia, 2000; 75(3): 311-319.
13. Rajka G. Essential aspect of atopic dermatitis. Berlin: Springer Verlag 1989; p. 45.
14. Megan M, Sheryl L, and Janet W. Perinatal predictors of atopic dermatitis occurring in the first six months of life. *INH Public Access*, 2004; 3: 468-474.
15. Blumenthal MN, and Amos DM. Genetic and immunological basis of the atopic response. *Chest,* 1987; 91: 176-184.
16. Holden CA and Parish WE. Atopic Dermatitis. In: Champion HR, Burton JL, Burns DA, et al. Rook Text book of dermatology, 6th ed., Vol. 1, Black Well Science, 1998; 18; 681-708.
17. Host A, Koletzko B, and Dreborg S. Breast feeding can modify the atopic dermatitis severity. *Arch Dis Child*, 1999; 8: 80-84.
18. Saarinen UM, Kajosaari M, Backman A, Siimes MA. Prolonged breast-feeding as prophylaxis for atopic disease. *Lancet*, 1979; 2(8135): 163-166.
19. Hauschildt E. Pediatric Effect of exclusive breast-feeding and early solid food avoidance on the incidence of atopic dermatitis in high-risk infants at 1 year of age. *Pediat Allergy Immunol*, 2002; 13: 234-242.
20. Goldman AS. Modulation of the gastrointestinal tract of infants by human milk. Interfaces and interactions. An Evolutionary Perspective. *J Nutrition*, 2001; 130(2): 426-431.
21. Businco L, Marchetti F, and Pellegrini G. Predictive value of cord blood IgE levels in "at-risk" newborn babies and influence of type of feeding. *Clin Allergy,* 1983; 13: 503-508.
22. Toma T, Mizuno K, and Okamoto H. Expansion of activated eosinophils in infants with severe atopic dermatitis. *Pediatr Int*, 2005; 47: 32-38.
23. Sandipan D, Rajib M, and Soumen C. Correlation of the severity of atopic dermatitis with absolute eosinophil counts in peripheral blood and serum IgE levels. 2005; 71: 246-249.

Correspondence to: Dr. Nawal B. Mahdi,
E-mail: nawalbahjat@yahoo.com
Received: 3rd Feb. 2009, Accepted: 21st Feb. 2010.

Value of C - Reactive Protein Measurements in Exacerbations of Chronic Obstructive Pulmonary Disease

Methaq A. M. Hussein *MBChB*, **Hashim M. Al-Kadhimi** *MRCP, FRCP*

Dept. of Medicine, College of Medicine, Al-Nahrain University

Abstract

Background The acute phase protein, CRP, when elevated, provides good evidence of an active tissue–damaging process. Thus; its measurement provides a simple screening test for active organic disease .Increased CRP production is a very early and sensitive response to most forms of bacterial infection..

Objective was to ascertain whether infective exacerbations of chronic obstructive pulmonary disease (COPD) and their successful treatment correlate with corresponding changes in CRP level.

Methods Fifty Patients (age 65 ± 6 years) diagnosed as COPD on the basis of clinical history and pulmonary function test were enrolled into the study. All those were admitted to Al-Kadhimya Teaching Hospital because of clinical exacerbations of their condition. Serum samples were obtained on admission from the patient for measurement of CRP and full blood count together with sputum sample for microbiological diagnosis (especially culture). CRP measured by semi quantitative method, the cutoff point of this test is 0.6 mg/L .So all positive values were ≥ 1.2 mg/L. All these patients with exacerbations were treated by conventional treatments. Blood samples for CRP, full blood count and forced expiratory volume in 1st second (FGV1) were repeated 4-5 days thereafter.

Results The levels of CRP were elevated ≥ 1.2 mg/L in 27 patients who were positive for bacterial culture .The average CRP level after adequate treatment was highly decreased (*p* value < 0.001) .There was a significant improvement in their measured FEV1 (*p* value < 0.001) . The peak CRP level and fall in CRP were significantly correlated with both the corresponding peripheral blood smear white cell count (r=0.57, *p* value < 0.001) and the correlation Coefficient between CRP and FEV1 was (r= -0.45, *p* value < 0.001).

Conclusions Since patients with acute exacerbations of COPD had their CRP levels elevated initially and had clinical improvement with lowering of the CRP levels after treatment, there is a strong possibility that CRP is a marker of exacerbation of COPD. We suggest that, in exacerbation of COPD, CRP estimation provides a useful and inexpensive early marker of the exacerbation and provides a useful guide to assess the efficacy of treatment.

Key words C, reactive protein. Chronic obstructive pulmonary disease with exacerbation.

Introduction

Chronic obstructive pulmonary disease (COPD) is defined as a disease state characterized by the presence of airflow obstruction due to chronic bronchitis or emphysema.

The airflow obstruction generally is progressive, may be accompanied by airway hyper-reactivity, and may be partially reversible [1]. Pathological changes in COPD occur in the large (central) airways, the small (peripheral) bronchioles, and the lung parenchyma [2].

Although primarily affecting the lungs, the chronic inflammatory process of COPD does have systemic effects [3]. There is increasing evidence that COPD is a multiorgan system disease. Skeletal muscle weakness and wasting and impaired exercise tolerance which are frequently occurring symptoms in advanced COPD appear to be linked to a systemic inflammatory response [4]. Several systemic inflammatory mediators such as TNF-**α** [5], the soluble TNF transmembrane receptor-75 (sTNF-R75) [6], soluble adhesion molecules [7], some interleukins, acute phase proteins (CRP, fibrinogen, lipopolysacceride-binding protein (LBP)) and leucocytes are increased in the systemic circulation of patients with COPD [4]. One of the markers of systemic inflammation that is consistently shown to be slightly increased in patients with COPD compared with healthy controls is CRP [8]. Exacerbation is a prominent feature of the natural history of COPD. Exacerbations are commonly considered to be episodes of increased dyspnea and cough and change in the amount and character of sputum. Exacerbations are more frequent as disease progresses and are most often triggered by respiratory infections, often with a bacterial component [9].

Studies have shown that an elevated CRP level is a useful indicator of exacerbation in cystic fibrosis, chronic bronchitis, and COPD [10-12].

Methods
Patients
Fifty Patients (age 65±6 years) with COPD on the basis of clinical history and pulmonary function test were enrolled into the study, were admitted to Al-Kadhimya Teaching Hospital because of clinical exacerbations of their condition with dyspnea and increased cough with expectoration of yellow-green sputum. All of them had baseline FEV1 of (0.7±0.2). Pneumonia was excluded by chest radiograph and clinical examinations.

Blood samples
Blood was withdrawn and serum was collected for measurement of CRP and full blood count together with sputum sample for microbiological examination (especially culture). CRP measured by using of Wellcotest Latex agglutination test (*Wellcome Diagnostics*) which is semi-quantitative method, the cutoff point of this test is ≤ 0.6 mg/dL, so all positive values were ≥ 1.2 mg/L.

Treatment and Follow up
All these patients with exacerbations were treated with antibiotics, bronchodilators, controlled low tension oxygen therapy, low dose diuretics for those associated with right sided heart failure with short course of steroids. Then blood sample for CRP and full blood count (Blood samples were collected for all patients before and after treatment. FEV1 is also repeated after 4-5 days after treatment mentioned above.

Statistical Analysis
The statistical analysis was done using *t test* and correlation coefficient (r). All the results are significant if the *p* value is < 0.005.

Results
CRP levels were elevated ≥ 1.2 mg/L in 27 patients who were positive for bacterial culture (group I), and 11 of the 23 patients with no clear bacteriological evidence of infection (group II); while those with CRP and culture negative were mentioned as group III (see Figure.1). The average elevated CRP level in group I was (11.2±6.8); while average

CRP level in those with culture negative (i.e. group II and III) was (3±2.9).

The average CRP level after adequate treatment was (3.2±2.9) (*p* value < 0.001). There was an improvement in their measured FEV1 from 0.78±0.2 before treatment to a mean of 1.4±0.2 after treatment (*p* value < 0.001).

The patient characteristics and their relationship to CRP were shown in table 1.

The peak level and fall in CRP were both correlated with the corresponding peripheral blood smear white cell count (r=0.57, *p* value < 0.001) as in Figure 2.

Also the correlation Coefficient between CRP and FEV1 was (r= -0.45, *p* value < 0.001) as shown in Figure 3.
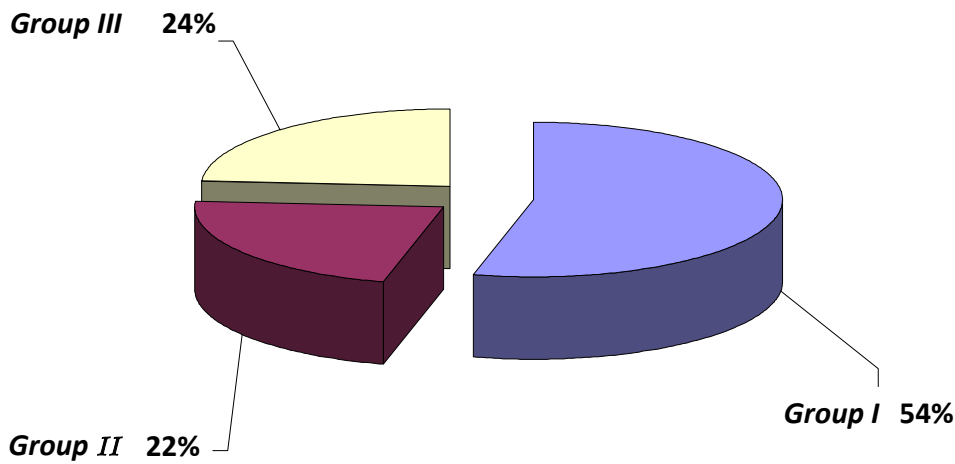
Figure 1: Groups of the patients in the study
Group I –Those with elevated CRP and culture positive (54%)
Group II- Those with deviated CRP and culture negative (22%)
Group III- Those with normal CRP and culture negative (24%)

Table 1: Characteristics of patients and their relationship to CRP

Characteristic of patients	Elevated CRP N=38	Normal CRP N=12	*p* value
Age (Mean ± SD)	63±6.14	68±5.8	0.359
Sex ♂/♀	30/8	10/2	0.741
FEV1 (Mean ± SD)	0.739±0.20	0.89±0.18	0.029
FEV1-5d (Mean ± SD)	1.423±0.23	1.32±0.18	0.193
WBC (Mean ± SD)	12.77±1.86	8.99±1.12	0.0001
WBC-5d (Mean ± SD)	8.27±1.86	4.88±0.68	0.0001
Culture P/N	27/11	0/12	0.0001

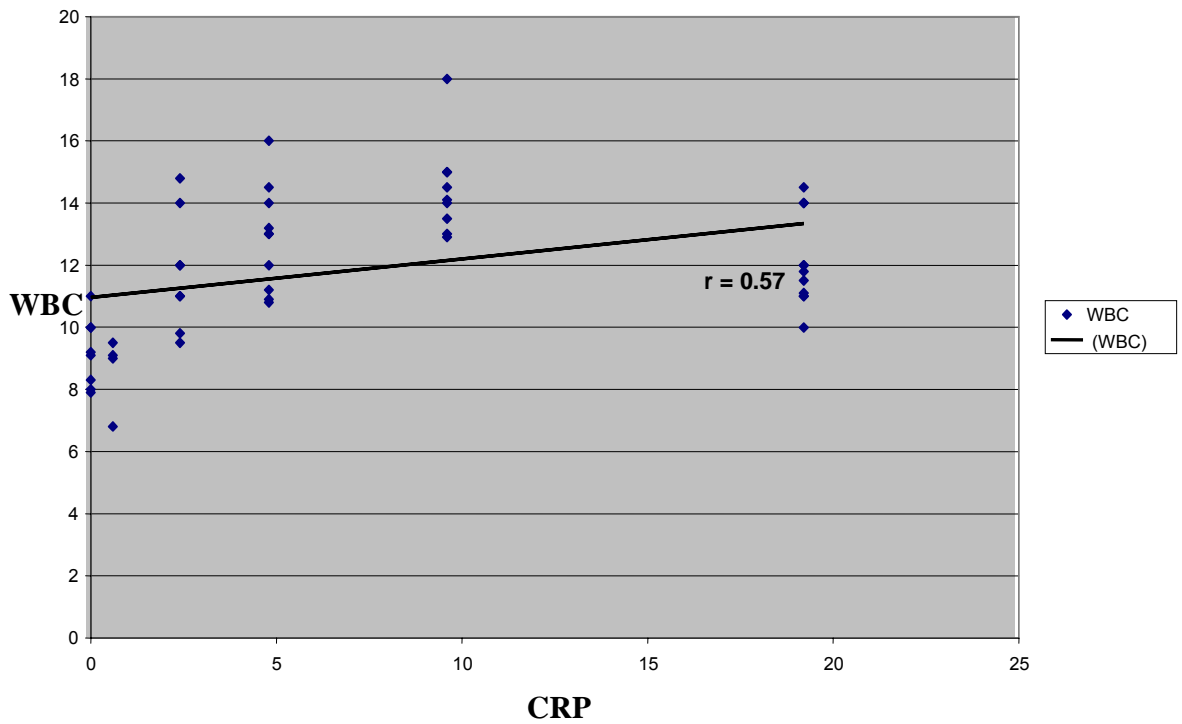**Figure 2: Correlation between CRP before and after treatment with corresponding peripheral blood white cell count**

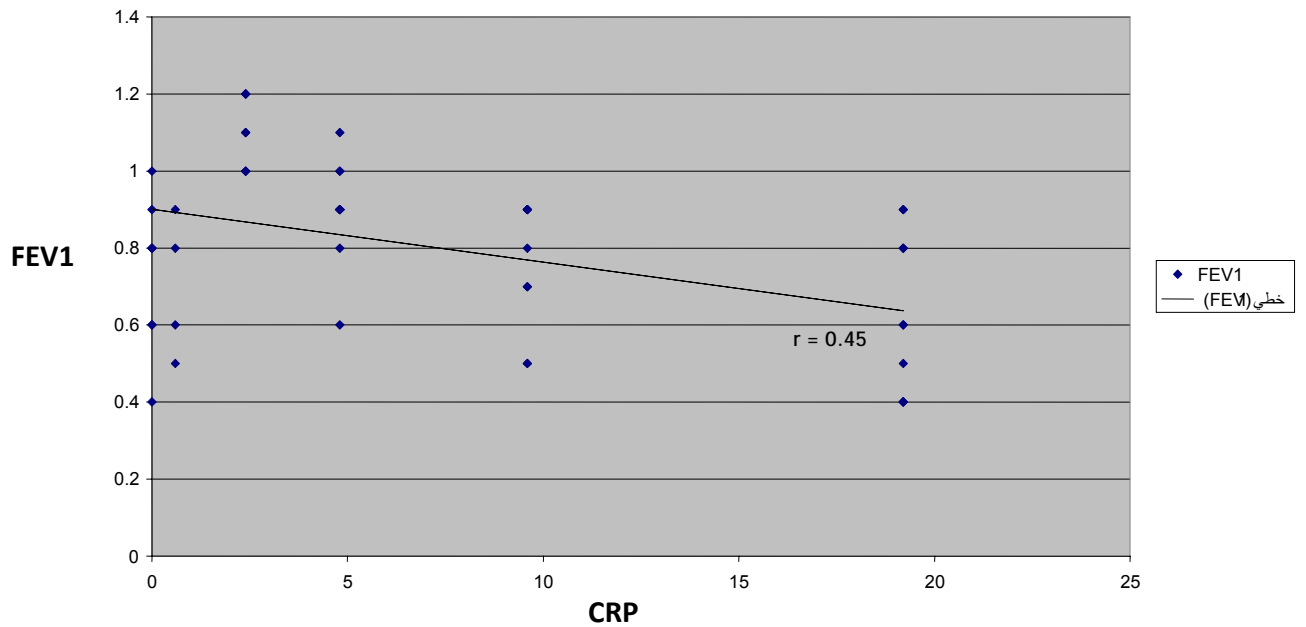r= 0.57, *p* value < 0.001

Figure 3: Correlation between CRP before and after treatment and corresponding FEV1

r= -0.45, *p* value < 0.001

Discussion

The acute phase protein, CRP, when elevated, provides good evidence of an active tissue-damaging process. Thus; its measurement provides a simple screening test for active organic disease. Increased CRP production is a very early and sensitive response to most forms of bacterial infection [11]. Studies have shown that it can be a useful indicator in differentiating between bacterial meningitis and viral meningitis [13], CRP levels also have been previously found to be of value in discriminating between bacterial and viral pneumonia [30].

In one study by Nel et al [31] showed a significantly increased level of CRP during infective exacerbations in emphysema. However, there has not been any assessment of the value of CRP in exacerbations of COPD, which is one of the commonest causes of hospital admissions. In this context, routine bacteriological analysis of sputum is often unreliable and slow. It is well established that the respiratory tract may be asymptomatically colonized; for example *Haemophilus influenzae* in chronic bronchitis [32,33]. Thus it may be difficult to distinguish active infection from colonization on the basis of sputum culture. In this situation, serial CRP assays which are cheap, sensitive and rapid to perform provide a useful quantitative measure of exacerbation in COPD.

There have been few studies to assess the value of measuring CRP in clinical exacerbations of COPD. Our study results were compatible with the study by Dev et al [34] in whom two group of patients have exacerbation of COPD, one with proven bacterial infection (by sputum culture – group I) and the other in which there is no bacterial cause of infection (group II). The results of our study showed that in both of these groups who had exacerbations of

COPD; there was an elevated CRP at the time of admission to the hospital. In group II, clinical improvement occurs following treatment during their hospital stay with an associated dramatic fall in their CRP levels. This is attributed to the following reasons:
1. Inadequate improper sputum sampling
2. Problems with the analysis of sputum
3. Unusual behavior of the strain
4. viral infection could be responsible pathogen in patients in whom sputum was negative for bacterial pathogens

On the other hand, some patients may be chronically colonized with potential bacterial pathogens. Therefore, microbial examination of sputum may not always be useful indicator of active infectious state.

Consequently, since both groups of patients with clinical exacerbations have their CRP levels elevated initially show clinical improvement with lowering of CRP levels after treatment, there is strong probability that CRP is a marker of an exacerbation of COPD, but not necessarily a marker of bacterial infection.

The fall in CRP level after treatment with the clinical improvement could be due to:
1. The antibiotics used to treat the bacterial infection
2. Several other factors such as steroid treatment, O_2 therapy, bronchodilator and other treatments used in the treatment of COPD exacerbations

However, twelve of patients from the culture-negative group did not show a rise in CRP levels despite the evidence of acute exacerbations (group III). These patients may have viral infection that does not cause a rise in CRP or several other physiological defects interfering with CRP response.

Since patients with acute exacerbations of COPD had their CRP levels elevated initially and had clinical improvement with lowering

of the CRP levels after treatment, there is a strong possibility that CRP is a marker of exacerbation of COPD.

Recommendation

We suggest that, in exacerbation of COPD, CRP estimation provides a useful and inexpensive early marker of the exacerbation and provides a useful guide to assess the efficacy of treatment

References

1. John J, Reilly JR, Edwin K, and Silverman SD. Chronic obstructive pulmonary disease. In: Kasper DL, Fauci AS, Longo DL, Braunwald E, Hauser SL, and Jameson JL, Harrison's Principles of Internal Medicine 16th ed. New York, McGrawHill, 2005; p. 1547-1554.
2. Senior RM, and Shapiro SD. Chronic obstructive pulmonary disease: Epidemiology, pathophysiology, and pathogenesis. In: Fishman's Pulmonary Diseases and Disorders, 3d ed. Fishman AD et al (eds). New York, McGraw-Hill, 1998; p. 659-681.
3. Barnes PJ. Chronic obstructive pulmonary disease. *N Eng J Med*, 2000; 343: 269-280.
4. ATS/ERS. Skeletal muscle dysfunction in chronic obstructive pulmonary disease. A statement of the American Thoracic Society and European Respiratory Society. *Am J Respir Crit Care Med*, 1999; 159: S1-40.
5. Gan WQ, Man SF, Senthilselvan A, Sin DD. Association between chronic obstructive pulmonary disease and systemic inflammation: a systematic review and a meta-analysis. *Thorax*, 2004; 59(7): 574-580.
6. Vernooy JH, Küçükaycan M, Jacobs JA, Chavannes NH, Buurman WA, Dentener MA, et al. Local and systemic inflammation in patients with Chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*, 2002; 166(9); 1218-1224.
7. Noguera A, Busquets X, Sauleda J, Villaverde JM, MacNee W, Agustí AG. Expression of adhesion molecules and G proteins in circulating neutrophils in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*, 1998; 158, 1664-1668.
8. Gan WQ, Man SF, Senthilselvan A, Sin DD. Association between chronic obstructive pulmonary disease and systemic inflammation: a systematic review and a meta-analysis. *Thorax*, 2004; 59(7): 574-580.
9. Kasper DL, Fauci AS, Longo DL, Braunwald E, Hauser SL, Jameson JL. Harrison's Manual of Medicine 16th ed. NewYork, McGrawHill, 2005; p. 675-678.
10. Glass S, Hayward C, and Govan JRW. Serum CRP in assessment of pulmonary exacerbation and microbial therapy in cystic fibrosis. *Paediatr*, 1988; 113: 76-79.
11. Raynor RH, Wiseman MS, Cordon SM, Hillar EJ, Shale DJ. Inflammatory markers in cystic fibrosis. *Respir Med*, 1991; 85: 139-145.
12. Dev D, Wallance E, Sankaran J, and Cunniffe J. Value of C-reactive protein measurement in exacerbation of COPD. *Respiratory medicine*, 1998; 92: 664-667.
13. Pepys MB. C-reactive protein: 50 years on. *Lancet*, 1981; 1: 653-657.
14. Peltola HO. C-reactive protein for rapid monitoring of infection of the CNS. *Lancet*, 1982; I: 980-982.
15. Shaw AC. Serum CRP and neoptrin concentrations in patients with viral or bacterial infection. *J Clin Pathol*, 1991; 44: 596-599.
16. Nel AE, Strachan AF, Welka HE, and de Beer FC. Acute phase responses in bronchiectasis and bronchial carcinoma. *Respiration*, 1984; 45:406-410.
17. Sodeman TM, and Colmer J. Microbiology of the respiratory tract. *Lab Med*, 1983; 14(2): 96-102.
18. Woods DE. Bacterial colonization of the respiratory tract: clinical significance. In: Respiratory infections: Diagnosis and Management, 2nd ed. New York, Raven Press, 1988.

Correspondence to: Dr. Methaq A.M. Hussein,
E-mail: m.hilalali@yahoo.com
Received: 16th Nov. 2008, Accepted: 29th Sept. 2010.

Metric study on depth of the sigmoid sinus plate in relation to suprameatal (Macewen's) triangle

Mohammad O. Selman *MBChB, FIBMS*

Dept. Human Anatomy College of medicine, Al-Nahrain University

Abstract

Background The lateral wall of the mastoid antrum, which offers the usual surgical approach to the tympanic cavity, is formed by the postmeatal process of the squamous part of the temporal bone. Since the suprameatal triangle covers the lateral wall of the mastoid air system, it is of importance to otologic surgeons during mastoidectomy.

Objectives To examine the relationships of the surgical landmarks on the lateral surface of the mastoid bone with the landmarks in a deeper location. Morphometric measurements were performed between the surgical landmarks (Macewen's triangle) and the sigmoid sinus plate.

Methods 30 dry human adult skulls were investigated. Three marks were allocated on the left and right sides of the norma lateralis of these skulls namely the tip of the mastoid process, the asterion, and the region of the suprameatal triangle (posterior border).

Results The measurements of (D1+D2), (D1+D3) and (D2+D3) showed statistical significant differences. The correlations of D1, D2 and D3 on the right and left sides showed statistical significant differences

Conclusion The assessment of the depth of sigmoid sinus plate may be used as a mark that possibly point toward avoidance of the bleeding possibility during mastoidectomy. The suprameatal approach eliminates the need for mastoidectomy and avoids the damage of sigmoid sinus plate. If the depth can be assessed by measuring D1 and D2 on lateral X-rays of the heads as this study prove that we can avoid the dangerous complications which occur in the normal mastoidectomy.

Keywords Suprameatal triangle, mastoidectomy

Introduction

Mastoiditis results from a middle ear infection that causes inflammation of the mastoid air cell. This dangerous condition may spread from the antrum to surrounding structures and cause life-threatening infection. In particular the infection may spread through the tegmen tympani to the dura mater of the middle cranial fossa, to cause an extradural collection [1].

The lateral wall of the mastoid antrum, which offers the usual surgical approach to the tympanic cavity, is formed by the postmeatal process of the squamous part of the temporal bone. The mastoid antrum is located 15 mm deep to the lateral surface of the mastoid bone.

In adults, the lateral wall of the antrum corresponds to the suprameatal triangle on the outer surface of the skull [2].

The superior side of the suprameatal triangle, the supramastoid crest, is level with the floor of the middle cranial fossa. The anteroinferior side, which forms the posterosuperior margin of the external acoustic meatus, indicates approximately the position of the descending part of the facial nerve canal [3]. The suprameatal approach eliminates the need for

mastoidectomy and posterior tympanotomy[4]. The area is entered through a retroauricular tympanotomy flap, and the electrode is introduced via a tunnel drilled in the suprameatal region superior to Henle's spine. The suprameatal approach is a simple and safe technique that does not endanger the facial nerve or the chorda tympani [5].

Since the suprameatal triangle covers the lateral wall of the mastoid air system, the suprameatal triangle is of importance to otologic surgeons during mastoidectomy [6]. The anatomical landmarks especially suprameatal crests on the outer surface of the squamous part of the temporal bone are of clinical importance as they are accepted as landmarks for some surgical approaches [7].

In vivo, identification of suprameatal triangle is by doing postural incision 0.5cm behind the retroauricular sulcus which is the exact site for operation on living patients in mastoidectomy. By removing the mastoid cortex with electric drill, the exact depth of the sinus plate is clarified. Drilling from suprameatal triangle was done in line of superior border of suprameatal

triangle [8]. The surface marking of the mastoid antrum is the suprameatal triangle (Macewen's triangle) Figure 1 [8].

The boundaries of the triangle are:
Anteriorly: The post-superior border of the bony external auditory canal along with the suprameatal spine of Henle. This spine is prominent landmark for the suprameatal triangle.

a) Superiorly: The supramastoid crest.
b) Posteriorly: a vertical line drawn tangential to the posterior margin of the external canal.

In adult this triangle lies 1.5 cm lateral to the mastoid antrum which the largest air cell in the mastoid bone [9].

Aim of this study
To measure the depth of the sinus plate that is an indicator for the distance between the site of the mastoidectomy and sigmoid sinus. The assessment of this plate may be used as a mark that possibly point toward avoidance of the bleeding possibility during the surgical mastoidectomy. This study was designed to assess the profitability of using simple parameters that could be obtained from the plan x-ray of the skull.
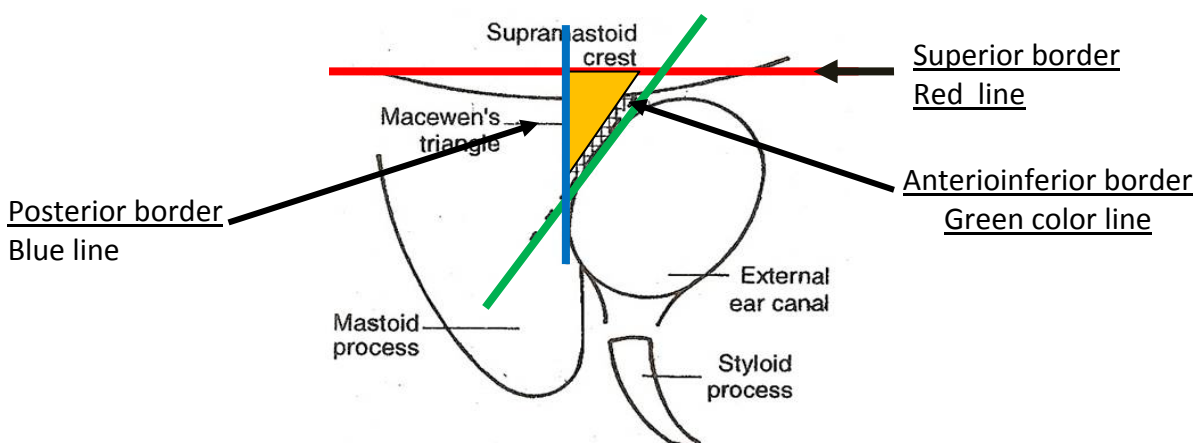

Figure 1: Diagrammatic illustration of suprameatal triangle (Macewen's) triangle [3].

Methods

Thirty dry adult human skulls were investigated. All the skulls were obtained from collection of the museum of anatomy in the College of Medicine Al-Nahrian University. Three marks were allocated on the left and right sides of the norma lateralis of these skulls namely the tip of the mastoid process, the asterion, and the region of the suprameatal triangle (posterior border). Three imaginary lines were considered in this study representing the connections of these three pointed landmarks. The lines were designated as following (figure 2):

D1= the distance between 'asterion' and the tip of mastoid process in the direction of posterior border of mastoid process.

D2 = the distance between asterion and the suprameatal triangle (posterior border).

D3 = Mediolateral mass of the mastoid. "The depth of the sigmoid sinus".

To measure the distance between surface of suprameatal triangle and the sigmoid sinus plate, the skull was oriented in the Frankfurt plane and the drilling was done between surface of suprameatal triangle and the sigmoid sinus plate in line of direction of maxillary process of temporal bone (its upper) border. Line D1 was considered as the representative for evaluation of the anteroposterior growth for the mastoid process.

Line D2 was considered as the representative for evaluation for the region of the suprameatal triangle.

Line D3 was considered as the representative for the evaluation of the depth of the sigmoid sinus plate from the surface of suprameatal triangle.

Measurements of D1 & D2 were done manually using Helios milimetric vernier (Inox). The anatomical landmarks were first defined and located, and then relevant measurements were done. D3 was estimated by measuring the length of a broom's bristle after passing it through the canal drilled into the suprameatal triangle.

The drilling was performed by using an electrical drill machine (Mizuho ika, kogyo co. Model No. m65-2c No. 79099 with drill size 2.8 mm).

Each of the artificial foramens which formed in the dry skulls was closed using the white cement powder to keep the normal skull morphology for teaching purposes.

The length of the three lines is demonstrated in 60 regions (right & left sides) of 30 human skulls. Statistical analysis was done using the statistical software package SPSS 16. The analysis was performed by using the chi square test for interpretation the results obtained.

The association between two categorical variables was assessed for statistical significance by chi-square test. P-values less than 0.05 were considered as statistically significant. The linear correlation between variables was measured by spearman's rank correlation coefficient. P values less than the 0.05 level of significance was considered statistically significant [10].
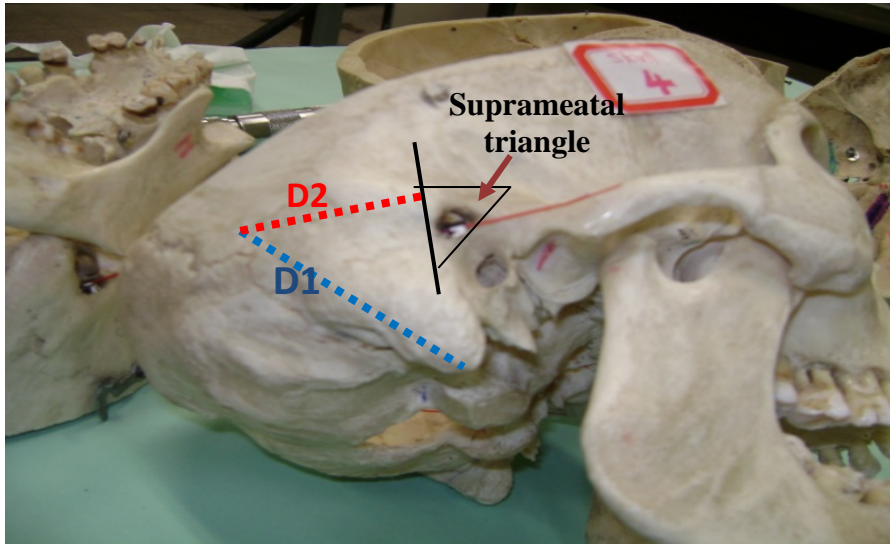
Figure2: Lateral view of a dry skull showing D1, D2 and the suprameatal triangle. D1: from asterion to the tip of mastoid tubercle, D2: transverse line perpendicular on posterior border of the suprameatal triangle.

Results

The values of D1, D2 and D3 was calculated in centimeters. The average of D1 (4.635 ±0.38), D2 (4.018±0.25) and D3 (1.189±0.16) of all skulls on both sides. The average of D1, D2 & D3 on right sides only are 4.610 (0.45), 4.050 (0.29) & 1.182 (0.17) respectively. The average of D1, D2 and D3 on left sides are 4.660 (0.32), 3.985 (0.20) and 1.196 (0.16) respectively as shown in table 1.

The measurements of (D1+D2), (D1+D3) and (D2+D3) on right side showed statistical significant differences. The probability (p <0.05) of high significant (p= 0.00001) as shown in table 2.

The correlations of D1, D2 and D3 on the right and left sides showed statistical significant differences. The probability (p <0.05) of high significant for D1 (P=0.007), and significant for D2 (P=0.029) and significant for D3 (P=0.012) as shown in table 3.

Data were analyzed using SPSS16 (statistical Package of Social Science, version 16 computer software). According to these results we can find the value of D1, D2 and D3 from following equations:

$$D3 = -0.548 + 0.375\ D1$$
$$D3 = -0.856 + 0.509\ D2$$
$$D2 = 1.534 + 0.536\ D1$$

From these equations if we find D1 or D2 radiologically, can calculate the approximate depth of sigmoid plate (D3) value.

Table 1: The variants of D1, D2 and D3 of adult skulls (Total no. 30) [average / (SD) on right and left sides]

	Adult skulls (Total no.=30)		
	Right side Average(SD)	Left side Average(SD)	All skulls Average(SD)
D1	4.610 (0.45)	4.660 (0.32)	4.635 (0.38)
D2	4.050 (0.29)	3.985 (0.20)	4.018(0.25)
D3	1.182 (0.17)	1.196(0.16)	1.189(0.16)

Table 2: Statistical Correlations and the probability (p values) of D1, D2 and D3 on the right side and its significance

	Correlation	p-value	significance
D1+D2(R)	0.824636598	P=0.00001	High-sign
D1+D3(R)	0.911606408	P=0.00001	High-sign
D2+D3 (R)	0.804874981	P=0.00001	High-sign

Table 3: Statistical Correlations and the probability (p values) of D1, D2 and D3 on the right and left sides and its significance

	Correlation	p-value	significance
D1 (R&L)	0.786915	P=0.007	High-sign.
D2 (R&L)	0.683902	P=0.029	Significant
D3 (R&L)	0.753688	P=0.012	Significant

Discussion

The measurements of (D1+D2), (D1+D3) and (D2+D3) showed statistical significant differences. By finding D1 or D2 radiologically, we can calculate the approximate depth of sigmoid plate (D3) value.

The complication of perforating the sigmoid venous sinus during surgical mastoidectomy may possibly be avoided by a preoperative assessment for the depth of the sinus plate.

It is very important to know the exact site of sigmoid sinus (depth of the sinus plate). If we irrigate or perform modified or radical mastoidectomy this may accidently damage the bone of the sinus plate leading to severe venous bleeding. If the depth (D3) can be assessed by measuring D1 and D2 on lateral X-rays of the heads, we may avoid this dangerous complication.

This study was designed to assess the profitability of using simple parameters that could be obtained from the plain x-ray of the skull as an indicator for the depth of the sinus plate. The statistical analysis demonstrates correlation between the depth of the sinus plate (D3) and the two other parameters namely D1 and D2 (pearson correlation). It was shown that the depth of the sinus plate could be speculated from the measurement of D1 and D2.

This is achieved by radiological assessment of Macewen's triangle as a surgical landmark [9]. Many morphometeric measurements were performed between surgical landmarks especially in the mastoidectomy [7].

The suprameatal approach is a simple and safe technique that does not endanger the facial nerve nor the chorda tympani [5].

If the depth can be assessed by measuring D1 and D2 on lateral X-rays of the heads as this study prove that we can avoid the dangerous complications which occur in the normal mastoidectomy.

Another point: the reason of using D1 and D2 to estimate D3, in other words, we built our hypothesis that measuring the first two lines might be an indicator to the third line, because many studies were done to clarify the topographic anatomy and clinical significance of the suprameatal triangle but no one mention the use of these criteria to know the depth of sigmoid sinus plate [2, 7, 11].

References

1. Williams PL and Warwick R. Gray's Anatomy. The Anatomical Basis of Clinical Practice. 36th ed. Churchill Livingstone. Edinbourgh, 2008; pp. 483-572

2. Aslan A, Mutlu C, Celik O, Govsa F, Ozgur T, Egrilmez M. Surgical implications of anatomical landmarks on the lateral surface of the mastoid bone. Surg Radiol Anat, 2004Aug; 26(4): 263-7.

3. McMinn RMH. Last's anatomy: Regional and applied. 9th ed. Churchill Livingstone. Edinburgh. 1998; pp.530

4. Baily CM. Surgical anatomy of the skull base. In: Scott Brown's Otolaryngology. 6th ed. Vol. 1. Kerr GA. Butterworth Heinemann, London.1997; pp. 1-15.

5. Kronenberg J, Baumgartner W, Migirov L, Dagan T, Hildesheimer M. The suprameatal approach: an alternative surgical approach to cochlear implantation. Otol Neurotol, 2004 Jan; 25(1): 41-4.

6. Peker TV, Pelin C, Turgut HB, Anil A, Sevim A. Various types of suprameatal spines and depressions in the human temporal bone. Eur Arch Otorhinolaryngol, 1998; 255(8): 391-5.

7. Turgut HB, Anil A, Peker T, Pelin C, Gulekon IN. Supra-articular, supramastoid and suprameatal crests on the outer surface of the temporal bone and the relation between them. Surg Radiol Anat, 2003 Nov-Dec; 25(5-6): 400-7.

8. Bhargava KB, Bhargava SK, and Shah TM. A short textbook of ENT diseases for students and practitioners. 7th ed, 2005; pp. 78.

9. Milton CM, Cousins VC. A radiological assessment of the usefulness of Macewen's triangle as a surgical landmark. J Laryngol Otol, 1986 Nov; 100(11): 1225-34.

10. Daniel WW; Hypothesis testing. In: Daniel WW. Biostatistics: A Foundation for Analysis in the Health Sciences, 8th ed., John Wiley & Sons, Inc. 2005; p. 222.

11. Luiz AS de Pavia; Marco Segre. Sexing the human skull through the mastoid process. Rev Hosp Clin Fac Med São Paulo, 2003; Jan-Feb 58(1):15-20.

Correspondence to: Dr. Mohammad O. Selman,
E- mail: mohammadoda@yahoo.com
Received: 25th July 2010, Accepted: 9th Dec. 2010.

Compliance with Good Practice in Prescription Writing at Private Clinics In Basra City; Southern of Iraq.

Dawood Ch. Helayel [1]*PhD*, **Jubran K. Hassan**[1] *MSc*, **Yasar A.H. Dawood**[2] *MSc*

[1]Dept. pharmaceutics and Clinical pharmacy, [2]Dept. Pharmacology, College of Pharmacy, University of Basra.

Abstract

Background Prescription order and information on it; represent the key for reach of safe and effective medications to the patient, and the clarity of its information is important to prevent medications errors that may be lethal to the patient.

Objectives Comparison of samples of prescriptions and their contents of physician; patient related information with standard information provided by WHO prescription writing.

Methods A sample of prescription orders received from private clinics by community pharmacies in Al Ashar of Basra city. The prescriptions were taken from several pharmacies randomly and about 1000 prescriptions were collected. The prescriptions represent about 40 physicians in different specialties and for a period extended from November 2009 until March 2010. The information of prescriptions were analyzed by simple statistics for calculation the percentage of adherence for WHO guideline in prescription writing.

Results The prescription information includes; prescriber's name, address, telephone number and signature were on 97.5%, 74.8%, 4.3% and 96.5% of prescriptions respectively. The patient's name, age and weight were on 96.6%, 15.5% and 2.2%. No prescription contained the patient's address and gender. The strength of medication and dose units were included in 1.7% and 1.4% of prescriptions. The prescriptions had only quantity indicated 2.4% and more than one third instructions for patient use (36.1%); the diagnosis was not included in more than two-thirds (85.2%). The prescriber's handwriting was illegible in 16.3% of prescriptions.

Conclusions There is severe deficiency in fitness of prescriptions written by the physician to WHO guidelines; so we recommend for administrative monitoring program for fitness of prescriptions to these guidelines and start education about this guideline for prescribers.

Key words Prescriptions, WHO, prescriber, patient, prescriber's name, address, telephone number, signature

Introduction

A medical prescription (R_x) is an instruction from a prescriber to a dispenser [1] or is a written order by a physician to a pharmacist for a treatment to be provided to the intended patient [2]. Commonly, the term prescription is used to mean an order to take certain medications. Prescriptions have legal implications, as they may indicate that the prescriber takes responsibility for the clinical care of the patient and in particular for monitoring efficacy and safety [3].

Prescriptions are handwritten on preprinted prescription forms that are assembled into pads, or alternatively printed onto similar forms using a printer; Preprinted on the form as text that identifies the document as a prescription [3].

A prescription is a legal regulated document used as a communication mechanism between pharmacists and physicians [3].

Regulations may define what constitutes a prescription, the contents and format of the prescription (including the size of the piece of paper) and how prescriptions are handled and stored by the pharmacist [3]. Although the prescription format may vary slightly from one country to another, most countries agree on the core elements that should be included in the prescription order. These are: prescriber's name, address, telephone number and signature; patient's name, address, age and weight (important at the extremes of age); prescription date; drug name, formulation, strength, dose, frequency of administration, quantity prescribed, reason for prescribing and instructions for use [4-7].

The community pharmacists have an important role in checking prescription to ensure they are appropriate to dispense.

It is not known how often prescribing errors occur. However it is known that patients can be harmed as a result of some prescribing errors. Prescribing errors can occur as a result of:

- Inadequate knowledge of the patient and their clinical status
- Inadequate drug knowledge
- Calculation errors
- Illegible handwriting
- Drug name confusion [8].

As good quality prescriptions are extremely important for minimizing errors in the dispensing of medications, physicians should adhere to the guidelines for prescription writing for the benefit of the patient [8].

The aim of this study was to detect the most frequent prescription errors by physicians in private clinics in Al Ashar area of Basra city in Iraq.
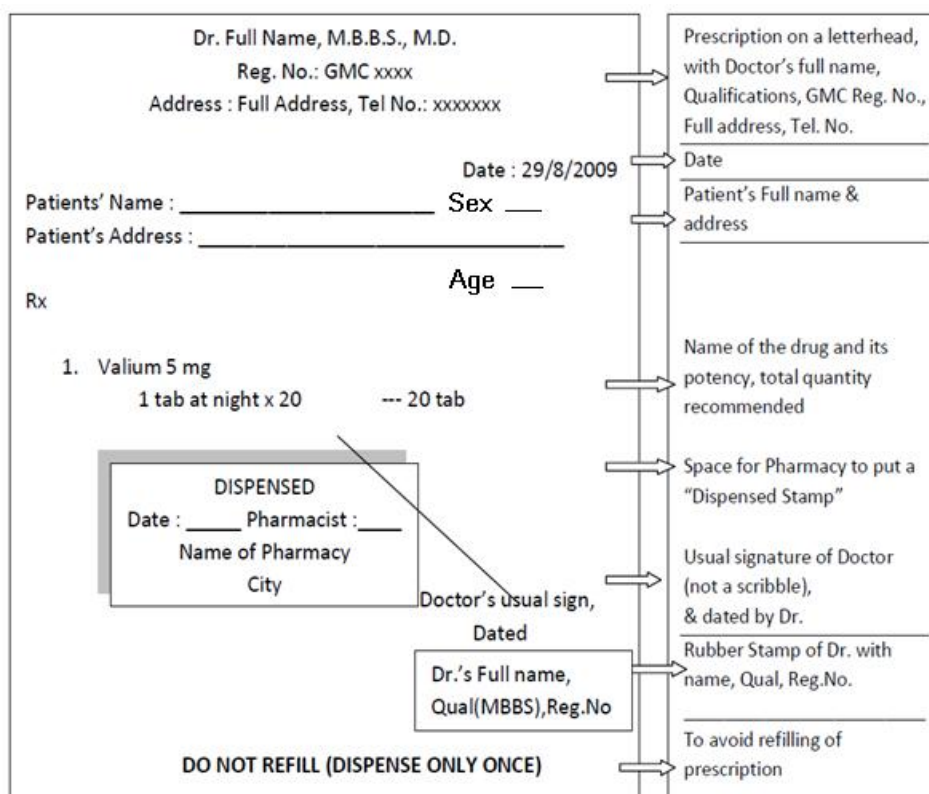

Figure 1: shows sample prescription from a private doctor, reprinted from WHO prescribing guidelines 2008

Methods

Private clinics prescriptions are collected from pharmacies located in the center of Basra city that is called Al ashar, the main referral private clinics in southern Iraq regions, that is visited by around 2 million patients per year.

The study was designed using simple randomization where; about 1000 prescriptions were collected from several different pharmacies in the area and. The prescriptions represent about 40 physicians in different specialties and for a period extended from November 2009 until March 2010.

The targets of the study are to evaluate all collected prescriptions to meet the standards of prescription writing; and identify the types and magnitudes of prescription writing errors. Prescriptions were analyzed for the essential elements to be included in the prescription order; where according to World Health Organization (WHO) recommendation; all prescriptions must include the following information [1]:

• The prescriber's name, address, registration number, and telephone number. This will allow either the patient or the dispenser to contact the prescriber for any clarification or potential problem with the prescription.

• Date of the prescription.

• Specific areas for filling in details about the patient including address, Patient Name and/or registration number (R/N), Date of Birth and/or Age, especially for children under 12 years and elderly over 75 years sex; weight;

• Name, form, Route of administration, and strength of the drug. The International Nonproprietary Name of the drug should always be used. If there is a specific reason to prescribe a special brand, the trade name can be added. Generic substitution is allowed in some countries. The pharmaceutical form (for example, "tablet", "oral solution", "eye ointment") should also be stated. The strength of the drug should be stated in standard units using abbreviations that are consistent with the System International (SI). "Microgram" and "Nanogram" should not, however, be abbreviated. Also, units' should not be abbreviated. Avoid decimals whenever possible. If this is unavoidable, a zero should be written in front of the decimal point.

• Frequency of administration or dosing interval e.g. three times a day or every 6 hours.

• The quantity of the medicinal product to be supplied should be stated such that it is not confused with either the strength of the product or the dosage directions. Alternatively, the length of the treatment course may be stated (for example "for 5 days").Wherever possible, the quantity should be adjusted to match the pack sizes available. For liquid preparations, the quantity should be stated in milliliters (abbreviated as "ml") or liters.

• Signature and initials of prescriber.

• When prescribing narcotics/controlled drugs, the strength, directions and the quantity of the controlled drug to be dispensed should be stated clearly, with all quantities written in words as well as in figures to prevent alteration. Other details such as patient particulars and date should also be filled in carefully to avoid alteration [1] (Figure 1).

Compliance with these elements was the degree to which the physician had met the obligation of including all the elements of a prescription in the prescription order. It is worthy to mention that Physicians did not know about this study.

Simple descriptive statistics were generated by the Microsoft excel 2007.

Results

The number of prescriptions sampled was 1000, the number of drugs prescribed ranged between 1 and 5 and around 50% of prescriptions included 3 drugs. Only 74.8% of prescriptions included the address of the prescriber. About 4.3% of the prescriptions included the telephone number of the prescriber; the name and signature of the prescriber were included in 97.5% and 96.5% of prescriptions, respectively (Table 1).The name of the patient was present on 96.6% of prescriptions, whereas the patient's age and weight were present in only 15.5% and 2.2 % respectively (Table 1). None of the prescriptions included the address and sex of patient.

Table 1: Review of 1000 prescription issues at Al Ashar area in Basra city; analysis of prescriber and patient information present on prescriptions

Information		No.	%
Prescriber	Name	975	97.5%
	Address	748	74.8%
	Phone No.	43	4.3%
	Signature	965	96.5%
Patient	Name	966	96.6%
	Age	155	15.5%
	Address	0	0%
	Weight	22	2.2%
	Sex	0	0%

Date of the prescription was provided in only 55.1% of prescriptions. The handwriting of the prescriber was not clear in 16.3 % of prescriptions (Table 2). With regard to the strength of medication, it was included in 1.7% of the prescriptions and was included for some drugs within the prescription in 60.6%. In the rest of prescriptions (37.7%), the strength of medication was missing. The dose units were not mentioned in 39.2% of prescriptions and mentioned for some drugs within the prescription 59.4%. The units were

mentioned for all drugs in 1.4% of prescriptions. Most of the prescriptions (47.5%) did not contain the quantity that the pharmacist should dispense. The directions for patient use were complete in only 36.1% of prescriptions, 34.5% prescriptions contained partial instructions either among the drugs prescribed or for certain drugs. The space provided for the diagnosis within the prescription was filled clearly in 14.8%, filled unclearly in 8.5% and unfilled in 76.7% of prescriptions (Table 2).

Table 2: Review of 1000 prescriptions issued at Al Ashar area in Basra city: analysis of information present on prescriptions

Element		No.	%
Date of prescription	Provided	551	55.1%
	Not	449	44.9%
Strength of medications	Included for all drugs	17	1.7%
	Included for some drugs	606	60.6%
	Not included for all drugs	377	37.7%
Dose unit	Included for all drugs	14	1.4%
	Included for some drugs	594	59.4%
	Not included for all drugs	392	39.2%
Quantity of medications	Included for all drugs	24	2.4%
	Included for some drugs	501	50.1%
	Not included for all drugs	475	47.5%
Instruction for patient use	Included for all drugs	361	36.1%
	Included for some drugs	345	34.5%
	Not included for all drugs	294	29.4%
Diagnosis	Present	148	14.8%
	Not clear	85	8.5%
	Missing	767	76.7%
handwriting	Clear	837	83.7%
	Not clear	163	16.3%

Discussion

The study was performed to identify the degree to which physicians confirm to guidelines for prescription writing during their clinical practice. A total of 1000 prescriptions were taken from Al Ashar area in Basra city were screened for the essential elements of prescriptions according to published WHO guidelines [4-7].

This is the first study reviewing prescriptions from private clinics in Basra city.

Our observations showed that prescriptions were deficient in the information as following:

In concern prescriber related information; about 4.3% of the prescriptions contained the telephone number of the prescriber and only 74.8% had the prescriber address. These elements should be included according to WHO prescription writing guidelines [4]. These deficiencies indicate how things are made difficult for the dispensing pharmacist to contact the prescriber in case of any clarification.

Concerning patient information; our finding patient's name, age and weight in were found in 96.6%, 15.5% and 2.2% of prescriptions respectively. Inclusion of weight is recommended for patients at the extremes of age [4-7], because of its implication on drug pharmacokinetics and pharmacodynamics. These finding differ slightly from another study like in Saudi Arabia that found 94.6%, 77.6% and 0.0% for patient's name, age, and weight[9] respectively.

None of the prescriptions we reviewed contained the address and sex of the patient; which is similar finding in Saudi Arabia study. The address of the patient is among the elements that should be included in the prescription according to WHO [4]. Omission of patient address from prescriptions is a serious deficiency when problems in the prescription are discovered and the patient needs to be contacted to correct the problem. This is even more serious when the name of the patient is also omitted. Our finding that less than half (44.9%) of prescriptions were not dated.

We found that more than one third (37.7%) of prescriptions did not include the strength of medication, this ratio was less than that reported in Saudi Arabia[9] the dose units were not included in 39.2% and the quantity of medications was not included in 47.5% of prescriptions. Apparently, these parameters are left to the pharmacist to decide upon and the implications for the duration of therapy will be dependent on the individual pharmacist. The strength of medication is particularly needed when the pharmaceutical product exists in more than ones strength. We did not look at the proportion of drugs which are available in only ones strength.

We also found that the prescriptions were included instructions for patient use in about 36.1% (2.3% in Saudi Arabia) [9], and about 34.5% contained only partial instructions, (90.7% in Saudi Arabia) [9] a finding that certainly will affect the adequacy of therapy. Which differ from finding in Saudi Arabia which were

Our finding that the diagnosis was missing or unreadable in more than two third (85.2%) of prescriptions; Also our finding that about (16.3%) of prescriptions suffered from poor handwriting, this percentage of poor handwriting we found could be due to the fact we considered the presence even of a single unclear word or a dose unit as poor handwriting for the whole prescription. Poor handwriting is a serious problem that might lead to dispensing the wrong medication to the patient with serious or even fatal results [10].Some reasons behinds prescription writing incompliance may include : Heavy load on physician specially the gynecologists, in addition to that; improper practice, by some physicians, like admission more than one patient in examination room, make physician hesitated in prescription writing , leading to poor hand writing, and / or errors or leads to missing of some information in

prescription. Also some patients may refuse to give true personal details to physician, which may be concern with social mores. Gender of patient may not be mentioned in the prescription, probably, it is easily to distinguish the gender from the name of patient, this may made physicians do not give attention to write gender in the prescription. There were some serious problems in some prescriptions so they are truly a mystery and not contain any clear information , and even can't be read by physician him self when a pharmacist inform from physician about the content of prescription.   And finally; unfortunately some doctors are ignorant of the standards required in a good prescription writing and may need to be educated about these standards.

In conclusion, the prescriptions we reviewed suffered from serious deficiencies and were not properly written. The need for physician education on appropriate prescription writing is obvious and follow-up on the matter is needed for newly qualified physicians. Furthermore, inclusion of tutorials about prescription writing in the final clinical year curriculum of medical students before graduation is necessary. Administrative monitoring of the prescription habits of physicians is needed both to improve the process and to maintain the improvement.

References

1. Mary R, Couper and Dinesh K Mehta; Prescription writing; WHO Model Formulary 2008 :p12

2. Http://www.wordiq.com/definition/Medical_prescription#Format_and_definition (May 1, 2010)

3. Http://en.wikipedia.org/wiki/Medical_prescription (May 1, 2010)

4. Vries TP. eds. Guide to good prescribing: a practical manual. Geneva, World Health Organization, 1995: 51–5 (WHO/DAP/94.11).

5. Lofholm PW, Katzung BG. Rational prescribing and prescription writing. In: Katzung BG, ed. Basic and clinical pharmacology, 8th ed. New York, McGraw Hill, 2001:1104–12.

6. Prescription writing. In: British national formulary, No. 41. London, British Medical Association & Royal Pharmaceutical Society of Great Britain, 2000:4–5.

7. Safe writing. In: Lacy CF et al., eds. Drug information handbook, 9th ed. Cleveland, Ohio, Lexi-Comp, 2001: 12.

8. Meyer TA. Improving the quality of the order-writing process for inpatient orders and outpatient prescriptions. *Am J Health-system Pharm*, 2000, 57(Suppl. 4): S18–2.

9. Irshaid YM, Al Homrany M, Hamdi AA, Adjepon-Yamoah KK, and Mahfouz AA. Compliance with good practice in prescription writing at outpatient clinics in Saudi Arabia. *East Mediter Health J*, 2005, 11 (5,6): 922-8.

10. Charatan F. Family compensated for death after illegible prescription. *Br Med J*, 1999, 319: 1456.

Correspondence to: Dr. Jubran K. Hassan,
E- mail: jubranhassan@gmail.com
Received: 21st Sept. 2010, Accepted: 24th Nov. 2010.

/   . .

/   . .

/   /   . . .

/   . .

/   . .

/   . .

/   . . .

/   . . .

/   . . .

/   . . .

/   . . .

/   . . .

/   . .

/   . . .

(   )   . .
    (   )   . .
(   )   . .
        (   )   . .
    (   )   . .
        (   )   . .
        (   )   . .
(   )   . .
(   )   . .
(   )   . .
    (   )   . .
        (   )   . .
    (   )   . .
    (   )   . .
        (   )   . .
(   )   . .
        (   )   . .
(   )   . .
        (   )   . .
    (   )   . .