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Iraqi Journal of Medical Sciences

A Medical Journal Encompassing All Medical Specializations

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Ageing and Senescence

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Senescence is the state orprocess of ageing. Cellular senescenceis a phenomenon where isolated cellsdemonstrate a limited ability to divide, while organismal senescence isthe ageing of organisms. After a periodof near perfect renewal (in humans,between 20 and 35 years of age),organismal senescence is characterized by the declining ability to respond tostress, increasing homeostatic imbalance and increased risk of disease. This currently irreversible series of changesinevitably ends in death [1].

Indeed, ageing is not an unavoidable property of life. Instead, it is the result of a genetic program. Numerous speciesshow very low signs of ageing ("negligible senescence"), the best known being trees like the bristlecone pine. In humans and other animals, cellular senescence has been attributed to the shortening of telomeres with each cellcycle; when telomeres become too short, the cells die. The length of telomeres is therefore the "molecular clock", Telomere length is maintained in immortal cells (e.g. germ cells and keratinocyte stem cells, but not other skin celltypes) by the telomerase enzyme. In the laboratory, mortal cell lines can be immortalized by the activation of theirtelomerase gene, present in all cells but active in few cell types. Cancerous cells must become immortal to multiplywithout limit. This important step towards carcinogenesis implies, in 85% of cancers, the reactivation of theirtelomerase gene by mutation. Since this mutation is rare, the telomere "clock" is seen by some as aprotective mechanism against cancer. Research has shown that the clock must be located in the nucleus of each cell andthere have been reports that the longevity clock might be located in genes on either the first or fourth chromosome ofthe twenty-three pairs of human chromosomes [2,3].

In this issue of the Iraqi Journal of Medical Sciences, there are 3 researches dealing with ageing, the first one presented by Dr.May and Dr. Salih, they studied the age-related changes in human skin as a histological and morphometric study. As skin ages, it becomes thinner and more easily damaged. Intensifying this effect is the decreasing ability of skin to heal itself as a person ages.

Among other things, skin aging is noted by a decrease in volume and elasticity. There are many internal and external causes to skin aging. For example: Aging skin receives less blood flow and lower glandular activity.

Another research regarding ageing is the study presented by Dr.Saadet al, as they studied the effect of ageing on testis. It is known that as men age, testicular function declines gradually and moderately. The similarities between the consequences of hypogonadism due to known disease and those of hypogonadism due to aging alone suggest that the decline of testicular function with aging does have consequences.

Several studies demonstrate that the serum testosterone concentration declines with increasing age. Some studies show a preservation of spermatogenesis with increasing age, and others show a decline.

The third study was done by Dr.Idris etal, as they studied the ageing changes of hypothalamo-pituitary gland but in rabbit not in human. The pituitary gland is essential for a normal life span. Certain pituitary hormones and posterior pituitary hormones maintain or prolong life of hypohphysectomised or old rats. These hormones are called life-maintaining hormones.

At present, the biological basis of ageing is unknown. Most scientists agree that substantial variability exists in the rates of ageing across different species, and that this to a large extent is genetically based. In model organisms and laboratory settings, researchers have been able to demonstrate that selected alterations in specific genes can extend lifespan (quite substantially in nematodes, less so in fruit flies, and less again in mice). Even in the relatively simple and short-lived organisms, the mechanism of ageing remain to be elucidated. Less is known about mammalian ageing, in part due to the much longer lives in even small mammals such as the mouse (around 3 years).

The US National Institute on Aging currently funds an intervention testing program, whereby investigators nominate compounds (based on specific molecular ageing theories) to have evaluated with respect to their effects on lifespan and age-related biomarkers in outbred mice[4]. Previous age-related testing in mammals has proved largely irreproducible, because of small numbers of animals, and lax mouse husbandry conditions. The intervention testing program aims to address this by conducting parallel experiments at three internationally recognized mouse ageing-centres, the Barshop Institute at UTHSCSA, the University of Michigan at Ann Arbor and the Jackson Laboratory.

Many have argued that life-span, like other phenotypes, is selected.

• Evolutionary Theories: Enquiry into the evolution of ageing aims to explain why almost all living things weaken and die with age. Exceptions such as rockfish, turtles, and naked molerat are highly informative.

• Telomere Theory: Telomeres (structures at the ends of chromosomes) have experimentally been shown to shorten with each successive cell division [5]. Shortened telomeres activate a mechanism that prevents further cell multiplication. This may be particularly limit in tissues such as bone marrow and the arterial lining where cell division occurs repeatedly throughout life . Importantly though, mice lacking telomerase enzyme do not show a dramatically reduced lifespan, invalidating at least simple versions of the telomere theory of ageing. Mice may be an exception for the theory, as they have long hypervariable telomeres [6], prolonging the period after which telomere shortening would affect life-span. But wild mouse strains do not, and telomere length in these breeds is unrelated to lifespan [7].

• Reproductive-Cell Cycle Theory: The idea that ageing is regulated by reproductive hormones that act in an antagonistic pleiotropic manner via cell cycle signaling, promoting growth and development early in life in order to achieve reproduction, but later in life, in a futile attempt to maintain reproduction, become dysregulated and drive senescence (dyosis)[8].

Some theories suggest that ageing is a disease. Two examples are

• DNA Damage Theory of Ageing: Known causes of cancer (radiation, chemical and viral) account for about 30% of the total cancer burden and for about 30% of the total DNA damage. DNA damage causes the cells to stop dividing or induce apoptosis, often affecting stem cell pools and hence hindering regeneration. DNA damage is thought to be the common pathway causing both cancer and ageing. It seems unlikely that

the estimates of the DNA damage due to radiation and chemical causes has been significantly underestimated. Viral infection would appear to be the most likely cause of the other 70% of DNA damage especially in cells that are not exposed to smoking and sun light. It has been argued, too, that intrinsic causes of DNA damage are more important drivers of ageing [9,10].

• Autoimmune Theory: The idea that ageing results from an increase in autoantibodies that attack the body's tissues. A number of diseases associated with ageing, such as atrophic gastritis and Hashimoto's thyroiditis, are probably autoimmune in this way [10].

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Effect of Aqueous Extract of Turmeric on Pathogenic Bacteria Isolated from Semen in a Sample of Iraqi Infertile Men

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Abstract

Background Contamination of semen with sexually and none sexually transmitted bacteria plays an important role in male infertility, contaminating bacteria should be eradicated by antibiotics, but most of bacteria become resistant to available antibiotics. Therefore most researchers search to find other antibacterial agents.

Objective To evaluate the frequency of bacteria in semen of infertile men and investigate the antibacterial activity of aqueous extract of turmeric (AET) on certain isolated bacteria from semen of infertile men compared with that of doxycycline as a standard antibiotic.

Methods Forty two semen samples obtained from infertile men attending The Higher Institute of Infertility Treatment and Assisted Reproductive Technology were evaluated bacteriologically using standard bacterial culture. Then investigated the antibacterial activity of AET on certain isolated bacteria from semen of infertile men compared with doxycycline using disc diffusion method.

Results Out of 42 semen samples of infertile men, 35(83.03%) were infected with different bacterial species (spp.) accompained with highest incidence rate. The overall frequency of *N.gonorrhea* and *S.epidermides* was 8(19%) and for remaining bacterial isolated from semen *(S.aureus, G.vaginalis* and *E.coli)* were 6(14.28%). Comparable antibacterial activity of ATE and doxycycline was found to be evident against most isolated bacteria ($P<0.001$) in mean inhibition zones of AET between Gram's positive and Gram's negative bacteria (23.35±0.68 and 8.35±1.52, respectively).

Conclusion Most of isolated bacteria from semen of infertile males had high frequency, which were generally accepted as pathogenic bacteria. Antibacterial activity of AET was comparable with doxycycline against most isolated bacteria and it was found more effective on Gram's positive than Gram's negative bacteria.

Keywords Turmeric, pathogenic bacteria, semen, infertile men

Introduction

The presence and profound influence of microorganisms in semen is evidence that pathological microorganisms play a significant role in male infertility [(1)]. Genital tract infection and inflammation have been associated with 8-35% of infertility cases [(2)]. Some possible pathomechanisms of the development of infertility linked with infection are considered: direct effect on sperm function (motility, morphology, etc), deterioration of spermatogenesis, autoimmune processes induced by inflammation and dysfunction of accessory sex glands [(3,4)].

Among the most common microorganisms involved in sexually transmitted infection that interfere with male fertility, are *N. gonorrhea* which has been reported in the colonization of human sperm and *G.vaginalis* that may assume a pathogenic role by extension to prostate. Less frequently, male infertility is due to none

sexually transmitted epdidmo-orchitis mostly caused by *E.coli* [5-7].

Opportunistic microorganisms including *S.aureus*, *S.fecalis* and *diphtheroids species* (spp.) responsible for semen contaminations generally originate from the urinary tract of patient or can be transmitted by the partner via sexual intercourse causing classical infection of urogenital tract and subclinical reproductive tract infection and could contribute to the deterioration of sperm quality of infertile men [3,8,9], therefore the presence of urogenital tract infection and inflammation must be eradicated by antibiotics and anti-inflammation treatment [2], but the continuous evolution of bacterial resistance to currently available antibiotic has necessitated the search for novel and effective antimicrobial component [10].

Curcuma longa commonly known as turmeric is widely used as spice and coloring agent and is well known for its medicinal properties such as antioxidant, anti-inflammatory, anti-platelet, cholesterol, antibacterial and antifungal [11]. More recently, alcoholic extract of turmeric was used for *in vitro* sperm activation of infertile patients [12].

However, due to limited information on its effective and practical application to bacteriospermia in infertile men, this study was designed to found the frequency of isolated bacteria from semen of infertile men and to investigate the effect of aqueous extract of turmeric on certain isolated bacteria from semen samples of infertile male.

Methods

Study population:

A total of 42 selected semen samples with ≥10 round cells /HPF were collected from infertile men attending Institute of Embryo Research and Infertility Treatment for examination from March to November 2010. The patients had no clinical signs or symptoms of infection of lower genital tract and negative history for receiving antibiotic treatment. Semen samples were collected by masturbation into sterile wide-mouth plastic container after 3-5 days of abstinence. Each infertile man was instructed to wash genitilia before masturbation.

Microbial Examination:

Collected semen samples were cultured after liquefaction both aerobically and anaerobically. For aerobic culture one drop of 0.1ml was inoculated into blood agar, MacConkey agar and sabrouaud dextrose agar (HiMedia laboratories, india) while the anaerobic culture was done on chocolate agar under 5% CO_2. All isolated microorganisms were identified by bacteriological feature of bacterial colonies and biochemical test and regarded as a cause of infection if the colony count was $\geq 10^3$ bacteria /ml of semen. Skin contaminant (*e.g coagulase-negative Staphylococcus, Streptococcus spp*. and *Corynebacterium spp.* were reported only if there were no more than two different species in the ejaculates.

Preparation of aqueous extract of turmeric:

Turmeric was purchased and cleaned from dust, it was crushed into powder using electrical mixer. Turmeric powder 1g was dissolved in 1L of D.W in a clean glass beaker, and a mixture was stirred for 4 hours at room temperature. Then, the mixture was filtrated using filter paper to collect aqueous extract of sterile turmeric in another glass beaker then it was filtrated by filter with 45 micrometer and 22 micrometer. Aqueous extract of turmeric with concentration 1mg/ml was considered as stock solution for other dose 25µg.

Sensitivity test:

Antibacterial activity of turmeric was tested by using disc diffusion method, 25µg concentration was chosen for this test. Sterile 6mm diameter filter paper discs were impregnated with these concentrations to prepare the turmeric discs. Certain isolated bacteria from semen of infertile men were tested, few colonies of overnight culture of every type of bacteria were mixed with normal saline, the turbidity of this suspension was adjusted to match a 0.5 Macfarland turbidity standard. Mueller-Hinton agar (HiMedia labrotories, India) surface was inoculated with

that bacterial suspension then turmeric disc and antibiotic disc with 30µg of doxycycline for all those bacteria were added on surface of plate using flamed forceps. The plate was incubated at 37°C. After 24 hours, the inhibition zones were measured in mm using a ruler

Statistical analysis:

Statistical package for social sciences (ssps) version 16 (13), and Microsoft office excel 2007 were used to analyze the results of this study using descriptive table and ANOVA test.

Results

Frequency of isolated bacteria from semen of infertile men

The frequency and type of organisms isolated from semen of infertile men are shown in table (1). Out of total number of 42 patients semen samples 35(83.03%) yielded bacterial growth with *N.gonorrhoea, S.epidermedis, S aureus G.vaginalis and E.coli,* as pure culture, having the highest incidence rate of 6 (14.29%) for both *N.gonorrhoea* and *S. epidermedis* 5(11.90%) for *S.aureus*,4(9.52%) for *G.vaginalis* and 3(7.14%) for *E.coli*. Those microorganisms were also detected as mixed culture therefore the overall their incidence rate were 8 (19%) for both *N.gonorrhoea* and *S. epidermedis* and 6(14.29%) for remaining spp. of bacteria (*S aureus, G.vaginalis and E.coli)* respectively.

Table 1. Frequency of isolated bacteria from semen of infertile men

Isolated bacteria	Frequency	%
N. Gonorrhea	6	14.29
S. Epidermedis	6	14.29
S. Aureus	5	11.90
G. Vaginalis	4	9.52
E. Coli	3	7.14
S. Fecalis	2	4.76
Corynebacterium spp.	3	7.14
N. Gonorrhea + E. Coli	1	2.38
N. Gonorrhea + S. Aureus	1	2.38
G. Vaginalis + E. Coli	2	4.76
Corynebacterium spp. + S. Epidermedis	2	4.76
Total	35	83.03

The results of antibacterial activity of (AET) showed zone of inhibition in millimeter for five of the highest incidence rates of bacteria in semen samples *(N.gonorrhoea, G.vaginalis, E.coli,* as Gram's negative and *S. aureus and S. epidermedis)* as Gram's positve in comparison with standard antibiotic (doxycycline) are presented in table 2 (a+ b).

From table 2 (a) no significant differences were shown in mean inhibition zones of AET concentration 25µg/ml against all isolated bacteria except for *Staph. epidermedis* (p=0.025) (23.50±1.00) as compared to mean inhibition zones of doxycycline (10.57±4.37).

From table 2(b), it was observed that AET had highly significant difference (P<0.001) in mean inhibition zones between group of Gram's positive and group of Gram's negative bacteria (23.35±0.68 and 8.35±1.52. respectively).

Table 2. Anti bacterial activity of turmeric on Gram's positive and Gram's negative bacteria compared with doxycycline

A. Comparison between different type of bacteria.

Antibacterial agent	Gram's positive		Gram's negative			p-value
	S. aureus	*S. epidermidis*	*N. gonorrhea*	*G. vaginalis*	*E. coli*	
Turmeric 25 µg/ml Doxycycline	23.16±0.98 15.40±4.11	23.50±1.00 10.57±4.37	9.87±2.29 19.00±4.14	9.50±3.18 10.33±5.36	5.16±2.53 9.16±5.83	<0.001 0.533
p-value	0.076	0.025	0.068	0.980	0.550	

B. Comparison between Gram's positive and Gram's negative bacteria.

Antibacterial agent	Gram's positive	Gram's negative	p-value
Turmeric 25 µg/ml Doxycycline	23.35±0.68 12.58±3.03	8.35±1.52 13.68±3.07	<0.001 0.805
p-value	0.005	0.134	

Discussion

In the current study table 1 shows that 35 (83.03%) out of 42 semen samples of infertile men yielded bacterial growth as pure and mixed culture. This result is in accordance with other study that found that bacterial growth from semen culture was 79% of total number of the study [14]. This high percentage of infected infertile patients may be due to stringent patient selection in the present study. Moreover it was shown from this table, that there were five spp. of bacteria resulted from semen culture had the highest incidence rate of *N. gonorrhoea and S epidermidis* 6(14.29%), *S. aureus* 5(11.90%), *G. vaginalis* 4(9.52%) and *E.coli* 3(7.14%) and the overall frequency of *N. gonorrhoea and S epidermidis* was 8(19%) and 6(14.29%) datively for remaining spp. of bacteria.This finding is in agreement with other studies which reported that those spp. had the highest frequency [1,15].

It is generally accepted that those five spp. of bacteria are regarded as pathogens because *N. gonorrhoea and G.vaginalis* are well recognized etiological agent of sexually transmitted diseases and, are related to male infertility [16]. Other common bacteria generally that were isolated from semen, *S. aureus and E. coli* were the main organisms with most negative influence on sperm quality [15,17], and *S.epidermidis* may play an important role in sperm impairment due to infertility [18]. Therefore those pathogenic bacteria should be eradicated by antibiotics or other antimicrobial agents from semen especially before using assistant reproductive technique.

Many researchers have studied antibacterial activity of various extracts of turmeric on different bacteria as food or clinical but not from semen samples isolates or standard strains [10,19,20]. Most of isolated bacteria, in the current study, were generally accepted as pathogenic bacteria because they showed negative influence toward reproductive potential and sperm quality leading to infertility [15-18]. Moreover those bacteria were found to have the highest incidence rate; therefore in vitro antibacterial activity of AET was investigated in comparison with standard antibiotic (doxycycline) against *S. aureus and S. epidermides* as Gram's positive bacteria and *N. gonorrhoea G. vaginalis and E. coli* as Gram's negative bacteria. Table 2a shows comparable antibacterial activity of AET to doxycycline against all isolated bacteria except for *S. epidermidis* which was more sensitive to turmeric (P=0.025). This finding is in agreement with another study [20], which reported that

AET shows comparable activity to standard antibiotic involving doxycycline.

The mechanism of action of doxycycline appears to be carried out by inhibiting protein synthesis by binding to 30S ribosom subunit and blocking the aminoacyl transfer RNA from entering the acceptor site on ribosom [21]. On the other hand, curcumin is a polyphenolic and hydrophobic agent which is considered a major active component of turmeric [22-25]. Curcumin to which have been attributed most of medical effect of turmeric such as antibacterial, anti-inflammatory and antioxidant activities [26,27], and hypothesis of mechanism of antibacterial action has proposed different workers which involve: hydrophobic and hydrogen bonding of phenolic compounds to membrane protein followed by partition in the lipid bilayer; perturbation of membrane permeability consequent to its expansion and increased fluidity causing the inhibition of membrane embedded enzyme; membrane disruption; destruction of electrons transport systems and cell wall perturbation [28]. So we can conclude there was relatively similar effectiveness of AET with doxycycline because both of them bind and acts on membrane protein of bacterial cell.

From Table 2b, it was observed that AET had more effectiveness against Gram's positive bacteria as compared to Gram's negative bacteria ($P<0.001$). This result is in accordance with other studies who reported that AET is more effective against Gram's positive bacteria compared to Gram's negative bacteria [10,19,20,29]. On the other hand it was found that the curcumin is soluble in ethanol and acetone, and alcohol was found to be better solvent for extraction of antimicrobially active substances compared to water [30,31]. Moreover it was found the curcumin was active against *S. aureus and S.epidermidis* whereas did not show antibacterial activity on *P.aeruginosa E.coli*, but *N.gonorrhoea and G.vaginalis* are Gram's negative bacteria [32], therefore, the antibacterial activity of AET on those bacteria may be similar to *E.coli and P.aeurginosa*. From the above results the reason of varying degrees of sensitivity between Gram's positive and Gram's negative bacteria may be related to low curcumin content in AET and may be due to the intrinsic tolerance of Gram's negative to curcumin which is responsible of antibacterial activity of turmeric.

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Age-related Changes in Human Skin: Histological, Morphometric and Immuncytochemical Study Using S100

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Abstract

Background Aging has many effects on a person's skin, from wrinkles and sags to increased risk of certain skin conditions, such as skin cancer. As people age, their skin begins to change due to environmental, genetic, nutrition and other factors.

Objectives Understand some of the changes that occur in aging skin including changes in the general morphological, histological and architectural arrangement, epidermal thickness, basement membrane and histochemical changes in melanocytes.

Methods Skin specimens were taken from the anterior abdominal wall of 30 human males at different ages. General histological preparation for paraffin blocks was performed and the blocks were sectioned at (5-6µ) and stained with H&E. S100 protein was used to demonstrate immunohistochemistry labeled melanocytes changes with age. Histometric measurement of epidermal thickness and basement membrane thickness, using eyepiece graticule was performed on these groups.

Results The young age group showed a uniform arrangement of cells in all stratums of the epidermis while the old age group showed diminished thickness of the epidermis. A significant difference between young- adult age group (A and B groups) and the old age group (C group) was recorded. The epithelial basement membrane thickness was increased with age significantly (P value ≤ 0.001). Melanocytes demonstration using S100 showed that these cells tend to be situated at the tips of rete pages, there number are generally low and didn't varied a lot between young and adult age groups. There was yet marked decline in the number of melanocytes in old age group.

Conclusion Aging as a process have a marked influences on skin morphology, thickness, cellularity and basement membrane.

Key Words Aging, skin, S100, morphometry and basement membrane

Introduction

The skin is one of the largest and widest organs of the body, accounting for about 16% of the body weight [(1)]. Skin performs many important functions; it protects the body organism from impact and friction injuries. It is a wide sensitive organ to receive special stimulus by touch, pressure [(2)]. Regulation of body temperature, because of skin elasticity, it can expand to cover large areas in conditions associated with swelling, such as edema and pregnancy, and formation of vitamin D which is necessary for metabolism of phosphor and calcium [(3)].

Aging can be defined as a progressive, generalized impairment of function resulting in a loss of adaptive response to a stress or as the 'latter part of animate life. Gerontologists use the term senescence to describe aging as a progressive deterioration of many body functions over time, marked by a decrease in fertility and an increased risk for diseases,

culminating in multi-organ failure and death [4].

There are two main processes that induce skin aging: intrinsic and extrinsic aging. Intrinsic aging reflects the genetic background and depends on time. Extrinsic aging is caused by environmental factors such as sun exposure, air pollution, smoking, alcohol abuse, and poor nutrition [5].

Skin aging is particularly important because of its social impact. It is visible and also represents an ideal model organ for investigating the aging process [6]. "Biological clock" affects both the skin and the internal organs in a similar way, causing irreversible degeneration [7].

We aimed in this study to follow up changes of the skin with aging on the following aspects:

- General epidermal changes and its architecture
- Epidermal thickness changes.
- Basement membrane changes.
- Immunolabelled melanocytes changes.

Methods

Skin specimens were taken from the anterior abdominal wall of 30 human males at different ages, in the operating theater in Al-Kadhimiya teaching hospital. Approval from all individuals was taken prior to the operation. Samples were grouped into three age groups each consisted of (10) individuals:

Group A (1-9) years,
Group B (12-30) years and
Group C (40) years and above.

All individuals selected at different age group, was disease free in regard to hypertension and diabetes mellitus. They were stained with:

Haematoxylin & Eosin (H&E): used with paraffin sections to demonstrate the morphological changes in the skin with age including epidermal and basement membrane.

Periodic Acid-Schiff Technique (PAS): used with paraffin sections to demonstrate the changes of basement membrane thickness with age.

S100 protein was use to demonstrate melanocytes changes with age.

Nuclear differentiation special stain (NDS)[8]: composed of two solutions:

Solution A: Basic fuchsine 0.4gm in 100ml of (2.5%) methanol.

Solution B: Prepared by mixing equal volumes of: Azure II, Methylene blue, Na_2Co_3 in ethanol alcohol

Histometricale measurement of basement membrane and epidermal thickness using eye-piece graticule was perform on these groups, for each age groups (10) sections were selected randomly for each age group, and for each section (10) readings was divided (5) for rete pages and (5) for area with distinct deep dermal papilla. Data were collected and the mean for epidermal thickness was calculated, standard deviation (SD) and ANOVA test were performed on these data.

Results

General histological study

Skin at different age groups showed that young group (A) had a uniform arrangement of cells in all strata of the epidermis. All five layers of the epidermis were easily identified with decrease in the thickness of stratum cornium. The dermis showed many areas of disorganised collagen fibres especially in papillary layer with high cellularity and diminished skin appendages as seen in (Figure 1).

Adult group (B) showed a thick epidermal layer with distinct five strata and a well recognized dermal papillae which have shown an increase in number and depth as seen in (Figure 2).

The thickness of the dermis was strikingly increased with uniformed thick parallel arranged collagen fibres with distinct skin appendages (Figure 3).

Diminished thickness of the epidermis was a sticking feature in old age group (C).

Distinction among the five strata of the epidermis was not easy yet they remained present in a uniform way, although they were diminished to some extent. Dermal papillae showed decrease in both; there number and shallowness (Figure 4 & 5).

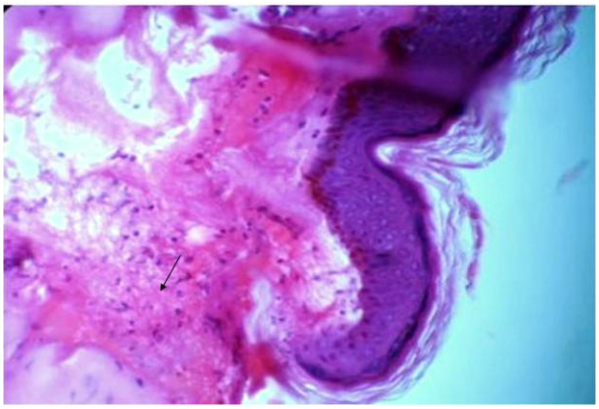

Figure 1. Section in the skin of young individual showing epidermis, the disarrangement & disorganized collagen (arrow) fibers with high cellularity of the dermis. Young age group (A), H&E, 400X

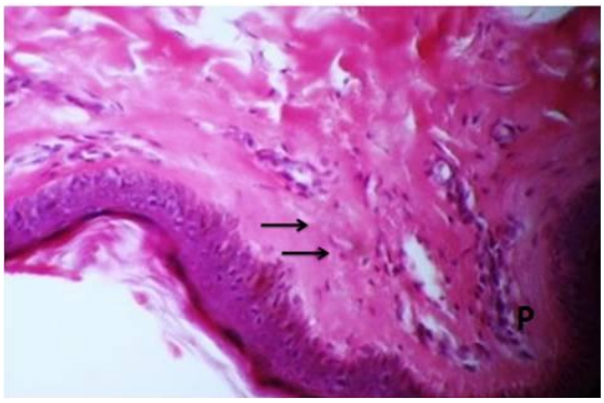

Figure 2. Section in adult skin showing the dermal papilla with well homogenized dermal fibers (arrow) and high cellularity of papillary layer (P). Adult age group (B), H&E, 400X

Old age group (group C) showed frequent appearance of well demarcated area of highly pigmented cell patches inside the epidermal layer (Figure 6).

Histometric measurement of epidermal thickness:

Histometric measurement of epidermal thickness using eyepiece graticule was perform on these groups, for each age groups (10) sections were selected randomly and for each section (10) readings was divided (5) for rete pages and (5) for area with distinct deep dermal papilla.

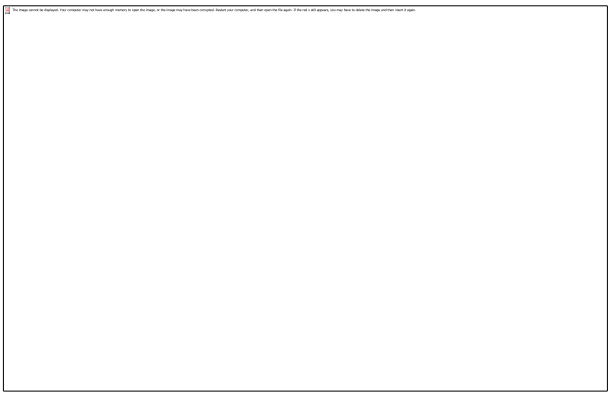

Figure 3. Section in adult Skin showing both epidermis and dermis with hair follicles (H) and sebaceous glands (S). Notice the thick dermis (D). Adult age group(B), H&E, 400X

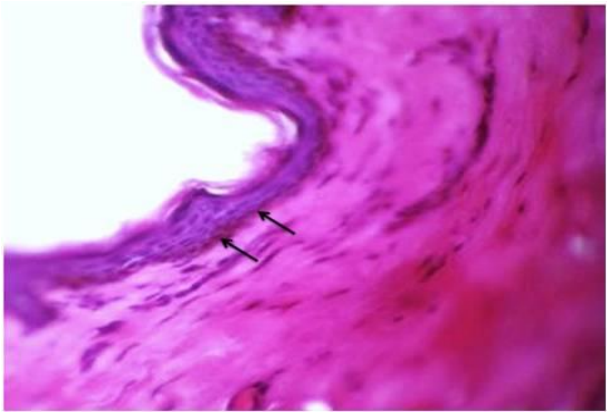

Figure 4. Decrease epidermal thickness with flatting epidermal-dermal ridges (arrow). Old age group(C), H&E, 400X

Figure 5. Decrease numbers of dermal papilla and flatting of the dermal-epidermal ridges (arrow) with diminished skin appendages and separation of collagen fibers (C). Old age group(C), H&E, 100X

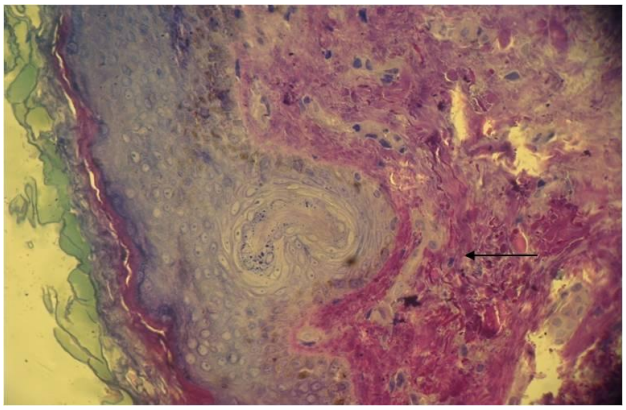

Figure 6. Epidermal patches, with disorganized collagen fibers below it (arrow). Old age group(C), NDS stain, 400X (Medium magnification)

Mean for each age group was calculate as show in (Table 1).

Table 1. Showing mean of epidermal thickness of each age groups.

Age groups	Mean ± SD of epidermal thickness
A (young age)	69.39 nm ± 2.85
B (adult age)	67.78 nm ± 3.07
Group C	10.90 nm ± 0. 40

Statistical analysis of the epidermal thickness was done using (Independent-sample t-test), there was a significant statistical difference between young and adult age group with old age group. The mean thickness of young age group (69.39 ± 2.85 nm), and adult age group (67.78 ± 3.07nm), while the old age group show a marked decrease in thickness of the epidermis with mean of (46.49 ± 2.33nm).
Independent-sample t-test show significant changes in thickness of the epidermis between each of group A & B with group C, with a (P value ≤ 0.001) as shown in (Table 2).

Changes and histometrical measurement of basement membrane:

Periodic acid Schiff's reagent was used to demonstrate and evaluate basement membrane changes. In all three age groups a well demarcated distinct basement membrane with clear boundaries was seen, thickness of basement membrane seem to changes as aging advance seen in (figure 7 and 8).

Table 2. The mean ±SD of epidermal thickness of the three age groups.

Age groups	Mean ± SD
Group A Group B	69.390 ± 2.858 (ns) 67.781 ± 3.076
Group A Group C	69.390 ± 2.858 * 46.491 ± 2.337
Group B Group C	67.781 ± 3.076 * 46.491 ± 2.337

* = P<0.001, ns= non significant

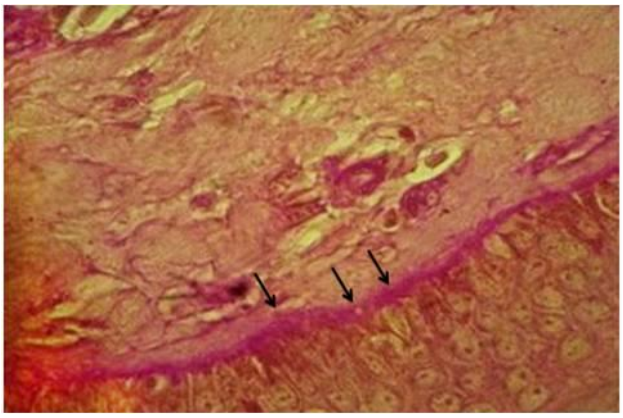

Figure 7. Young age group skin showing a distinct basement membrane (arrow) underlying the epidermis. Young age group, PAS, 400X

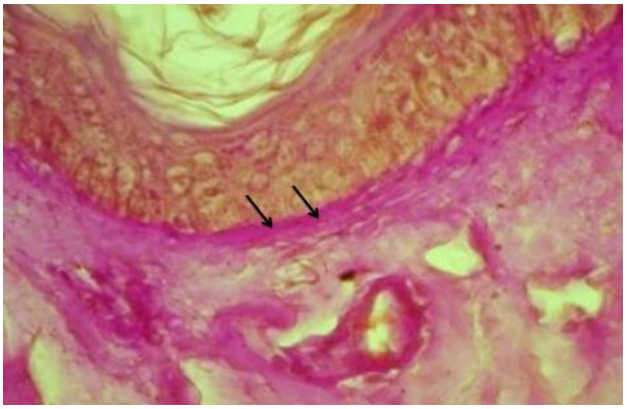

Figure 8. Old age individual skin showing the basement membrane (arrow) in PAS stain. Old age group, PAS, 400X

The mean thickness of basement membrane showed gradual increase in thickness as seen in (Table 3).

Table 3. Showing mean of basement membrane thickness of each age groups.

Age groups	Mean ± SD of basement membrane thickness
A	5.41 nm ± 0.3
B	8.30 nm ± 0.380
C	10.90 nm ± 0.40

Statistical analysis showed that there was significant statistical difference between the three age groups. The mean thickness of basement membrane of young age group (5.41± 0.31 nm) and adult age group (8.30± 0.380 nm), while the old age group show a marked increase in thickness of basement membrane with mean of (10.90 ± 0.40) as show in (Table 4). There was a gradual increase in thickness of the basement membrane starting from young age group, reaching the maximum thickness in old age group.

Statistical analysis showed significant changes in thickness of basement membrane between all age groups (A, B & C) with P value of (P≤0.001) as shown in (Table 4).

Table 4. The mean ±SD of basement membrane thickness in three age groups.

Age groups	Mean ± SD
Group A Group B	5.416 ± 0.31* 8.300 ± 0.38
Group A Group C	5.416 ± 0.31* 10.900 ± 0.40
Group B Group C	8.300 ± 0.38* 10.900 ± 0.40

* = P<0.001

Immunohistochemical study:

Studied section of S100 showed a number of melanocytes characterized by elongated or ovoid nuclei surrounded by clear space, they are usually smaller than neighboring basal keratinocytes, and appear associated between keratinocytes of stratum basale with darkly stain nuclei and obvious cytoplasmic granules (Figure 9). Melanocytes tend to be situated at the tips of rete pages, their numbers are generally low and did not vary a lot between young and adult age group, no significant differences was found between young and adult age groups (A and B) (figure 9).

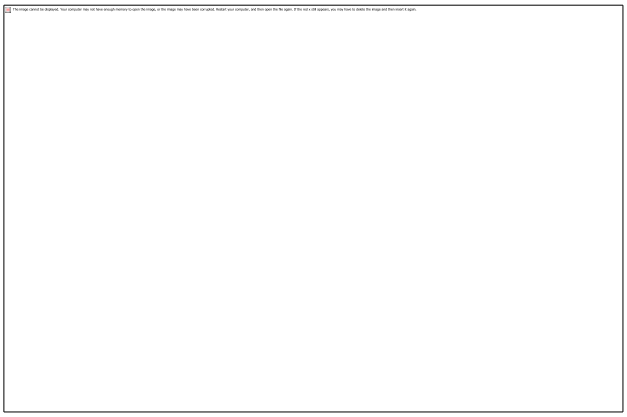

Figure 9. Melanocytes cells (M) at the tips of rete page in young age group. S100, 1000X (High magnification)

There was a marked decline in the number of melanocytes in old age group; they were difficult to be identified in the stratum basale as it was seen in (Figure 10).

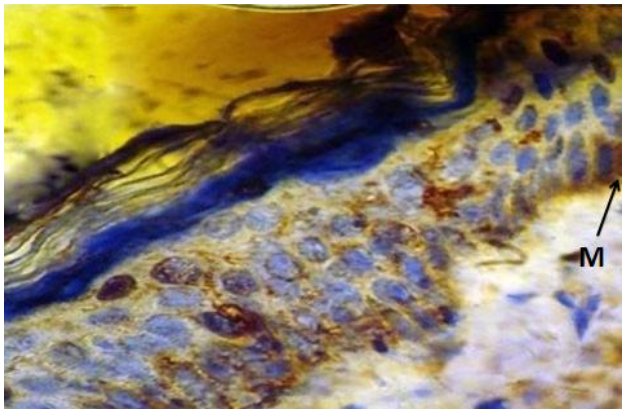

Figure 10. Melanocytes cells (M) in the stratum basale with darkly stain nuclei and obvious cytoplasm granule. S100, 1000X (High magnification)

Discussion

The skin covers the entire external surface of the human body; it is the principle sit of interaction with surrounding world. It serves as a protective barrier that prevent internal tissue from trauma, UV radiation,

temperature extreme, toxins and bacteria. Skin changes are among the most visible signs of aging. Evidence of increasing age includes wrinkles and sagging, along with the obvious graying of the hair, and with aged skin, histological and biochemical as well as function changes occur [9].

Collagen density in skin decrease annually with an average of 2% but Raine-fenning et al [10] calculated the number of collagen fibers in the skin diminished by (30-35%) after first (5years) of menopause.

The mean fractional volume of collagen fibers determined from stereological data is reported to be between (66 and 69%) for both papillary and reticular dermis for all age groups studied by [11,12].

In this study, it was found that aging Aging have influence the dermo-epidermal junction profoundly, dermal papillae flatten with the loss of the rete pegs, resulting in increased slippage between the epidermis and dermis.

The dermo-epidermal junction seem to be affected by an inter reaction of anchoring fibers, during the entire period of hormonal activity which they well affected collagen type IV and collagen type VII, the lack of hormonal stimulation causes decrease in production of these fibers, by the fibroblast of the corium, the shortening of collagen type IV in many structural protein may cause flatting of central epidermal border, which result in disturbance in the exchanges of nutritional component and also will caused thinning of the skin [13].

Epidermal thickness and its relation to age was investigated in this study as it formed the common study in dermatology research nowaday, The mechanical properties of the skin are due to the thickness and qualitative properties of epidermis and dermis. During age qualitative and quantitative change occur in the skin, lack elasticity and collagen content, increase wrinkling and aging lesions [14].

Epidermal thickness showed wide changes with aging in this study, a sticking diminished epidermal thickness was features of the old age group. The mean epidermal thickness measurement showed no significant differences between epidermal thickness in young and adult age group, with mean of (67-69 nm), while old age group showed significant decrease in epidermal thickness with mean of (45nm).

Many authors had reported the relation of epidermis and its interaction to variance factors, including aging without drowning a firm conclusion. Light microscopy may still be considered the ‘‘gold standard’’ for measurement of epidermal thickness, and by which other methods are compared [15]. Others have found difference in the epidermal thickness using different sites in the body [16]. Sun light exposure has been proved to induce thickening of skin corniume ([17] found thinner skin corniume in sun protected body sites compared to exposure body sits. In the current study a significant difference in epidermal thickness was found between (young- adult age group) and between the old age group but, no convincing difference in the epidermal thickness between young and adult age group.

Basement membranes are directly involved in the important biological process; it represents an extracellular scaffold that is necessary for morphological differentiation of thickness [18]. No previous study regarding measurement of basement membrane thickness and its changing during aging was found.

To determine the relationship between skin basement membrane and skin changes during age, hisometrical measurement of the basement membrane was performed, the outcome yields a significant difference between the three age groups.

Epithelial basement membrane thickness increased with age significantly with a (P value ≤ 0.001). We did not find previous study that has dealt with measurement of basement membrane thickness and its changing during aging.

Although changes of basement membrane in the other body parts were investigate by many authors, researcher seems to be more concerned with the structure, functional and architecture of basement membrane, due to the direct relationship between basement membrane and much significant biological process in the body [18].

Type IV Collagen fibres being one of the constituents of basement membrane of the skin had been study by (Vazquez et al [13], who evaluated type IV collagen fibres by immune labelling and morphometrical study. It was found that the thickness of Type IV fibres, plotted against age, showing a highly significant positive correlation. The increment in thickness was found to be significant in old age group 50 years and above.

Melanocytes are commonly distributed along the basal zone of the epidermis. It is one of the cells that cannot be identified by routine histological stain. Using S100 protein marker we were able to observe melanocytes changes.

Immunohistochemical labelling of melanocytes using S100 was performed in this study to demonstrate them. Melanocytes are commonly distributed along the basal zone of the epidermis, they represents type of cells that are difficult to be identified by routine haematoxylin and eosin stain.

S100 is a protein marker that has the ability to localize melanocyte cells and their changes in different conditions, and it is considered as the most frequently used marker in clinical practices. Monoclonal antibody labelled to S100 is a calcium binding portion that is usually originated by isolated from the brain and its sensitive marker that reacts with abroad rang of benign and malignant neoplasm as well as normal melanocytes, therefore is considered as a highly specific melanocytes marker [19].

Melanocytes tend to be situated at the tips of rete pages, there number are generally low and didn't vary a lot between young and adult age groups. Yet there was a marked decline in the number of melanocytes in old age group.

Examining melanocytes using S100 we did not depended on the density of the reaction but we depend upon the presence of cytoplasmic granules in the melanocytes cytoplasm, a features that is approved by all histopathologist.

Melanocytes number and its capability of synthesising melanosomes pigment is a highly affected by the process of aging, melanocytes had shown decrease in the life span as well as a decrease in the response to growth factor [20].

Aging seemed to reduce the immune functions in a naturally aged skin a function that can be referred to UV radiation in non aged skin. The numbers of melanocytes are proved to be highly effected in long standing sun exposure [21].

Conclusion

In the light of these findings of the present study, it was concluded that:

a) Many morphological and histological changes may be observed on skin with age advance.
b) The epidermal layer showed decreased on its thickness with age. This decreasing was non significant between young and adult age groups, but was highly significant between adult and old age groups.
c) The basement membrane of skin showed significant increasing in its thickness in the three age groups.
d) Immunolabelled melanocytes appeared to be affected with skin aging.

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Immunohistochemical Expression of MMP-3 and MMP-8 in Breast Carcinoma. A Clinicopathological Study

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Abstract

Background Matrix metalloproteinases are enzymes that are involved in the digestion of the components of the extracellular matrix (ECM), cell surface receptors for soluble factors and junctional proteins and physiological processes such as tissue remodeling, but also in the stimulation of tumor growth, invasion, and metastasis.

Objectives To assess the immunohistochemical expression of MMP-3 and MMP-8 in breast carcinoma and to correlate this expression with clinicopathological parameters including patient's age, tumor size, grade, subtype, lymph node status and lymphovascular invasion.

Method Sixty-two tissue blocks of breast carcinoma specimens were collected from Al-Kadhimiya Teaching hospital and Teaching Laboratories of the Medical City Center. Three sections of 5µm thickness were taken from each block and stained with H&E and Immunohistochemically for MMP-3 and MMP-8.

Results MMP-3 and MMP-8 expression were statistically correlated with patient's age, tumor grade, tumor size, histological subtype, lymph node involvement, and lymphovascular permeation with the exception of MMP-8 and age. Strong expression for MMP-3 was noticed in invasive carcinoma, high grade tumors, large size tumors, cases associated with positive lymph nodes and lymphovascular permeation (positive correlation). Negative expression for MMP-8 was noticed in most of the cases associated with lymphovascular permeation and positive lymph node(s) involvement by the metastatic cells (inverse relationship). Also a negative expression for MMP-8 was noticed in most cases associated with high grade, large size tumors.

Conclusions Assessment of MMP-3 in breast carcinoma reflects the grade of tumors and can predict progression of insitu to invasive cancer, lymph node involvement and lymphovascular permeation so that it may be useful additional prognostic factor. Expression of MMP-8 correlates with a lower incidence of lymph node metastasis and lymphovascular permeation and can be utilized as a marker indicating a good prognosis to these patients.

Keywords Breast carcinoma, MMP-3, MMP-8.

Introduction

Breast cancer is the most common cancer affecting women in the world today. It is the leading cause of cancer related death for women aged between 35 and 55 years worldwide. One in nine women will suffer from breast cancer during her life and in excess 130 thousand women die from breast cancer each year [(1)]. In Iraq, cancer of the breast is the commonest cancer in females, constituting 31% of all other malignancies in women [(2)]. Matrix Metalloproteinases (MMPs) family consists of

more than 26 endopeptidases that share homologous protein sequences, with conserved domain structures and specific domains related to substrate specificity and recognition of other proteins [3].

Considering the main action mechanisms, MMPs roles may be discussed in terms of tissue destruction, cancer invasion and metastasis, angiogenesis, apoptosis, escaping mechanisms, and antitumor defensive mechanism, and as a pivotal role in the pathogenesis of arthritis, atherosclerosis, pulmonary emphysema, and endometriosis. Tissue inhibitors of metalloproteinases (TIMPs) may act in the tissue environment to neutralize used proteinases thereby preventing excessive and unwanted degradation away from the sites of metalloproteinase production [4].

Stromelysins (MMP-3 or Stromelysin 1 and MMP-10 or Stromelysin 2) digest a wide array of substrates, including aggrecan, fibronectin, nidogen, laminin, type IV, IX and X collagens, tenascin, vitronectin and decorin [5]. Studies have shown that the expression of MMP-3in the mammary glands of transgenic mice causes the production of invasive carcinomas by stimulating epithelial mesenchymal transition (EMT), acting as a natural tumor promoter and enhancing cancer susceptibility in mammary glands of transgenic mice [6]. In humans EMT is associated with the most aggressive breast cancers. A particular molecule involved in cell-cell contact (E-cadherin) is known to be lost in EMT. Stromelysin-1 induces cleavage of E-cadherin, a process that may be the initial step in EMT and subsequent tumor formation [7].

Collagenases (MMP-1 or collagenase-1, MMP-8 or collagenase-2 and MMP-13 or collagenase-3) can digest major fibrillar collagens in their triple-helical domain at physiological pH [5]. Analysis of MMP-8 in breast cancer patients revealed that the expression of this metalloproteinase by breast tumors correlates with a lower incidence of lymph node metastasis and confers good prognosis to these patients. However, to date, no information is available about the molecular mechanisms underlying the putative role of MMP-8 in the regulation of the metastatic process [8].

The aim of the present study is to assess the immunohistochemical expression of MMP-3 and MMP-8 in breast carcinoma and to correlate this expression with clinicopathological parameters including patient's age, tumor size, grade, subtype, lymph node status and lymphovascular invasion.

Methods

A retrospective study included the collection of 62 formalin fixed, paraffin embedded tissue blocks from archived material at Al-Kadhimiya Teaching Hospital and Teaching Laboratories of the Medical City Center covering for the period from January 2009 to November 2010. These blocks represent the mastectomy specimens of breast carcinoma cases. Clinicopathological parameters such as (age, size, grade histological subtype, lymph node involvement and lymphovascular permeation) were obtained from the available histopathological reports and patients' files. An ethical approval was obtained from the institution in which the study was carried out in order to enable us to record patients' clinical data from their files.

Three sections of 5µm thickness were taken from each block, the first was stained by hematoxylin and eosin stain (H&E) for revision of the histopathological diagnosis, the rest two sections were stained immunohistochemically using three steps- indirect streptavidin method for MMP-3 and MMP-8.

Technical negative controls were obtained by omitting the primary antibody for the two markers under identical test condition, respectively.

Immunohistochemical expression of MMP-3 in ductal epithelial cells of the breast is cytoplasmic (brown color) and is better evaluated by the

intensity of the staining as to classify the result to negative (0 score), weak positive (+ score) (Figure 1), and strong positive (++ score) (Figure 2) depending on an intensity in control cases of endometroid endometrial carcinoma as weak positive MMP-3 is expressed in Grade I endometroid endometrial carcinoma and strong positive MMP-3 expression is detected in Grade III endometrial carcinoma [9-11].

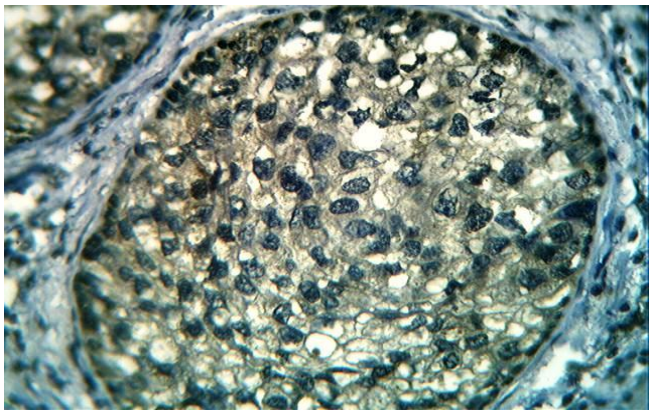

Figure 1. Ductal carcinoma *insitu* showing weak cytoplasmic immunohistochemical expression of MMP-3 by the malignant cells (X40).

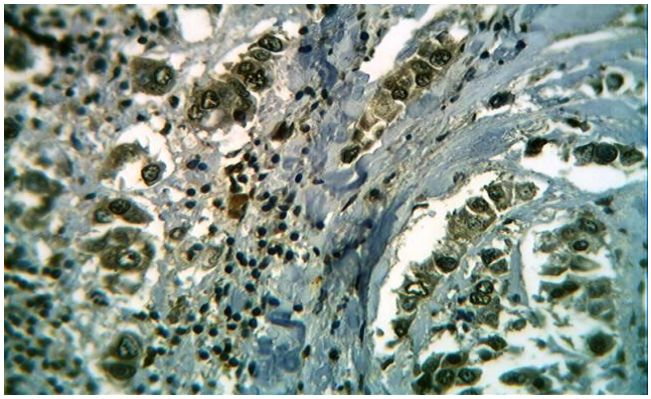

Figure 2. Invasive ductal carcinoma(NOS), Grade II, showing strong cyoplasmic immunohistochemical expression of MMP-3 (X40).

Immunohistochemical expression of MMP-8 in malignant ductal epithelial cells of the breast is cytoplasmic (brown color) and it is either negative (0 score) (Figure 3) or positive (+ score) (Figure 4) [12,13]. The positive control for MMP-8 is neutrophils in sections of acute supportive appendicitis according to the leaflet instructions. Statistical analysis was performed using SPSS V.17 (statistical package for social sciences) and Microsoft Excel 2007 programs. Data analysis was done using chi –square test and ANOVA. P-value is considered statistically significant when it is less than 0.05.

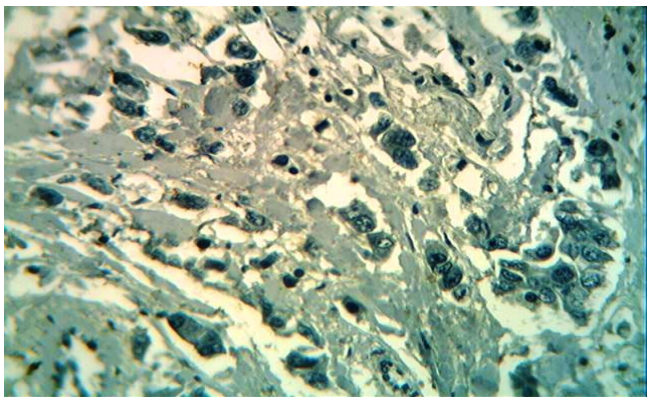

Figure 3. Invasive ducal carcinoma (NOS), Grade II, with negative IHC expression of MMP-8 (X40).

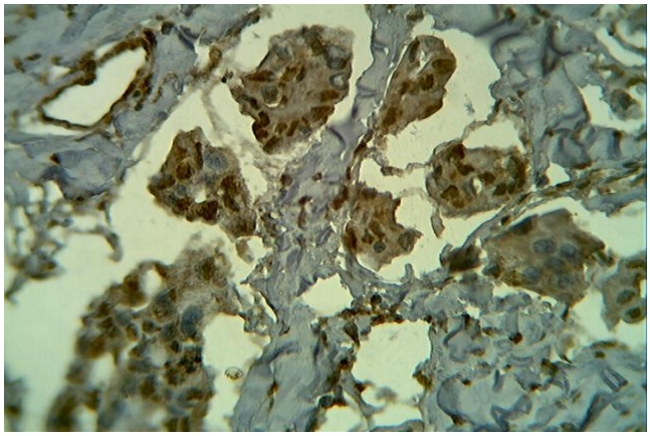

Figure 4. Invasive ductal carcinoma (NOS), showing positive cytoplasmic IHC expression of MMP-8 by the malignant cells (X40).

Results

The age of patients ranges between 30-70 years with a mean of (48.55±1.3 year). Regarding grade of the tumor, 64.5% of cases were Grade II, 25.8% Grade III and 9.7 % were Grade I breast carcinoma. The majority of the studied cases (46 cases) (74.2%) were invasive ductal carcinoma

(IDC), not otherwise specified (NOS). Forty four cases (70.96%) were associated with lymph node involvement by metastatic tumor cells while 18 cases (29.03%) were negative for lymph node metastasis. Forty six cases (74.19%) were associated with lymphovascular permeation while 16 cases (25.8%) negative for lymphovascular permeation. There was a statistically significant correlation between the lymphovascular permeation and lymph node(s) involvement (P<0.001) with an Odd ratio of 23(95% CI). Thirty two cases (51.6%) were with T2 tumor size (2-5 cm), 18 cases (29%) were with T3 (>5 cm), and 12 cases (19.4%) were T1 (<2 cm).

In the present study the overall expression of MMP-3 in breast carcinoma cases was 90.31% (56 cases) while MMP-8 was positively expressed in 29.03% (18 cases).

There was a statistically significant correlation between MMP-3 expression and age of the patients, tumor grade, tumor size, histological subtype (Figures 1 and 2), lymph node involvement, and lymphovascular permeation. Strong expression for MMP-3 was noticed in invasive carcinoma (Figure 2), high grade tumors, large size tumors, cases associated with positive lymph nodes and lymphovascular permeation (positive correlation) (Tables 1-3).

There was a statistically significant correlation between MMP-8 expression and tumor size, grade, histological subtype, lymph node involvement, and lymphovascular permeation, while there was no statistically significant correlation between MMP-8 expression and age of the patients. Negative expression for MMP-8 was noticed in most of the cases associated with lymphovascular permeation and positive lymph node(s) involvement by the metastatic cells (inverse relationship). Also a negative expression for MMP-8 was noticed in most cases associated with high grade, large size tumors (inverse or negative correlation) (Tables 1-3).

Table 1. Correlation of MMP3 and MMP8 immunohistochemical expression with age of patients, tumor size and grade

Marker Expression	Age range (Years)	P-value	Size <2 cm	Size 2-5 cm	Size >5 cm	P-value	Grade I	Grade II	Grade III	P-value
-ve MMP3	51.67±2.58		2	4	0		4	2	0	
Weak MMP3	43.08±8.31	0.003	10	14	0	<0.001	2	18	4	<0.001
Strong MMP3	52.06±10.78		0	14	18		0	20	12	
-ve MMP8	50.05±9.87	0.072	4	26	14	0.006	2	28	14	0.044
Positive MMP8	44.89±10.47		8	6	4		4	12	2	

Table 2. Correlation of MMP3 and MMP8 immunohistochemical expression with tumor subtypes

Marker Expression	Comedo carcinoma	DCIS*	IDC+DCIS**	IDC(NOS)***	P-value
Negative MMP3	0	2	0	4	
Weak MMP3	2	2	2	18	0.016
Strong MMP3	0	0	8	24	
Negative MMP8	2	4	10	28	0.032
Positive MMP8	0	0	0	18	

*ductal carcinoma insitu ** combined invasive ductal carcinoma and insitu carcinboma ***invasive ductal carcinoma not otherwise specified

Table 3. Correlation of MMP-3 and MMP-8 immunohistochemical expression with Lymph node(s) involvement and Lymphovascular permeation

Marker Expression		LN involvement		P-value	LV permeation		P-value
		Negative	Positive		Negative	Positive	
MMP3	Negative	4	2	<0.001	4	2	<0.001
	Weak	14	10		12	12	
	Strong	0	32		0	32	
MMP8	Negative	6	38	<0.001	6	38	0.001
	Positive	12	6		10	8	

Discussion

In the present study there was a statistically significant positive correlation between MMP-3 expression and the age of the patients with a P-value of 0.003. MMP-3 has been implicated in overall age-associated risk of cancer development.

senescent stromal fibroblasts secrete soluble and insoluble factors that can, at least in principle, disrupt the architecture and function of the surrounding tissue and stimulate (or inhibit) the proliferation of neighboring cells. These factors include inflammatory cytokines (e.g. IL1), epithelial growth factors (e.g. heregulin) and matrix metalloproteinases (e.g. MMP-3). Thus, senescent cells may create a tissue environment that synergizes with mutation accumulation to facilitate the progression of epithelial malignancies. Consistent with this idea, human and rodent cells with senescent characteristics accumulate in vivo with age and at sites of age related pathology, including hyperplastic and premalignant lesions. Moreover, senescent human fibroblasts can promote the proliferation and tumorigenic conversion of premalignant (non-tumorigenic, but bearing potentially oncogenic mutations), but not normal, epithelial cells in culture and in vivo [(14)].

In a study conducted by Nakopoulou et al reported no significant correlation of MMP-3 expression with age of patients with breast cancer, a finding which disagrees with this study due to difference in sample size, technique or population [(15)].

In the present study, the relation between the MMP-8 expression and patient's age was not statistically significant with a P- value of 0.072. This finding goes with a study done by Decock et al which recorded that the higher percentage of positive MMP-8 was expressed in pre-menopausal age; however the correlation of MMP-8 expression with age in breast cancer was not significant [(16)].

Regarding tumor grade, the current works revealed a statistically significant correlation between MMP-3 expression and the grade of breast carcinoma ($p< 0.001$). It is obvious that the expression of MMP-3 is more in higher grade tumors that all cases of Grade III were positive for MMP-3 expression. MMPs have also been shown to be involved in malignant transformation of the mammary gland. Overexpression of MMPs like MMP-3 (stromelysin-1) and MMP-7 (matrilysin-1) in the mammary gland of transgenic mice, results in premature differentiation and increased incidence of mammary tumor formation. A potential molecular basis for such an effect has been elucidated by the demonstration that some MMPs like MMP-3 and MMP-7 promote epithelial to mesenchymal transition (EMT), an early step in malignant transformation of epithelial cancers [(17)]. In humans EMT is associated with the most aggressive breast cancers [(7)]. However, studies by McGowan and

Duffy [18] and by Krippl et al [19] revealed no significant correlation of MMP-3 with tumor grade. This discordance could be attributed to environmental, racial and geographical differences, in addition to the difference in the sample size and antibodies used for detection of MMP-3 antigen.

In the current study there is a statistically significant correlation between MMP-8 expression and the grade of the carcinoma with P -value of 0.044, but the opposite to MMP-3, here we can notice that most of the higher grade cases (Grades II, III) were negative MMP-8, i.e., poorly differentiated breast carcinomas associated with negative MMP-8 expression while positive MMP-8 expression is detected mainly in low grade carcinomas. MMP-8 is not expressed in normal breast tissue while a low positive expression of MMP-3 is noticed in normal breast tissue. The unexpected finding that MMP-8 might play tumor-defying functions first derived from studies of cancer susceptibility in a murine model of MMP-8 deficiency. The absence of MMP-8 strongly increased the incidence of tumors in male $MMP^{-}/^{-}$ mice .Bone marrow transplantation studies provided additional evidence that neurtophil-derived MMP-8 is sufficient to restore the antitumor protection mediated by this metalloproteinase [20]. A study by Decock et al agrees with the present work [16]. Other studies revealed discordant results due to technical, statistical, racial or sample size differences [1,18].

The present study reported a statistically significant correlation between MMP-3 expression and the histological type of the tumor with P- value of 0.016. The expression of MMP-3 is more intense with invasive breast carcinoma and less with the in situ carcinomas. A particular molecule involved in cell-cell contact (E-cadherin) is known to be lost in EMT. Stromelysin-1 induces cleavage of E-cadherin, a process that may be the initial step in EMT and subsequent tumor formation [7]. Holliday et al [21] reported similar results with the current study; however, Nakopoulou et al disagrees with these findings [15].

This study showed a statistically significant correlation between MMP-8 expression and the histological type of breast carcinoma with P -value of 0.032 . The positive MMP-8 expression is found only in invasive carcinomas and is not detected in *in situ* tumors. Analysis of this negative regulation of cell invasiveness mediated by MMP-8 revealed that it is associated with an increased adhesion of cells expressing MMP-8 to different extracellular matrix components, such as type I collagen and laminin-1 and actin fiber reorganization, consistent with the increased adhesion of cells expressing MMP-8 [22,23].

There is a statistically significant correlation between the lymphovascular permeation and lymph node involvement with P- value of less than 0.001 and an Odd ratio (95% CI) of 23, which means that the patient with lymphovascular permeation is 23 times more risky to have a positive lymph node(s) than a patient without lymphovascular permeation. In the present study we found that there is a statistically significant correlation between MMP-3 expression and lymph node(s) involvement ($P < 0.001$) and with lymphovascular permeation ($P < 0.001$).

MMP-3 expression was more intense (strong) with positive lymphovascular permeation and positive lymph nodes. Lymphangiogenesis plays an important role in tumor biology; it is directly linked with the formation of lymphatic metastases. MMP-3 plays a role in activation of MMP-9 which is important in the modulation of the Vascular Endothelial Growth Factor (VEGF) bioavailability (the most potent inducer of tumor Lymphangiogenesis) and making sequestered VEGF bioavailable for its receptor VEGFR2, in turn, promotes dissemination of metastases into the lymph. So increase MMP-3 is linked with lymphatic invasion and lymph node metastases[24]. Krippl et al [19] reported also a

significant correlation between MMP-3 expression and lymph node involvement; however, the current study disagrees with a study done by McGowan and Duffy [18] due to similar reasons mentioned above.

In the present study there was a statistically significant negative correlation between MMP-8 expression and Lymph node(s) involvement (P <0.001) and also significant negative correlation with lymphovascular permeation (P = 0.001). MMP-8 expression was more with negative nodal metastases and negative lymphovascular permeation while the majority of positive nodal cases and positive lymphatic permeation were negative for MMP-8. MMP-8, like other MMP enzymes, is secreted as a proenzyme, which can subsequently be activated by a number of other enzymes including MMP-3 and serine proteases, which themselves can be inactivated by specific tissue inhibitors. The interplay between these potential activators of MMPs and their inhibitors plays a significant role in the function of these enzymes. Therefore, in addition to the differential expression of MMP-8 in tumor cells, activation of the procollagenase could be an important regulatory step in its inhibitory effect on metastasis, also MMP-8–expressing cells had an increased adhesion to type I collagen and laminin-1 so potentiates cell adhesion, and this might be a candidate mechanism by which this protease reduces cell invasion and metastasis, but (to date) the exact mechanism by which MMP-8 protect against lymph node metastasis is not clear [23,25]. Pennington *et al*. found that there was a significant correlation between MMP-8 expression and lymph node involvement and that the reduced expression of MMP-8 equating to greater nodal spread and suggested that the function of MMP-8 antagonizes metastasis of breast carcinomas[26] Decock et al revealed also a significant correlation of MMP-8 with nodal involvement and that MMP-8 is less expressed with node positive patients [16]. However, McGowan and Duffy [18] disagrees with this result.

When tumor size is taken into consideration, the present work found that there is a statistically significant correlation between MMP-3 expression and the tumor size (P value< 0.001) and that the staining is more intense with large tumors (T2 and T3), the strongest expression is in T3 tumors, and no strong expression in T1 carcinomas. Possible mechanisms by which MMP3 contributes to tumor cell growth include promotion of angiogenesis (which is necessary for a tumor to grow to a size greater than approximately 2mm in diameter, MMP-3 have been shown to breakdown endothelial-derived perlecan, releasing basic fibroblast growth factor (FGF), a potent endothelial mitogen, activation of stimulating growth factors or their receptors, and inactivation of inhibitory growth factors **[27]**. Other studies found no significant correlation of MMP-3 with tumor size [18,19].

In the present study there is a statistically significant correlation between MMP-8 expression and the tumor size (P = 0.006) and that MMP-8 expression in the majority of large tumors (T2 and T3) is negative (the opposite to MMP-3). The mechanism by which MMP-8 act as tumor defying agent is still unclear, but the possible explanation for increase expression of MMP-8 in small size tumor is that it develop it`s functions by targeting TNF (Tumor Necrosis Factor) which decreases tumor size by apoptosis, also this enzyme may target substrates distinct from collagens or other matrix components. The potential proteolytic processing activity of MMP8 on inflammatory mediators, which contribute to the host antitumor defense system, could help to explain this [23,25]. Studies by McGowan and Duffy [18], and Decock et al [16] recorded different results. This disagreement could be caused by difference in sample size, antibody used, methods of quantifying immunohistochemical staining(manual versus automated) and racial differences.

In conclusion, assessment of MMP-3 in breast carcinoma reflects the grade of tumors and can predict progression of *insitu* to invasive cancer, lymph node involvement and lymphovascular permeation so that it may be useful additional prognostic factor. Expression of MMP-8 correlates with a lower incidence of lymph node metastasis and lymphovascular permeation and can be utilized as a marker indicating a good prognosis to these patients.

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The Proliferative Pattern of the Ventricular Neuro-epithelium of Embryonic Rat Fourth Ventricle

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Abstract:

Background Studies have shown that the ventricular neuroepithelium (VN) of the dorsal metencephalonrepresents the site of the cerebellar GABAergic neurons progenitors' production. These studies used different histological techniques but all have provided qualitative information regarding the biosynthesis and cell mitosis.

Objective Quantifying the proliferative activity of the cells at the (VN) during the embryonic period.

Methods Nine age groups from day 13 to day 21 albino rat embryos *Rattusrattusnorvegicusalbinus* were investigated with Ag-NOR staining technique.

Results There was a statistically significant difference ($p<0.01$) between cellular activity at different age groups with a maximum proliferative activity on the embryonic day 15.

Conclusion Ag-NOR staining technique provided a valuable quantitative index of cell proliferation at the (VN) of the developing cerebellum.

Key words Ventricular Neuroepithelium, Rhombencephalon, Ag-NOR, Quantitative

Introduction

The term rhombencephalon is widely used to designate the region of the neuroaxis that surrounds the fourth ventricle and its recesses. The developing rhombencephalon is subdivided into two parts, the myelencephalon, giving rise to the medulla, and the metencephalon, which is said to give rise to the cerebellum and the pons. The dorsal surface plates of the rhombrncephalon fail to fuse medially. Instead, a membrane, the medullary velum, part of which becomes the telachoroidea, spreads over the enlarged rhomboid cavity, the fourth ventricle. This membrane interconnects the edges of the caudal and the rostral portions of the classical dorsal rhombencephalon. The bridgeheads are the dorsal metencephalonrostrally and the precerebellarneuroepithelium caudally [(1)].

Different morphological descriptions and delineations have been given to the roof plate of the developing rhombencephalon, the dorsal metencephalic anlage [(2,3)] and the cerebellar analage[(4)].

The dorsal metencephalonisis the site of development of the cerebellar and extracerebellar structures. This is in agreement with the current findings of embryogenesis in the roof plate of the metencephalon[(5)].

The dorsal metencephalon is lined by the ventricular neuroepithelium (VN) that begins its productive activity on the embryonic day 13 that is considered to be the onset of the formation of the cerebellar primordium in the rat[(6)]. Both morphological and genetic approaches suggest that VN is the source of all GABAergic types of cells in the developing cerebellum [(7,8)].These GABAergic neurons

consist of at least five different neuronal subtypes and are generated in three sequential but overlapping waves, the first-born are small Deep Cerebellar Nuclei neurons which eventually settle in the white matter beneath internal granule neurons. Purkinje cell progenitors are second to be generated and they become postmitotic then migrate dorsally along guiding radial glial processes to their final destination beneath the external granule layer. A third population of neurons, which consists of GABAergic interneurons of the Deep Cerebella Nuclei, stellate, basket, Lugaro, and Golgi cells, is generated during late embryonic and postnatal development[9].

In the proliferating cells, nucleolar organizer regions (NORs) are loops of DNA which contain ribosomal RNA genes important for the synthesis of proteins. These NORs are stained with silver colloid technique, and the result is known as Ag-NOR dots [10].AgNOR (Argyrophilic nuclear organizers regions) technique is simple, rapid, inexpensive and can be performed on paraffin- embedded tissue including archival material. Therefore, unlike most available 'proliferation' techniques, it does not require special processing of tissue [11]. By using this technique these NOR-associated proteins are selectively stained; and the number and area of Ag-NORs are an accurate index of activity and cell proliferation in terms of protein synthesis [12,13]. Hence, Ag-NOR stain is used to measure the biosynthetic profile and cell mitotic activity by demonstrating the amount of rRNA that increases during cell replication[14]. It can be used as marker for both cell proliferation and malignancy [15].The Ag-NOR staining technique was employed as a quantitative method as it is recommended for the investigation of protein biosynthesis and cellular activity in the developing CNS [16].

This work aims at assigning a quantitative proliferation index for the cells of the VN during their embryonic development by the application of Ag-NOR staining technique and correlate it to other qualitative studies.

Methods

A sample of eighteenth albino rats *Rattusrattusnorvegicus*albinus was divided into nine age groups from embryonic day 13 to embryonic day 21 and brain tissue specimens were obtained by decapitation. Tissue blocks were immersed in Bouin's fixative for 16 hours at room temperature (25°C) and parasagittal paraffin sections of 6 micrometer thickness were prepared for embryonic age day 13 through day 21.Sections were stained according to the method of Ploton[17]. Dewaxingin xylene was done for 3-5 minutes then pre-incubation in glycine solution (made by dissolving glycine powder (AnalaR) in 99% ethanol alcohol) for 10-20 minutes followed by rehydration in descending concentrations of ethanol alcohol (100%, 90%, 80% and 70%) each for 3 minutes.

Colloidal developer solution was made by dissolving 2 g of gelatin powder (Agar LTD) in 100 ml of double deionized distilled water (2% w/v). This was added to 1% aqueous formic acid. Developer solution was mixed 1:2 volumes with 50 g/dl aqueous freshly prepared double deionized silver nitrate (M & B) solution filtered through mini-pore filter paper under dark room conditions.Histological sections were left in silver colloid solution for 45 minutes at 37°C in an air incubator. Background stain was reduced through holding the slides perpendicularly in Coplin's jars where the precipitate remains at the bottom.Sections were washed in running double deionized distilled water for 10-15 minutes then treated with 10% nitric acid solution (Fluka) for 30 seconds, washed well with flowing double deionized distilled water and immersed in 5% sodium thiosulphate (AnalaR) (w/v) solution for 5 minutes to provide a permanent preparation.Finally, dehydration was achieved by ascending concentrations of ethanol alcohol (70%, 80%, 90% and 100%), each for 3 minutes, then clearing with xylene and mounting with Eukitta mounting medium.

Examination of 45 sections (5 sections/ age group) was done under light microscope

(1250X oil immersion).Five fields showing the region of VNper section were examined and simple random sampling of 20 cells in each field was done for the number of Ag-NOR dots per cell (Figure 1). Hundred cells of VN with Ag-NOR stained nuclei were recorded in each section and the average number of staining dots per each cell was obtained[14].

Results

A view of the developing Rhombencephalon is seen in figure1 that shows the fourth ventricle roofed by the dorsal metencephalon and the medullary velum. The dorsal metencephalon is the site where the cerebellum develops, and it is lined by VN.

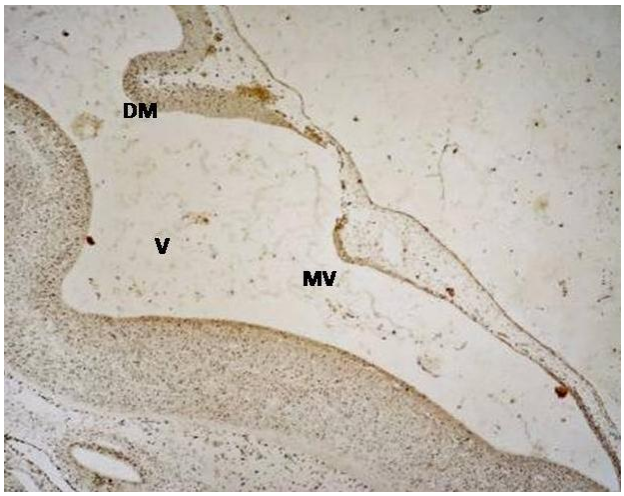

Figure 1.Rat embryo at day 15.Aparasagittal section in the developing Rhombencephalon showing the fourth ventricle (V) roofed by theDorsal Metencephalon (DM) and the Medullary Velum (MV). Paraffin, Ag-NOR stain. 40 X.

Another orientation view of the developing cerebellum is seen in figure 2 that shows a compact cellular layer at the VN that lines the cerebellar primordium. Purkinje cells are observed as a less packed stratum deep to the External germinal layer that spreads from the region of the Rhombic lip to cover the surface of the developing cerebellum, while fronds of cellular projections from the medullary velum mark the development of the choroid plexus at the roof of the fourth ventricle.

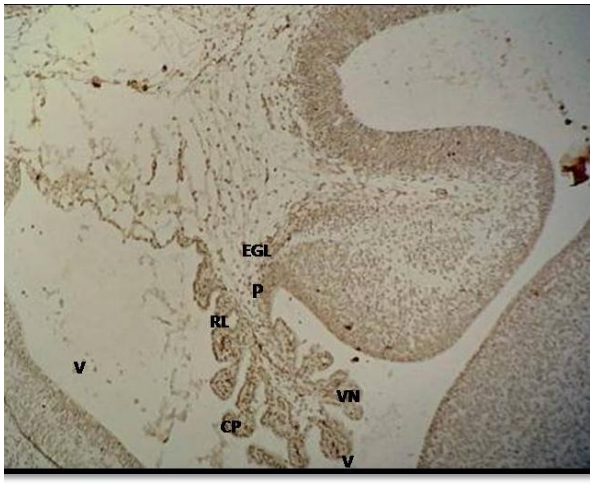

Figure 2.Rat embryo at day 17. A parasagittal section in the developing Cerebellum showing the Ventricular Neuroepithelium (VN), the Purkinje cell layer (P), External germinal layer (EGL), Rhombic lip (RL), Choroid plexus(CP), and the fourth ventricle (V). Paraffin, Ag-NOR stain.100X.

In figure 3, the cells of VN are magnified to reveal the Ag-NOR dots within the nuclei.

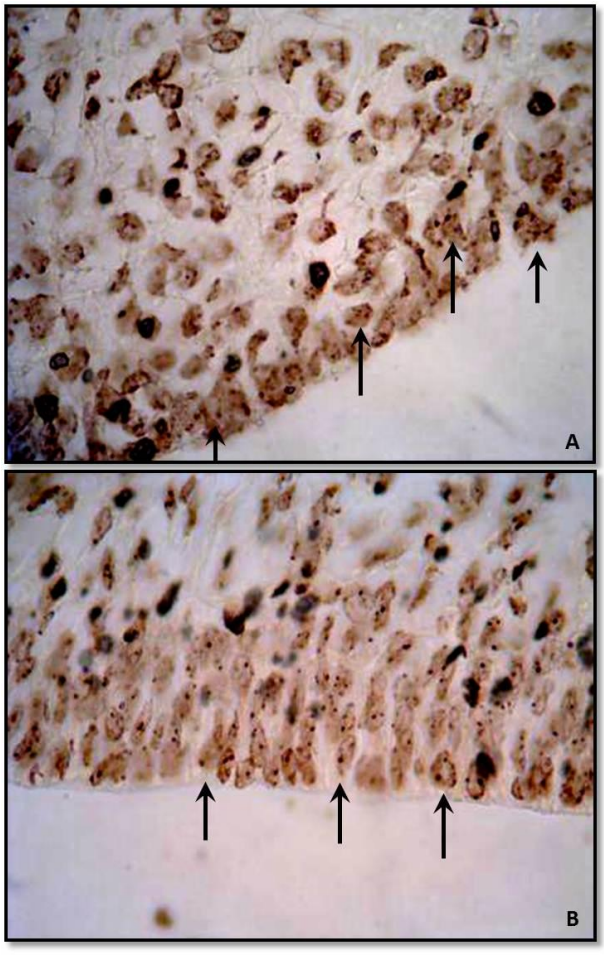

Figure 3. Cells of the ventricular neuroepithelium of the roof plate of the fourth ventricle with maximum mitotic activity at E15 (A) and minimum mitotic Activity at E18 (B) reflected by the number of "dots" (arrows) in the proliferating cells.Paraffin, Ag-NOR stain. 1250 X Oil immersion.

The Average number of Ag-NOR dots per cell nucleus for 100 cells within five fields per age section of VN in nine different age groups of rat embryo are shown in table 1. The results showed an increment in the average numbers of Ag-NOR dots per cell nucleus during E13,E14 to reach its maximum on E15 then they decline on E16-E18 to show another increment again on E19 till birth.

In order to analyze the differences between the various age groups, a single factor ANOVA was applied regarding the Ag-NOR parameter evaluated: the mean Ag-NOR number per cell. The results show a statistically significant difference between the various age groups studied ($P<0.01$).

Table 1. Average numbers of Ag-NOR dots per cell nucleus for 100 cells within five fields per age section of the ventricular neuroepithelium of the roof plate of the fourth ventricle in nine different age groups of rat embryos.

section	Age (Days)								
	E21	E20	E19	E18	E17	E16	E15	E14	E13
1	2.93	2.81	4.79	2.74	2.81	2.83	5.88	5.3	3.32
2	2.54	3.32	3.87	2.4	2.23	2.98	6.47	6.12	4.12
3	2.89	3.1	4.43	2.34	2.88	4.16	7.04	4.71	3.65
4	2.21	3.22	3.98	1.94	3.1	3.13	7.12	5.55	2.98
5	3.1	2.91	4.14	2.62	2.45	2.64	6.64	4.91	3.34
Mean	2.73	3.07	4.24	2.41	2.69	3.15	6.63	5.32	3.48
± SD	±0.36	±0.21	±0.37	±0.31	±0.35	±0.59	±0.5	±0.56	± 0.43

Discussion

Different methods (lectinhistochemistry[18], short-term and long-term survival thymidine autoradiograms[19], enzyme histochemistry [2], and immune histochemistry[9])have studied VNof the developing cerebellumin terms of cellular production and migration. These studies have shown that this region is a germinal zone that undergoes temporal variation in the production of different types of cells that contribute to the cerebellar neurons; none of these have studied the proliferative activity of the cells of this region throughout the prenatal development of the rat CNS.

Many studies performed on various regions of the CNS using the Ag-NOR technique alone or in combination with other histological stains have revealed quantitative associations between cell proliferation and different aspects of functions [12], neoplastic changes [13, 15], and cell production [16].

The results showed a significant increment in cell proliferative activity in terms of Ag-NOR dots per cell nucleus during the embryonic days E13, E14 to reach its maximum in the embryonic day E15. Such results conform to observations made by other birth dating studies indicate that all projection neurons are generated between E13 and E15 in the developingcerebellum of the rat[20, 21].

Previous studies have found that the neurons of the deep cerebellar nuclei are generated from the layer of VN between embryonic days (13-15) with peak production on day 14. The Purkinje cells are also generated from the layer of the VN between embryonic days (13-16) but with a peak on day 15 [22, 23]. The peak of proliferative activity noticed on E15 in this study agrees with the peak of purkinji cells' production noticed on E15 by the previous studies, but it's not the case with the deep cerebellar nuclei.

The results of this work support the results of the recent studies demonstrating that only the GABAergic component of the DCN (neucleo-olivary projection neurons and the

interneurons) are derived from VN, while the glutamatergic components are derived from the rhombic lip [24,25,26].

The proliferative activity of VN cells is seen to be increased again during the embryonic days E19-E21. This period of embryological development is the time of GABAergicinterneuronsproduction (interneurons of the DCN, stellate, basket, Lugaro, and Golgi cells) that are generated during late embryonic and postnatal development [9, 20, 25].

The proliferative activity of VN cells in the early stages of cerebellar formation (E13-E15) is shown to be higher than in the late embryonic stages (E19-E21).Recent studies have revealed that the two main classes of GABAergic neurons are generated in VN according to distinct strategies. Theprojections neurons are produced within VN at the onset of cerebellar neurogenesisand are committed to their fate at early ontogenetic stages. In contrast, inhibitory interneurons derive fromsingle pool of multipotent progenitors that delaminate from the VN during late embryonic life and continue to divide in the prospective white matter up to postnatal development[24, 27].

This work demonstrates that the temporal variation in the proliferative activity of the cells at VN of the developing cerebellum coincides with the birth-dating timetable of the different types of cells produced in this region.

In conclusion, the Ag-NOR staining technique provides a quantitative index of cell proliferation at the ventricular neuroepithelium of theRhombencephalon roof plate to support previous qualitative cellular production and birth-dating studies.

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The Effect of Aging on Human Testis: Anatomical and Histological study

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Abstract

Background Aging of testis is gradual dysfunction of body organs and tissues due to reduction of cell number and loss of cell capacity for reproduction and regeneration of its structural elements.

Objective To study the age related changes of the human testes anatomically and histologically.

Methods Forty testes of twenty adult Iraqi male cadavers, with age ranged from 20-69 years were taken from the Forensic Medicine Department of Tikrit and Azadi Teaching Hospital in Salahddin and Kirkuk province, respectively during the period from November 2006 to September 2007. The testicular specimens were divided into five groups according to cadaver age. Tunica albuginea was removed to investigate the lobular and tubular structures of the testis, by anatomical and histological procedures.

Results The gross metrical measurement and anatomical inspection revealed that a negative correlation was existed between the process of aging and the weight of the postmortem, testis. A strong directly proportional positive correlation was found between age and the thickness of basement membrane, interstitial spaces between seminiferous tubules. Tubular sclerosis was observed in some testis specimens of senescent cadavers associated with a decrease in the number of the lining epithelial cells of the seminiferous tubules and sclerotic changes in the walls of testicular blood vessels.

Conclusion The process of aging leads to testicular changes such as tubular diverticula and decrease in number of leydig cells.

Key words Aging, Leydig cells, senescent testis, seminiferous tubules.

Introduction

The aging is gradual dysfunction of body organs and tissues due to reduction of cell number and loss of cell capacity for reproduction and regeneration of its biochemical structural elements [1-4].

Testes as being small until puberty when they grow very quickly and reach maximum development in the period of the sexual activity and decreasing the volume and weight with age over 50 years old [3,5-7].

Age related change is a complex multifocal process as a result of gradual loss of the cell capacity for reproduction and regeneration of its biochemical structural elements [8-11]. Volume of the testis is a rough indicator of spermatogenesis remains constant over long period of life and reduction in paired testicular weights, total volume and seminiferous tubule volume, seminiferous epithelium and length of tubules [12-16]. Reduction of type-A dark spermatogonia, increased occurrence of multinucleated spermatogonia, megalospermtocytes as well as giant spermatids and leydig cells are the characteristic feature of senescence [17-19]. The average decreases in the production of testosterone in men usually over 40 years of

age, but in men the testes remains functional throughout life by spermatogenesis, as well as the synthesis of testosterone [20,21]. The diminished androgen production or spermiogensis might be reflected by a rise in gonadotropine serum levels and a testosterone decline with age [2224].

Aim of the study

To study the age related changes of the human testes anatomically and histologically.

Methods

Twenty normal adult Iraqi male cadavers, with age ranging from 20-69year's were taken during the period from November 2006 until June 2007 at the Forensic Medicine Department of Tikrit and Azadi Teaching Hospital in Salahddin and Kirkuk province, respectively. The agreement consent (permission paper) from the relatives of cadavers was performed for medico legal purposes. The cadavers were divided into five groups according to age and four in number for each group as follows:

- Group (A): ranged from 20–29 Years (control group).
- Group (B): ranged from 30–39 years.
- Group (C): ranged from 40–49 years.
- Group (D): ranged from 50–59 years.
- Group (E): ranged from 60–69 years.

Anatomical study: A longitudinal incision downwards through the skin of the anterolateral aspect of the scrotum. The testicular fascia and tunica albuginea were shelled out from the testis. This provides excellent exposure of testes, in order to examine extensions, Lobular structures of the testis (Figure 1).

The weight of the right and left testes was measured by an electronically weighing scale (Mettler AE 200, Japan). The total testis weight was then calculated by adding together the weight of both testes.

Histological Study: Fixation of the specimens was made using 10% formalin saline (100 ml of 40% formaldehyde, 9gm Sodium chloride and 900 ml tap water) for 24-48 hour [25]: Routine staining of sections was performed using haematoxylin and eosin stains (H&E) and Periodic Acid Schiff's Technique "PAS Technique":

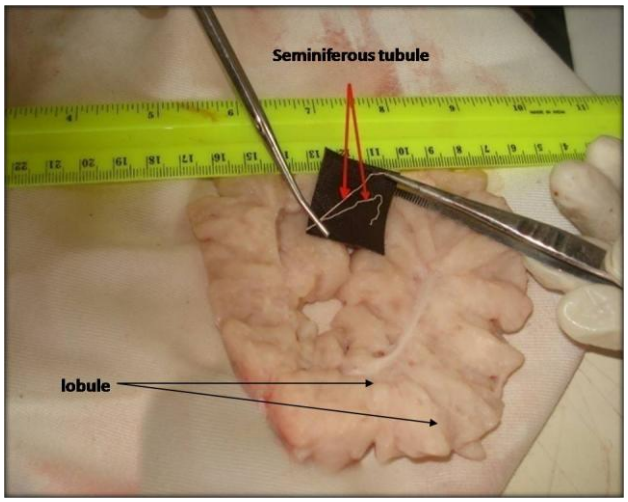

Figure 1. The highly convoluted seminiferous tubules (red arrows) and lobules (black arrows)

Measuring techniques by measuring the diameter of seminiferous tubules, and the interstitial space between the seminiferous tubules distance was measured by calibrated ocular lens and the calibrated stage was consisting of 100 minute lines is equal to (10 µm) and was performed, at 40 X objective lens, each line of the calibrated ocular lens is equal to 2.4 µm.

Results

Each testis consist of about (250- 300) lobules and each lobules has 1-4 seminiferous tubules which are highly convoluted tubules (40-70) cm length (Figure 1).

In groups B, C, D, and E the means weight of the right testes were 20.41±2.66, 20.64±2.24, 19.45±2.24 and 17.20±1.26 gm, respectively, whereas those of the left testes of the same groups were, 20.44±2.68, 20.60±2.25, 19.46±2.24 and 17.10±1.26 gm, respectively groups D and E had significant values compared with those of groups A (17.30 ± 1.53 gm) on right side, and 17.28±1.56 on left side

as control group (P<0.01) of both side (Table 1).

Table 1. Weight of testes according to age groups.

Group	No.	Rt. testis weight (gm)	Lt. testis weight (gm)
A	4	17.3 ± 1.53	17.28 ± 1.56
B	4	20.41 ± 2.66*	20.44 ± 2.68*
C	4	20.64 ± 2.24*	20.6 ± 2.25*
D	4	19.45 ± 2.24*	19.46 ± 2.24*
E	4	17.20 ± 1.26**	17.10 ± 1.26**

Group A is the control group
*= P<0.05, **= P<0.01 as compared to group A

It was appearing that the number of the lining epithelial cells (sertoli and spermatogonial cells) of the seminiferous tubule were decreased in testes specimens of groups D and E compared with those of groups A as control group (Figures 2&3).

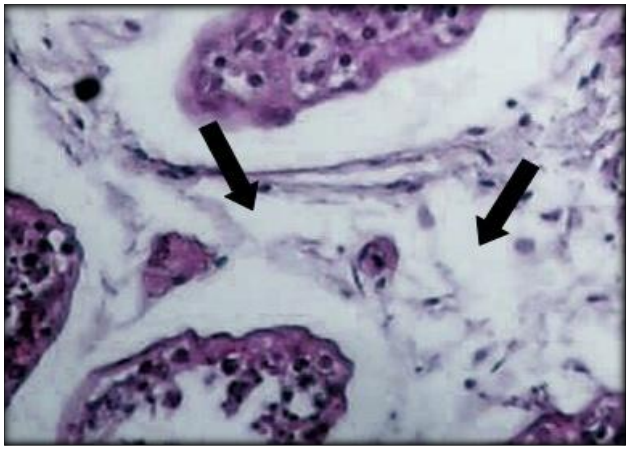

Figure 2. Testicular tissue showing the parenchyma with diffused small clusters of adipose cells (arrow), forming great part of testicular parenchyma in group D (H&E, 40X).

The adipose cells were few, scattered as a single cell in the parenchyma of groups A and B, while in group C, D and E were arranged as small clusters of adipose cells composed from many cells. The presence of adipose cells within the interstitial connective tissue gives a deep yellow appearance of testicular specimen section in these groups.

Thickening of the intertesticular – arterial walls was found in tissue sections of group E which appeared as a homogenous pink hyaline thickening in about 50% of small testicular vessels associated with narrowing of lumen and there was obvious thickening of basement membranes of seminiferous tubules at same groups (Figure 3).

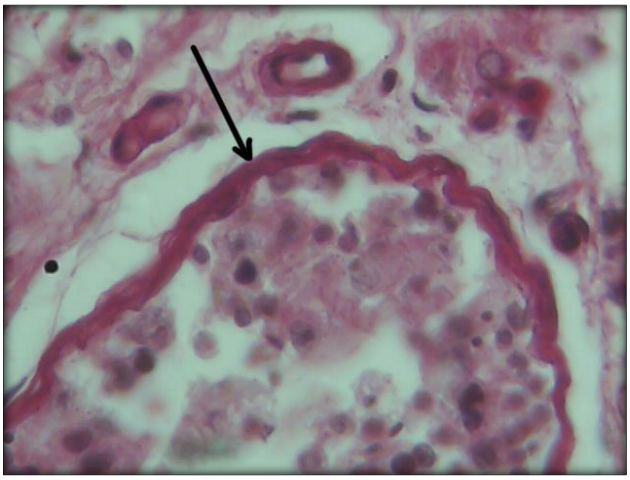

Figure 3. Thickening of the basement membrane of the seminiferous tubules in group E. (PAS, 100X).

The mean values of the seminiferous epithelial cells thickness in testis specimens were (52.27±2.74, 54.15±2.78 and 54.05±3.46 µm) in group A, B, and C, respectively, but means values reached values 44.10±4.35, 38.45±3.57 µm in groups D and E, respectively which is significant as compared with those of group A (control group) (P<0.01) as seen table 3.

Table 2. Mean and SD of the Thickness of the Sem. Epith. Cells and wall thickness of blood vessels according to age groups.

Group	No.	Thickness of the Seminephrous Epith. Cell	Wall thickness of Blood vessels
A	4	52.27 ± 2.74	11.58 ± 0.60
B	4	54.15 ± 2.78^{n}	12.85 ± 0.69*
C	4	54.05 ± 3.46^{n}	14.05 ± 0.75*
D	4	44.10 ± 4.35*	15.05 ± 0.49**
E	4	38.45 ± 3.57**	17.20 ± 0.42**

Group A is the control group
*= P<0.05, **= P<0.01 as compared to group A

The means of the interstitial spaces among the seminiferous tubules were, 27.78±1.63,

29.60±1.48, 39.22±3.47 and 44.72±1.75µm in group B, and C and D, respectively which revealed significant values as compared to group A (25.55±0.96) (P<0.01) as noticed in table 3.

Table 3. Mean and SD of the seminiferous Interstitial Space and Basement membrane` thickness of seminiferous Tubules according to age groups.

Group	No.	Seminiferous ISS between Tubules	BM thickness of Seminiferous Tubules
A	4	25.55 ± 0.96	2.97 ± 0.45
B	4	27.87 ± 1.63*	3.30 ± 0.20*
C	4	29.60 ± 1.58*	3.87 ± 0.29*
D	4	39.22 ± 3.47**	4.07 ± 0.40**
E	4	44.72 ± 1.75**	4.62 ± 0.38**

ISS = interstitial space, BM = basement membrane.
Group A is the control group
*= P<0.05, **= P<0.01 as compared to group A

The means of Basement membrane thickness in group B,C,D and E were, 3.30±0.20, 3.87±0.29, 4.07±0.40 and 4.62±0.38 µm, respectively which revealed significant values as compared to control group A (2.97±0.45), (P<0.01) as sown in table 3.

The measuring of blood vessels wall thickness revealed that there was an increment in the thickness of the blood vessels as, 12.85±0.69, 14.05±0.75, 5.05±0.49 and 17.20±0.42µm in groups B,C,D and E, respectively and statically significant as compared to control group A, (11.58±0.6) (P<0.01) as demonstrated in table 2.

The mean values of the diameter of seminiferous tubules in testes specimens of present study at magnification power 400 X, were 225.70±5 and 232.75±7.37 µm in groups B and C, respectively, but these means showed a marked decrease and reached significant values to 228.50±13.29, 187.75±11.47µm, in groups D and E, respectively, as compared to control group A, (190.50±1.2) (P<0.01) as shown in table 4.

Table 4. Diameters of seminiferous tubules according to age groups.

Group	No.	Age (year)	Diameter of seminiferous tubules (µm)
A	4	20-29	190.50±1.29
B	4	30-39	225.70±5.00**
C	4	40-49	232.75±7.37**
D	4	50-59	228.50±13.29**
E	4	60-69	178.75±11.47*

Group A is the control group
*= P<0.05, **= P<0.01 as compared to group A

Discussion

There was a tendency of human testes to contain more adipose with aging and they could be a factor that would accelerate aging process, this observation agrees with other authors [4,8], stated that there is an increase in connective tissue and adipose tissue by aging .

Regarding the weight of the testes specimens, it has been found that a good negative correlation was existed between age progress and the testes weights. other authors [3,5-7,16], mentioned that there was a slight decrease of absolute testicular weight starting at the age of 42.5 years, due to tubule involution which associated with an enlargement of the tunica propria leading to progressive sclerosis parallel to a reduction of the seminiferous epithelium with complete tubular sclerosis as an end point.

The histological finding showed that the diverticula appeared in some seminiferous tubules in aged groups as evagenations of the seminiferous epithelium towards testicular interstitium. The diverticula were connected to the seminiferous tubules by a narrow neck or by a wide base. Other study which mentioned that peritubular myoid cells may be affected by hormone alterations that take place with increasing age [18, 34], whereas Laporte and Gillet [35] stated that besides the peritubular cells, Sertoli cells might also be involved in the formation of diverticula. Since human sertoli cells undergo morphologic alterations with aging leading to a progressive decline, it is

probable that these alterations may compromise sertoli cell function [28].

In addition to the diverticula appearance in some seminiferous tubules in aged groups, is a reduction of the seminiferous epithelium (spermatogonium and sertoli cells). This finding was in agreement with other authors [29, 30] who mentioned that the thickening of tunica propria related to the tubular involution leading to the progressive sclerosis parallel to a reduction of seminiferous epithelium with complete tubular sclerosis as an end point.

The histomorphometric study of the testicular tissue specimens showed a distinct negative correlation between age and number of seminiferous tubules. This finding can be attributed to the tubular involution of the testes as age- related dependent change which was shown in present study and was generally similar to the finding of other authors [8,10,24,30], they reported that there was a reduction of the seminiferous tubules due to age-related reduction of blood supply to the testicular parenchyma, and sclerotic changes in the walls of the testicular blood vessels.

A marked decrease in thickness of the seminiferous epithelial cells was observed in testis specimens of groups D and E. According to Johnson [17] and Holstein [20] this finding was because of degenerative changes due to physiological germ cells loss observed in the germinal epithelium of elderly men. Sertoli cells also can undergo degenerative changes by accumulation cytoplasmic lipid droplet and multinucleated patterns. Harbitz [21] and paniagua *et al* [22] who mentioned that the process of aging leads to decrease in number of the seminiferous epithelial cells.

A highly significant positive correlation was found between the process of aging and the interstitial space between seminiferous tubules and due to tubular involution, and other reason that underlies this increase in tubule-interstitial spaces was the interstitial fibrosis which occurs mainly due to age-related increased content of collagen in parenchyma of testis [8, 15, 29, 31].

Regarding the thickening of basement membrane of seminiferous tubules of testes in specimens of the present study showed a distinct positive correlation with age, this can be attributed to the increase of collagen, and the increased content of various laminin isoforms with in the basement membrane of seminiferous tubules and gets thicker with age [33].

The sclerotic changes seen in the walls of testicular blood vessels in correlation to age can be attributed to the age-related sclerotic changes in the arteries wall of the testis parenchyma take place; This finding was in consistent with findings of authors which mentioned that testicular sclerosis was associated with defective vascularization of testicular parenchyma and with systemic arteriosclerosis of aged men with arteriographic studies [10,28].

It was evident in the present study a good negative correlation between age and the diameter of the seminiferous tubules. other authors [15, 34], with respect to old age revealed collapsed seminiferous tubules lined by Sertoli cells, incomplete spermatogenesis and the seminiferous tubules, and efferent ductules were devoid of spermatozoa. Whereas Wolf [33] cited that, mean diameter of seminiferous tubules in young men was (189±2.8 µm), and it reduced in the elder men to (150±3.7 µm).

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The Frequency of FLT3 Mutation in Fifty Five Iraqi Adult Patients with Acute Myeloid Leukemia

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Abstract

Background Mutations within the *FLT*3 gene, which code for the class-III-receptor kinase FLT3, ranked within the most frequent recurrent known genetic markers in acute myelocytic leukemia (AML). Internal tandem duplication (ITD) mutations in the juxtamembrane domain of FLT3 gene occur in 20-25% of AML.

Objectives This study designed to detect the frequency of FLT3-ITD mutation in adult AML patients, and to correlate the prevalence of this mutation with the clinical presentation of the patients and their response to induction therapy.

Methods The study comprised 55 AML patients and 33 healthy controls. For each patient, complete blood picture, blood film, bone marrow aspiration and biopsy was done. FLT3-ITD mutation was detected by conventional Polymerase Chain Reaction technology. Complete hematological remission achievement after induction chemotherapy was assessed by clinical examination and full laboratory investigations.

Results Out of 55 AML patients 8 (14.54%) had FLT3-ITD mutation and all of them presented as *de novo* AML. Moreover, 6 (75%) out of 8 mutated patients were newly diagnosed whereas 2 out of 8 were in relapse and were not on any therapy. The mean age of patients who had the mutation was lower than those without the mutation; also the majority of patients with mutation were male. The mean WBC count in mutated patients was not significantly higher than non-mutated patients. Higher bone marrow blast cell percent was found in mutated patients. FLT3-ITD mutation was mostly detected in M3 (37.5%) followed by M2 (25%), and lastly in M1 and M4 subtypes (12.5% for both subtypes) of FAB classification. Four out of 8 mutated patients failed to response to induction therapy although they were with good compliance to drug and 1/8 died throughout the induction therapy.

Conclusion Since FLT3-ITD mutation was associated with higher WBC count, significantly higher bone marrow blast cell percent and low rate of response to induction therapy; therefore it had been considered one of poor prognostic factor. It is a factor in defining risk stratification of AML patients.

Keywords AML, FLT3-ITD mutation, conventional PCR, FAB sub-types

Introduction

Acute myeloid leukemia (AML) is a genetically heterogeneous disease with accumulation of acquired genetic alterations in hematopoietic progenitor cells that disturb normal mechanisms of cell growth, proliferation and differentiation [(1)]. Its heterogeneity results from a complex network of cytogenetic aberrations and molecular mutations [(2)].

Limited prognostic and predictive ability of traditional morphological, immunophenotypic, and cytogenetic tests has driven research to define more subtle nucleotide-level alterations that not only shed light on pathogenesis but also serve as tumor markers and, in some

cases, impart valuable prognostic information. As a consequence, genetic characterization of all AML patients at presentation is nowadays regarded as mandatory to determine treatment choices [3].

About half of all adult patients with acute myelogenous leukemia (AML) lack any detectable cytogenetic abnormalities and thus display a normal Karyotype [4], and those patients are considered as an intermediate risk group and their clinical outcome is quite variable. So, additional markers with prognostic importance are required in order to detect clinically relevant subgroups in AML patients with normal karyotype [5].

FLT3 is a cell surface tyrosine kinase receptor with important roles in hematopoietic stem/progenitor cell survival and proliferation[6]. The human FLT3 gene is over 1,000 kilobases in length and is composed of 24 exons located on chromosome 13 (13q12) [7].

It is one of the most mutated genes in leukemia, and the internal tandem duplication (FLT3-ITD) which occur in the juxtamembrane domain-coding sequence of the FLT3 gene, is found in approximately 20-25% of cases of adult AML [8]. Additionally, point mutations which may occur in codons 835 and 836 in the tyrosine kinase domain (TKD mutations) can be detected in 7% of AML patients [9]. Clinical and experimental evidence both indicate that FLT3 is a proto-oncogene with the capacity to enhance survival and proliferation of leukemia blast cells [10].

FLT3-ITD has been identified in all FAB subtypes, with the highest frequency in the M3 subtype [11].

Studies had demonstrated very high levels of FLT3 mRNA and protein in adult and pediatric AML patients, without FLT3 mutations, and this over expression may have an unfavorable prognostic impact on overall survival [12].

FLT3 ITDs regarded as an adverse prognostic factor in AML, and because of its importance, inhibitors of FLT3 signaling have been developed and are in clinical trials in AML [13]. The mutant to wild-type allelic ratio AR (FLT3/AR) often increased at relapse and a retrospective analysis of minimal residual disease (MRD) status for AML patients indicated an increased tendency for relapse in those with FLT3-ITD positive [14].

Methods

This prospective study was conducted on 88 subjects including 55adult patients with AML and 33 healthy controls, who were selected randomly in relation to age and sex. Fifty patients were diagnosed as *de novo* AML and 5 patients were presented as secondary AML after other hematological diseases. Thirty six patients were newly diagnosed, and 19 patients were relapse. Forty nine out of them attending Baghdad Teaching hospital, and other patients were taken from Al-Kadhimiya Teaching hospital and Al-Yarmook teaching hospital. From each patient and control subject 2 ml peripheral blood sample divided in 2 EDTA tubes was collected one for analysis of hematological parameters by automated coulter and the other for DNA analysis in the Microbiology Department, Al-Nahrain Medical College. The Tubes were kept in deep freeze (-70^{o}C) until the day of analysis. Peripheral blood and bone marrow aspirate smears of the patients were examined by two hematology consultants for diagnosis of AML and their sub classification according to FAB classification.

Detection of FT3- ITD Mutation

High molecular weight DNA was extracted according to the kit protocol (Promega) following instruction manual [15]. All samples were analyzed for FLT3 mutation in exon 11using PCR method. The use of exon 11 specific primers allowed covering the whole juxtamembrane and the first part of tyrosine kinase-1 domain where most of the reported mutations are located [16].

Fifty to 100 nanogram of DNA(5 µl)was amplified in a 50 µl reaction mixture containing 1.5 mM MgCl, 50 mM KCl, 200 µM each deoxy ribonucleotide triphosphate (dNTP), 2.5 units Taq polymerase, 40 picomol of each primer have the following sequences

(Forward Primer 11F: 5'-CAATTTAGGTATGAAAGCC-3', Reverse Primer 12 R: 5'-CAAACTCTAAATTTTCTCT-3'). A positive reaction was assessed in duplicate and a negative control was included in each reaction. PCR amplification was performed using PCR Thermal cycler (Eppendorf Master Cycler, France). Amplification process consisted of 40 cycles of 30 sec at 94^{o}C for denaturation, 45 sec at 50^{o}C for annealing, 1 minute at 72^{o}C for extension and 1 cycle of 7 minutes at 72^{o}C for the final extension. [16]

Twenty µl of the PCR product was electrophoresed on 2.5% agarose gel (Promega), using 100bp DNA ladder (Promega) as molecular weight marker and stained with ethedium bromide (Promega).

Follow up of patients: Patients had received induction chemotherapy consisting of doxorubicine (Adriamycin) 25 mg/m2 I.V. for 3 days and cytosine arabinoside (Ara-C); 100mg/m2 I.V. infusion over 24 h for 7 days, (The 3 and 7 protocols).

The initial response to induction chemotherapy was assessed in each patient whether there is complete hematological remission (CR), treatment failure, or early death. Complete remission was defined as apparent recovery of hematopoiesis with< 5% blast cells on aspirate and near normal peripheral blood counts(hemoglobin >10.0g/dl; neutrophil counts>1.5 x10^{9}/l) [17].

Statistical analysis: Statistical analysis was done using SPSS version 16 & Microsoft Office Excel 2007.Numerical data were expressed as Mean±SD whereas nominal data were expressed as frequency. Analysis of numeric variables was done using one way ANOVA or t-test, whereas analysis of nominal data was done using Chi-squire. P-value < 0.05 was considered significant.

Results

This study was conducted on 55 AML patients along with 33 healthy control subjects who were cases age and sex matched (p value > 0.05). Most of the patients enrolled in this study were males (54.5%), with a male to female ratio 1.2:1 (Table 1).

Table 1. Characteristics of persons involved in the study

Characteristics		Control	AML Patients	P-value
Age/Year		37.03±9.78	39.81±19.24	1.000
Gender	Male	23	30	0.160
	Female	10	25	
Total		33	55	

In the current study by using conventional PCR, the amplified DNA product of the wild type from the patients and healthy control was about 133 bp band while the mutated type showed additional band > 133bp, (Figure 1). The presence of any PCR fragment longer than the wild-type allele was considered positive for FLT3-ITD.

FLT3-ITD mutations were found in 8 patients (14.54%). All the eight mutated cases were *de novo*-AML cases and no mutation was detected in the five secondary AML cases, (p-value 0.501). Furthermore 6 out of 36 newly diagnosed cases (16%) were mutated whereas 2(10.5%) of the relapsed cases had FLT3-ITD mutation, (p value 0.333) as shown in table 2.

Patients with *FLT3-ITD* mutation were younger than non mutated patients, (31.12±14.06; 41.29±19.73, respectively), but of no significance (p-value 0.169), (Table 2).

Out of 55 patients 54.5% were males and higher percentage of them had the mutation, (p-value 0.209).

Regarding the distribution of FLT3-ITD mutation within the FAB subtypes; the mutation was higher in patients with M3

followed by M2, and lastly by M1 and M4, (p value 0.169), (Table 2). Moreover, most of the patients presented with fever 90.9% followed by pallor 70.5% with no specific relation to FLT3-ITD mutation, (p value 0.386), (Table 2).

Table 2. FLT3-ITD Mutation relation to clinical presentation

Clinical Presentation		FLT3-ITD -ve	FLT3-ITD +ve	Total	%	P-value
Type of AML	*De novo*	42	8	50	89	0. 501
	Secondary	5	0	5	11	
Gender	Male	24	6	30	54.5	0.209
	Female	23	2	25	45.5	
Age/Year		41.29±19.73	31.12±14.06	55	100	0.169
FAB subtype	M1	21	1	22 (40%)	12.5	0.153
	M2	12	2	14 (25.45)	25	-
	M3	3	3	6 (10.9%)	37.5	-
	M3v	2	1	3 (5.5%)		
	M4	5	1	6 (10.9%)	12.5	-
	M5	3	0	3 (5. 5%)	0	-
	M6	1	0	1 (1.8%)	0	-
Lymphadenopathy		16	4	20	36.4	0.386
Splenomegaly		31	4	35	63.6	0.386
Hepatomegaly		22	4	26	47	0.867
Pallor		43	7	50	90.9	0.717
Fever		34	7	41	74.5	0.363
Weight loss		11	1	12	21.8	0.490
Total		47	8	55	100	-

Regarding the relation of FLT3-ITD mutation to hematological parameters of the patients enrolled in the study, the mean WBC count in mutated patients was 58.44±50.29 $X10^9$/L which was higher than non mutated patients 38.24±31.24, (p value 0.703), whereas platelet count and hematocrit were lower in patients with mutation, (56.75±59.27; 24.62±5.78, respectively) than in patients without mutation (62.70±44.24; 26.80±5.60, respectively), (P-value 0.316), (Table 3).

Table 3. FLT3-ITD mutation and hematological parameters of patients with AML

Hematological Indices	FLT3-ITD -	FLT3-ITD +	P-value
WBC count $X10^9$/L	38.24±31.24	58.44±50.29	0.303
Platelet count $X10^9$/L	62.70±44.24	56.75±59.27	0.703
Hematocrit %	26.80±5.60	24.62±5.78	0.316
Periphral blood blast %	63.22±27.35	74.75±17.46	0.257
Bone marrow blast %	70.11±24.25	86.12±8.21	**0.003***
Total	47	8	----

Table 3 show that the mean peripheral blood blast cell percent (74.75±17.46; 63.22±27.35, respectively) was higher in FLT3-ITD positive cases than FLT3-ITD negative cases, but it did

not reach level of significance, (P value 0.257), (Table 3). The mean bone marrow blast cells percent in mutated patients (86.12±8.21) was significantly higher than non mutated patients, (70.11±24.25) (P-value 0.003), (Table 3).

Furthermore, 4 out 8 mutated cases showed failure of response to induction therapy, however; this correlation was insignificant, (p-value 0.53), (Table 4).

Table 4. FLT3-ITD and the response to induction therapy

Response to induction therapy	FLT-ITD -ve	FLT-ITD +ve	N	%	P-value
Remission	19	3	22	40	0.53
Failure	26	4	30	54.5	-
Death	2	1	3	5.5	-
Total	47	8	55	100	-

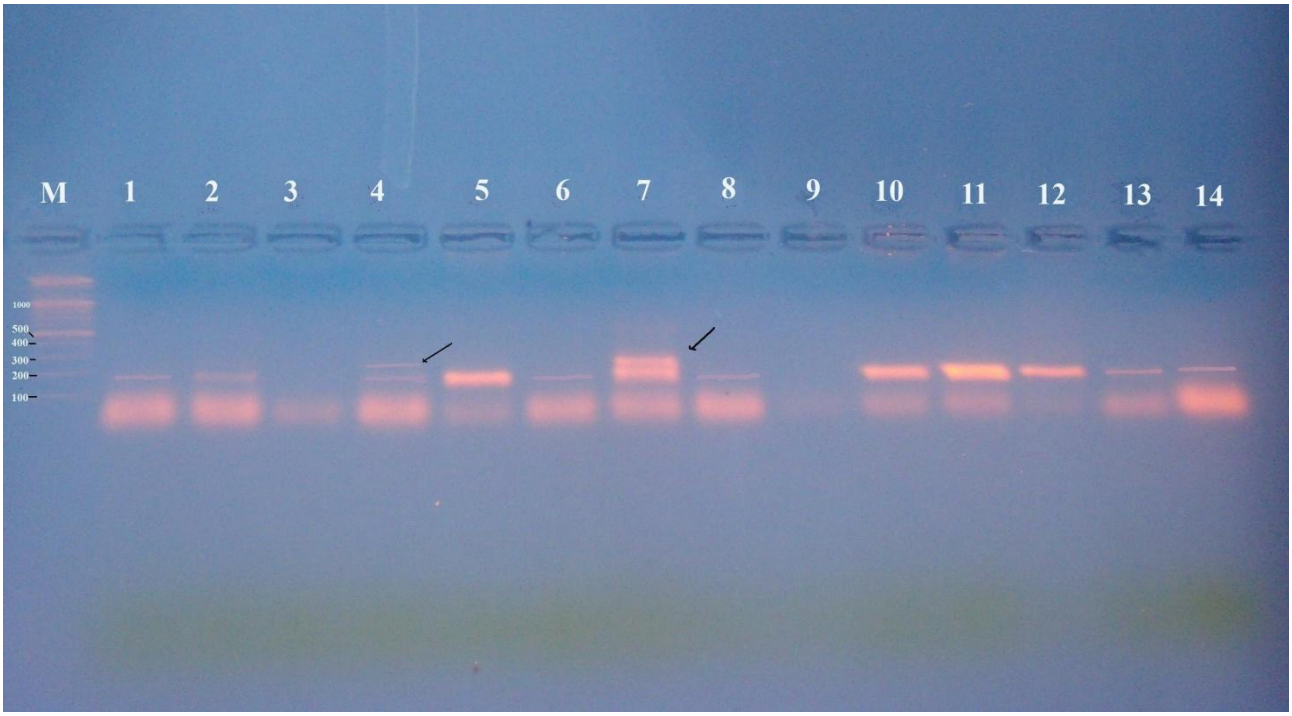

Figure 1. Detection of FLT3-ITD mutation using PCR in adult AML patients. Lane 1: Amplified product from healthy control. Lanes 2,5,6,8,10-14 amplified product from patients wild type (about 133 bp). Lanes 4 and 7 amplified products from patients show extra mutated band (> 133bp arrows) of FLT3-ITD. Lane 9 negative control (no template). M: Molecular weight marker. Electrophoresis was carried in 2.5% agarose gel at (4V/cm) for 60 min.

Discussion

Acute myeloid leukemia in general has a poor prognosis [18]. It is infrequent, yet highly malignant neoplasm responsible for a large number of cancer-related deaths. In Iraq leukemia ranks the 4th cancer among the commonest ten cancers according to Iraqi Cancer Registry 2005. It constitutes 6.4% of all cancers with an annual incidence of 3.34 per 100000 populations. [19] In this study 54.5% of the patients were males with a male to female ratio1.2:1 which was in accordance with the last statistic reported by the Iraqi ministry of health 2010 [20] and other Iraqi study [21].

In this study FLT3 gene mutation was detected in 8 (14.54%) of AML cases, which was lower

than Schnittger et al study [22] which had reported that the incidence of the mutation was 18% and this may be explained by the difference in PCR circumstances and difference in the primer set which was applied.This result was consistent with a study done by Emami et al [23] and Vahid et al [24] who reported that FLT3-ITD frequency was 16%. Also Gari et al [25] had reported that the incidence of the mutation was 11.6%.

Since the incidence of FLT3-ITD in adult patients with AML in different studies done in Arab countries and Iran was lower than that discovered in other parts of the world [26-28], thus we may propose that this difference may be due to ethnic and geographical differences.

Furthermore the present study found that FLT3-ITD mutation in females was lower than in males and this was in agreement with other studies applied [23]. On the other hand Gari et al [25] had reported higher frequency of the mutation in females; this difference may be due to larger sample size, and different method of screening, using conformation sensitive gel electrophoresis.

This study showed that patients with FLT3-ITD mutation were non significantly younger than patients without mutation, this result was in agreement to other studies.[22] Also, the mean WBC count at the time of diagnosis of those patients with FLT3-ITD was non significantly higher than that in patients without this mutation ,which may be due to that FLT3-ITD mutation can cause constitutive activation of the receptor tyrosine kinase leading to autonomous cytokine independent cellular proliferation, leading to leukocytosis [29]. This result was consistent with the result of other studies [21,30]. Moreover, the mean blast cells percent in bone marrow in patients with FLT3-ITD mutation was significantly higher than in patients without this mutation which was similarly found by Thiede et al [31]. FLT3 expression may play a role in the survival or proliferation of leukemic blasts, and FL (FLT3 Ligand) induced dose-dependent proliferation of leukemic blasts [32].

FLT3-ITD mutation was not exclusively correlated with any certain FAB sub-type, however it occur mostly in M3 sub-type, which was similarly reported by other studies [33,34]. Similar to Kiyo et al [30], the current study found that hepatosplenomegaly or lymphadeno-pathy, pallor; fever and weight loss was not affected by the presence of this mutation.

The present study found that FLT3-ITD mutation was detected only in *de novo* AML cases and not in secondary AML cases, this was in concordance with Hayakawa et al [34] study which stated that there was higher frequency of this mutation in de novo AML cases as compared to secondary and therapy related AML. Also higher frequency of mutation was found in newly diagnosed cases as compared to relapsed cases, which may be explained by the fact that those patients with relapse had received chemotherapy which may have altered over all pathophysiology. Similarly other study found that the mutation which was detected at the time of diagnosis had disappeared on relapse [35]. Also this study showed that FLT3-ITD mutation had no influence on the remission rate in mutated cases as compared to non mutated cases. This result was assisted by Thiede et al who had reported that FLT3-ITD mutation had only inferior disease free survival in AML patients with this mutation [31].

Conclusion

FLT3-ITD mutation was detected in 14.54% of AML patients and since it was associated with higher WBC count, significantly higher bone marrow blast cell percent and low rate of response to induction therapy, therefore; it had been considered one of poor prognostic factor. It is a factor in defining risk stratification of AML patients.

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Immunohistochemical Expression of CD34, Smooth Muscle Actin and Type IV Collagen in Breast Carcinoma. A clinicopathological Study

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Abstract

Background Tumoral angiogenesis is essential for the growth and spread of breast cancer cells.

Objective To evaluate angiogenesis by measuring microvessel density (MVD) with CD34 and its maturity with smooth muscle actin (SMA) immunohistochemistry and to study invasion of basement membrane by tumor cells using type IV collagen.

Methods In the present study microvascular quantification was undertaken on 52 cases of breast carcinoma and 5 cases of benign breast lesions after immunohistochemical staining of tumor vessel, using CD34 antibody and SMA antibody. Microvessel quantification was performed at x400 magnification in the three most vascular areas of the tumors (hot spots).

Results The difference in MVD between benign and malignant cases is significant (P= 0.001). MVD is significantly correlated with L.N. involvement (P=0.004) and lymphovascular permeation (P=0.001), no statistical significant correlation between MVD and age of patient (P=0.656), tumor size (P=0.052), tumor grade (P=0.324).

Conclusion Measurements of angiogenesis may have clinical utility in the evaluation of breast cancer, particularly for estimation of metastatic risk. A high MVD may be a poor prognostic marker of breast carcinoma and a target for antiangiogenic therapy.

Key words Angiogenesis, CD34, SMA, Collagen IV, breast carcinoma.

Introduction

Breast cancer is the most common cancer affecting women in the world today. It is the leading cause of cancer related death for women aged between 35 and 55 years worldwide [(1)]. In Iraq, breast cancer is the commonest type of female malignancy, accounting for approximately one-third of the registered female cancers [(2)].

Tumor angiogenesis is the development of new blood vessels from an existing vascular network; it is a prerequisite for tumor growth beyond 2 mm in diameter and plays an important role in metastasis and prognosis of the tumors including breast cancer [(3)]. Angiogenesis can be quantitated by staining histological sections with antibodies that specifically identify endothelial cells. CD34 is a sensitive marker for vascular endothelium. The maturity of blood vessels can be assessed by using SMA which is a marker that stains smooth muscles covering mature vessels. The number of microvessels can be estimated by many methods including hot spot method [(4)]. Type IV collagen is a marker used to study the invasion of basement membrane by tumor cells. In the present study we concentrated on

the immature blood vessels which are the target of antiangiogenic (anticancer) therapy.

Methods

Fifty seven cases were included in this retrospective study, 5 benign and 52 malignant (female not receive any therapy). All formalin fixed paraffin embedded tissue blocks representing mastectomy specimens of breast carcinoma were retrieved from archived files of department of pathology of Al-Kadhimiya Teaching Hospital (for the period between Jan. 2010-Nov. 2010) and Teaching Laboratories of Medical City Hospital (for the period between Jan. 2009-Oct. 2009).

Clinical information regarding age, tumor size, grade, histological subtype, lymph node involvement, and lymphovascular permeation were studied. For each case, 4 sections of 5 μm thickness were taken, one for H&E and others were processed for immunohistochemical analysis to determine the MVD by CD34, vascular maturity by SMA and basement membrane invasion by Collagen type IV (all from Dako) using LSAB (labeled strept-avidin-biotin peroxidase) technique on paraffin-embedded sections. After deparaffinization with xylene, the slides were put in antigen retrieval then in buffer (3006) then incubated with peroxidase blocking reagent, then with primary antibody for 24 hours then with biotinylated link antibody then incubated with streptavidin\perixidase then with DAB chromogen.

Vascular count: We chose special areas of tumor in 40× magnification that did not have necrosis, ulceration or inflammation, as vascular hot-spot. we counted the number of vessels(stained by CD34) in three hot spots with 400× magnification field (area of 0.79 mm2) after counting the number of mature blood vessels in the same spot stained by SMA, we substract them from total count by CD34 then obtained average value of MVC of the three spots so that only immature vessels are included in the vessel count.

Isolated endothelial cells or cords of non-perfused endothelial cells , without lumen , for which the reaction for CD34 is positive and negative for SMA represent the immature vessels (Figure 1).

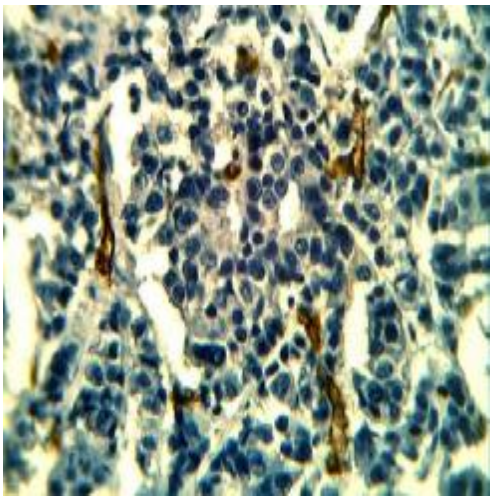

Figure 1. Invasive ductal carcinoma of breast (NOS) grade II immunostained with CD34 showing immature sprouting blood vessels (vascular hot spot)(X40).

They do not have pericytes or smooth muscle cells in their walls. The mature vessels were characterized through the co-expression of CD34 and SMA, the presence of lumen and sometimes containing RBCs (Figure 2).

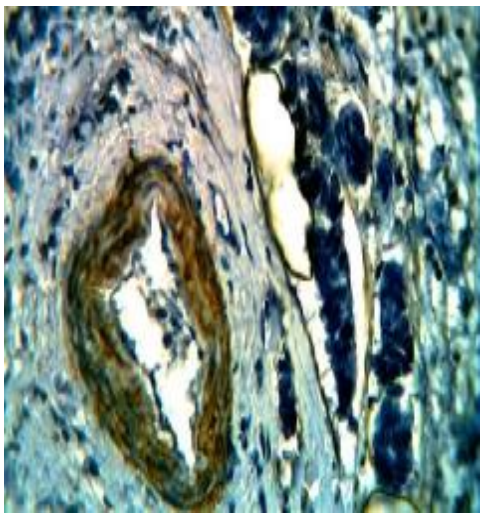

Figure 2. Invasive ductal carcinoma (NOS) grade II immunostained with SMA showing mature blood vessel with no immature blood vessels (X40).

The microvessel counts per field were converted to microvessel per square millimeters for subsequent statistical analysis.
Any endothelial cells or endothelial cell cluster positive for CD34 and separate from an adjacent cluster was considered a single countable microvessel [4].

MVD=mean MVC\ high power field area (0.79) mm^2

Collagen IV for assessment of BM
In normal section of breast tissue there is strong and well defined staining of immunoreactive type IV collagen. In carcinoma insitu, the continuity of type IV collagen staining at the interface between cancer cells and stroma was noted (Figure 3).

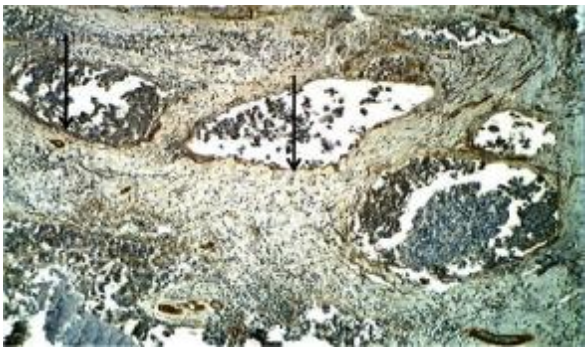

Figure 3. Invasive ductal carcinoma(NOS) with comedo carcinoma *insitu* immunostained with collagen IV showing continuity of the basement membrane at the interface between malignant cells and stroma (arrows)(X10).

Focal interruptions indicate areas of invasion. In invasive carcinoma, there is limited, very weak or absent type IV collagen staining (Figure 4).

The data were statistically analyzed using SPSS V.17 (statistical package for social sciences). The student t-test was used to assess the correlation between two parameters. Pearson correlation coefficient (r) was used to study correlation be. P-value of less than 0.05 was considered statistically significan.

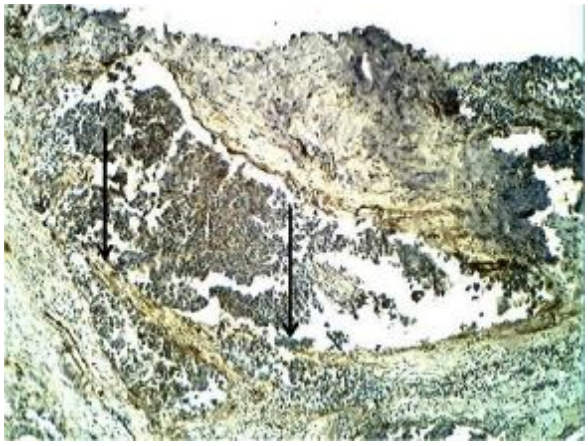

Figure 4. Invasive ductal carcinoma(NOS) immunostained with collagen IVshowing distuption of the basement membrane(arrows)(X10).

Results

The correlation between MVD and the clinicopathologic parameters are summarized in table 1. There was a significant correlation between MVD and lymph node involvement and lymphovascular permeation, MVD not significantly correlated with age, tumor size and grade. ANOVA test cannot applied to study correlation between histological subtypes and mean MVD because to 2 of the types includes 1 case only, so P-value is not calculated for this parameter.

Discussion

The ability of tumors to induce a vascular stroma is a critical requirement for tumor progression at all stages of breast cancer development [5]. Although angiogenic activity is associated with tumor proliferation and metastasis, vessel maturation also plays an essential role in the later stages of tumor development [6]. Recruited pericytes and smooth muscle cells form a protective barrier that stabilizes blood vessels and reduces apoptotic events [7,8]. Thus, there is heterogeneity in blood vessels present in tumors [9].
In the present study, more neovascularization in malignant than in benign lesions was noticed. The results were in agreement with Hammoudi, 2005 [10]. MVD values in *insitu*

carcinoma are lower than in invasive carcinoma which means more blood vessels are required by the tumor for invasion. A dense microvascular rim adjacent to the basement membranes of DCIS associated with invasive ductal carcinoma was seen in the present work. This finding is consistent with previous publications which suggested that periductal vascularization may be caused by the direct release of angiogenic factors by neoplastic cells and could be important in determining the transformation from in situ to invasive disease [(11,12)].

Higher MVD in higher grade tumors indicates more aggressive tumor although the relation was statistically not significant. Thus, tumor de-differentiation would seem to be associated with an increased pro-angiogenic activity. The results were in agreement with El-Moneim et al, 2008 [(13)].

MVD in T2 is higher than in T1 and T3, this is in agreement with El-Moneim et al [(13)], 2008 and disagree with Hammoudi, 2005 [(10)]. This could be due to the interstitial pressure in tumor would lead probably to compression closure of capillaries, and then to ischemia and transport problems that ultimately result in necrosis. Moreover, active angiogenesis occurs mainly in the tumor periphery, while maintenance of the inner vascularization is a result of continuous remodeling. Therefore, it is not surprising that the importance of angiogenesis is diminished as the tumor grows [(14)]. Also related to that the determination of MVD in the areas of highest vascular density (hotspots), in which systemic dissemination of cancer cells is more likely in the areas of highest vessel density. Since identification of hotspots is of great importance for the method, the chance of identifying the "hottest-spots" would most likely be influenced by the potential variation of vascular density between different parts of a tumor, so tumor heterogenicity play a role in variation of vascular counting [(15)].

There was a statistically significant correlation between the MVD and lymph node status, this is in agreement with El-Moneim et al, 2008 [(13)] and disagree with Rishil 2009 [(16)]. The increase in microvessel density (MVD) in and around tumors is thought to increase the chance that invasive tumor cells enter the lymphatic vasculature. In turn, it is suggested that this promotes the formation of lymph node metastases, as increased numbers of disseminating tumor cells are transported to regional lymph nodes [(17)].

The correlation between MVD and lympho-vascular permeation was statistically significant, this disagree with Johan 2002 [(15)]. The explanation of this is related to the architecture of microvessels that makes them more amenable to the entry of invasive tumor cells, they have loose overlapping cell–cell junctions, and end capillaries have no or only an incomplete basement membrane. Invasive tumors can permeate into the lymphatic vasculature locally as strings of cells, but generally traffic as emboli [(18)].

Measurements of angiogenesis may have clinical utility in the evaluation of breast cancer, particularly for estimation of metastatic risk.MVD might also have predictive value with regard to benefit from adjuvant chemotherapy, or by specific antiangiogenic drugs.

In conclusion ,MVD is significantly correlated with L.N. involvement and lymphovascular permeation. No statistical significant correlation between MVD and age of patient, tumor size, and tumor grade. Assessment of tumor vascularity by CD34, SMA immunohistochemistry is valuable in quantifying angiogenesis and its maturity in breast carcinoma. Measurements of angiogenesis may have clinical utility in the evaluation of breast cancer, particularly for estimation of metastatic risk. A high MVD may be a poor prognostic marker of breast carcinoma and a target for antiangiogenic therapy.

Table 1. Summary of clinicopathologic parameters and correlation of them with MVD of studied cases

Parameters		No. of cases	Mean MVD	P* value
Behavior	Benign Malignant	5 52	45.60±7.54 91.55±3.93	0.001
Age (27-80) year Mean = 46.52+1.46 year Median=40 year		52 (malignant)	91.55±3.93	0.656
Histological type	DCIS DCIS+LCIS IDC(NOS) IDC+comedo IDC+DCIS Mucinous	1 1 35 8 6 1	59 66 93.74 96.25 92.8 91	
Grading	Grade II Grade III	36 16	88.94±4.35 97.43±8.27	0.324
Size	<2 cm (T1) 2-5 cm (T2) >5 cm (T3)	10 22 20	78.40±3.43 102.09±6.57 86.55±6.37	0.052
LN involvement	Positive Negative	35 17	97.60±5.46 79.11±2.46	0.004
Lymphovascular permeation	Positive Negative	43 9	95.20±4.52 74.11±3.55	0.001

DCIS: ductal carcinoma *in situ*; LCIS: lobular carcinoma *in situ*; IDC (NOS): invasive ductal carcinoma not otherwise specified. P* value <0.05 is significant.

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Sub Thalamic Nucleus Deep Brain Stimulation: Iraqi Case Series

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Abstract

Background Sub thalamic nucleus (STN) Deep brain stimulation (DBS) electrodes are implanted into STN and programmed by external pulse generator. DBS alleviates the cardinal Parkinson disease symptoms and reduce the need for levodopa and related drugs and eventually reduces levodopa-related motor complications in advanced Parkinson's disease.

Objective To evaluate the STN DBS implantation in Parkinson disease patients.

Methods A retrospective evaluation of data base of the patients operated on for STN DBS between Jan. 2010 and Jan. 2011. The study involved 11 patients (10 males and 1 female) with an age range between 39 and 65. Surgical implantation was done in the Neurosciences Hospital in Baghdad. Unified Parkinson's disease Rating Scale was reported before surgery and 3 monthly after implantation. Paired t test was used to test the significance of difference between 2 means.

Results Highly significant differences ($P < 0.0001$) in the activities of daily living, Tremor, Rigidity, Bradykinesia and Gait parameter. There was no difference in Postural stability before and after. There was 65% of the patients reduced their levodopa medication dosage after STN DBS. One patient out of 11 (9%) developed intracerebral hemorrhage.

Conclusions STN DBS is very successful in managing motor clinical manifestations in advanced Parkinson disease and reducing levodopa medication.

Key worlds Parkinson, Deep brain stimulation, subthalamic

Introduction

Deep brain stimulation (DBS) is a Stereotactic surgical treatment in which a device called a neurostimulator delivers tiny electrical signals to the areas of the brain that control movement [(1,2)]. It was first reported for treatment of Parkinson disease treatment in 1993 from Benabid's clinic and widespread use of DBS began after FDA approval for essential tremor on 1997 [(3,4)]. It has provided remarkable therapeutic benefits for advanced Parkinson disease. It makes pulses of titratable electrical stimulation at the target site in the brain leading to interference with neural activity, creating a reversible lesion in the implanted nucleus .DBS results in improvement of motor features of Parkinson disease (bradykinesia, rigidity and tremor) [(4)].

The keys for successful DBS procedure were proper patient selection, proper preparation of the patient, accurate electrode positioning and after implantation care. This multiple steps process involves team of neurologist, neurosurgeon, neurophysiologist, and bio medics engineer [5].

DBS was admitted first in Iraq on 2007; and all implantations were done in the neurosciencesshospital in Baghdad [6].

The aim of this study is to compare the results of the Unified Parkinson's Disease Rating Scale (UPDRS11 and 111) before and after the sub thalamic DBS (STN DBS) implantation during on period; also to record the side effects and to assess the best Stimulation polarity Settings in Parkinson disease patients inneurosciences hospital in Iraq.

Method

A retrospective evaluation of data base of the patients operated on for STN DBS between October 2007 to June 2009.

We have evaluated the patientsand reviewed their past data from the neurosciences hospital file system. The study was conducted between Jan 2010 and Jan 2011 in neurosciences hospital in Baghdad; the study enrolled 11 patients [10males and 1 female], their age was ranged between 39 and 65.

The patients were considered as Parkinson disease when fulfilling the United Kingdom Parkinson's Disease Society Brain Bank Diagnostic Criteria [7]. The inclusion criteria were failure of all drugs regimen, Good response to Levo Dopa therapy, fairly normal level of function during on period, Normal brain MRI, more than five years disease duration.

The criteria for exclusionwere dementia, major psychiatric illness, blood dyscrisias and any history of stroke. Patients' written consent was taken to be enrolled in this study. The study was approved by ethical committee of the directorate of neurosciencesshospital.

Operative procedures

The sub thalamic nucleus (STN) was anatomically localized streotactically using leksel stereotactic frame system of Elekta through a series of brain MRI (0.25 TESLA) scanning axial images in 2 mm volumetric thickness which were integrated using a frame (4 software Medtronic). Physiological localization was done by using microelectrode recording. 5 electrode contact sites were inserted (anterior, posterior, medial, lateral and central); Recording and stimulation of those 5 sites with monitoring for the best response by neurologist was used to determine the permanent electrode for stimulation. Thereafter macro electrode stimulation was done to assess the best response and the least side effects. Surgery was done in 3 stages for each patients; under local anesthesia electrode implantation (Medtronic model 3389, Medtronic, Minneapolis) on one side and within a week implantation of the other side electrode were done; then under general anesthesia implantation of the pulse generator (kinnetra7428 Medtronic) on the left subclavicular 1 day from implantation of the electrode.

Each patient was evaluated by using The Unified Parkinson's Disease Rating Scale (UPDRS) [8] before surgery and 3 monthly after implantation. Electrode contact (four sites), polarity (monopolar or bipolar), frequency, voltage, and pulse width were assessed for the best response 1 week after implantation.

Statistical analysis

Subclasses of (UPDRS) Results were transformed into (means±standard deviation) and comparison was done using graph pad was used for data input and analysis. Paired t test was used to test the significance of difference between 2 means. A p-value less than 0.05 were considered the cutoff point to determine significant findings.

Results

Table 1 showed the basic demographic features of the patients in the present study; also showed that the duration of the disease was 8.3±1.7 years. The best electrodes were the anterior one in 50 % and medial in 50 %; no lateral or posterior electrodes were present in this study.

The starting mode of stimulation was monopolar in 100% of the patients; after 12 months the monopolar stimulation was the mode in only 27% versus 73% bipolar mode (Table 1).

Table 2 showing a highly significant difference (P value < 0.0001) in the activities of daily living, tremor, rigidity, bradykinesia and p value < 0.0004 in gait parameter.

Also table 2 was showing no difference in Postural stability before and after STN DBS (p value = 0.8). Table 3 showing 65% of the patients reduced their levodopa medication dosage after STN DBS (p < 0.004. We have one patient out of 11 (9%) developed intracerebral hemorrhage. We did not report infection or other side effects.

Table 1. Demographic features and stimulation setting of the patients

Variables					
Sex	Male Female	10 1			
Age	Range (39- 65)	57.273±7.4			
Duration of the illness prior to DBS	8.3±1.7				
Electrode chosen		Medial	Anterior	lateral /posterior	Total
Right side Left side Total		8/11 (73%) 3/11 (27%) 11/22 (50%)	3/11 (27%) 8/11 (73%) 11/22 (50%)	0/11 0/11 0/22	11/11 11/11 22/22
Mode of stimulation		Monopolar	Bipolar		
Starting mode 3 months 6 months 9 months 12 months		11/11 (100%) 9/11 (81%) 8/11 (72%) 5/11 (45%) 3/11 (27%)	0/11 2/11 (19%) 3/11 (28%) 6/11 (55%) 8/11 (73%)		

Table 2. UPDRS motor parameters differences before and after DBS implantation

UPDRS motor parameters	T test	95% CI	SE of Deviation	P value
Activities of daily living (range, 0-52)	6.5	9.63-19.64	2.25	0.0001
Tremor (range, 0-28)	8.4	7.48-12.88	1.20	0.0001
Rigidity (range, 0-20)	8.0	5.77-10.23	1.00	0.0001
Bradykinesia (range, 0-32)	8.9	6.88-11.49	1.03	0.0001
Gait (range, 0-4)	5.2	0.58-1.42	0.19	0.0004
Postural stability (range, 0-4)	1.9	-0.04-0.59	0.14	0.0816

Table 3. Levodopa therapy dosage pre and post DBS

Levodopa medication dose	Post implantation
No change	5 (55%)
Reduce the dose	6 (65%)

P-Value = 0.004

Discussion

The present study was retrospective evaluation of the data base of the operated patients one year ago; the patients were selectively randomized to undertake the operation during the last 3 years.

DBS implanted patients in the present study shows statistically significant improvements of activities of daily living, tremor, rigidity, hypokinesia and gait; the above results were consistent with results of deep brain stimulation groups [9-16].

We have used the UPDRS scale to assess the disease severity and clinical tool to assess the disease clinical manifestation; these tests were more detailed than the Hohen and yaher scale [17].

Regarding the side effects we record only one patient with intracranial hemorrhage approximating other studies [9,12]. The above result of intracerebral hemorrhage in the present study [9%] was higher than that was reported by Park et al which was 5% [18]; this higher result was related to the difference of surgical method used in both studies as well as the MRI used in our study was 0.25 tesla. Also, the use of multiple microelectrode insertion (MMI) was associated with higher risk of hemorrhage than the circumferential paired microelectrode insertion (CPMI). We did not report other side effects like infection or cognitive deficits or Persisting adverse effects included eyelid opening apraxia, weight gain, psychiatric disorders, depression, dysarthria, dyskinesia, and apathy [19-21].

We did not report suicide attempt in our patients although its rate were very high in other studies that showed the suicide rate following deep brain stimulation was 13 times higher in the first postoperative year [22,23].

The present study showed significantly lowered dosage of levodopa medication after bilateral STN DBS, this result is in accordance with previously published international findings [19-21].

Unipolar stimulation usually had a significantly higher efficacy than bipolar stimulation; however, also with a higher rate of side-effects (19% vs. 0%) [24]. In our study the starting mode of stimulation was monopolar in all patients, this is to avoid patients' exposure to a disabling dyskinesia [24]; after one year of the DBS implantation 73% of the patients were changed to bipolar mode , this is consistent with Obeso et al study [9].

Our study showed no significant benefit of DBS implantation on postural stability, this in accordance to Indian experience of DBS [14] this may be related to fear of fall after surgery [24], also gait and balance anatomical motor connections in STN involved is more diffusely distributed [25].

The challenges and problems facing our work were many, of them was using 0.2 tesla open MRI; all other studies were used 1.5 tesla MRI. Other was the limited number of patients included in the study because of the limited popular information of this treatment modality

Conclusion

Bilateral STN DBS in Iraqi patients was very successful in managing motor clinical manifestations in advanced Parkinson disease and reducing levodopa medication; we have higher intracerebral hemorrhage than other

international studies, this will be subdued in the future through introducing more advanced generation of MRI like 3 tesla, using more advanced more accurate technique for STN localization and also using newer frameless DBS technique. The mode of stimulation was similar to other studies.

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PCNA Expression in cagA Strain H. pylori Gastritis: Immunohistochemical and *Insitu* Hybridization Study

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Abstract

Background Carriage of *Helicobacter Pylori (H. Pylori)* in the human stomach is associated with increased risk of peptic ulcer disease, distal gastric adenocarcinoma and gastric B-cell mucosa associated lymphoid tissue lymphoma. Several studies have shown increased evidence of increased cell proliferation in the gastric mucosa both in human carrying *H. Pylori, and* animal model of *H. Pylori* infection.

Objective To study the immunohistochemical expression of Proliferating cell nuclear antigen (*PCNA*), as a proliferative marker in the gastric mucosa of patients infected with *CagA Helicobacter Pylori* demonstrated by *insitu* hybridization method.

Methods Gastric antrum and corpus biopsies from 99 patients with dyspeptic symptoms (50 men, 49 women, and median age 40) were analyzed for *H. pylori*, presence of chronic inflammation, intestinal metaplasia, and atrophy according to updated Sydney system. *Insitu* hybridization technique was done to detect cagA *H. pylori*. Immunostaining for PCNA (Avidin- Biotin method) was performed on paraffin embedded tissue specimens.

Results Forty four patients (44.44%) had *H. Pylori* cagA positive strain. Atrophy of gastric mucosa was present in 14 (14.14 %) patients. Intestinal metaplasia was present in 8 (8.08%) patients. The frequency of atrophy was significantly higher in cagA *H. Pylori* gastritis than non-cagA *H. Pylori* gastritis (p=0.041). The frequency of intestinal metaplasia was significantly higher in cagA *H. Pylori* gastritis than non-cagA *H. Pylori* gastritis (p=0.023). PCNA labeling index (LI) of the gastric glands was significantly higher in presence of atrophic alterations (p <0.001), intestinal metaplasia (p <0.001) and in cagA strain *H. Pylori* positive gastritis (p<0.001).

Conclusion The rates of gastric glandular atrophy, intestinal metaplasia, and epithelial proliferation increase in the presence of *H. Pylori* infection, and are further increased when *H. Pylori* is of cag A strain.

Key words cag A *H. pylori* gastritis, PCNA immunohistochemical expression.

Introduction

Carriage of *Helicobacter Pylori* in the human stomach is associated with increased risk of peptic ulcer disease, distal gastric adenocarcinoma and gastric B-cell mucosa associated lymphoid tissue lymphoma [(1)].

In developed countries, strains of *Helicobacter Pylori* that carry the cag Pathogenesity Island, a 35-40 Kb DNA fragment encoding a series of virulence-related gene associated with an extracellular secretory apparatus, are associated with a greater risk of peptic ulcer and adenocarcinoma than strains that are negative

for cag island [2,3]. Several studies have shown increased evidence of increased cell proliferation in the gastric mucosa both in human carrying *Helicobacter Pylori* [4-10] and animal model of *Helicobacter Pylori* infection [11,12]. After eradication therapy, increased proliferation returns to normal levels, which suggests that *Helicobacter Pylori* or the associated inflammatory response is responsible for the increased proliferation observed [4,6-9].

Proliferating cell nuclear antigen (PCNA) is a 36 KDa intranuclear polypeptide protein whose expression is associated with DNA synthesis and cell proliferation [13,14]. It is a useful immunohistochemical marker of cell proliferation because its expression and distribution correlate with cellular proliferation rate and DNA synthesis [15].

The aim of this study is to study the immunohistochemical expression of PCNA, as a proliferative maker in the gastric mucosa of patients infected with *Helicobacter Pylori* demonstrated by *insitu* hybridization method.

Methods

A total of 99 adult patients presented with dyspeptic symptoms referred to the OGD (oesophagogastroduodenoscopy) unit at Al-Kadhimiya teaching Hospital in Baghdad with an age range of 19-70 years (median 40 years) for upper endoscopy between June 2009 and March 2010 were included. In this study patients who had received anti-ulcer agents or antibiotics for up to two months before the examination and those who had histories of gastric cancer, gastric or duodenal ulcer, or gastric surgery, were excluded. The study was approved by the committee of ethical approval in the College of Medicine, Al-Nahrain University.

Three tissue biopsies were obtained from each patient, two from the antrum and corpus and one from the corpus. Rapid urease test was performed on one of the antral biopsies. The medium used for the test was urea broth. It consists of urea, phenol red indicator and distilled water. One biopsy piece from each sample was inoculated immediately after collection into 1.5ml to 2ml of urea broth. It was incubated at 37°C in the incubator for one and a half hour. The change in color of the broth from pale yellow to deep pink was taken as a positive reaction. The other biopsy specimens were paraffin embedded and processed. One section from each block was stained by H&E to study the histopathological features and grading of gastritis was done according to the updated Sydney system. One section was used for In situ hybridization (ISH) method to identify Cag-A starin *H. pylori*, and one section was stained immunohistochemically for PCNA (Dakocytomation-Mouse monoclonal primary antibody).

Two methods were used to identify *H. Pylori* infection status; rapid urease test and histological sections stained with H&E stain. Patients were considered to be infected with *H. pylori* if one or two of the tests were positive: rapid urease test, or histology. Patients were considered infection free when both of the two tests were negative.

Cell proliferation in gastric epithelium was examined by PCNA labeling indices. The number of positive cells/100 gastric mucosal epithelial cells was counted and was considered to be the proliferation index [16].

In-situ hybridization technique uses biotinylated cDNA probe (for *H. Pylori* cagA gene detection) together with Maxim's ISH detection kit. This complete hybridization and immune detection system, incorporates the biotin-streptavidin amplified technology to provide consistent results and maximum sensitivity to ensure economical and efficient use of the nucleic acid probes. A dark blue signal appears at specific site of the hybridized probe.

Statistical analysis was performed using SPSS 16 and Microsoft Excel 2007. Numeric variables were expressed as mean±SD. Chi-square test

was used to study association between two discrete variables. T-test was used to compare the mean of numeric variables. A P-value of less than 0.05 was considered significant.

Results

Histopathological assessment of gastritis: the assessment was done according to the revised Sydney system [17] (Figure 1).

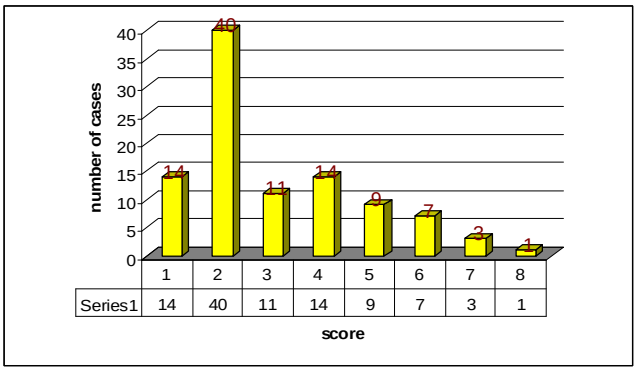

Figure 1. Distribution of cases according to total Sydney score

The results were as follow:

1. Chronic inflammation was mild in 46 (46.46%) patients, moderate in 37 (37.37%) patients and severe in only 16 (16.16%) patients.
2. Active inflammation was present in only 34 out of 99 patients (34.34%), and it was mild in most of the cases (29 patients {29.29%}).
3. Atrophy of gastric mucosa was present in only 14 out of 99 patients (14.14%) and was of mild degree.
4. Intestinal metaplasia was present in 8 out of 99 patients (8.08%), and it was of mild degree.
5. *H. Pylori* infection was present in 69 out of 99 patients (69.69%) (44 were cagA positive), and it was of mild degree in most of the case.

Relation between various histopathological parameters and cagA *H. Pylori* status (cagA versus non cagA):

1. Chronic inflammation and CagA: The degree of chronic inflammation in the presence of cagA strain was significantly higher than that in the absence of cagA strain (mean score 2.11±0.65 versus 1.00±0.00; p<0.001) as shown in table 1.
2. Activity of inflammation and cagA: The activity of inflammation in the presence of cagA strain was significantly higher than that in the absence of cagA strain (mean score 0.90±0.64 versus 0.00±0.00; p<0.001) as shown in table 1.
3. Atrophy and cagA status: The degree of atrophy was significantly higher in cagA *H. Pylori* gastritis than non-cagA *H. Pylori* gastritis (0.22±0.42 versus 0.16±0.37; p=0.041). Also, the distribution of atrophy was more frequent among cagA *H. Pylori* gastritis than non-cagA *H. Pylori* gastritis (13/44 versus 1/25; p=0.011) as shown in tables 1 & 2.
4. Intestinal metaplasia and cagA: The degree of intestinal metaplasia was significantly higher in cagA *H. Pylori* gastritis than non-cagA *H. Pylori* gastritis (0.18±0.39 versus 0.00±0.00; p=0.023). Also, the distribution of intestinal metaplasia was more frequent among cagA *H. Pylori* gastritis than non-cagA *H. Pylori* gastritis (8/44 versus 0/25; p=0.044) as shown in tables 1 & 3.

Table 1. Correlation between various histopathological parameters and cag A H. pylori gastritis (cag A versus non cag A)

Histological parameter Mean score±SD	Cag A (present) No. = 44	Non cag A (absent) No. = 25
Ch. inflammation	2.11±0.65	1.00±0.00*
Activity	0.90±0.64	0.00±0.00**
Atrophy	0.22±0.42	0.16±0.37***
Int. metaplasia	0.18±0.39	0.00±0.00****

*= P< 0.001, ** = P< 0.001, ***& = P< 0.04,**** = P< 0.023

PCNA immunohistochemical expression

PCNA LI of the corpus glands was significantly higher in presence of atrophic alterations (9.71±3.60) as compared to the non-atrophic

mucosa (0.75±1.47) (p <0.001). Also, PCNA LI of the antrum glands was significantly higher in presence of atrophic alterations (13.71±3.60) as compared to the non-atrophic mucosa (2.54±2.99) (p <0.001), as shown in table 4. PCNA LI of the corpus glands was significantly higher in presence of intestinal metaplasia (9.00±5.09) as compared to the non-atrophic mucosa (1.40±2.81) (p <0.001). Also, PCNA LI of the antrum glands was significantly higher in presence of intestinal metaplasia (13.00±5.09) as compared to the non-atrophic mucosa (3.34±4.15) (p <0.001) as shown in table 5.

Table 2. distribution of atrophy according to cagA H. pylori infection

P= 0.011		cagA H. Pylori		
		Positive	Negative	Total
Atrophy	Absent	24	31	55
	Present	1	13	14
	Total	25	44	69

Table 3. The distribution of Intestinal metaplasia according to Cag A H. pylori infection

P= 0.044		cag A H. Pylori		
		Positive	Negative	Total
Intestinal Metaplasia	Absent	25	36	61
	Present	0	8	8
	Total	25	44	69

The PCNA LI in the gastric antrum mucosa was significantly higher in cagA strain *H. Pylori* positive gastritis than cagA strain *H. Pylori* negative gastritis (7.79±4.47 and 2.6±3.51 respectively; p<0.001). The PCNA LI in the gastric corpus mucosa was significantly higher in cagA strain *H. Pylori* positive gastritis than cagA strain *H. Pylori* negative gastritis (4.00±4.52and 0.96+2.18 respectively; p<0.001) as shown in table 6.

Table 4. Comparison of PCNA LI between atrophic and non-atrophic mucosa

	Atrophy	No.	Mean±SD	P value
PCNA Corpus	Present	14	9.71±3.60	<0.001
	Absent	85	0.75±1.47	
PCNA antrum	Present	14	13.71±3.60	<0.001
	Absent	85	2.54±2.99	

Table 5. Comparison of PCNA LI in the presence and absence of intestinal metaplasia

	Meta-plasia	No.	Mean±SD	P value
PCNA Corpus	Present	8	9.00±5.09	<0.001
	Absent	91	1.40±2.81	
PCNA antrum	Present	8	13.00±5.09	<0.001
	Absent	91	3.34±4.15	

Table 6. Comparison between corpus and antrum mucosa in regard to PCNA LI

	Cag A	No.	Mean±SD	P value
PCNA LI Antrum	Positive	44	7.79±4.73	<0.001
	Negative	25	2.60±3.51	
PCNA LI corpus	Positive	44	4.00±4.52	<0.001
	Negative	25	0.96±2.18	

Discussion

The sequence of events that have been suggested in the development of gastric carcinoma is chronic inflammation, mucosal atrophy, intestinal metaplasia, dysplasia and carcinoma [16]. The incidence of the precancerous lesions (atrophy and intestinal metaplsia) is variable in different studies. The percentage of atrophy, in those studies, ranged from 9 to 15 % [18-20]; while the percentage of intestinal metaplasia ranged from 35 to 42 % [18-20]. In the

current study the percentages of intestinal metaplasia and atrophy were 8.8% and 14.14% respectively. Several studies have shown a significant positive association between *H. pylori* infection and development of gastric atrophy [21-24], and this finding is in accordance with the result of the current study. Other studies claimed that infection with *H. pylori* is responsible for higher rates of intestinal metaplasia [21,24-26].

Table 7. Correlation between Sydney score and PCNA expression

Histological parameter	PCNA antrum		PCNA corpus	
Chronic inflammation	r *P*	0.401 <0.001	r *p*	0.392 <0.001
Activity	r *P*	0.556 <0.001	r *P*	0.444 <0.001
Atrophy	r *P*	0.787 <0.001	r *P*	0.856 <0.001
Intestinal metaplasia	r *P*	0.532 <0.001	r *P*	0.567 <0.001
H. pylori score	r *P*	0.550 <0.001	r *P*	0.365 <0.001

Again this finding is in accordance with the result of the current study. Some studies concluded that cag A strain is the main pathogen behind the higher rates of gastric atrophy and atrophic gastritis [21]; which again supports the result of the current study. Also those studies had attributed the higher rates of intestinal metaplasia to cag A strain [21]. This is also in accordance with the finding of the current study. All of the eight cases of intestinal metaplasia were seen in chronic atrophic gastritis, and no intestinal metaplasia was present in chronic non-atrophic gastritis. According to these data, it can be concluded that *H. pylori* existence is an important factor for the development of atrophy and that atrophy can cause intestinal metaplasia. This finding is in accordance with Derya *et al* [16].

PCNA:

The current study has revealed that severity of gastritis and presence of *H. pylori* has different impacts on gastric epithelial cell proliferation. A significantly higher proliferation activity was established in the gastric mucosa in atrophy. These findings are in accordance with some other studies [23,27], which suggest higher proliferative activity of epithelial cells of gastric mucosa in the presence of atrophy.

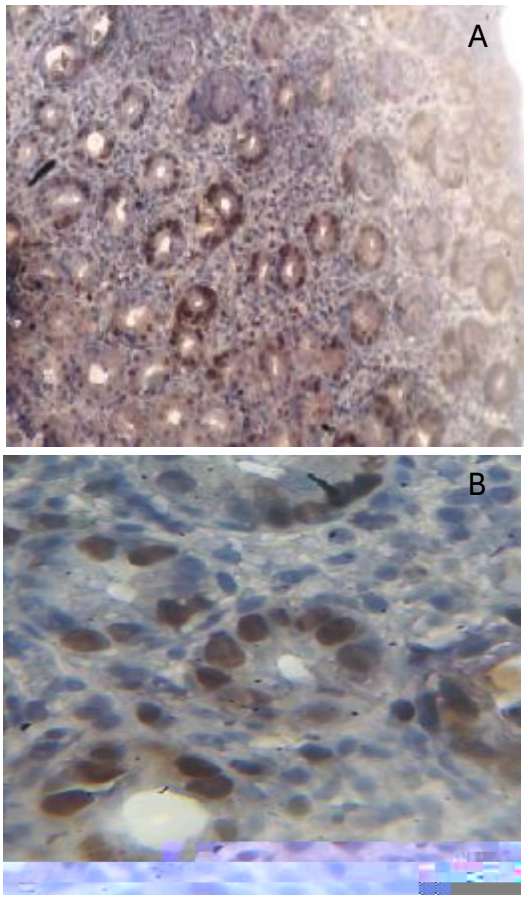

Figure 2. *A*. Positive immunohistochemical expression of PCNA (10X). *B*. Positive immunohistochemical expression of PCNA (40X).

There are data from other authors, which show enhancement of proliferation index with increasing degrees of gastritis both in the antrum and in corpus mucosa [28, 29]. Our data are in concordance with the study of Lynch et al. who have shown also a strong correlation between

epithelial proliferation and chronic inflammatory cell infiltrate [10].

Increased proliferative activity in the atrophic gastric mucosa suggests that the gland cell populations maintain an active cell turnover despite reduction in cellular mass as established through grading criteria.

Some studies showed a positive correlation between the severity of chronic inflammation and the expression of proliferative marker (PCNA) [30]. This is in accordance with findings of the current study.

Steven *et al* showed an increased rate of gastric epithelial cell proliferation in the presence of *H. pylori* infection than in the absence of infection; and that the rate is further increased in the presence of cag A stain *H. pylori* [31]. These data again are in accordance with the results of the current study. Indeed an increase in mucosal cell proliferation increases the likelihood of a neoplastic clone of epithelial cells emerging where there is chronic epithelial cell injury associated with *H. pylori* gastritis and in this way may play a part in gastric carcinogenesis.

Conclusions

The rates of gastric glandular atrophy, intestinal metaplasia, and epithelial proliferation increase in the presence of *H. pylori* infection, and are further increased when *H. pylori* is of cag A strain.

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Morphological and Hormonal Studies Related to Ageing Changes of Hypothalamo-pituitary Gland in Rabbits

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Abstract

Background The hypothalamic - pituitary axis is an auto-regulating system that realizes a tight integration among the endocrine, nervous and immune systems.

Objective To identify anatomical and microscopical age changes of hypothalamus-pituitary gland.

Methods Eighteen female rabbits are divided into 3 groups, group A (4-6 months), group B (12-24 months) and group C (36-60 months). The rabbits are sacrificed and dissection of hypothalamus, pituitary gland is done, anatomical position, their weight, measurements and histological study of their sections regarding the number of different cell types.

Results The weight and number of cells in different parts of hypothalamus and pituitary gland are negatively correlated with age.The serum thyroid stimulating hormone (TSH), growth hormone (GH), prolactin hormone (PRL), and adrenocorticotrophic hormone (ACTH) hormones almost remain unchanged while the level of leutinizing hormone (LH) and follicular stimulating hormone (FSH) were increasing with age, and the prolactin hormone level primarily increases with age then decreases with advancing age.

Conclusion The weight of the Hypothalamus and pituitary gland is primarily increased before age of 24 months, and then it started to decrease. The effects of aging inversely on the function and structure of hypothalamus and pituitary gland.

Key words Aging, hypothalamus, pituitary, HPA axis

Introduction

Epithelial cells of pituitary gland are of 2 types, the chromophobes and the granule containing chromophils which can be further subdivided into acidophil cells and basophil cells [(1-3)]. Posterior lobe is an extension of the brain, composed primarily of nerve fibers (axons) which originate from nerve cell bodies in the hypothalamus to the pars.

The aging is biophysical and biochemical changes of cell matter, physico-chemical changes of cell structure, and the gradual loss of the cell capacity for reproduction and regeneration [(4)]. Endocrine system was thought to play a critical role in aging[(5,6)] . The intension of this study is to detect changes in hormone levels during age progress of hypothalamus and pituitary gland.

Methods

Eighteen female rabbits (Oryctolaguscuniculus), local breed growing their ages between 4 months and 5 years are used in this study. They were divided according to age into 3 groups, six rabbits in each group. The three groups are:

1. Group A: their age ranged between 4-6 months (control group).
2. Group B: their age ranged between 1-2 years.
3. Group C: their age ranged between 3-5 years.

The animals are sacrificed by using intensive dose of chloroform in sealed glass box.Skull cap was removed then the brain was removed in one piece. Dissection of hypothalamus by making a horizontal cut through interventricularforamen (Figure 1).

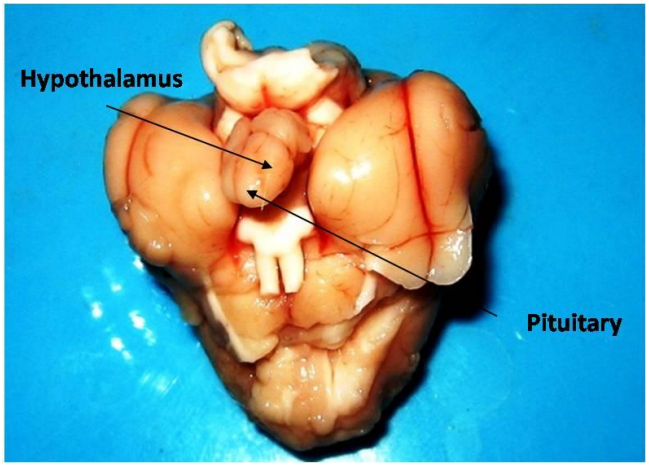

Figure 1. Location of the brain parts (pituitary and hypothalamus)

The weight of each individual gland is measured by an electronically weighing scale. Histological Preparation for Light Microscopy [8] is performed by Fixation of the specimens is made by using 10% isotonic formalin saline for 24-48 hours. Routine staining of sections is performed by using H&E stains.

The standard counting unit is expressed per microscopic field, by micrometer calibrated ocular lens. The numbers of cells are counted in twenty microscopic fields for each specimen and then the mean values of these numbers were calculated.

The hormonal measurements are performed by radioimmunoassay (RIA) method, using the Mini VIDAS instrument (BioMeriux 69280-France model). Then serum is obtained and kept at -20^{o}C in a deep freezer some days in order to use it for measuring the level of LH, FSH, PRL, GH, TSH and ACTH. The SPRs is used for measuring each of them. Specific strip contains 10 wells, the first well for serum sample. Insert the strip and SPRs of each hormone inside the Mini VIDAS instrument and then initiate the radioimmuno-assay technique [9, 10].

Results are analyzed statistically using correlation analysis and t tests between different age groups.

Results

Weight of hypothalamus:

The mean of total Hypothalamus weights are 0.03±0.01, 0.05±0.01 and 0.04±0.01, for group A, B and C, respectively. This reveals a non significant differences ($p>0.05$) are existed between B and C group in comparison with control group (A) (Table 1).

Weight of pituitary gland:

The Weight of Pituitary gland is 0.0085±0.0073, 0.03±0.005 and 0.025±0.01 in groups A, B and C, respectively. There is increase in weight of pituitary gland in groups B in comparison with control group (A) with statically high significant difference ($p<0.01$). There is also increase in weight of pituitary gland in groups C in comparison with control group (A) with statically significant difference ($p<0.05$) (Table 1).

Table 1.Weight of different Glands types

Age group	Gland weight mean±SD	
	Pituitary	Hypothalamus
A	0.0085±0.0073	0.03±0.01
B	0.03±0.01**	0.05±0.01^{ns}
C	0.025±0.005*	0.04±0.01^{ns}

*= P<0.05, **= P<0.01, ^{ns} = not significant (comparison of B and C groups with group).

Number of Supraopticand paraventricularcells of hypothalamus gland

The mean values of the number of supraoptic cells are 15.6±4.39, 22.0±3.94 and 10.2±3.54 of A, B and C. There is a highly significant difference between age group B in comparison with control group (A) p<0.01, and a significant reduction in number of supraoptic cells in age groups C and control group (A) p<0.05 (Table 2).

Table 2. Number of cells in Hypothalamus Gland

Group	Hypothalamus Gland mean±SD	
	supraoptic	paraventricular
A	15.60±4.39	17.80±4.21
B	22.00±3.94**	24.40±4.51**
C	10.20±3.54*	12.00±1.79*

*= P<0.05, **= P<0.01 (comparison of B and C groups with group A).

The mean values of the number of paraventricularcells (Figure 2) are 17.8±4.21, 24.4±4.51 and 12.0±1.79 in age group A, B and C. There is a highly significant difference between age group B in comparison with control group (A) p<0.01, and a significant reduction in number of cells in age groups C in comparison with control group (A) p<0.05 (Table 2).

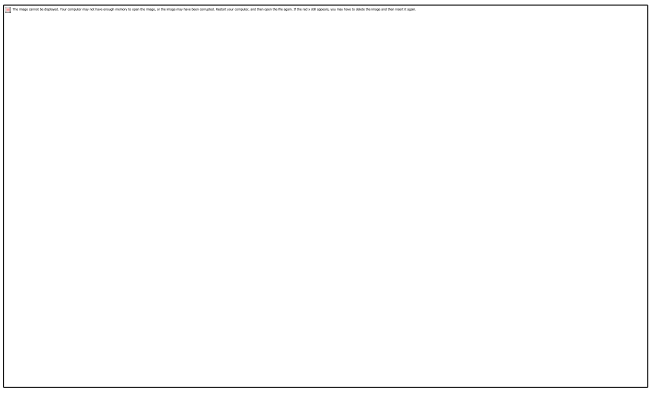

Figure 2.Hypothalamus, shows paraventricular nucleus, neurons and tract fibers of the nucleus (H&E,400X).

Number of the Acidophil, Basophile and Chromophope cells in Pituitary Glands

The mean values of the Acidophil are 14.2±1.64, 23.0±6.0 and 07.2±1.92 in age groups A, B and C, respectively. These results reveals an increase in number of Acidophilsin group B(young adult) in comparison with control group (A), with a significant difference (p<0.05) and a decrease in number of Acidophils in group C in comparison with control group (A), which shows a significant difference (p<0.05) (Table 3).

The mean values of Basophil cells number were 44.0±3.94, 55.0±7.91 and 21.8±6.76 in groups A, B and C, respectively. these results showed B group(young adult) reveals an increase in number of basophile cells in comparison with control group (A), with a significant difference (p<0.05) and a decrease in number of basophile in group C (aged group) in comparison with control group (A), with a high significant difference (p<0.01) (Table 3).

The mean values of cells numbers of chromophope are 10.2±4.32, 14.6±6.73 and 05.8±1.48 in age groups A, B and C, respectively. these results showed B group(young adult) reveals an increase in number of chromophope cells in comparison with control group (A), with a significant difference (p<0.05) and a decrease in number of chromophope in group C (aged group) in comparison with control group (A), with a significant difference (p<0.05) (Table 3).

Table 3. Number of cells in pars distalis of Pituitary gland

Group	Type of cell mean±SD		
	Acidophil	Basophil	Chromophobe
A	14.20±1.64	44.0±3.94	10.2±4.32
B	23.0±6.0*	55.00±7.91*	14.6±6.73*
C	07.2±1.92*	21.80±6.76**	05.8±1.48*

*= P<0.05, **= P<0.01 (comparison of B and C groups with group A).

Hormones measurement in age groups

The mean values of ACTH in different age groups are 0.056±0.018, 0.060 ± 0.016 and 0.053± 0.017 nmol/L in groups A, B and C, respectively. these results showed group B(young adult) reveals an increase in mean values of ACTH in comparison with control group (A), with a non significant difference (p>0.05) and a decrease in mean values of ACTH in group C (aged group) in comparison with control group (A), with a non significant difference (p>0.05) (Table 4).

The mean values of LH in different age groups are increased as following, 1.22±0.01, 1.38±0.02 and 1.62±0.02 nmol/L in groups A, B and C, respectively. These results showed that group B(young adult) reveals an increase in mean values of LH in comparison with control group (A), with a significant difference (p<0.05) and aincrease in mean values of LH in group C (aged group) in comparison with control group (A), with a significant difference (p<0.05) (Table 4).

The mean values of FSH in different age groups are increased as following, 2.5±0.361, 3.5±1.1 and 5.6± 0.1 nmol/L, in groups A, B and C, respectively. These results showed that group B(young adult) reveals an increase in mean values of FSH in comparison with control group (A), with a significant difference (p<0.05) and aincrease in mean values of FSH in group C (aged group) in comparison with control group (A), with a high significant difference (p<0.01) (Table 4).

The mean values of PRL in different age groups are 1.61±0.02, 1.69±0.01 and 1.6±0.01 noml/L in groups A, B and C, respectively. These results showed group B(young adult) reveals an increase in mean values of PRL in comparison with control group (A), with a significant difference (p<0.05) and a decrease in mean values of PRL in group C (aged group) in comparison with control group (A), with non significant difference (p>0.05) (Table 4).

The mean values of GH in different age groups are 1.95±0.1, 1.97±0.02 and 1.92±0.02 nmol/L in groups A, B and C, respectively. these results showed that group B(young adult) reveals an increase in mean values of GH in comparison with control group (A), with a non significant difference (p>0.05) and a decrease in mean values of GH in group C (aged group) in comparison with control group (A), with non significant difference (p>0.05) (Table 4).

The mean values of TSH in different age groups are increased as following, 2.4±0.2 nmol/L, 3.6±0.436nmol/L and 6.1±0.1nmol/L in age groups A, B and C, respectively. These results showed group B(young adult) reveals an increase in mean values of TSH in comparison with control group (A), with a significant difference (p<0.05) and aincrease in mean values of TSH in group C (aged group) in comparison with control group (A), with a high significant difference (p<0.01) (Table 4).

Table 4. Level of Hormones in nmol/ml of different age group

Hormone	Age Group		
	A	B	C
LH	1.22±0.01	1.38±0.02*	1.62±0.02*
FSH	2.5±0.361	3.5±1.1*	5.6±0.1*
PRL	1.61±0.02	1.69±0.01*	1.6±0.1^{ns}
GH	1.95±0.01	1.97±0.02^{ns}	1.92±0.02^{ns}
TSH	2.4±0.2	3.6±0.436*	6.1±0.1**
ACTH	0.056±0.018	0.06±0.016 ^{ns}	0.053±0.017^{ns}

*= P<0.05, **= P<0.01, ^{ns}= non significant (comparison of B and C groups with group A).

Discussion

Weight of Hypothalamus gland

The specimens reveal that there is a non-significant differences in group B and C then the weight decreases beyond that age this finding was in disagreement with Zietz[11], Treier[12], Oliver [13]and Calogero[14].

Concerning the weight of Pituitary gland also underwent early increment in young and then revealed a decrease in weight in aged group(C)

and this is due to reduction of cell number and this result agreed with Saxton[15], who found that there is a reduction of weight and cells number in pituitary in old rodents compared to young, also Stein[16], Wolfe[17], Payne[18], Hanke[7] and Oliver [13]are got same results.

Hypothalamus Glands

The number of cells in the supra optic nucleus was increased in early (group B) but it decreased in later months (group C) and the paraventicular cells undergo same variation in number of cells according to age, also the cell number of paraventricular nucleus is more than supra optic nucleus. These findings may be due to death of cells of the hypothalamic nuclei without replacement of dead cells in aged animal which lead to defiantly and these findings are in agreement with Zietz[11], Treier[12] and Calogero[14].

Pituitary gland

The number of different cells of pars distalis of pituitary gland during early months of age, increased in number but later beyond the age of 24 months. These cells start to decrease in number and basophil cells form the predominant type (60-65%) and the chromophope is the least cell number (15-16%). These findings may be due to difficulty in replacing the dead cells in aged animal and these finding are in agreement with Saxton[15], Stein[16], Wolfe[17], Hanke[7] and Payne[18].

The level of PRL hormone tend to increase during the early months of life then the level was reduced later after the age 24 months and this result is in agreement with Saxton [15] who found that in rabbits with pituitary glands ,serum PRL levels are increased in rabbits of 25-29-month-old, in comparison to rabbits of 15-24 months old female rabbit and then shows a decline of PRL level with further aging. This rise and decline during aging correlated with changes in PRL cell volume, density.

In present study, the serum concentration of thyroid stimulating hormone increases modestly. The variations in TSH concentration between young and elderly subjects are statistically high significant ($P<0.01$) between group C and control group. In contrast, studies reported the decreased in the level of TSH in the elderly men but not in women Olsson[19]. Others found that TSH decreased in aging women [10].

The level of GH has no correlation with age progress in the current study. This disagrees with studies that suggested the onset of aging might be under the control of GH, since primary importance of GH is in the regulation of growth during the maturation process, [20].

In the current study the level of ACTH remain unchanged throughout age. Aging of the anterior pituitary's capacity to ACTH would have significance because of this hormone's mediating role in mobilizing the body's energy reserves in stressful situations. There are however, apparently no age effects on the concentration of ACTH in the blood and these results are agreed with Blichert[21] and disagreed with Thieme[22] who found that ACTH level is positively correlated with age. The HPA axis would become less resilient with age in responding to stimulations.

The current study reveal that the level of FSH hormone is positively correlated with age and this result is in agreement with Tatone[23] and Chand [24] who stated that High levels of Follicle-Stimulating Hormone are indicative of situations where the normal restricting feedback from the gonad is absent, leading to an unrestricted pituitary FSH production. Whereas this is normal in women leading up to and during post menopause, it is abnormal during the reproductive years.

The current study reveal positive correlation between the level of LH hormone and age, just like the FSH hormone and this result is in agreement with Ahmed[25] who stated that there was a significant progressive increase in FSH levels as early as age 29-30 years which was continued throughout the 30s and become more

marked in the early 40s. The Increase In basal FSH (and later LH) may represent the earliest endocrine marker of reproductive aging and can be used as hormonal markers to counsel patients as to the likelihood of their reproductive potential[25].

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Surgical Revision of Ventriculoperitoneal Shunt in Hydrocephalus Patients with Intracranial Tumors

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Abstract

Background Patients with intracranial tumors are predisposed to persistent hydrocephalus, often requiring a permanent CSF diversion procedure with shunts.

Objective This study reviews the long-term experience with ventriculoperitonealshunts for the management of hydrocephalus in patients with intracranial tumors.

Methods Patients with intracranial tumors who underwent ventriculoperitoneal shunt placement for hydrocephalus from January 1999 to January 2009 were included in this study from four neurosurgical centers in Baghdad/Iraq. During the 10-year period, medical charts, operative reports, imaging studies, and clinical follow- up evaluations were reviewed and analyzed retrospectively for all patients. A total of 187 intracranial tumor patients with hydrocephalus were included. The median follow up was 391 days. Malignant tumors were present in 40% of the patients.

Results Overall shunt failure was 27.8%. Single shunt revision occurred in 13% of the patients and 14% had multiple shunt revision. Tumor histology, age and a procedure prior to shunt placement (ventriculostomy/ Ommaya reservoirs) were significantly associated with the shunt revisions. Shunt system replacement and proximal shunt complication were significantly attributed to multiple shunt revisions. The overall shunt revision within 3 months, 6 months, 1 year and 2 years was 17.7%, 18.7%, 19.8% and 24.1%, respectively.

Conclusions The results of the studydemonstrate that VP shunting is an effective procedure for the management of hydrocephalus in patients with intracranial tumors. Age, tumor histology, and a procedure prior to shunt placement (ventriculostomy/Ommaya reservoirs) were significantly associated with the shunt revisions.

Key words Brain neoplasm, Cerebrospinal fluid, Surgery, Shunt

Introduction

Hydrocephalus is a common disorder that results from a disturbance of formation, flow, or absorption of cerebrospinal fluid (CSF), leading to an accumulation of this fluid in the central nervous system (CNS) [1]. It encompasses heterogeneous group of disorders including intracranial tumors, brain hemorrhage, head injury, congenital anomalies, and infections [2,3]. Tumors arising from CNS can block CSF pathways or lead to excessive production of CSF and frequently cause hydrocephalus (Figure 1). Thus, patients with intracranial tumors are at risk of developing hydrocephalus.

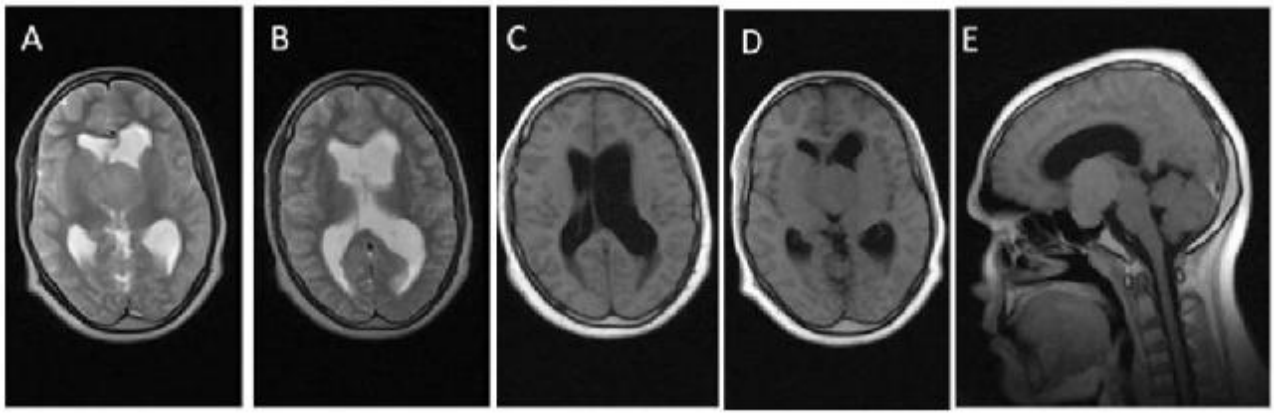

Figure 1. MRI of the brain showing a suprasellar meningioma causing hydrocephalus (A & B T2 axial views, C & D T1 axial views, E T1 sagittal view).

In general, management of hydrocephalus associated with intracranial tumors is a growing concern in neurosurgery. A permanent CSF diversion procedure has been indicated in these patients prior to or after surgical resection of tumor [4]. To date, no consensus exists regarding the management of hydrocephalus in patients with intracranial tumors before, during and after tumor surgery. Some favor [5] preoperative placement of a permanent shunt prior to surgical resection of tumor and others [6] have advocated transitory shunt and steroids to control symptomatic hydrocephalus as a consequence of the subsequent tumor surgery. This would reduce tumor-excision-related morbidity and mortality.

Implantation of a ventriculoperitoneal (VP) shunt is the most widely used treatment for the management of hydrocephalus [7-9]. Although CSF shunting reduces the morbidity and mortality of hydrocephalus, it is associated with potential complications that may require multiple surgical procedures, as well as shunt revisions, during a patient's lifetime [10-13]. Causes for shunt complication and shunt failure include obstruction, infection, mechanical disconnection, and over drainage[10, 11, 12, 13].Thus, the management of hydrocephalus in patients with multiple VP shunt failures is still a challenging problem in neurosurgery.

Earlier studies reveal that an increasing number of previous revisions and shorter time to first revision are associated with the cumulative risk of shunt complications in hydrocephalus patients [12, 14, 15]. The factors that influence the shunt failures or the risk of shunt complications have yet to be fully investigated in hydrocephalus patients with intracranial tumors.

Methods

Patients with intracranial tumors who underwent primary shunt implantation were included in this study between January 1999 and January 2009 in 4 neurosurgical centers in Baghdad/Iraq. The details of the patients' selection for the study are summarized in Figures 2 a and b.

For the 10-year period, medical charts, operative reports, imaging studies, and clinical follow- up evaluations were reviewed retrospectively. Information on each patient, including age, gender, etiology of hydrocephalus, date of shunt placement, date of first and subsequent shunt replacement or revisions, date of last follow-up, and cause of shunt malfunction or failure, were collected from patient's records.

The primary outcome of interest was the

overall shunt revision rate and shunt survival (revision free) in hydrocephalus patients with intracranial tumor. The overall shunt failure was defined as either revision or replacement of an existing VP shunt occurring during the follow-up period.

Statistical Analysis

Multiple logistic regression analysis was used to determine independent risk factors for shunt failure, death, and having multiple revisions (among patients with shunt failure). The Wilcoxon rank-sum test was used to compare groups that are significantly different on shunt failure rate on average number of shunt revisions or failures. The Cox proportional hazards regression model was used to determine independent significant factors for 6-month shunt survival. The Kaplan–Meier method of survival analysis was used to estimate the shunt survival (revision-free) rate and to determine significant factors for shunt failure. The log rank test was used to compare shunt survival rate between categories of identified risk factors for shunt failure; also to compare 2-year patient survival rate between the malignant and benign tumor groups.

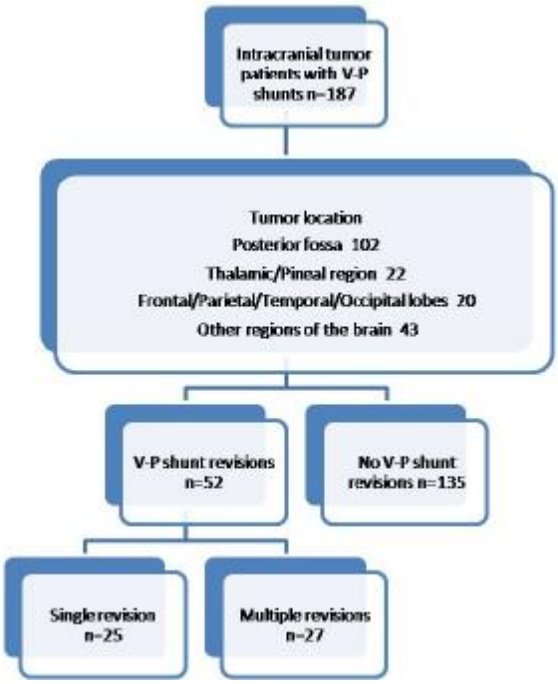

Figure 2a. Flowchart depicting the selection of patients for the study

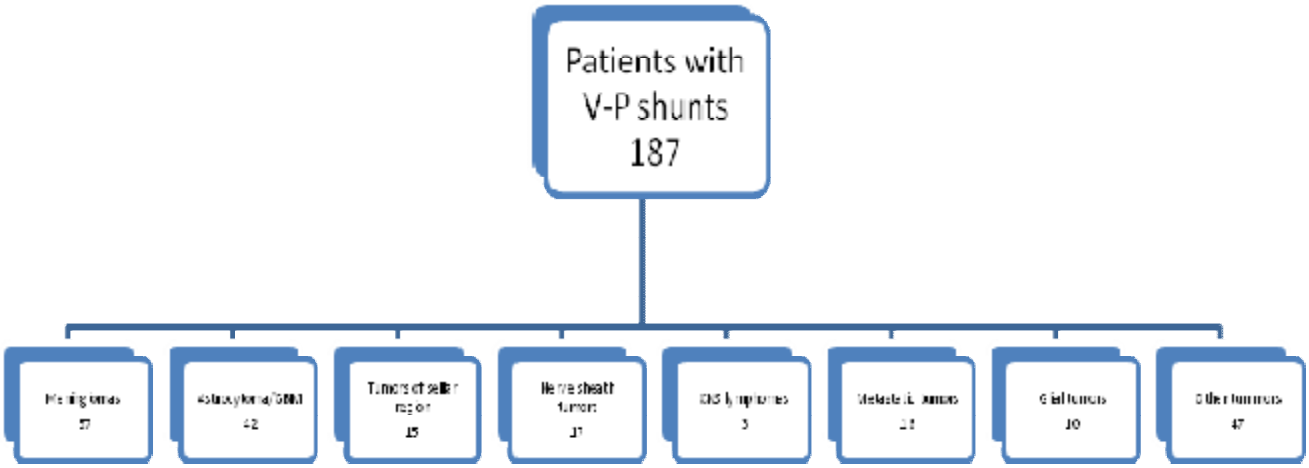

Figure 2b. Flowchart depicting the hydrocephalus patients with intracranial tumors

Results

187 intracranial tumor patients with VP shunt placement were included for the evaluation (Figures 2a, flow chart).

All surgeries were done under general anesthesia and in prone position with proper antisepsis, draping and per-operative and postoperative antibiotic cover and meticulous dressing.

Of the 187 patients, 85 (45%) were male and 102 (55%) were female. Of the 187 patients, 168 (90%) were adults and 19 (10%) were children. The majority of the tumors (54%) were located in the posterior cranial fossa region and tumor size ranged from 0.7 to 10. 7 cm. Benign tumors were present in 112 (60%) patients and 75 (40%) had malignant tumors. There were 131 (70%) patients who had a shunt placement prior to tumor removal and 56 (30%) had a shunt placement after tumor surgery. Of the 187 patients, the majority of the patients (89%) had no prior procedures prior to shunt placement. There were 18 patients who had ventriculostomy and three other patients who had Ommaya reservoirs as a procedure prior to shunt placement.

The site of the burr-hole for shunt placement, depending on the surgeons preference and the site of the tumor, were frontal 33(17.64%), parietal 60 (32.08%), and occipital 94 (50.26%) (Table 1).

Table 1. Demographics of hydrocephalus patients with intracranial tumors

Parameter		No. (%)
Total patients		187 (100%)
Mean age	<18	19 (10.2%)
	>18	168 (89.8%)
Gender	Male	85 (45.4%)
	female	102 (54.6%)
Tumor type	Malignant	75 (40.1%)
	benign	112 (59.9%)
Tumor location	PF region	102 (54%)
	T/P region	22 (12%)
	F/P/T/O lobes	20 (11%)
	Other regions	43 (23%)
Shunt insertion	Before tumor removal	131 (70%)
	After tumor removal	56 (30%)
Procedure prior to VP shunt insertion	Yes	21 (11.2%)
	No	166 (88.8%)
Site of the burr hole for the VP shunt placement	Frontal	33 (17.64%)
	Parietal	60 (32.08%)
	occipital	94 (50.26%)

PF = posterior fossa, T/P = thalamic/pineal, F/P/T/O = frontal/parietal/temporal/occipital.

The median follow up time for all patients was 391days. Of the 187 patients with VP shunt

placement, 52 (28%) experienced one or more shunt failures requiring shunt revision(s). Single shunt revision occurred in 27 (14.4%) patients and multiple shunt revisions occurred in 25 (13.4%) patients after the initial shunt placement (Table 2). Overall, there were 33 (17.7%), 35 (18.7%), 37 (19.8%) and 45 (24.1%) patients experienced shunt failures requiring shunt revisions within 3 months, 6 months, 1 year and 2 years, respectively, after initial shunt placement (Table 2).

Table 2 Shunt revision in hydrocephalus patients with intracranial tumors

Shunt revision	Patients	
	No.	%
Single	25	13.4%
Multiple	27	14.4%
Within 3 months	33	17.7%
Within 6 months	35	18.7%
Within 1 year	37	19.8%
Within 2 year	45	24.1%
Total	52	27.8%

The results in Table 3 list the most common reasons for shunt revisions in hydrocephalus patients with intracranial tumors. A total of 113 shunt revisions occurred in 52 patients, due to various causes such as obstruction, infection, over drainage, mechanical and other shunt complications.

Obstruction caused a total of 45 shunt revisions in 30 (16%) patients. Infection accounted for a total of 16 revisions in 12 (6.4%) patients. Proximal shunt complication caused a total of 43 revisions in 30 (16%) patients. Shunt system replacement accounted for 29 total revisions in 25 (13%) patients.

The findings in Table 4 reveal the risk factors that are independently associated with shunt failure in hydrocephalus patients with intracranial tumors. Among various possible risk factors, tumor histology, procedure prior to shunt placement (ventriculostomy/Ommaya reservoirs), and age were significantly associated with shunt failure.

Table 3. causes of shunt revision among patients and among shunt revisions

Cause	Total patients (n = 187)	Shunt revisions patients (n = 113)
Infection	12 (6.4%)	16 (14.2%)
Obstruction	30 (16.0%)	45 (39.82%)
Overdrainage	3 (1.6%)	4 (3.5%)
Prox. shunt complication	30 (16%)	43 (38.1%)
Dis. shunt complication	13 (7.0%)	16 (14.2%)
Shunt system replacement	25(13.4%)	29 (25.7%)
Valve replacement	16 (8.6%)	17 (15.0%)
Externalization of shunt	13 (7.0%)	14 (12.4%)

Prox. = proximal, Dist. = distal

Table 4. Independent risk factors for shunt failure

Risk factors	OR	95% CI for OR	P value
Benign *vs* malignant	2.56	1.20-5.44	0.015*
Procedure prior to VP shunt (Yes *vs* No)	6.33	2.33-17.24	<0.01**
Age (shunt placement)	0.98	0.97-0.99	0.049

* p = < 0.05, p = < 0.01, OR = odd ratio

The odds for shunt failure among patients with benign tumors were 2.56 times higher than those for patients with malignant tumors; odds for shunt failure among patients who had a procedure prior to their VP shunt placement (ventriculostomy/Ommaya reservoirs) were 6.33 times higher than those for patients with no such prior procedures; for every year increase in age at shunt placement there was a 2% decrease in odds for shunt failure, which indicates that younger patients have a higher risk for shunt failure. Adjusted for the effects of one another on shunt failure, these factors are significantly associated with a patient having

shunt failure or revision. Interestingly, insertion of shunt prior to or after tumor extraction showed no association with shunt failure.

The data in Table 5 show the comparison on average number of shunt failures or revisions between categories of the risk factors for shunt failure. The average number of shunt failures was significantly higher among patients with benign tumors than among those with malignant tumors (0.8 *vs* 0.4, p = 0.02); among pediatric patients than among adults (1.8 *vs* 0.5, p = 0.03); and among patients with a procedure prior to their VP shunt placement (ven-triculostomy/Ommaya reservoirs) than among those with no such prior procedure (1.5 *vs* 0.5, p <0.01).

Table 5. Comparison on average number of shunt revisions among patient groups by factors significantly associated with shunt failure

Group		No.	Shunt failures mean (range)
Malignancy	Benign	112	0.8 (0-7)
	Malignant	75	0.4 (0-5) *
Age group	Pediatric	19	1.8 (0-7)
	Adult	168	0.5 (0-5) *
Procedure prior to VP shunt	Yes	21	1.5 (0-7)
	No	166	0.5 (0-7) **

* = p < 0.05, ** = p < 0.01

Risk factors for multiple shunt failures/revisions were determined among the 52 patients who had a total of 113 shunt revisions. The independent significant factors for multiple revisions are shunt system replacement and proximal shunt complication (Table 6). Adjusted for the effects of other factors, odds for multiple revisions among patients with shunt system replacement(s) were 24.39 times higher than those among patients with no shunt replacement (p < 0.01); odds for multiple revisions among patients with proximal shunt complication were 14.49 times higher than those among patients with no proximal shunt complication (p < 0.05).

Table 6. Independent risk factors for multiple shunt revisions among patients with shunt failure

Risk factors	OR	95% CI for OR	P value
Shunt replacement yes *vs* no	24.39	2.92-200.0	0.003**
Proximal revision yes *vs* no	14.49	1.72-125.0	0.014*

* p = < 0.05, p = < 0.01, OR = odd ratio

Since patients with malignant intracranial tumors are associated with a significantly shorter overall survival rate, we examined the risk factors that are independently associated with 3 and 6 month shunt survival using multivariate analysis. The results indicate that the independent significant factors for 3- and 6-month shunt survival were gender, malignancy and procedures prior to shunt placement such as ventriculostomy or Ommaya reservoirs (Table 7) as determined by multivariate analysis. Male sex, patients with benign tumors and patients with procedure prior to shunt placement (ventriculostomy / Ommaya reservoirs) had significantly lower 3 or 6 month shunt survival rates than female sex, patients with malignant tumors and those with no procedures prior to shunt placement, respectively (Table 7).

Figures 3 through 5 show the 6-months shunt survival rate by gender, tumor status (benign or malignant), and the presence or absence of procedures prior to shunt placement (ventriculostomy/Ommaya reservoirs).

In this study, we observed that the overall shunt revision rate is significantly higher among the patients with benign tumors than those

with malignant tumors. The higher shunt revision rate in patients with benign tumors could simply be due to shorter overall survival rate among the patients with malignant tumor as most of these patients died before they had a chance for their shunts to fail. Therefore, we assessed the mortality rate in patients with malignant tumors and compared to those with benign tumors.

Table 7. Factors significantly associated with 3- and 6-months shunt survival

Factor/Category		3 month survival rate (%)	6month survival rate (%)
All patients (n=187)		82.3	80.7
Gender	Male (n=85)	77.6	74.1
	Female (n=102)	86.3*	86.3*
malignancy	Benign (n=112)	77.6	75.9
	Malignant (n=75)	89.3*	89.3*
Procedure prior to shunt insertion	Yes (n=21)	54.3	52.4
	No (n=166)	86.1**	84.9**

* p = < 0.05, p = < 0.01

The Chi-square analysis indicated that the mortality rates within 3 months, 6 months, 1 year and 2 years of shunt placement were significantly higher among patients with malignant tumors than those with benign tumors (Table 8). These findings clearly indicate that most patients with malignant tumors died before they had a chance for their shunts to fail and thus had significantly lower shunt revision than those with benign tumors. Among various potential risk factors that were analyzed, only the tumor histology is independently associated with mortality of the patients with hydrocephalus. The odds for death among patients with malignant tumors are 2.16 (95% CI 1.19-3.9) times higher than those with benign tumors (p=0.011).

Discussion

The management of hydrocephalus in patients with surgically resectable intracranial tumors remain great challenge and controversial. Some surgeons favor permanent placement of shunts and others recommend external ventricular drains. Although the placement of permanent VP shunts is effective for the management of hydrocephalus, they are associated with myriad potential complications from the shunt itself, including infection, mechanical obstruction, and disconnection. Thus shunt removal, or revision is inevitable in these patients [9,12].

Table 8. Mortality of hydrocephalus patients with intracranial tumors

Time	Malignant tumors (n=75)	Benign tumors (n=112)
3 months	18 (24.0%)	14 (12.5%)*
6 months	20 (26.7%)	15 (13.4%)*
1 year	28 (37.3%)	21 (18.8%)**
2 years	40 (35.3%)	35 (31.2%)**

* p = < 0.05, p = < 0.01

In this study, we retrospectively evaluated the incidence of shunt failures, overall shunt survival and risk factors associated with shunt failures in a cohort of 187 patients with intracranial tumors who underwent VP shunt placement for hydrocephalus in the period between January 1999 and January 2009.

The results from this study show that the overall incidence of shunt revision was 27.8% in hydrocephalus patients with intracranial tumors. The shunt revision rate was similar at 3-months 6 months and 1 year (18, 19 and 20%, respectively) but increased to 24% by 2-years after initial shunt placement. The incidence of VP shunt revision varies considerably among patients with various etiologies of hydrocephalus [12,16-19]. Moreover, pediatric patients experience a high rate (40-50%) of shunts failure compared with adult patients (29%) within the first year of shunt placement [15,20,21]. Although our findings on

the incidence of shunt revision are consistent with these reports, the patient population in this study includes both adults (90%) and children (10%). Furthermore, the etiology in our study is confined to intracranial tumors. However, our results are closely comparable with the study by Hoh et al [16], in which they reported that 26 (30%) of the 87 adult tumor patients with hydrocephalus experienced shunt revisions. In addition, the 6 month revision rate (18.7%) observed in this study is well in agreement with the recent findings reported by Farahmand et al [14] in which they found that 12 (18.5%) of the 65 tumor patients with hydrocephalus experienced shunt revisions within the 6 months of shunt placement.

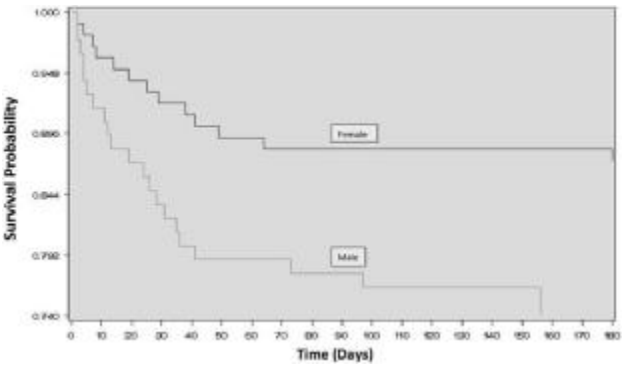

Figure 3. Analysis of shunt survival in hydrocephalus patients with intracranial tumors according to the gender. The Kaplan–Meier plot shows significant differences in 6-month shunt survival between male and female patients (log rank test, p <0.001)

Interestingly, we found that the malignant tumor patients experienced significantly less shunt revisions than benign tumor patients indicating benign tumor patients have a higher risk in developing shunt complications. This could simply be due to the higher death rate in malignant tumor patients. Similar findings have been reported by Hoh et al [16] where the authors found that tumor (non-hemorrhage) patients experienced higher shunt revision than non-tumor (hemorrhage) patients. Moreover, our results revealed that children and patients with a procedure prior to shunt placement (ventriculostomy / Ommaya reservoirs) experienced significantly higher shunt revisions than adults and patients with no procedure prior to shunt placement, respectively.

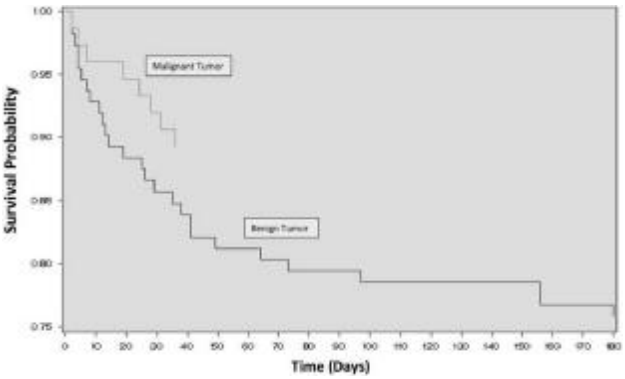

Figure 4. Analysis of shunt survival in hydrocephalus patients with intracranial tumors according to the tumor histology. The Kaplan–Meier plot shows significant differences in 6-month shunt survival between benign and malignant tumor patients (log rank test, p <0.0 01)

Conversely, the results of this study indicate that among various independent risk factors only shunt system replacement and proximal shunt complication are significantly attributed to multiple shunt failures in hydrocephalus patients with intracranial tumors. Previous studies have shown that proximal obstruction contributes to a greater extent to early shunt failure than late failure [22-24]. Currently, it is unclear why shunt system replacement and proximal shunt complication are associated with multiple shunt revisions. Perhaps tumor patients may have tumor growth with resultant obstruction of the shunt by tumor cells or infiltrate, causing proximal shunt malfunction requiring multiple shunt revisions. A thorough analysis of factors affecting shunt system replacement and proximal obstruction is beyond the scope of this study.

It is well known that the risk of death is significantly higher in patients with malignant

tumors than benign tumors. Therefore, we assessed the various risk factors such as gender (male *vs* female), tumor histology (benign or malignant), and a procedure prior to shunt placement (ventriculostomy / Ommaya reservoirs) in relation to 3 and 6 month shunt survival in the patients. Among various risk factors, gender (male), tumor histology (malignant), and a procedure prior to shunt placement (ventriculostomy/Ommaya reservoir) emerged as independent risk factors for 3- and 6-month shunt survival. Similarly, Wu et al [(12)] observed that the male sex is independently associated with increased risk of shunt complications in patients with hydrocephalus.

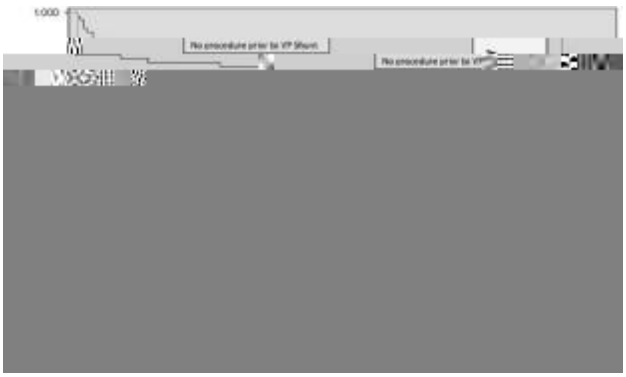

Figure 5. Analysis of shunt survival in hydrocephalus patients with intracranial tumors according to the procedure prior to shunt placement. The Kaplan-Meier plot demonstrates significant differences in 6-month shunt survival between the patients with or without a procedure prior to shunt placement (log rank test, p<0.001)

Overall, the findings of this study demonstrate that VP shunting is an effective neurosurgical procedure for the management of hydrocephalus in patients with intracranial tumors. Several studies are focused on improving shunts by developing material and valve mechanisms [(25-27)].

Furthermore, endoscopic neurosurgery has been developed as an alternative to avoid invasive surgery or shunt insertion related adverse events. Currently, we are exploring the clinical benefits of endoscopic third ventriculostomy as an alternative treatment option for certain hydrocephalus patients with intracranial tumors.

This study is subject to a number of important limitations. One important shortcoming of this investigation is the retrospective nature of the study that explores the long-term management of hydrocephalus in patients with intra-cranial tumors. Although uniform technique for VP shunt placement was used, the overall treatment was chosen by a number of neurosurgeons. Moreover, the variables included in this study could not be analyzed in a controlled way. Also, many of the variables were dependent on the decisions of individual neurosurgeons involved in shunt placement.

Conclusion

In summary, the results of this study show that the VP shunting is a valuable treatment option for the management of hydrocephalus in patients with intracranial tumors. The overall shunt revision rate observed in this study was comparable to the earlier published reports. Age, benign tumor, and a procedure prior to shunt placement (ventriculostomy/Ommaya reservoirs) were significantly associated with the shunt revisions. In addition, shunt system replacement and proximal shunt complication were significantly attributed to multiple shunt failures.

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Risk of Facial Paralysis Following Parotidectomy

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Abstract

Background The facial nerve should be sacrificed only if there is strong indication. Sometimes it is possible to sacrifice only part of the facial nerve and this termed "semiconservative parotidectomy". The commonest operation performed is superficial conservative parotidectomy, which is removal of the parotid superficial to the facial nerve with nerve preservation. A total conservative parotidectomy was performed only if clearly indicated by the pathological condition, since the complete freeing of the facial nerve in this operation increases the incidence of nerve paralysis.

Objective To demonstrate under what circumstances is the surgeon likely to be called upon to sacrifice the facial nerve deliberately, and mentioning what be done to reduce the risk of functional facial paralysis following conservative parotidectomy.

Methods The material comprises 30 cases of parotidectomy of all types. We analysed the incidence and degree of functional facial paralysis following conservative parotidectomy and also we reported some experimental work attempting to elucidate its etiology. We classify the degree of facial nerve paralysis to grade I, absent or slight, grade II moderate, grade III complete.

Results We did superficial parotidectomies for 22 cases, 19 had grade I, two had grade II and only one had grade III facial nerve paralysis. Conservative total parotidectomies done for 2 cases, one had grade I and one had grade III facial nerve paralysis. Semiconservative parotidectomies done for 4 cases all had grade I facial nerve paralysis and lastly radical parotidectomies done for 2 cases, the results had grade III for two cases facial nerve paralysis.

Conclusion To reduce the incidence of facial paralysis after conservative parotidectomy: carrying total parotidectomy only when clearly demanded by pathological condition by avoiding washing out the wound, and by measures designed to preserve the blood supply of the trunk of the facial nerve. The present study support that ischemia is the principal factor in post-parotidectomy functional facial paralysis.

Key words Parotidectomy, Facial nerve, Mixed tumor

Introduction

The thought that they may wake up with a paralyzed face is probably the chief anxiety of most patients who have been advised to undergo operations on the parotid. Such paralysis may theoretically occur in three ways. First, the surgeon may inadvertently cut or otherwise grossly interrupt the anatomical continuity of the facial nerve. Secondly he may deliberately sacrifice the nerve as a necessary step in removing the pathological process. Thirdly, although the surgeon preserves the nerve anatomically, the patient may develop a functional facial paralysis after operation, which is fortunately almost always temporary - [1-6].

The modern operation of conservative parotidectomy, that is parotidectomy with conservation of the facial nerve, is so well worked out that an advertent major

anatomical interruption of the nerve should be and experience shows is rare. The principal questions thus remaining are; first, under what circumstances is the surgeon likely to be called upon to sacrifice the facial nerve deliberately? And secondly can anything be done to reduce the risk of functional facial nerve paralysis following conservative parotidectomy? [7-11].

So an obvious principle of parotid surgery is that the facial nerve should be sacrificed only if clearly demanded by the conditions. In some cases it is possible to sacrifice only part of the facial nerve and this termed "semi-conservative parotidectomy" [12-17].

Misplacement of the suction drains may also lead to neurapraxia. The suction drains should be placed in such way that they do not overlie the trunk or any branch of the facial nerve and secured to the bed of the wound with 4:0 catgut sutures. In parotidectomy due to chronic parotitis, the duct is best tied as it may contain muco-purulent saliva [18-20].

Finally, there was a small modification in the technique of exposure of the trunk of the facial nerve based on the work of Blunt who found that the main blood supply of the trunk of the nerve in its extra cranial course came from two small branches of the stylomastoid artery which entered the nerve close to the stylomastoid foramen. In an attempt to preserve this blood supply it is better to identify the trunk of the facial nerve nearer to its main division rather than near stylomastoid foramen. Of purely surgical factors ischemia was thought to be the most important with edema and stretching, particularly of the finer branches of the nerve as possible subsidiary factors [20-24].

Methods

Total number of patients was 30 (13 males and 17 females; the age range was 13-75 years) whom diagnosed clinically, histopathology and treated surgically by parotidectomy of all types at Al-Kadhimiya Teaching Hospital from 2006 till 2010.

In all cases of parotidectomy, preauricular incision "Modified Blur incision" was used and the operation was classified into:

1. Superficial conservative Parotidectomy which include removal of the parotid superficial to the facial nerve with complete anatomical preservation of the nerve.
2. Total Conservative Parotidectomy involves sub-facial dissection. The sub facial tumors are those lying deep to the facial nerve which may arise in either the superficial or deep lobes and occasionally involve both lobes. It was performed only if clearly indicated by the pathological condition, since the complete freeing of the facial nerve and its branches has been shown to increase the incidence of functional facial paralysis.
3. Semi-conservative Parotidectomy in which an important part of the facial nerve was involved in the growth and was deliberately sacrificed.
4. Radical Parotidectomy in which the whole facial nerve was deliberately sacrificed

Regarding the facial nerve identification, the nerve lies at a point midway between the tip of the mastoid process and the lower bony auditory meatus, and these points of anatomy are identified with the index finger. The main trunk of the facial nerve is readily distinguished from surrounding tissues by its texture, color, position and direction.

The dissection proceeds forward and with minimal flanking movements, using gentle retraction and fine curved artery forceps. The technique involves laying the artery forceps immediately above the nerve and then opening it and carefully dividing the bridging tissue over the nerve. Avoid repeated heavy pressure on the dissected facial nerve by way of a dry swab, the assistant's sleeve or an excessively hot pack used in the interest of hemostasis.

We analyzed the incidence and degree of functional facial paralysis following conservative parotidectomy and also we reported some experimental work attempting

to elucidate its etiology. In our cases we have tried to eliminate the factors which seemed to predispose to functional facial paralysis from the previous studies.

We classify the degree of facial nerve paralysis:

1. Grade I (absent or slight) Recovery from any grade I functional paralysis is usually complete within a few months.
2. Grade II (moderate) Recovery from a grade II functional paralysis does not usually begin for three months and may take six months to complete
3. Grade III (complete) resulting from anatomical interruption of the nerve and is permanent unless anatomical continuity is in some way restored.

All the data were analyzed using SPSS version 15 (2006) computer program.

Results

A total of 30 patients who had undergone parotidectomy at Al-Kadhimiya Teaching Hospital were studied prospectively from November 2006 till November 2010 with a mean period of follow-up of 1.65 years. The study comprised 13 (43.3%) males and 17 (56.7%) females. Their ages ranged from 13 to 75 years with a mean of 47 years. The demographic features of the patients was shown in table 1.

Table 1. Demographic features of the studied patients

Case	Age (years)	Gender	Histopathology	Procedure	Grade
1	75	♂	Adenoid cystic carcinoma	Radical	III
2	45	♀	Pleomorphic adenoma	=	III
3	54	♂	=	Semi-conservative	I
4	43	♂	=	=	I
5	65	♀	Adenoid cystic carcinoma	=	I
6	56	♀	Muco-epidermoid carcinoma	=	I
7	40	♀	Pleomorphic adenoma	Superficial conservative	I
8	50	♂	=	Total conservative	I
9	54	♀	Muco-epidermoid carcinoma	=	III
10	43	♀	Pleomorphic adenoma	Superficial conservative	I
11	47	♀	=	=	I
12	53	♀	=	=	I
13	37	♀	=	=	I
14	29	♀	=	=	I
15	37	♀	=	=	I
16	41	♀	=	=	I
17	50	♀	=	=	I
18	35	♀	=	=	I
19	28	♂	=	=	I
20	33	♂	=	=	I
21	48	♂	Warthin's tumor	=	I
22	13	♂	Vascular malformation	=	I
23	54	♀	Myo-epithelioma	=	I
24	27	♀	Calculus	=	I
25	55	♂	Warthin's tumor	=	I
26	63	♂	=	=	I
27	68	♂	=	=	I
28	54	♂	Muco-epidermoid carcinoma	=	II
29	52	♀	Adenoid cystic carcinoma	=	II
30	60	♂	=	=	III
Mean age :47 years			SD : (13.51946)		
Malignancy 7 cases (23.3%)			Benign 23 cases (76.7%)		
(♂:♀) (13:17)		♂ 43.3%		♀ 56.7%	

Type of Operation and Histopathogic Results
Superficial conservative parotidectomy was the most commonly done operation (22 cases, 73.3% of cases). With regard to the pathology, twelve cases were pleomorphic adenoma, four cases were Warthin's tumor, two were adenoid cystic carcinoma, one was inflamed parotid due to calculus, one was myo-epithelioma, one case was a muco-epidermoid carcinoma and one vascular malformation.
Semi-conservative parotidectomy done in 4 cases (14%). The pathology examination showed that one cases was muco-epidermoid tumor, another case was adenoid cystic carcinoma, while the remaining two cases were of pleomorphic adenoma.
Total conservative parotidectomy done in 2 (7%) cases; one case was a muco-epidermoid tumor and the other was a recurrent pleomorphic adenoma. Radical parotidectomy done in 2 (7%) cases; one case with carcinoma and the second case of pleomorphic adenoma (Figure 1 and Table 2).

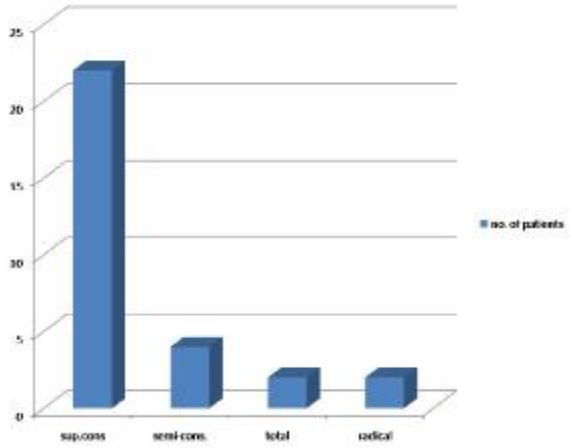

Figure 1. Type of surgery

Out of the total 30 patients, 7 patients (23.3%) had malignant diseases and 23 patients (76.7%) had benign diseases.
Grade of facial paralysis
In our series, most of the cases (24 cases 80%) was classified as grade I in which no facial paralysis results, 2 (7%) patients with grade II, 4 (13%) patients with grade III. The mean grade = 1.3%

Table 2. Histopathology Results of the entire group

Histopathology	patients	
	No.	%
pleomorphic adenoma	16	53.40%
adenoid cystic carcinoma	4	13.30%
mucoepidermoid carcinoma	3	10%
warthins tumor	4	13.30%
vascular malformation	1	3.30%
Calculus	1	3.30%
Myoepithelioma	1	3.30%

Radical Parotidectomy was done in 2 cases and both of them had grade III facial paralysis. Semi-conservative Parotidectomy done in 4 cases and all of them had grade I facial paralysis. Total Conservative Parotidectomy done in 2 cases; one case had grade I and the other had grade III. Superficial Conservative Parotidectomy done in 22 cases, 19 case (86.5%) showed grade I, 2 cases (9%) grade II and 1 case (4.5%) grade III facial paralysis (Table 3).

Discussion

Superficial Conservative Parotidectomy: 19 of the 22 cases of this group showed little or no functional facial paralysis (grade I). There was one case (adenoid cystic carcinoma) of grade III paralysis and 2 cases of grade II (one was adenoid cystic carcinoma and one was a muco-epidermoid carcinoma with adhesion to both skin and masseter).
Total Conservative Parotidectomy: One case had grade III, it was muco-epidermoid tumor was arise in the sub-facial parotid and it presented retro-pharyngeally. The superficial parotid was merely reflected for access but not resected, but since the facial nerve had to be mobilized on both superficial and deep aspects, we have considered the operation as equivalent to a total parotidectomy. The other

case of this group (recurrent pleomorphic adenoma) had grade I, here, although the sub-facial parotid was not resected, the facial nerve was completely freed on both aspects under the impression subsequently proved false, that there was a secondary primary tumor in the sub facial parotid.

Semi-conservative Parotidectomy: In 2 cases the lower main divisions of the nerve was resected and in one case the upper main division. In the remaining one case, growth had infiltrated in the region between the two main branches, and a leash of intermediate branches was involved and resected. In the case of resection of the upper main division the only movements lost were those of the forehead, and in the 2 cases of resection of the lower main division those of the angle of the mouth. In the case of intermediate facial nerve resection there was slight weakness of the cheek and upper lip. In all cases in this group the good facial function was present immediately after operation and the functional paralysis has therefore been classified as grade I.

Radical parotidectomy One of the two cases was a case of carcinoma. The second was a case of mixed parotid tumor (pleomorphic adenoma) twice recurrent. The recurrences involved both skin and masseter, portions of both of which were removed in continuity with multiple adherent tumor masses (both of them located in the deep lobe of the parotid gland).

The discussion will be limited to the question of facial paralysis following parotidectomy. The first point worthy of comment is that sacrifice of the facial nerve, complete or partial was thought necessary in no fewer than 5 out of the 30 cases. Two points in this connection may be made, firstly; that the only primary tumor requiring major sacrifice of the facial nerve were highly malignant tumors, secondly, that of the recurrent tumors [25- 28].

A recurrent pleomorphic tumors does not necessarily demand partial or complete sacrifice of the facial nerve, but recurrence clearly increase the danger of some sacrifice of the facial nerve being necessary. In grade II and III cases in this group the operations involved some technical difficulty. The problem is not only that a recurrent tumor may show infiltrative characters but also that, even if it does not do so, if the original operation has been in the neighborhood of nerve, tumor and nerve may be so bound together by fibrous adhesions that separation is impossible. Resection of the nerve in these cases may fairly be regarded as the price that had to be paid for an inadequate primary operation [29-30].

Never assume that a centrally placed tumor is entirely superficial simply because it seems so on palpation. In some apparently fairly superficial and movable tumors the growth may be found to extend beneath the seventh nerve. In case of superficial tumors much of the superficial parotidectomy is achieved as possible before the nerve is gently mobilized from the surface of the tumor. Once mobilization is completed we place fine vascular slings beneath the nerve and very gently lift it away from the tumor and continue dissection.

The surgeon should resist the impulse to stimulate the nerve repeatedly to confirm movements of the mimetic musculature. The nerve stimulator and bipolar diathermy is good servant and bad masters and we should never forget the advice of Hughes et al "that repetitive direct stimulation both at one site and multiple sites of the same nerve produced significant myelin and axon degeneration" [10].

Never use also unipolar diathermy because this will lead to damage to the nerve. A striking feature in our study was slight degree of facial paralysis which followed sacrifice of up to half of the facial nerve particularly of the lower half. The terminal branches of the facial nerve have inter-communicating branches and it is presumably on the preservation of all these that the good result depends.

There is a striking reduction in the incidence and severity of functional facial paralysis in our

study as compared with the Patey and Moffat series (the same type of surgery). Thus if we group together the grade II and grade III paralysis and regard them as major functional facial paralysis, there were 44 such paralysis in 95 cases in the Patey and Moffat series as compared with 4 in 24 cases in our study [22 superficial parotidectomies and 2 total parotidectomies] [and 4=2 grade II and 2 grade III] (P = 0.002).

Our percentage of post-operative facial weakness therefore was 16% and that of Maynard 2000 21%. Maynard compares the complications rate of primary parotidectomy for mixed tumors (number = 155) with parotidectomy for chronic and obstructive parotitis (number = 94), he indicate 21% of postoperative facial weakness. Norman found 26% (number = 100) postoperative weakness with duration of between 24 hours and 9 months. The indications for surgery include obstruction parotitis, benign and malignant tumors [11-12].

Gunn reports a partial and temporary facial paresis occurred in 47% of primary parotidectomy and recovery usually took place in 3-6 months. Zan Mra et al in 1993 reported 9 cases out of 10 that the marginal mandibular branch shows weakness postoperatively. In general temporary facial nerve paresis involving all or just one or two branches of the facial nerve and permanent total paralysis have occurred respectively in 9.3% to 64% in the literatures [35, 36].

The cases of transient facial nerve paresis resolved within 6 months with 90% within 1 month. Temporary paresis usually resolves according to Laccourrey within the 18th post-operative month [8].

The incidence of facial nerve paralysis is higher with total than with superficial parotidectomy which may be related to stretch injury or as a result of surgical interference with the vasa nervosum.

In our study the avoidance of washing out the wound with powerful antiseptics combined with the limitations in the indications for total parotidectomies provide an obvious explanations for the reduced incidence for major functional paralysis.

Table 3. Types and numbers of cases classified according to facial nerve paralysis

Types of Parotidectomy		No. Of cases	Degree of Facial Paralysis					
			Grade I		Grade II		Grade III	
			No	%	No	%	No	%
Conservative	Superficial	22	19	64%	2	7%	1	3%
	Total	2	1	3%	0	0	1	3%
Semi-conservative		4	4	31%	0	0	0	0
Radical		2	0	0%	0	0	2	7%
Total		30	24	80%	2	2%	4	13%

Table 4 Gives the incidence of the different degrees of functional paralysis in the comparable superficial parotidectomies in three studies.

The reduction of the incidence of major functional paralysis from 18 in 49 cases (37%) in Patey and Moffat to 3 in 22 (14%) cases in our study is statistically significant (P-value:0.002).

In our study the trunk of the facial nerve was identified whenever possible some way in front of rather than at the stylomastoid foramen on the hypothesis that in this way the blood supply to the trunk of the facial nerve might be preserved. Our study thus provides evidence both for the value of this maneuver and to support Patey and Moffat's conclusion that

ischemia is the main factor in functional facial paralysis after conservative parotidectomy.

Detailed analysis of the cases of our study suggests however that ischemia may not be the only factor. Thus two superficial parotidectomies were followed by major functional paralysis though it was thought that the blood supply to the trunk of the facial nerve had not been interfered with, both were cases in which the gland was swollen and inflamed the time of operation and it is possible that edema played a part in the facial paralysis. Again, we formed the impression that functional paralysis was predisposed to in cases in which branches of the facial nerve were stretched around a tumor or cyst. In these circumstances the freeing of the nerve branches might stretch them still further and thus interfere with their conductivity. Finally, there was the grade III functional paralysis which has already been mentioned and which followed an uneventful superficial parotidectomy. In this case again it was thought that the blood supply to the trunk of the facial nerve had not been interfered. Whatever the cause of the functional paralysis, the case is important in emphasizing that in spite of all precautions the risk of major functional facial paralysis cannot be entirely eliminated [31-34].

Table 4. Types of tumors and their grading in the present study and those reported others

Tumor Type	Grading	David & Patey (1993)	Patey & Moffat (1995)	Present study
Mixed parotid tumor	Grade I	38	31	19
Mucoepidirmoide carcinoma & adenoid cystic carcinoma	Grade II	4	16	2
Sarcoma, mucoepidirmoid carcinoma, adenoide cystic carcinoma, recurrent mixed parotid tumor	Grade III	2	2	1

The results confirmed that of (Patey and Thackray) [11] that the facial movements after these partial resection may be surprisingly good, Most cases of nerve injuries occurred in adenoid cystic carcinoma [24].

As a conclusion, the incidence of major functional facial paralysis after conservative parotidectomy has been significantly reduced by carrying out total parotidectomy only when clearly demanded by the pathological condition, by avoiding washing out the wound, and by measures designed to preserve the blood supply of the trunk of the facial nerve. The present study support that ischemia is the principal factor in post-parotidectomy functional facial paralysis.

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Antiphospholipid Antibody in Serum of Guillain-Barre Syndrome Patients

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Abstract

Background Studies have provided convincing evidence that Guillain-Barre syndrome [GBS] is caused by an infection-induced aberrant immune response that damages peripheral nerves. Despite intensive research over the past two decades, the immune target is still unknown in patients with acute inflammatory demyelinating polyradiculoneuropathy [AIDP], the most frequent variant of GBS.

Objective Measuring of immunoglobulin G [IgG] and immunoglobulin M [IgM] antiphospholipid antibodies [aPL] of incidental untreated GBS patients and comparing them with that of normal population.

Methods This is an age and gender matched paired case-control study at Al-Kadhimiya Teaching Hospital between 1-Dec-2008 and 31-Jan-2010. Each patient was paired with the age and sex matched control which was useful in controlling the confounding effect of age and gender on possible case-control differences. The aPL were measured by Immunometric Enzyme Immunoassay.

Results Eleven patients with GBS (cases) and eleven age and gender matched controls included in current study. The GBS cases have higher IgM and IgGaPL titers than healthy controls [P=0.026, P=0.13 respectively]. The GBS cases IgMaPL titers have negative correlation with duration of illness [r=-0.494, P=0.12], while the cases IgGaPL titer have positive correlation with duration of illness [r=0.243, P=0.47]. The GBS cases that need mechanical ventilation have lower IgM and IgGaPL titers than cases that do not need mechanical ventilation [P=0.1, P=0.06 respectively].

Conclusion GBS cases have statistically significant higher IgMaPL titers during the first week [p=0.028] and the first two weeks [p=0.026]of illness than healthy controls, and aPL may have a protective effect in GBS.

Keywords Guillain-Barre syndrome, Demyelination, Antiphospholipid antibodies and autoantibodies.

Introduction

Until 2010, GBS has remained a descriptive diagnosis of a disorder for which there are no specific diagnostic tests. The combination of rapidly progressive symmetrical weakness in the arms and legs with or without sensory disturbances, hyporeflexia or areflexia, in the absence of a CSF cellular reaction, remains the hallmark for the clinical diagnosis of GBS [(1)].

Based on well-controlled population-based studies the incidence of GBS in Europe is 1.2-1.9 cases per 100 000, while worldwide, the incidence is 0.6-4 cases per 100 000 [(2,3)]. Despite the effect of intravenous immunoglobulin [IV Ig] or plasma exchange [PE] treatment, 10% to 20% of patients are left with disabling motor deficits and 4% to 15% of patients die by 1 year after onset [(4)]. Up to one-third of GBS patients need to make substantial changes in their job, hobbies or social activities due to the residual functional deficit [(5)]. Even 3-6 years after onset, GBS has a large impact on social life and the ability to perform activities

[6,7,8]. GBS often remains a severe disease for which better treatment is required, at least in some patients.

About two-thirds of GBS cases have an antecedent infection within 6 weeks prior to symptom onset, generally an upper respiratory tract infection or gastroenteritis [9]. Autoimmune diseases such as graft-vs.-host disease, systemic lupus erythematosus, scleroderma, and sicca syndrome are sometimes associated with GBS [10]. Some of these are also characterized by the presence of aPL. This relationship between the above autoimmune diseases and GBS raises the question of whether aPL are encountered in patients with this syndrome or not.

Nearly any neurological manifestation may occur in patients have aPL[11]. Non-thrombotic manifestations were described in relation to the presence of aPL like epilepsy, chorea, transverse myelitis, multiple sclerosis, GBS, dementia, and psychiatric disease [12]. Also subtle abnormalities in neurocognitive function may be found in patients have a PL, such as memory loss or behavioral disturbances [13].

The aim of study is to reveal if IgG and IgM aPL associated with GBS and if there a significant deference in the titers of IgG and IgM aPL between incidentals untreated GBS patients without known autoimmune disorders and normal population.

Methods

Study design: Age and gender matched paired case-control study. Patients with GBS [cases] were recruited from patients admitted to Al-Kadhimiya Teaching Hospital between 1-Dec-2008 and 31-Jan-2010 [14 months] who fulfilled the following eligibility criteria:-

1. Patient is examined and diagnosed as GBS according to Asbury and Cornblath criteria [1].
2. Patient with no signs of improvement.
3. The period from appearance of first symptom of GBS till time of blood sample aspiration is less than two weeks.
4. The patient did not receive steroidal therapy since the appearance of first GBS symptoms.
5. The patient did not receive intravenous immunoglobulin since the appearance of first GBS symptoms.
6. The patient did not start plasmapheresis sessions since the appearance of first GBS symptoms.
7. Patient is not a known case of autoimmune disorder.
8. Patient is not diabetic

The controls were recruited from Al-Kadhimiya Teaching Hospital medical staff and patient's relatives who were matched with cases according to age and sex on an individual bases.

The serum have been separated from each blood sample and stored in the refrigerator of Hospital's Blood Bank at temperature range from -35°C to -40°C till time of analysis.

The patients that fulfill the inclusion criteria during the period of this study were only eleven patients, (Table 1), with an age and gender matched healthy control was paired with each case, (Table 2). The selected controls showed evidence of effective case-control age matching, (Table 3).

The presence of aPL antibodies was investigated using the ORGENTEC Anti-Phospholipid Screen IgG/IgM kit [ORG 529] for Immunometric Enzyme Immunoassay (EIA) for the quantitative determination of the sum of autoantibodies against Cardiolipin, Phosphatidyl Serine, Phosphatidyl Inositol, Phosphatidic Acid and ß2-Glycoprotein I (IgG and/or IgM class).

Statistical analysis of data was done using SPSS version 13 computer software (Statistical Package for Social Sciences). Frequency distributions for selected variables were done first. The primary outcome for the present study was the IgG and IgM aPL titers, which were non-normally distributed continuous quantitative variables. Such variables are conveniently described by median and inter-quartile range. The non-parametric tests of significance, which do not require the

assumption of normal distribution, are applicable here. The statistical significance of paired case-control differences in median was assessed by Wilcoxon Signed Ranks test. The statistical significance of difference in median between two groups (like males and females) was assessed by Mann-Whitney test. The paired t-test was used to assess the statistical significance of paired case-control difference in mean age (a normally distributed variable). P value less than the 0.05 level of significance was considered statistically significant. The statistical significance, strength and direction of linear correlation between two quantitative variables (one of which being non-normally distributed) were assessed by Spearman's rank linear correlation.

Table 1. Demographic features of the studied cases

No.	Sex	Date of 1st symptom [Day zero]	Day of blood aspiration [D]	Significant history	Need mechanical ventilation	Age (years)	aPL titer	
							IgG U/ml	IgM U/ml
1	Male	05-12-2008	D11	NS	No	55	0.489	0.154
2	Female	11-12-2008	D10	Resp. inf.	No	3.5	16.363	6.311
3	Male	17-12-2008	D6	GIT inf.	No	5.5	11.331	7.03
4	Female	13-12-2008	D10	Resp. inf.	No	60	2.543	2.127
5	Male	28-12-2008	D2	Resp. inf.	No	8	0.498	12.295
6	Male	27-12-2008	D8	Resp. inf.	No	6	6.071	0.829
7	Male	05-09-2009	D5	NS	No	33	5.094	7.009
8	Male	05-11-2009	D4	Resp. inf.	Yes	30	0.093	0.119
9	Female	18-11-2009	D6	Resp. inf.	No	14	0.098	3.254
10	Female	31-12-2009	D4	Resp. inf.	No	6	0.799	9.049
11	Male	27-12-2009	D9	Resp. inf.	Yes	30	0.109	1.15

Table 2. Demographic features of the controls

No.	Sex	Date of Blood aspiration	Note	Age years	Control aPL titer	
					IgG U/ml	IgM U/ml
1	Male	22-12-2008	Hospital analyzer	54	0.042	0.004
2	Female	10-01-2008	Miner head trauma	3	0.97	9.058
3	Male	28-12-2008	Child of a patient	5.5	0.074	0.028
4	Female	03-01-2008	Relative of a patient	60	0.744	0.014
5	Male	22-01-2008	Miner head trauma	8	0.017	0.017
6	Male	24-01-2008	Relative of a patient	6	0.01	0.01
7	Male	12-09-2009	Medical staff	33	0.305	4.69
8	Male	10-11-2009	Doctor	30	0.069	0.041
9	Female	27-11-2009	Relative of a patient	16	8.505	1.363
10	Female	14-01-2010	Candidate of elective tonsillectomy	6	0.075	0.057
11	Male	07-01-2010	Doctor	30	4.909	0.033

Table 3. Case-control age matching

Age in years	Cases	Control	Case-control age difference	P (paired t-test)
Range	3.5-60	3-60	-2 to 1	
Mean	22.82	22.86	-0.05	
SD	20.4	20.21	0.72	0.84[NS]
SE	6.15	6.095	0.218	
N	11	11	11	

Results

The cases that needed mechanical ventilation showed IgG and IgM aPL medians less than that of cases that did not need mechanical ventilation, but the difference between medians failed short of statistical significance, (Table 4).

The cases were also classified according to time between onset of symptoms [Day zero] and blood aspiration into two groups, first week and second week group. By comparing these two groups, the IgG aPL median of second week cases was higher than that of first week cases but the difference between medians was not significant. While the IgM aPL median of first week cases was higher than that of second week cases but the difference between medians failed short of statistical significance, (Table 5).

Table 4. The aPL titer medians difference between cases who need and cases who don't need mechanical ventilation

Cases		Need for mechanical ventilation		P (Mann-Whitney)
		Negative	Positive	
Serum IgG aPL titer	Range Median Inter-quartile range Number of cases	0.098-16.363 2.543 0.494-8.701 9	0.093-0.109 0.101 0.093-** 2	0.06[NS]
Serum IgM aPL titer	Range Median Inter-quartile range Number of cases	0.154-12.295 6.311 1.478-8.04 9	0.1-1.15 0.635 0.119-** 2	0.1[NS]

"**" refers to "cannot be calculated"

Table 5. The aPL titer medians difference between 1st week and 2nd week cases

Cases		1st week cases	2nd week cases	P (Mann-Whitney)
Serum IgG aPL titer	Range Median Inter-quartile range Number of cases	0.093-11.331 0.649 0.097-6.653 6	0.109-16.363 2.543 0.299-11.217 5	0.47[NS]
Serum IgM aPL titer	Range Median Inter-quartile range Number of cases	0.119-12.295 7.02 2.47-9.861 6	0.154-6.311 1.15 0.492-4.219 5	0.1[NS]

The cases IgG aPL median was higher than that of controls in both time strata, but the median case-control difference was not significant, (Table 6). The cases IgM aPL median was also higher than that of controls in both time strata, but the median case-control difference in the total and first week groups was statistically significant, while in the second week group was not significant, (Table 7).

The cases positive correlation with duration of symptoms, while the cases IgM aPL showed a statistically insignificant moderately strong negative correlation with duration of symptoms, (Table 8).

By comparing IgG aPL with IgM aPl, the controls IgG aPL showed statistically significant strong positive correlation with their IgM aPL, while the cases IgG aPL showed a statistically

insignificant moderately strong positive correlation with their IgM aPL, (Table 8).

Table 6. The IgGaPL titer difference between cases and controls

Case-control group		Serum IgG aPL titer			P (Wilcoxon Signed Ranks Test)
		Cases	Controls	case-control difference	
1st week	Range Median Inter-quartile range Number	0.093-11.33 0.649 0.097-6.65 6	0.017-8.50 0.075 0.056-2.35 6	-8.40-11.25 0.603 -2.08-6.40 6	0.25[NS]
2nd weeks	Range Median Inter-quartile range Number	0.10-16.36 2.543 0.29-11.21 5	0.01-4.90 0.744 0.026-2.94 5	-4.8-15.39 1.799 -2.17-10.72 5	0.23[NS]
Total	Range Median Inter-quartile range Number	0.093-16.36 0.799 0.10-6.07 11	0.01-8.50 0.075 0.042-0.97 11	-8.40-15.39 0.724 0.024-6.06 11	0.13[NS]

Table 7. The IgM aPL titer difference between cases and controls

Case-control group		Serum IgG aPL titer			P (Wilcoxon Signed Ranks Test)
		Cases	Controls	case-control difference	
1st week	Range Median Inter-quartile range Number	0.11-12.29 7.02 2.47-9.86 6	0.017-4.69 0.049 0.023-2.19 6	0.078-12.27 4.661 1.43-9.81 6	0.028
2nd weeks	Range Median Inter-quartile range Number	0.154-6.31 1.15 0.492-4.21 5	0.004-9.05 0.014 0.007-4.54 5	-2.74-2.11 0.819 -1.29-1.61 5	0.5[NS]
Overall	Range Median Inter-quartile range Number	0.119-12.295 3.254 0.829-7.03 11	0.004-9.05 0.033 0.014-1.36 11	-2.74-12.27 1.891 0.15-7.002 11	0.026

Table 8. The linear correlation coefficient between aPL titer, age, and duration of symptoms

Group		Serum IgG aPL titer	Serum IgM aPL titer
Control	Serum IgM aPL titer Age (years)	r= 0.636 P = 0.035 r= 0.087 P = 0.8[NS]	r=-0.324 P = 0.33[NS]
Cases	Serum IgM aPL titer Age (years) Duration of symptoms (days)	r= 0.409 P = 0.21[NS] r=-0.516 P = 0.1[NS] r= 0.243 P = 0.47[NS]	r=-0.457 P = 0.16[NS] r=-0.494 P = 0.12[NS]

Note: r = 0–0.2: very weak. r = 0.2–0.4: weak. r = 0.4–0.6: moderately strong. r = 0.6–0.8: strong. r = 0.8–1.0: very strong

To determine the best cut-point values by which the aPL test shows the highest accuracy, we use the ROC (Receiver Operator Characteristic) Curve analysis, which determined the best cut-off value for IgG aPL as 0.084U/ml with 77.3% accuracy and for IgM aPL as 0.088 U/ml with 86.4% accuracy, (Table 9 and Figure 1).

Table 9. Validity parameters of IgG and IgM aPL test at selected cut-off values when used to diagnose GB syndrome differentiating it from healthy controls.

Positive if ≥ cut-off value	Sensitivity	Specificity	Accuracy	PPV at pretest probability =50%	PPV at pretest probability =90%	NPV at pretest probability =10%
Serum IgG aPL titer						
0.084	*100.0*	*54.5*	*77.3*	*68.7*	*95.2*	*100.0*
0.096	90.9	54.5	72.7	66.6	94.7	98.2
0.104	81.8	54.5	68.2	64.3	94.2	96.4
0.207	72.7	54.5	63.6	61.5	93.5	94.7
0.397	72.7	63.6	68.2	66.6	94.7	95.4
0.494	63.6	63.6	63.6	63.6	94.0	94.0
0.621	54.5	63.6	59.1	60.0	93.1	92.6
0.772	54.5	72.7	63.6	66.6	94.7	93.5
0.885	45.5	72.7	59.1	62.5	93.8	92.3
1.757	45.5	81.8	63.7	71.4	95.7	93.1
3.726	36.4	81.8	59.1	66.7	94.7	92.0
5.002	36.4	90.9	63.7	80.0	97.3	92.8
5.583	27.3	90.9	59.1	75.0	96.4	91.8
7.288	18.2	90.9	54.6	66.7	94.7	90.9
9.918	18.2	100.0	59.1	100.0	100.0	91.7
Serum IgM aPL titer						
0.088	*100.0*	*72.7*	*86.4*	*78.6*	*97.1*	*100.0*
0.137	90.9	72.7	81.8	76.9	96.8	98.6
0.492	81.8	72.7	77.3	75.0	96.4	97.3
0.990	72.7	72.7	72.7	72.7	96.0	96.0
1.257	63.6	72.7	68.2	70.0	95.4	94.7
1.745	63.6	81.8	72.7	77.8	96.9	95.3
2.691	54.5	81.8	68.2	75.0	96.4	94.2
3.972	45.5	81.8	63.7	71.4	95.7	93.1
5.501	45.5	90.9	68.2	83.3	97.8	93.8
6.660	36.4	90.9	63.7	80.0	97.3	92.8
7.020	27.3	90.9	59.1	75.0	96.4	91.8
8.040	18.2	90.9	54.6	66.7	94.7	90.9
9.054	9.1	90.9	50.0	50.0	90.0	90.0
10.677	9.1	100.0	54.6	100.0	100.0	90.8

When aPL test used to differentiate GBS cases from healthy controls, the ROC area analysis showed that IgG aPL test showed a statistically significant moderately strong ROC area, while the IgM aPL test showed statistically significant test of high validity, (Figure 1).

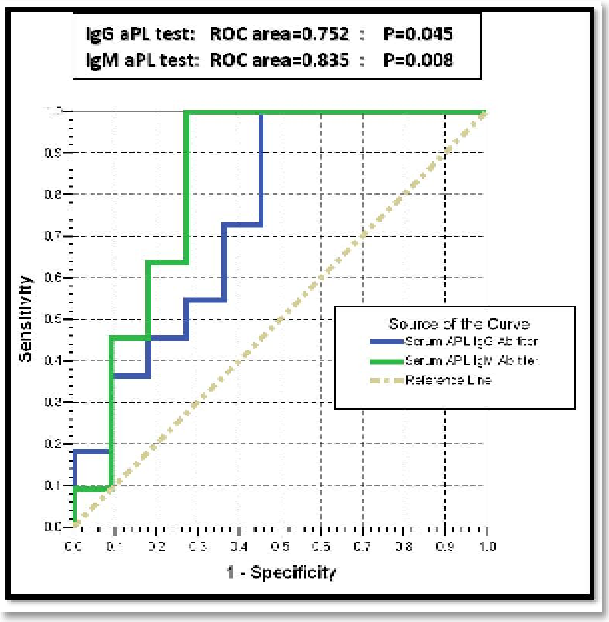

Figure 1. ROC curves for IgG and IgM aPL tests differentiating GBS cases from healthy controls

Using aPL test to differentiate cases with <8 days symptoms from cases with longer duration, the IgG aPL test was not significant, while the IgM aPL test showed high validity, but failed short of statistical significance, (Table 10).

Table 10. The aPL test ROC area and P value for different positive finding

	Test				
	Symptoms duration < 8 days differentiating them from longer duration		Males differentiating them from females		Need for mechanical ventilation Vs not needing
	Serum IgG aPL	Serum IgM aPL	Serum IgG aPL	Serum IgM aPL	
ROC area	0.633	0.8	0.571	0.643	
P value	0.47[NS]	0.1[NS]	0.71[NS]	0.45[NS]	

Note: ROC area = 0.5–0.7: weak. ROC area = 0.7–0.8: moderately strong. ROC area = 0.8–0.9: strong. ROC area = 0.9–1: very strong. ROC area = 1: perfect

Discussion

The current study findings about the elevated IgM aPL titers in cases and it's negative correlation with duration of illness have been reported by Nakos *et al* 2005 [xiv]. The important difference between results of our research and that of Nakos *et al*is that, the later investigates GBS cases treated with IV Ig, and explain the negative correlation of IgM aPL with time from onset of GBS as a result to the blocking effect of IV Ig that have been given to the cases which resulted in the decrement of IgM aPL titers with time. While current study showed this negative correlation as part of the natural history of the GBS without the effect of any given drug.

Our observations can be explained by the history of recent common infections [Respiratory infection, GIT Infection] that are usually reported within two weeks before the onset of GBS. A recent study identified aPL as a common phenomenon in patients with

common infections, independent from the type of infection. These aPL have pathogenic abilities that can be assumed by the presence of prolonged activated Partial Prothrombin Time [aPTT] in the infected patients and these aPL may be part of the immunoreactions against the inoculated pathogen [(xv)].

It is crucial to investigate if there is a direct relation between aPL and specific antigens in Schwann cell membrane or not. Keeping in mind that there is no known antiganglioside antibodies associated specifically with AIDP subtype of GBS [which is the most common subtype] till 2005 [(16-19)].

The findings of current study about IgG and IgM aPL titers difference between cases who need mechanical ventilation and who do not need, may point out to a protective effect for aPL in GBS. This can be explained by two ways. First the ventilated cases immune system; for unknown host factor; cannot synthesize the reactionary aPL in response to the common pathogens which other cases immune system can synthesize aPL in response to them. Second, the ventilated cases have been infected with different pathogen which is not known to be associated with production of reactionary aPL.

The IgM aPL test showed statistically significant very good sensitivity and acceptable specificity when used to differentiate between GBS cases and healthy controls at the cut-off value suggested by ROC curve analysis. These test performance characteristics suggest its use as screening test for GBS among subjects with unclear neurologic symptoms and as a very early additional marker of diagnostic significance for GBS cases in the first two weeks of illness, given the fact that both electromyographic techniques and cerebrospinal fluid biochemical findings lag for days or even weeks in the early diagnosis of the syndrome.

Both IgG and IgM aPL tests have very good overall test accuracy reflected by the tests ROC areas which failed short of statistical significance, when used to differentiate between ventilated and not ventilated GBS cases. Although the possibility of chance effect cannot be excluded as an alternative explanation for any statistically insignificant finding due to the very small number of cases of our research, but these results is still very important. If these results are confirmed by other larger studies, the aPL test can be used as early test to detect cases that will need mechanical ventilation.

Conclusion

From this study it was concluded that:-

1. GBS cases have statistically significant higher IgM aPL titers during the first week and the first two weeks of illness than healthy controls. This reflects a more extensive immune reaction beyond the well known antiganglioside production, which has been related to the demyelination of the peripheral nerves.
2. There is a statistically not significant negative time trend for IgM aPL titer and duration of illness in GBS.
3. The aPL may have a protective effect in GBS.

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