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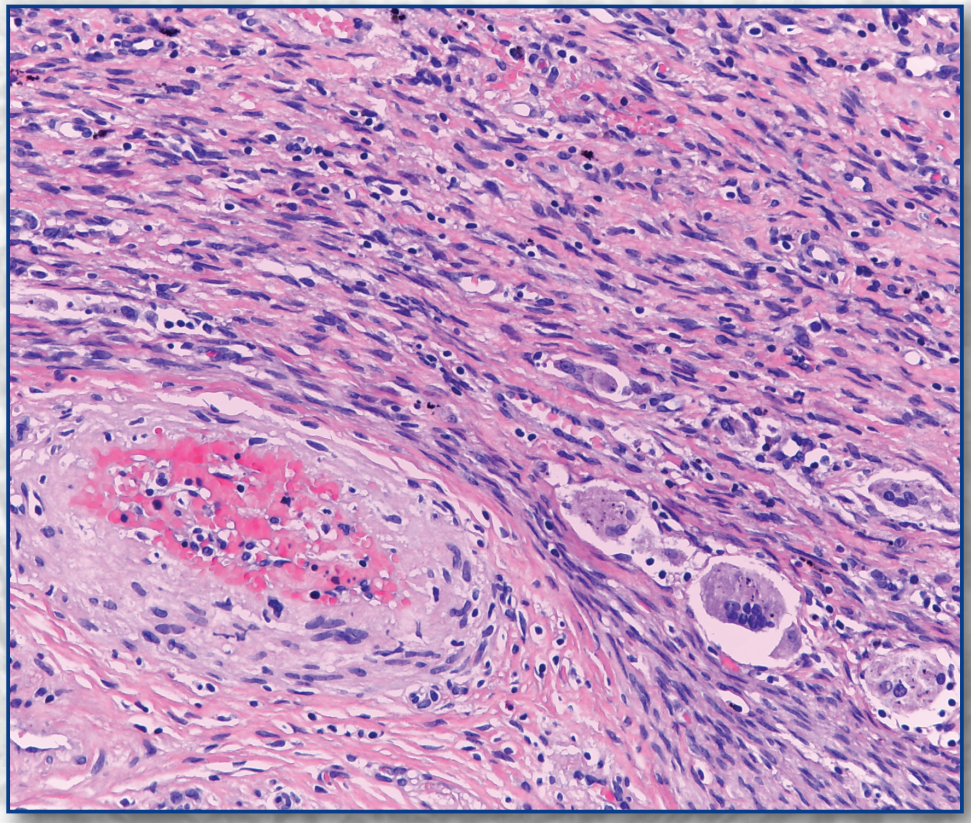

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MORPHOLOGY 30 (1)

Original Articles

Morphometric Properties of the Myenteric Ganglia in the Rat Colorectal Region

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The aim of the present study was to determine the sectional area of ganglia and the sectional area of neuronal perikarya in the proximal colon, distal colon and rectum of the rat. As a result of the conducted morphometric studies, we found that ganglia have similar sectional size in the three regions of the large intestine. In the proximal colon, 42% of the ganglia had sectional area sizes in the range of 1000 μm^2 to 2000 μm^2, whereas in the distal colon (32%) and rectum (21%), ganglia with sizes in the field of 500 μm^2 to 1500 μm^2 were most frequent. In the proximal colon and rectum, neuronal perikarya with a smaller sectional area (an average value of 50 μm^2) were represented more frequently at 52% and 48%, respectively. Neuronal bodies with an average value of 100 μm^2 occur in the distal colon with the highest frequency of 39%.

Key words: myenteric plexus, morphometry, rat, colon, rectum

Introduction

The enteric nervous system (ENS) is a web of neurons embedded in the wall of the gastrointestinal tract [1]. Multiple functions of the ENS related to gastrointestinal

motility, gastric acid secretion, blood flow, nutrient uptake, and interaction with the intestinal immune and endocrine systems have been described in the literature [2]. An essential part of the enteric nervous system is Auerbach's plexus (Myenteric plexus). This myenteric plexus exists between the longitudinal and circular layers of the extrinsic muscle in the intestinal tract [5]. The ganglia that make up the myenteric plexus have been described in the literature being of various sizes and to be interconnected by nerve fibers [4] and have been shown to have properties that are very similar to those of the central nervous system. These properties include the presence of interneurons, glia, extracellular space, synaptic neuropil, multiple synaptic connections and neurotransmitters. The morphology of the ganglia in the plexus myentericus varies in length, width, and area. It has been shown that there is a difference in the morphology of the ganglia at different levels of the large intestine [6]. The average area of neuronal perikarya in different regions of the large intestine can vary widely [3].

The present study aimed to determine at the light microscopic level the sectional area of myenteric ganglia and the sectional area of neuronal perikarya in the rat proximal colon, distal colon and rectum. 2D images of thin paraffin sections from the three regions of interest were examined and compared.

Material and Methods

The scientific studies were carried out on six adult (3-month-old) male Wistar rats with an average weight 250-280 g, delivered from the vivarium of the Faculty of Medicine at Trakia University – Stara Zagora. The animals were housed under an artificial 12-h light/dark cycle and at a temperature of 22°C. Water and food pellets were supplied *ad libitum*. The experiments in this study were approved by the Research Ethics Committee at the Medical Faculty of Trakia University and the Commission for Ethical Treatment of Animals at the Bulgarian Food Safety Agency. All the experiments were carried out in full agreement with the Directive 2010/63/EU on the protection of animals used for scientific purposes. For the purposes of morphometric analyses all rats were anesthetized with 87 mg ketamine/kg of body weight and 13 mg xylazine/kg after simultaneous intraperitoneal injection and transcardially perfused first with cold 0.05 M phosphate buffered saline (PBS) and after that cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.36. Three tissue segments were collected from the proximal colon, distal colon, and rectum. The tissue samples were postfixed in the same fixative for 24 h and then were first washed in tap water, followed by distilled water, dehydrated, and embedded in paraffin. Paraffin blocks were cut into 6 μm tissue sections and mounted on chrome-gelatinized glass slides and then processed for hematoxylin and eosin staining. The slides were examined and photographed with a research microscope Leica DM1000 equipped with a digital camera Leica DFC 290 and the images were processed with Adobe Photoshop CC software.

The graphic analyzing software ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to perform the morphometric analysis of the ganglions and the neurons. As ganglion, we defined an element of the plexus containing at least one nucleated nerve cell profile according to the described methodology of Gabella and Trigg [3]. The cross-sectional area of ganglia and neurons was determined using the ImageJ program by precisely delineating neuronal bodies and well-distinguished ganglion boundaries.

Statistical analysis was performed by GraphPad Prism®6 software (San Diego, CA, USA) and Kruskal-Wallis One Way Analysis of Variance followed by post-hoc pairwise multiple comparison Dunn tests. Statistically significant differences were considered if p-values were <0.05.

Results

In the present study, we measured the sectional area of the ganglia and the area of the neuronal perikarya that form these ganglia in the myenteric plexus of the colorectal region. Transverse and tangential sections of the proximal colon, distal colon and rectum were analyzed. The ganglia of the myenteric plexus were clearly visible and delineated with the classic histological stain hematoxylin and eosin, which allowed us to better define the shape, nucleus and type of the nerve cell (**Fig. 1**). The non-parametric Kruskal–Wallis test to investigate the sectional area of the ganglia with Dunn's multiple analysis was used to compare medians (Med) between the three segments of the large intestine [in the proximal colon (Med = 1701 μm^2), distal colon

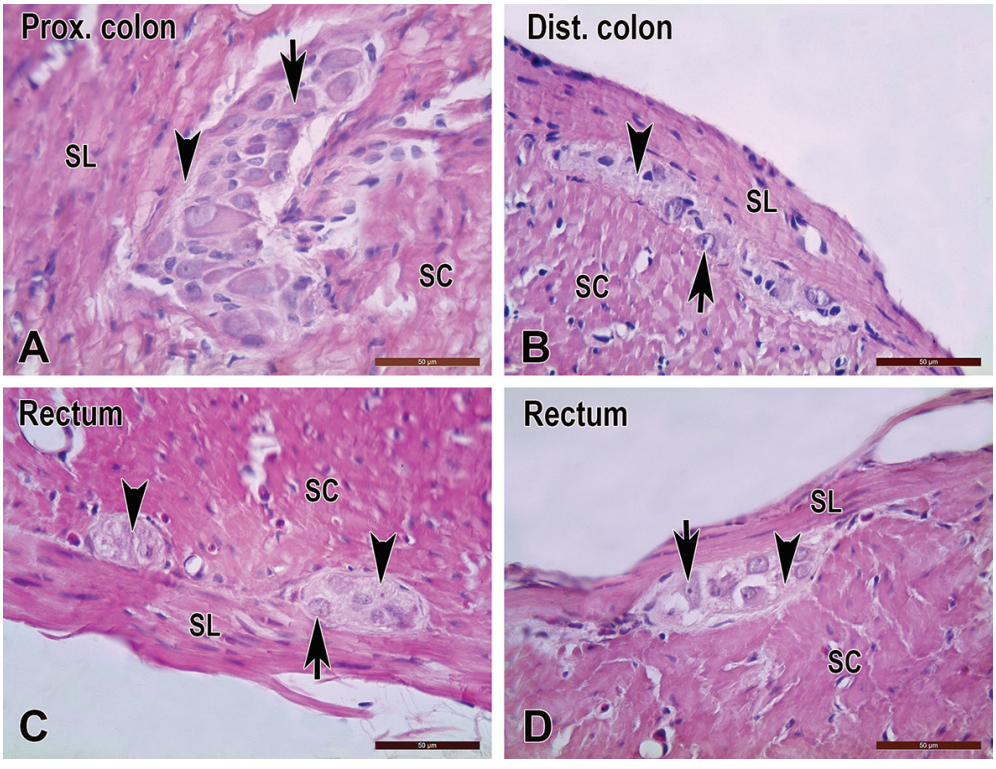

Fig. 1. Hematoxylin and eosin stained sections visualizing the myenteric ganglia and the neuronal perikarya in the proximal colon **(A)**, distal colon **(B)**, and rectum **(C, D)**. Arrows indicate neurons, while arrowheads indicate ganglia. Scale bars: 50 μm.

(Med = 1399 μm²) and rectum (Med = 1893 μm²)]. The sectional area of the ganglia in the three regions of the large intestine did not differ statistically significantly, H(2) = 1.738, p = 0.4194 (**Fig. 2A**). Ganglia with an average cross-sectional area of 1500 μm² (in the range of 1000 μm² to 2000 μm²) occur in the proximal colon with the highest frequency of 42% (**Fig. 2B**). In the distal colon and rectum, ganglia with a smaller cross-sectional area size (an average value of 1000 μm² and the range of 500 μm² to 1500 μm²) were represented more frequently at 32% and 21%, respectively (**Fig. 2B**).

Morphological examinations of the perikarya had also been carried out. We have examined more than 138 – neuronal cell bodies (perikarya) from ganglions of all three segments. The perikarya were well-defined, and in the ganglions that we measured, all of the neuronal bodies were nucleated and easily distinguished from the glial cells. A Kruskal-Wallis test showed statistically significant differences in the cross-sectional area of neuronal perikarya size in the three examined areas of the colorectal region, H(2) = 16.75, p = 0.0002. The medians in all three study regions were different and

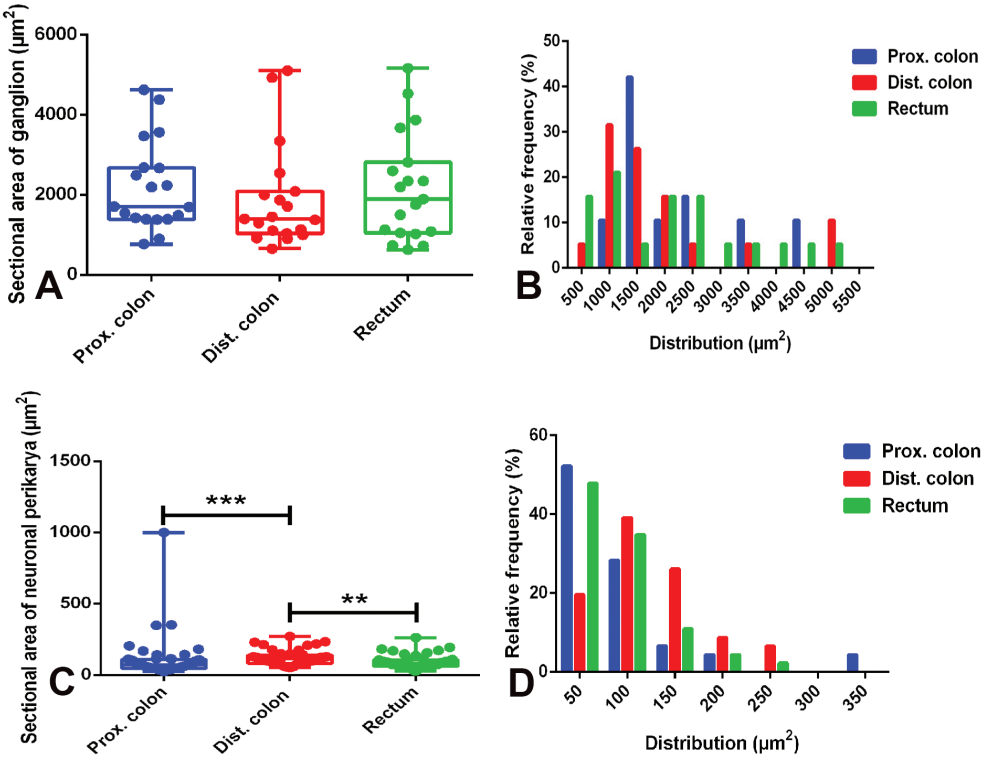

Fig. 2. Box plot diagrams showing statistical comparison of the sectional area of ganglions **(A)** and the sectional area of neuronal perikarya **(C)** in proximal colon (boxes outlined in blue), distal colon (boxes outlined in red) and rectum (boxes outlined in green) in the rat. The data are presented as box plots, where the line within the box represents the median and the boxes represent the second and third quartiles (25–75%). Individual points are the corresponding values. The data compared using the Kruskal-Wallis test, where p** < 0.01; p*** < 0.001. Histograms showing the relative frequency in percent of a distribution of values for the cross-sectional area of ganglia **(B)** and cross-sectional area of neuronal perikarya **(D)**.

as follows: proximal colon (Med = 72.25 μm^2), distal colon (Med = 116.99 μm^2) and rectum (Med = 80.749 μm^2) (**Fig. 2C**). A post hoc pairwise multiple comparison test using Dunn's test showed significant sectional neuronal perikarya size differences between the proximal colon and distal colon, $p < 0.001$ and between the distal colon and rectum, $p < 0.01$ (**Fig. 2C**). In the proximal colon and rectum, neuronal perikarya with a smaller sectional area (an average value of 50 μm^2) were represented more frequently at 52% and 48%, respectively. Neuronal bodies with an average value of 100 μm^2 occur in the distal colon with the highest frequency of 39% (**Fig. 2D**).

Discussion

In this study, we attempt to verify previous data regarding the size of the ganglia and the sectional area of the neurons that make up Auerbach's plexus in the rat colorectal region. Our results showed a slight morphological difference in the sectional area of the ganglia forming the myenteric plexus in the rat's proximal colon, distal colon and rectum, but these differences were not statistically significant. We did not obtain sufficient evidence that a specific pattern of increase or decrease in the size of the ganglia from the upper to the lower parts of the large intestine. This gives us reason to agree with the conclusions drawn by Elżbieta Nowak and her colleagues [6].

The most common size of Auerbach's plexus neuronal bodies in the colon and rectum is between 200 and 400 μm^2 [7]. In the guinea pig myenteric plexus, the average sectional area of neuronal perikarya varies between 300 μm^2 per colon and from 157 μm^2 to 278 μm^2 for the rectum [3]. In our study, however, the mean size of the neuronal bodies we examined in the colon and rectum was generally smaller than that reported by other authors.

The method we used to conduct our morphometric analysis could be criticized. Cross section method was preferred because it was less time-consuming, easier to prepare and better for obtaining more data. Whole mount preparation could have given better opportunities to get more relevant data for the ganglions at the different levels of the large intestine. The sections we mainly used were tangential as they were known to give better accuracy of the size of neuronal cells and ganglions [3]. It had been determined [3] that the tangential sections were visualizing the ganglion much better and would be given better opportunities to examine the neurons, the neuronal nuclei and the glial cells.

Conclusions

The detailed examination provides new scientific data for the overall size difference of the ganglions and neuronal perikarya at the different levels of the large intestine.

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Incidental Isolated Vasculitis of the Uterine Cervix: Morphological Findings and Clinical Significance

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Gynecologic vasculitis has been reported as single-organ vasculitis and less frequently in the context of systemic vasculitis. The cause and pathogenesis of the isolated small vessel vasculitis of the uterine cervix, remain unknown. The purpose of this work is to research clinical, epidemiological, and morphological characteristics of isolated vasculitis of the cervix in long series of cases in order to make valid conclusions.

This is a case study of twelve patients with isolated small vessel vasculitis discovered incidentally in surgical specimens of the female genital tract. The latter are verified histologically, supported with immunohistochemistry, and studied in a retrospective analysis. The awareness of this rare type of cervical pathology is important for the daily diagnostical practice of the pathologist and the gynecologist, to exclude the further need of follow up or treatment.

Key words: vasculitis, cervix, uterus, conisation

Introduction

Systemic vasculitis is a group of heterogeneous conditions characterized by inflammation of the blood vessels, commonly affecting various vascular territories and organs [8]. Gynecologic vasculitis has been reported as single-organ vasculitis and less frequently in the context of systemic vasculitis. The significance of isolated vasculitis and whether isolated cases represent part of true systemic vasculitis, or a local inflammatory process is unclear, but in both cases the microscopical picture reveals PAN or PAN- like vascular lesions [12].

The cause and pathogenesis of the isolated small vessel vasculitis of the uterine cervix, remain unknown.

In this study we reviewed twelve cases of vasculitis of the uterine cervix diagnosed on pathology specimens in correlation with previous diagnostic and

surgical intervention, aiming to assess the significance of isolated vasculitis identified histopathologically in the gynecological tract.

Materials and methods

A retrospective study of twelve cases with isolated vasculitis involving the uterine cervix was performed at the Departments of Pathology, Jossigny Hospital, Jossigny, France and St. George University Hospital of Plovdiv, Plovdiv, Bulgaria for a 21-year period (2002–2022). Clinical and follow-up data were obtained from medical records and surgical pathology files. We inspect the clinical data for performed diagnostical and surgical interventions and history about autoimmune disorders and systemic vasculitis.

The study was approved by the local Ethics Committees of the hospitals. There is no patient – identifying information in any of the materials presented, and hence patient consent was not obtained.

All specimens were routinely fixed in 10% buffered formalin and embedded in paraffin for histological evaluation. Standard 4-μm-thick sections were cut from paraffin blocs.

Retrospectively, tissue sections from each case were observed independently by two pathologists. Sections were stained with haematoxylin-eosin (HE) and haematoxylin-eosin-saffron (HES).

The immunohistochemical study was performed using standard avidin-biotin peroxidase complex technique. The following primary antibodies (Dako, Caripenteria, California, USA, Leica Biosystems and Diagomics, France, Diagomics, France) were used: anti-CD3 (1:150, clone SP7); anti-CD 4 (1:50, clone RBT-CD4), anti-CD8 (1:100, clone SP16), anti-CD20 (1:100, clone L26); and anti-PD-L1 (1:200, clone QR1).

Results

Epidemiological, clinical and pathological data is summarized in **Table 1**.

In eleven of twelve cases, we found isolated, more or less, necrotizing small vessel vasculitis and in one we detected lymphocytic vasculitis (**Fig. 1A**).

All of the vascular findings were sustained with positive immunohistochemical markers CD3/C8, spotting the T-cell population in the lesions, while CD20, dentifying B-cells, is scantily positive (**Fig. 1B, 1C**).

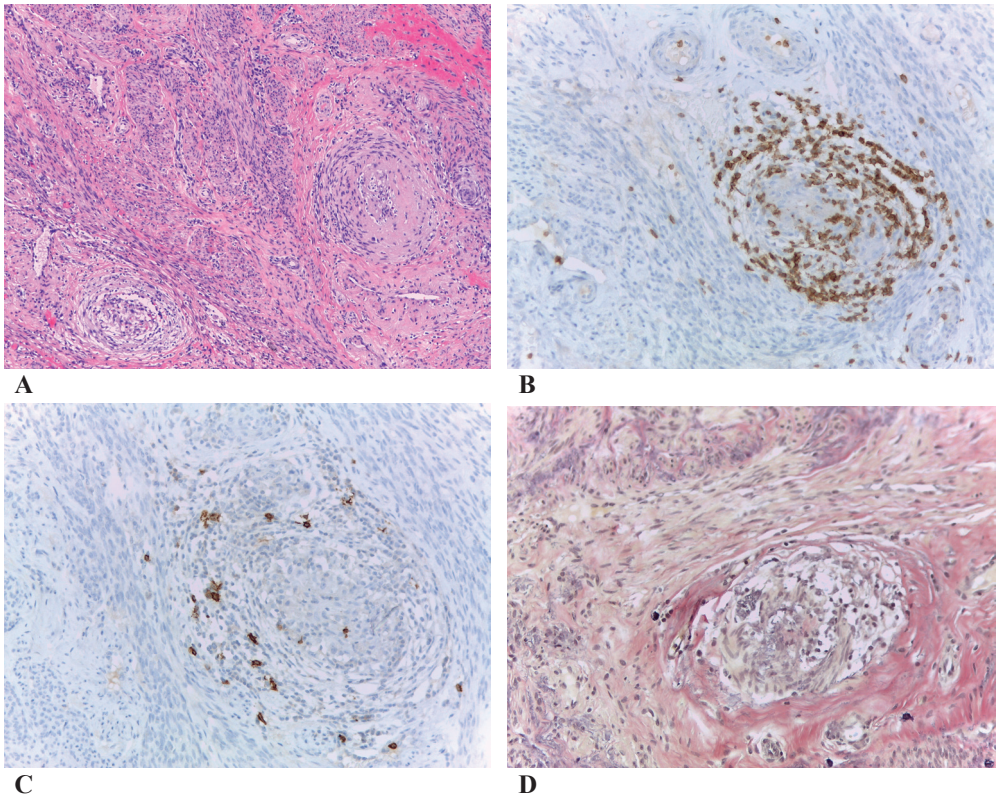
Staining with orcein showed thickening and focal dissociation of internal elastic membrane, which proof the arterial localisation of the lesion (**Fig. 1D**).

Other histological changes that we found in the cervix are resorptive inflammation with formation of foreign body granuloma in five of the patients (**Fig. 2A**), and stromal cervical fibrosis in the other seven (**Fig. 2B**). Absence of residual epithelial lesions were found in three of the speciments.

The age of the patients ranged from 28 to 82 years (mean age 45.5 years).

The main cervical pathology is the high-grade dysplastic changes of the epithelium, four cases with CIN III and one with carcinoma in situ. Once in a case we diagnosed CIN II, chronic cervicitis, isthmic cervical sclerosis and cervical polyp.

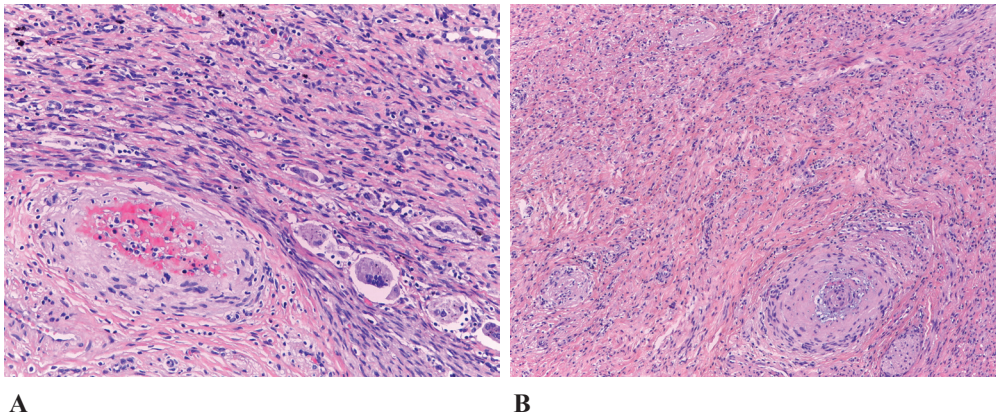
Eight of the cases have data for targeted diagnostical (smears, colposcopy-guided cervical biopsy) or surgical intervention (conisation) in the past months.



A **B**

C **D**

Fig. 1. Morphological and immunohistochemical characteristics of incidental isolated vasculitis of the uterine cervix. Necrotizing small vessel cervical vasculitis (right) or lymphocytic vasculitis (left) **(A)**. Immunohistochemically, transmuralniat inflammatory infiltrate rich of CD3+/ C8+ T-lymphocytes **(B)**, while CD20+ B – cells, is scantily positive **(C)**. There are thickening and focal dissociation of internal elastic membrane **(D)**. *Haematoxylin-eosin-saffron, × 100 (A); anti-CD3 and anti CD8, × 200 (B and C); elastica van Gieson staining, × 200 (D).*



A **B**

Fig. 2. Associated histological changes near to the incidental isolated vasculitis of the uterine cervix. Resorptive inflammation with formation of foreign body granuloma **(A)** and/or stromal fibrosis **(B)**. *Haematoxylin-eosin-saffron, × 200 (A) and × 100 (B).*

The mean time between the interventions and the apearance of the vasculitis is found to be 13,1 months.

Preoperative diagnosis and the main cervical pathology matched in the majority of the cases, in the others the previous diagnostical intervention had therapeutic outcome. After investigating the uterine corpus, we found adenomyosis in three, endometrial polyp in two and leyomyomas in one and absence of pathological changes in four of our cases.

No data for systemic vasculitis or other autoimmune diseases were found, exept one case with lymphocytic colitis without vasculitis.

Discussion

The clinical course of systemic PAN is mainly influenced by the involvement of circulation of brain, kidney or heart, whereas the severe prognosis of PAN results in acute cerebral hemorrhage or mostly in acute or chronic heart and renal failure. Treatment of choice is a combination of steroids and cyclophosphamide, but in cases of severe hemorrhage surgery is mandatory [10]. Isolated necrotizing arteritis of the PAN-type localized to the female genital tract is rare. In 1932, Plauth published the concept of isolated vasculitis of the uterine cervix [11]. Since then, 50 cases of isolated necrotizing vasculitis of the female genital tract have been reported [1,3,5,7,9,14,15,16].

Information about other etiological factors causing vasculitis of the female genital tract, like cytomegalovirus infection or secondary to local pressure exerted by a pessary, are found in the literature [2,4]. Drugs, applied during cone biopsy and used systemically may cause isolated vasculitis of the cervix [13,14].

Although it has been encountered as a self-limited lesion confined to a single organ, it can also be the first manifestation of systemic PAN, after which involvement of other organs is discovered. The lesions seen in isolated necrotizing arteritis are histologically indistinguishable from those of classic PAN, which are characterized by a segmental or circumferential fibrinoid necrosis with inflammatory infiltrate composed mainly of mononuclear cells [10], a finding that we report in our work. A study with 46 cases representing vasculitis affecting the female genital tract concluded that most of the cases appear to be examples of isolated vasculitis similar in histology and outcome to isolated arteritis at other sites [6]. It has been mentioned that foreign materials introduced by cone biopsies have been proposed to induce immune-complex mediated responses, with formation of foreign body reaction. They report 15 patients with a previous gynecological operations, but no comment about connection with developing of vascular lesions was made [6,11]. A clinicopathologic and immunohistochemical study of eleven cases of isolated necrotizing arteritis of the female genital tract report and support the relationship of the vascular lessions with hypersensitivity reaction to foreign materials after cone biopsy or a curettage [5].

We describe a series of cases of necrotizing vasculitis of the cervix. In all of the cases we found accompanying stromal changes: resorptive inflammation with formation of foreign body granuloma or stromal cervical fibrosis. In the majority of the cases we have data for previous surgical procedure. Our contribution to the literature about the etiology of isolated PAN-like cervical arteritis is the relationship between the vascular changes and previous surgical intervention, supporting the hypothesis for the role of hypersensitivity reaction caused by past procedures.

Our study enriches the literature with more information, relating the isolated necrotizing arteritis of the cervix caused by previous surgical or diagnostical intervention. The mean time between the interventions and the diagnosis of the vasculitis is found to be 13,1 months.

Diagnosis of true vasculitis is very important since true vasculitis requires systemic treatment. Presence of clinical data about previous diagnostical and theraptical gynecological operations on the cervix and absence of systemic vasculitis or other autoimmune diseases, exclude the further need of follow up or treatment.

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Table 1. Clinical details, pathological findings and follow-up (12 cases).

Number	Age	Previous surgical intervantions conisation,biopsy, ets. / months ago	Recent surgical intervantion	Preoperative diagnosis	Type of found vasculitis
1	53	conisation/18	total hysterectomy	Ca in situ	necrotizing vasculitis
2	45	cervical biopsy/12	total hysterectomy	adenomyosis, pelvix pain with metrorrhagia	necrotizing vasculitis
3	52	conisation/14	total hysterectomy	CIN II	necrotizing vasculitis
4	32	conisation/1	total hysterectomy	Ca in situ with microinvasion	necrotizing vasculitis
5	82	non	total hysterectomy	isthmic cervical sclerosis	necrotizing vasculitis
6	57	endometrial biopsy	cervical popypectomy	metrorrhagia postmenopausal	lymphocytic vasculitis
7	28	cervical biopsy/2,24,60	conisation	CIN III	necrotizing vasculitis
8	40	conisation/17	total hysterectomy	CIN I and AIS	necrotizing vasculitis
9	42	cervico-vaginal smears, vaginal biopsy/24,2	conisation	ASC-H,HSIL, VIN III	necrotizing vasculitis
10	40	cervical biopsy and conisation/2,60	conisation, cervical stenosis	ASC-H,HSIL, VIN III	necrotizing vasculitis
11	45	abortion, curretage/ 72	total hysterectomy	menometroragia, myoma utery	necrotizing vasculitis
12	30	conisation	total hysterectomy	invasive squamous cell carcinoma pT1b	necrotizing vasculitis

Number, number of cases; Age, age in years; CIN, cervical intraepithelial neoplasia; AIS, adenocarcinoma HSIL, high grade squamous intraepithelial lesion; VIN, vulvar intraepithelial neoplasia

IHC	Main cervical pathology	Additional cervical pathology	Pathological changes in the uterine corpus	History of diseaces with immune genesis
CD3+,CD8＋ CD20±	CIN III	stromal fibrosis	adenomyosis	non
CD3+,CD8＋ CD20±	chronical cervicitis	stromal fibrosis	adenomyosis	non
CD3+,CD8＋ CD20±	CIN II	resorptive inflamation with granuloma type foreign body	endometrial polyp	non
CD3+,CD8＋ CD20±	Ca in situ	resorptive inflamation with granuloma type foreign body	non	non
CD3+,CD8＋ CD20±	isthmical scrlerosis	inflamatory stromal fibrosis and hyalinosis	leiomyomas	microscopic colitis
CD3+,CD8＋ CD20±	cervical polyp	inflamatory stromal fibrosis	endometrial polyp	non
CD3+,CD8＋ CD20±	CIN III	inflamatory stromal fibrosis	non	non
CD3+,CD8＋ CD20±	no residual epithelial lesions	resorptive inflamation with granuloma type foreign body	endometrial polyp	non
CD3+,CD8＋ CD20±	CIN III	stromal fibrosis	non	non
CD3+,CD8＋ CD20±	CIN III	resorptive inflamation with granuloma type foreign body	non	non
CD3+,CD8＋ CD20±	non	stromal fibrosis	adenomyosis	non
CD3+,CD8＋ CD20±	no residual tumor lesions	resorptive inflamation with granuloma type foreign body and stromal fibrosis	non	non

in situ; ASC-H, atypical squamous cells, cannot rule out high grade squamous intra-epithelial lesion;

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Influence of Cholesterol and Hydrogen Peroxide, Alone and in Combination, on Sperm Morphology

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We studied the effects of increased cholesterol on the morphology of ejaculated human spermatozoa by manipulating cholesterol content of the medium. The results, surprisingly, showed an increase of head and neck abnormalities similar to that caused by hydrogen peroxide, a known source of oxidative damage. The highest rate was observed in samples exposed to both substances, showing that cholesterol did not protect spermatozoa from the damaging action of H_2O_2, and maybe even exacerbated it. We could hypothesize that conferring excessive rigidity to sperm cell membrane by cholesterol beyond a certain point is no longer beneficial to the integrity of the membrane, and may actually predispose it to new types of damage. The observed increased rate of sperm decapitation could indicate that the reduced fluidity of cell membrane, combined with the active motility of ejaculate spermatozoa, could lead to breakage in the neck region as a focal point of mechanical strain.

Key words: Spermatozoa, oxidative damage, cholesterol, morphological defects

Introduction

Sperm cells have a characteristic pattern of changes in the membrane cholesterol content: it increases during epididymal storage to ensure stability [8], remains relatively high in the ejaculate, and then decreases during capacitation to prepare the cell membrane for acrosome reaction and gamete fusion [3]. Despite the importance of this dynamics, still little is known about the effects of cholesterol membrane content on sperm structure and function. Some authors have found higher levels of cholesterol in seminal plasma of patients with teratozoospermia than in men with normal sperm morphology [4], while others have reported a positive association between the

percentage of morphologically normal spermatozoa and the amount of cholesterol in seminal plasma [1]. These contradictions may be due to the complexity of processes associated with the natural secretion and regulation of cholesterol, and may be resolved by experiments directly exposing sperm cells to different concentrations of cholesterol. So far, the influence of higher cholesterol content in the medium has been investigated by researchers trying to improve cryopreservation of spermatozoa of farm animals. Cholesterol addition has been shown to increase viability and motility of cryopreserved spermatozoa by improving their membrane integrity and preventing apoptosis [10]. However, little is known about the effects of cholesterol on sperm cells outside the context of cryodamage. Among the questions that should be addressed are whether cholesterol would have a similar protective action in physiological conditions, and whether it could partially prevent the harmful effects of other agents. A major source of damage to sperm cells are reactive oxygen species such as hydrogen peroxide [7]. The aim of the present study was to evaluate the morphology of human spermatozoa treated with cholesterol, hydrogen peroxide, and a combination of the two.

Materials and Methods

Cells used in this study were ejaculate spermatozoa provided by eight healthy volunteers aged 20-40. Informed consent was obtained from all volunteers. Fresh semen samples were incubated at 37°C for 30 minutes in order to achieve semen liquefaction. Sperm count, motility and morphology were determined. Only normozoospermic samples (according to WHO criteria, and Kruger strict criteria) were used for further investigation, therefor two of the volunteers' samples were no longer used in the study. The remaining semen samples were diluted with gamete buffer (COOK Medical, USA) to a concentration of 2 million cells/mL and divided into four groups. The first group was used as a control and was incubated at 37°C for 3.30h in gamete buffer. Spermatozoa in the second group were incubated for 30 min at 37°C in gamete buffer, and then H_2O_2 was added to 400 μM and incubation continued for another 3h in order to induce oxidative stress. Spermatozoa in the third group were incubated with cyclodextrin cholesterol (Sigma-Aldrich, Germany) added to a ratio of 2 mg per 120 million spermatozoa [6], for 3.30h. Spermatozoa in group 4 were treated with cyclodextrin cholesterol for 30 min., and then H_2O_2 was added to 400 μM and incubation continued for another 3h. Total incubation time for all samples was 3.30h. After incubation all samples were centrifuged for 10 min at 1400 rpm, and diluted in fresh gamete buffer. Spermatozoa were stained as described before [5], with slight modifications. Briefly, they were fixed on slides with cold ethanol for 5 min, air-dried, stained with 0.4% buffered stock solution of Giemsa (Sigma-Aldrich, Germany) diluted 1:20 with distilled water for 15 min, rinsed with distilled water and air-dried before observation. For each slide, 2×100 spermatozoa were counted and their morphology was evaluated. Based on their morphological features, sperm cells were subdivided into four categories: normal, with head or neck abnormalities, with middle piece abnormalities, and with abnormalities of the tail distally of the middle piece. In cases of multiple abnormalities, only the predominant defect was taken into account.

Results and Discussion

The proportions of the four morphological categories of sperm cells in untreated and treated samples are shown in **Table 1**. In the control group, no major changes in morphology were observed after the incubation (**Fig. 1A**). In all other groups, the tail distally of the neck seemed largely unaffected by the treatments, but abnormalities in the head and neck regions were more common (**Fig. 1B, C, D**). In cholesterol-treated samples, the proportions of abnormally sized heads (too large or two small) were increased, and spermatozoa with head detached from the tail, a very severe defect of the neck, were often observed (**Fig. 2**). In most cases the detached head and tail were visible in a single vision field which is indicative that the detachment occurred during the spermatozoa manipulations (**Fig. 3**). To our knowledge, such effects have not been previously reported. They could be a direct result of cholesterol's ability to change thickness, rigidity, compressibility, and curvature of biological membranes [11]. In spermatozoa these effects could be more substantial due to membrane composition specifics, namely significant amount of poly-unsaturated fatty acids (PUFA). In model membrane systems cholesterol leads to the segregation of phospholipids containing PUFAs' resulting in the formation of disorganized membrane regions and rearrangement of the lipid rafts [9]. Presence of cholesterol in sperm cell membranes in concentrations exceeding the physiological could improve cryopreservation survivability [6] but according to our results, prolonged exposure and incubation leads to generation of significant morphological defects and membrane stress points that result in decreased cell stability.

Table 1. Proportions of different morphological categories of sperm cells (in percentages) in control samples and samples treated with cholesterol, H_2O_2 and cholesterol plus H_2O_2, respectively.

	Normal morphology	**Abnormal head or neck**	**Abnormal middle piece**	**Abnormal distal tail**
Control	45%	25%	18%	12%
Cholesterol	35%	33%	19%	13%
H_2O_2	33%	36%	19%	12%
Cholesterol + H_2O_2	17%	57%	17%	9%

In the group treated with H_2O_2, the results were predictable. PUFAs are particularly sensitive to oxidative stress due to the double bonds. Lipid peroxidation leads to loss of membrane integrity and morphological abnormalities with a strict dose dependent trend [2]. H_2O_2 damage is usually associated with damage to the middle piece and loss of motility, while our results demonstrate increased morphological abnormalities in the head and neck, which could be explained by the higher H_2O_2 concentrations used. Compared to the cholesterol group, the H_2O_2 group had more misshaped heads and necks, and fewer enlarged heads and decapitated spermatozoa.

The group treated with both cholesterol and H_2O_2 showed the least amount of normal spermatozoa (17%), and the largest percentage of spermatozoa with head and neck defects (47%) (**Table 1, Fig. 2D**). Results indicate that the added cholesterol is unable to protect sperm cell membranes from oxidative stress at high H_2O_2 concentrations, and furthermore, leads to increased damage and subsequent changes in morphology. This could be caused either by the cholesterol induced segregation of PUFAs and a resulting massive oxidative damage to highly disorganized membrane domains that are not present in untreated spermatozoa, or cholesterol itself becomes a target of H_2O_2 which leads to the formation of cholesterol-hydroperoxyde and intensification of oxidative damage. Further research is needed to determine the cause of the observed damage aggravation.

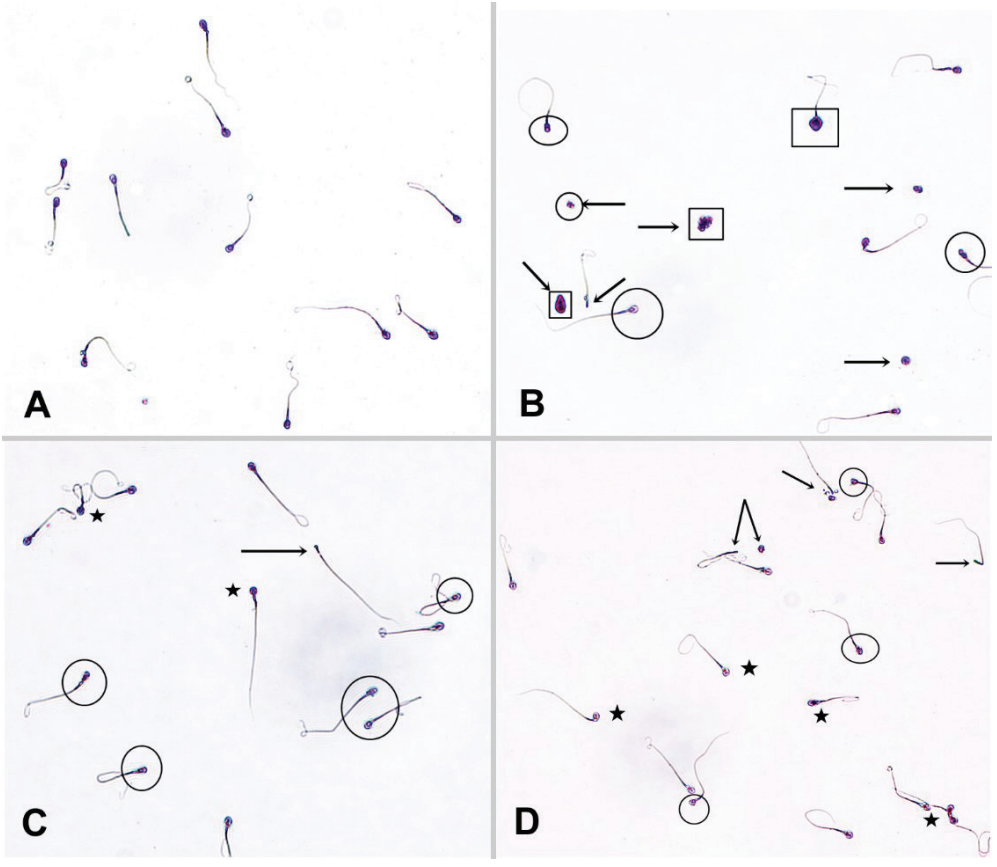

Fig. 1. A – control group, B – cholesterol treated spermatozoa, C – H_2O_2 treated spermatozoa, D – spermatozoa treated with both cholesterol and H_2O_2. Designations: → severed head/tail; ○ small head; □ large head; ★ abnormal morphology. Original magnification 400×.

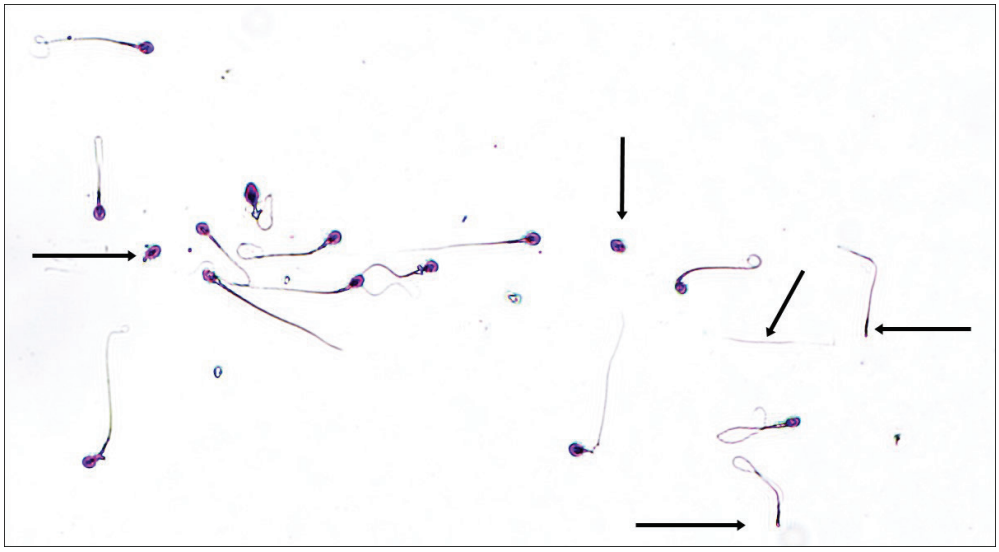

Fig. 2. Abnormal morphology and spermatozoa breakages in cholesterol treated samples. Designations and original magnification as in **Fig. 1**.

Conclusions

While cholesterol may be protective for sperm cells during cryopreservation, under physiological conditions it causes morphological abnormalities at a rate comparable with that of the known damaging agent H_2O_2, and provides no protection against H_2O_2 damage.

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Immunochemical Evaluation of Biomarkers of Carcinogenesis, Angiogenesis, Neuro-Cancer Interactions and Demyelination in Cadmium Chloride-Induced Testicular Toxicity in Rats.

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Cadmium is a carcinogen. Neurotransmitter-cancer interaction and tissue-innervation impact cancer survival. This study examined repro-protective and neuro-protective potentials of MO11 (isolated from *Moringa oleifera* leaves) and MS06 (isolated from *Musa sapientum* suckers) in cadmium chloride ($CdCl_2$)-induced testicular toxicity. Twenty-four adult male rats were randomly divided into 6 groups. Group 1 was control. Groups 2-4 and 6 received intraperitoneal single-dose of $CdCl_2$ (Day 1). Groups 3, 4 and 6 were post-treated with MO11-dose, MO11+MS06-doses and Doxorubicin-dose respectively, while Group 5 received Olive Oil-dose (vehicle) from Days 1-17. Quantitative tissue enzyme-linked-immunosorbent-assays of biomarkers of carcinogenesis and neuro-cancer interaction in testicular homogenates were evaluated. Data were analysed using Mann-Whitney-U test ($p \leq 0.05$). Results showed downregulations of MBP, Caspase-3 and sVEGFR, but upregulations of Dopamine, Glutamate and Cytochrome-p450 in Groups 3, 4 and 6, compared with Group 2. Overall, $CdCl_2$-induced testicular toxicity, angiogenesis and neuro-cancer interaction were ameliorated by post-treatments with MO11 and MS06.

Key words: Cadmium, testicular toxicity, drug candidates, neuro-protection, anticancer effects

Introduction

Cadmium (Cd) is one of the 10 chemicals of concern for human health and a human carcinogen as categorized by the World Health Organization, National Toxicology program and the International Agency for Research on Cancer [7, 16, 37]. Commercially, Cd is used in home appliances such as television screens, lasers, batteries, paint pigments and cosmetics [8], hence Cd is an environmental toxin of concerns. Cd is equally an established carcinogen in animals [7, 16, 37]. Cd-exposure resulted in decreased testicular weight [7, 30], necrosis of spermatogenic epithelium and degenerations of testicular seminiferous tubules [7], depletion of germ cells, testicular necrosis, carcinogenesis and sterility in *in vivo* models, and adverse effects on testicular cells in *in vitro* models [1, 33]. Specifically, Cd-induced testicular toxicity resulted in significant decreases of sperm count, sperm motility, testosterone synthesis, spermatogonia, Sertoli cells and Leydig cells in rat testis [23, 40]. In addition, human Cd-exposure was linked with nervous system dysfunctions resulting in impaired learning capacity, headache and vertigo, decreased cognitive functions, olfactory dysfunction, poor vasomotor functioning, peripheral neuropathy, poor equilibrium and balance coordination, and developments of neuro-degenerative diseases (such as Parkinson's disease and Alzheimer's disease) [16].

Neurotransmitters (such as Dopamine and Glutamate) released by nerve fibres within tumour micro-environment influence cancer metastasis via complex neurotransmitter-cancer interactions; and have become targets for therapeutic interventions [19]. Both Dopamine [29] and Glutamate [35] are expressed in the testis. Myelination is required for proper functioning of nerve fibres supplying tissues, and Myelin Basic Protein (MBP) is a biomarker of de-myelination [3]. The mechanism underlying metastasis remains poorly understood. Hence, the biology of innervation of tissues is relevant in the search for anticancer drugs from plants or other sources.

Moringa oleifera (MO) and *Musa sapientum* (MS) are ethno-medicinal plants which are well grown across the world [5]. 'MOF6' is fractionated from ethanolic extract of MO leaves. *In vivo* neurobiological analyses showed that MOF6 possessed significant antioxidant and neuro-protective potentials against Cuprizone-induced cerebellar damage in rats [28], and ameliorated Sodium arsenite-induced neurotoxicity and dysregulations of Acetylcholinesterase concentrations in rats [4]. Furthermore, MOF6 and 'MSF1' (fractionated from ethanolic extract of MS suckers) exhibited hepato-protective and anticancer potentials in 7,12-Dimethylbenz[a]anthracene-induced hepato-toxicity in rats [5].

Will Cd-induced toxicity and decreased testicular weight of the testes change testicular levels of biomarkers of Dopamine and Glutamate? Is de-myelination associated with Cd-induced toxicity and decreased testicular weight? Is angiogenesis associated with Cd-induced toxicity and decreased testicular weight? What are the effects of post-treatments with MO and MS on possible mechanisms underlying Cd-induced toxicity and decreased testicular weight?

Single intraperitoneal administration of 1.5 mg/kg body weight of $CdCl_2$ resulted in significantly decreased diameters of seminiferous tubes and thickness of the germinal layer, and decreased numbers of spermatogonia, Sertoli and Leydig cells in rat testis compared with control group which received sterile distilled water on Days 13, 25 and 49 [23]. In addition, single intraperitoneal administration of $CdCl_2$ resulted

in significantly decreased sperm motility, sperm count and testosterone levels in the $CdCl_2$-only treated group compared to control group on Days 13, 25 and 49 in rats [23]. Furthermore, there was significantly increased level of Malondialdehyde in the $CdCl_2$-only treated group compared to control group on Days 13, 25 and 49 in rats [23]. Sustained increased Malondialdehyde level implied increased oxidative stress which is implicated in carcinogenesis [3, 22].

Toxin-induced cyto-toxicity resulting in decreased tissue or organ weight is associated with necrosis which is further associated with carcinogenesis [22]. Cytochrome p450 plays a role in steroidogenesis [34], and it is an established biomarker of drug metabolism and carcinogenesis [29]. Caspase-3 plays regulatory roles in spermatogenesis [36], and it is a biomarker of apoptosis and carcinogenesis [3]. In addition, sVEGFR is opined to be a biomarker of testicular inflammation and toxicity [14], and it is an established biomarker of angiogenesis [23] while MBP is a biomarker of myelination [3]. Furthermore, Dopamine and Glutamate are neurotransmitters of importance in evaluation of neuro-cancer interactions [19], Cadmium exists as a divalent cation, complexed with other elements, such as cadmium chloride ($CdCl_2$) [7, 8, 16, 37]. Therefore, in-order to answer stated research questions, this study examined the mechanisms underlying *in vivo* $CdCl_2$-induced testicular toxicity and the ameliorative potentials of MO11 (isolated from *Moringa oleifera* leaves) and MS06 (isolated from *Musa sapientum* suckers) on tissue levels of Cytochrome-p450, Caspase-3, sVEGFR, MBP, Dopamine and Glutamate in the testes of adult male rats in $CdCl_2$-induced testicular toxicity.

Materials and Methods

Ethics approval

Ethical approval for this study was sought and received from the Ethical Review Committee of the University of Ilorin, Nigeria. Appropriate measures were observed to ensure minimal pain or discomfort of rats used in this study. The ethical approval number is UERC/ASN/2018/1161. Furthermore, this research study was conducted in accordance with the internationally accepted principles for laboratory animal use and care as provided in the European Community guidelines (EEC Directive of 1986; 86/609/EEC), the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and the Guidelines of the U.S. Public Health Service and NIH regarding the care and use of animals for experimentation (NIH publication #85-23, revised in 985).

Authentication and deposition of MO leaves and MS suckers

Freshly cut MO leaves and MS suckers were obtained from forest reserves in Ilorin, Kwara State of Nigeria. The plants' samples were authenticated and assigned Herbarium Identification Numbers: UILH/001/1249 and UILH/002/1182 respectively at the Department of Botany of study institution.

Evaluations of antioxidant and antimicrobial activities of MO and MS fractions

Antioxidant activities of plants' extracts were evaluated using modified 2,2- diphenyl-1-picrylhydrazyl method as previously described [9], Antimicrobial activities of plants' extracts were evaluated by testing cyto-toxic potentials of each fraction against growths of *Escherichia coli* and *Salmonella tiphimurium* as previously described [12].

Extractions of MO11 and MS06 from MO leaves and MS suckers

MO11 and MS06 were extracted as final therapeutic isolates from MO leaves and MS suckers following series of antioxidant analyses, antimicrobial cyto-toxicity potentials, column chromatography and Liquid chromatography-mass spectrometry as previously reported [5, 6].

Animals

Twenty-four (24) adult male Wistar rats (average weight of 155 g and 2 months of age) were purchased from a colony breed at Badagry in Lagos state, Nigeria. The rats were acclimatized for a week, and randomly divided into 6 groups with 4 rats per group. The rats were kept under standard conditions. The body weights of rats in grams were computed on daily bases using electronic SF-400C compact weighing scale (Valid Enterprise, Mumbai, India).

Experimental design

MO11 and MS11 were dissolved in Olive Oil (vehicle). Rats of Control Group 1 received physiological saline only for 17 Days (Days 1 – 17). The dose of 1.5 mg/kg body weight $CdCl_2$ was used to induce testicular toxicity as determined from a previous study which investigated the effects of low dose of $CdCl_2$ on the testis [23].

Each rat of Experimental Groups 2 – 4 and 6 received single intra-peritoneal administration of 1.5 mg/kg body weight $CdCl_2$ (Sigma-Aldrich, Japan Co.) on Day 1. Rats of Group 2 (Toxic Control) were left untreated throughout experimental procedure for 17 Days (Days 1 – 17). Thereafter, rats of Group 3 were post-treated with oral administration of 15 mg/kg body weight of MO11 for 17 Days (Days 1 – 17). Rats of Group 4 were post-treated with oral administration of combined mixture of 15 mg/kg body weight of MO11 and 7 mg/kg body weight of MS06 for 17 Days (Days 1 – 17). Rats of Group 5 received only oral administration of 1 ml/kg body weight of Olive Oil (vehicle) for 17 Days (Days 1 – 17), and were not exposed to administration of $CdCl_2$. Rats of Group 6 were post-treated with oral administration of 3.35 mg/kg body weight of Doxorubicin (standard anticancer drug – Positive Control) for 17 Days (Days 1 – 17).

Completion of experimental procedures

No anesthesia were used for animal sacrifice as approved by the University Ethical Review Committee after evaluation of experimental protocol based on the fact that the biomarkers to be examined include enzymes such as Caspase-3 and metabolic agents such as Cytochrome p450, which may be endogenously altered by anesthetic agents requiring post-experimental control of confounding factors. Hence, the rats were sacrificed by cervical dislocation as previously applied [28].

Morphometric analyses of Gonado-Somatic Index (GSI)

The testes of each rat were excised and separately weighed in grams. GSI was computed for each rat using the formula: Gonads Weight (g)/Body weight (g) × 100 [32].

Tissue-ELISA analyses of levels of Dopamine, Glutamate, Myelin Basic Protein (MBP), Cytochrome p450, Caspase-3 and sVEGFR in the testes

The excised and isolated testes of rats of Groups 1–6 were thoroughly homogenized using porcelain mortar and pestle in ice-cold 0.25 M sucrose. 1 g of testicular tissue was homogenized in 4 ml of 0.25 M sucrose solution. The tissue homogenates were additionally filled up to 5 ml with sucrose in a 5 ml serum bottle. Testicular homogenates were consequently centrifuged at 3000 revolution per minute for 15 minutes using a centrifuge (Model 90-1). The supernatant was collected with Pasteur pipettes and placed in a freezer at -20°C, and thereafter assayed for concentrations of Dopamine, Glutamate, MBP, Cytochrome p450, Caspase-3 and sVEGFR in the testes of all rats of Control Group 1 and Experimental Groups 2 – 6 using ELISA technique as previously described [5]. ELISA kits were products of CUSABIO Technology LLC, Houston, USA). AgileReader™ ELISA plate reader was employed with absorbance read at the wavelength of 450 nm.

This Tissue-ELISA assay uses the quantitative sandwich enzyme immunoassay method. Dopamine, Glutamate and Myelin Basic Protein specific antibodies were pre-coated onto the microplate. Samples and Standards were tuneled into the wells while presenting Dopamine, Glutamate, MBP, Cytochrome p450, Caspase-3 and sVEGFR were bound by the immobilized antibody. A biotin-conjugated antibody specific for each of Dopamine, Glutamate, MBP, Cytochrome p450, Caspase-3 and sVEGFR was added to the wells following removal of unbound substances. Thereafter, Avidin conjugated Horseradish Peroxidase was transferred into the wells after washing. A substrate solution was introduced into the wells resulting in colour development in relationship with the quantity of each of Dopamine, Glutamate, MBP, Cytochrome p450, Caspase-3 and sVEGFR bound in the initial step following removal of any unbound avidin-enzyme reagent via washing. Finally, colour intensity was measured and the development of colour was stopped. The mean absorbance against the protein concentration was plotted and the curve best fitting the standard result was drawn, and the absorbance of samples were interpolated to the curve to calculate the concentration for each biomarker.

Data analysis

Computed data of concentrations of each biomarker was expressed as arithmetic means ± standard error of mean. Mann-Whitney U test (Wilcoxon-Mann-Whitney Test, 2016) was used for statistical comparison of the concentration of each biomarker between two groups. Significant difference was confirmed at 95% confidence interval with associated p value of less than 0.05 ($p \leq 0.05$).

Results

GSI: Group 2 versus Groups 1 and 3 – 6

Results showed significant lower ($p \leq 0.05$) levels of GSI in rats of Group 2, when compared with Group 1. In addition, results showed statistically non-significant lower ($p \geq 0.05$) levels of GSI in rats of Group 2, when compared with Groups 3 – 6 (**Table 1**).

Concentrations of Dopamine, Glutamate and MBP in testes of rats

Results showed statistically significant lower ($p \leq 0.05$) levels of Dopamine and Glutamate, but statistically non-significant higher ($p \geq 0.05$) levels of MBP in homogenates of testes of rats of Group 2, when compared with Control Group 1 (**Table 1**). Furthermore, results showed statistically non-significant lower ($p \geq 0.05$) levels of Dopamine, but statistically significant lower ($p \leq 0.05$) levels of Glutamate in homogenates of testes of rats of Group 2, when compared with Groups 3 – 5. In addition, results showed statistically non-significant higher ($p \geq 0.05$) levels of MBP in homogenates of testes of rats of Group 2, when compared with Groups 3 – 5. Results showed statistically non-significant lower ($p \geq 0.05$) levels of Dopamine, but statistically significant lower ($p \leq 0.05$) levels of Glutamate in homogenates of testes of rats of Group 2, when compared with Group 6. In addition, results showed statistically non-significant higher ($p \geq 0.05$) levels of MBP in homogenates of testes of rats of Group 2, when compared with Group 6.

Concentrations of Cytochrome p450, Caspase-3 and sVEGFR in testes of rats

Results showed statistically significant lower ($p \leq 0.05$) level of Cytochrome p450, but statistically significant higher ($p \leq 0.05$) levels of Caspase-3 and sVEGFR in homogenates of testes of rats of Group 2, when compared with Control Group 1 (**Table 2**). Furthermore, results showed statistically significant lower ($p \leq 0.05$) level of Cytochrome p450 in homogenates of testes of rats of Group 2, when compared with Groups 3 – 5. In addition, results showed statistically significant higher ($p \leq 0.05$) levels of Caspase-3 and sVEGFR in homogenates of testes of rats of Group 2, when compared with Groups 3 – 5. Results showed statistically significant lower ($p \leq 0.05$) level of Cytochrome p450 in homogenates of testes of rats of Group 2, when compared with Group 6. In addition, results showed statistically significant higher ($p \leq 0.05$) levels of Caspase-3, but insignificantly higher ($p \geq 0.05$) levels of sVEGFR in homogenates of testes of rats of Group 2, when compared with Group 6.

Discussion

GSI is used in the assessment of increase or decrease of testicular weight. Results of this study showed significant reduction of GSI in rats of $CdCl_2$-treated only Group 2, when compared with Normal Saline-only treated Control Group 1 and Groups 3, 4 and 6 (**Table 1**). This observation suggests that $CdCl_2$-exposure resulted in shrinkage of the testes with associated significant reduction of GSI and testicular weight in rats. The findings of this study are in agreement with those of previous studies which reported that Cd-exposure resulted in decreased testicular weight in animal models [1, 33].

Post-treatments with MO11, MS06 and Doxorubicin resulted in non-significant increase of GSI in rats of $CdCl_2$-exposure + MO11 post-treated Group 3, $CdCl_2$-exposure + MO11 + MS06 post-treated Group 4 and $CdCl_2$-exposure + Doxorubicin post-treated Group 6, when compared with $CdCl_2$-only treated Group 2 (**Table 1**). These observations suggests that MO11, MS06 and Doxorubicin ameliorated $CdCl_2$-induced decreased weight of the testes.

Dopamine is involved in regulations of arousal, motor control, motivation, reinforcement, reward, sexual gratification, nausea and lactation [27]. Dopamine-induction of apoptosis occurred via Cytochrome C/Caspase-dependent pathway and Dopamine possessed the capability to inhibit tumour growth [19]. However, Dopamine levels are downregulated in tumours [17, 37]. In addition, Cd-exposure resulted in decreased Dopamine levels in rats [13, 15] with accompanied motor dysfunctions [15], low energy, lack of motivation and depression [13].

Furthermore, Glutamate is the major excitatory neurotransmitter of the central nervous system, and it is at the cross-road of several metabolic pathways and could cause excito-toxicity when excessively excited. Hence, too little or too much Glutamate is harmful to the body system requiring cells to have the right glutamate-sensitivity, withstand normal glutamate-stimulation and remove Glutamate at normal rates from the right places [38]. Metabolism-related genes are mutated in cancers, making cancers to be glutamate-dependent. Hence, dysregulation of glutamate levels promotes tumour growth [39].

The findings of this study showed significant downregulations of Dopamine and Glutamate in Group 2, when compared with Control Group 1 (**Table 1**). These observations are in agreement with previously reported Cd-induced downregulations of Dopamine [15] and Glutamate [18] in rats. Both Dopamine [29] and Glutamate [35] are expressed in the testis. Hence, Cd-induced downregulations of Dopamine and Glutamate in the testes of rats of Group 2 possibly implied adverse effects on neurotransmitter levels required for normal innervation of the testes.

Post-treatments with MO11, MO11+MS06 and Doxorubicin in Groups 3, 4 and 6 respectively resulted in upregulations of Dopamine and Glutamate, when compared with Group 2 (**Table 1**). Hence, these observations suggest that MO11, MS06 and Doxorubicin ameliorated $CdCl_2$-induced dysregulations of Dopamine and Glutamate in the testes, and possess testicular-protective and neuro-protective potentials.

MBP is a membrane actin-binding protein and the second most abundant protein of myelin after proteolipid protein. It transmits extracellular signals to tight junctions of myelin and to the cytoskeleton of oligodendrocytes [26]. Astrocytes' depletion result in breach of the glial-limiting membrane, Schwann cells' invasion for myelin sheath repair, dissociation of MBP from the plasma membrane and consequent loss of myelin sheath (de-myelination) in response to axonal degeneration and consequent oxidative stress [2]. Therefore, MBP-upregulation is associated with demyelination [16].

Will $CdCl_2$-induced testicular toxicity be associated with demyelination via increased MBP levels of the testis? Results showed non-significant upregulation of MBP levels in the testes of Group 2, when compared with Control Group 1 (**Table 1**). This observation suggests that Cd-induced toxicity did not result in evident de-myelination of nerve fibres supplying the testes of rats of Group 2. In addition, post-treatments with MO11, MO11+MS06 and Doxorubicin in Groups 3, 4 and 6 respectively resulted

in non-significant decrease of MBP levels, when compared with Group 2 (**Table 1**). These observations suggest no significant changes in MBP levels either with $CdCl_2$-exposure or further post-treatments with MO11, MO11+MS06 and Doxorubicin.

Cytochrome p450 (CYPs) are monooxygenases that oxidize fatty acids, steroids and xenobiotics thereby enhancing the water-solubility and expulsion of foreign compounds. Cytochrome p450 thus plays regulatory roles in the clearance of drugs and compounds, detoxification of drugs and xenobiotics, vitamin D metabolism, synthesis of cholesterols and hormones, cellular metabolism and homeostasis [25, 31]. Cytochrome p450 is involved in activation/inactivation of carcinogen as well as activation/inactivation of anticancer drugs, and clearly plays strong roles in cancer therapy [25]. Furthermore, Cytochrome p450 promotes steroidogenesis [34].

Will $CdCl_2$-induced testicular toxicity result increase or decrease Cytochrome p450 levels in the testes? Results showed significant downregulations of Cytochromes p450 levels in the testes of Group 2, when compared with Control Group 1 (**Table 2**). This observation suggests that the previously reported Cd-inductions of depletion of germ cells, testicular necrosis and carcinogenesis [7, 30, 33] in *in vivo* models as well as significant decreases of sperm count, sperm motility, testosterone synthesis, spermatogonia, Sertoli cells and Leydig cells in rat testis [23, 40] may involve adverse effects on testicular levels of Cytochrome p450.

Post-treatments with MO11, MO11+MS06 and Doxorubicin in Groups 3, 4 and 6 respectively resulted in significant increase of Cytochrome p450 levels, when compared with Group 2 (**Table 2**). These observations suggest that MO11, MS06 and Doxorubicin ameliorated $CdCl_2$-induced decreased levels of Cytochrome p450 in rat testes.

Caspase-3 is the major executioner protease amongst the reported 14 caspases implicated in the human apoptotic pathway mechanism [36]. Hence, the resolution of cytotoxicity via apoptosis involves the activation of Caspase-3 in both the intrinsic mitochondrial pathway and the extrinsic death-receptor pathway of apoptosis [16, 22]. Furthermore, the several mitotic divisions and clonal expansion of germ cells in spermatogenesis require apoptotic control mechanism to match the number of germs cells with functional capacity of available number of nursing Sertoli cells [36]. Hence, over-activation of Caspase-3 in the testis may drive depletion of germ cells.

Will $CdCl_2$-induced testicular toxicity result in upregulation of Caspase-3 levels in the testis of rats? Results of this study showed significant upregulations of Caspase-3 in the testes of Group 2, when compared with Control Group 1 (**Table 2**). These observations suggest that previously reported Cd-induction of depletion of testicular germ cells, Leydig cells and Sertoli cells [7, 23, 30, 33] may involve over-activation of Caspase-3 levels in the rat testis.

In addition, Cd is an established carcinogen [1, 8, 30, 33], while angiogenesis is a significant component of carcinogenesis and consequent associated metastasis [7, 37]. VEGF is an established angiogenic factor, and abnormal VEGF upregulation is associated with increased angiogenesis [25]. Furthermore, VEGF upregulation is a promising biomarker of testicular inflammation [14].

Will $CdCl_2$-induced testicular toxicity result in upregulation of sVEGFR levels in the testis of rats? Results of this study showed significant upregulation of sVEGFR in the testes of Group 2, when compared with Control Group 1 (**Table 2**). These observations suggest that Cd-induction of testicular toxicity is possibly associated with increased angiogenesis and inflammation in the testes of rats of Group 2.

Post-treatments with MO11 and MO11+MS06 in Groups 3 and 4 respectively resulted in significant downregulation of sVEGFR levels, when compared with Group 2 (**Table 2**). These observations suggest that MO11 and MS06 ameliorated $CdCl_2$-induced testicular associated angiogenesis and inflammation, and possess anticancer potentials.

Contrari-wise post-treatments with Doxorubicin in Group 6 resulted in non-significant downregulations of Caspase-3 and sVEGFR levels, when compared with Group 2 (**Table 2**). These observations suggest that Doxorubicin ameliorated $CdCl_2$-induced testicular angiogenesis. However, Doxorubicin possesses lesser anti-angiogenesis potentials when compared with MO11 and MS06. In addition, the findings of this study showed no adverse effects of Olive Oil (the vehicle used to dissolve MO11 and MS06) on weight of the testis and levels of biomarkers of drug metabolism, carcinogenesis, angiogenesis and neuro-cancer interactions, when compared with $CdCl_2$-only treated group.

Which factor underlies the repro-protective potentials of MO11 and MS06 as observed in this study? Spectroscopic analyses showed the presence of Glutamic acid, Guanine, Phenylalanine, Leucine, and other anticancer compounds in MO11 and MS06 isolates [6]. Glutamic acid [11], Guanine [10], Phenylalanine [21, 41], and Leucine [20] are established anti-inflammatory, anti-cancer and antioxidant compounds. Therefore, Leucine, Glutamic acid, Guanine, Phenylalanine and other anticancer compounds in MO11 and MS06 isolates could have been responsible for their observed repro-protective and anticancer potentials.

Conclusion

Overall, the findings of this study suggest that $CdCl_2$-induced testicular toxicity resulted in downregulation of Cytochrome p450 which may possibly be associated with inhibition of steroidogenesis. In addition, $CdCl_2$-induced testicular toxicity resulted in upregulations of Caspase-3 and sVEGFR which may possibly be associated with increased testicular angiogenesis and inflammation as well as depletion of germ cells. Furthermore, study findings showed that $CdCl_2$-induced testicular dysregulation of neurotransmitters occurred via downregulations of levels of Dopamine and Glutamate in the testes of rats.

However, post-treatments of $CdCl_2$-induced testicular toxicity with MO11, MS06 and Doxorubicin conferred neuro-protection and repro-protection against $CdCl_2$-induced testicular damage via upregulations of Dopamine, Glutamate and Cytochrome p450, but downregulations of Caspase-3 and sVEGFR in the testes of rats. These observations indicate that MO11, MS06 and Doxorubicin possess neuro-protective, repro-protective, anti-angiogenesis and anticancer potentials. Hence, MO11 and MS06, are recommended for further evaluations as potential drug candidates for the treatments of $CdCl_2$-induced repro-toxicity and angiogenesis.

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Table 1. Gonado-Somatic Indices and Concentrations of Dopamine, Glutamate and MBP in testes of rats

Drug/Extract →	**Normal Saline only Group 1**	**$CdCl_2$ only Group 2**	**$CdCl_2$-exposure + MO11 post-treated Group 3**	**$CdCl_2$-exposure + MO11 + MS06 post-treated Group 4**	**Olive Oil only Group 5**	**$CdCl_2$-exposure + Doxorubicin post-treated Group 6**
Gonado-Somatic Index	**0.65±0.03	0.29±0.05	0.35±0.08	0.47±0.09	0.55±0.09	0.35±0.10
p–value	p<0.001		0.55	0.09	0.03	0.56
Dopamine (pg/ml)	**6.25±0.74	3.19±0.32	4.34±0.03	4.90±0.15	4.13±0.03	3.80±0.10
p-value	p<0.01		0.10	0.07	0.25	0.70
Glutamate (ng/ml)	**137.77±0.16	112.50±1.50	**137.22±0.39	**138.06±0.17	**130.27±1.04	**134.20±0.26
p–value	p<0.01		p<0.01	p<0.01	p<0.01	p<0.01
Myelin Basic Protein (ng/ml)	4.26±0.02	4.97±0.32	4.27±0.01	4.16±0.01	3.80±0.07	4.32±0.01
p–value	0.22		0.23	0.07	0.06	0.20

p – value at $p \leq 0.05$: Group 2 versus Groups 1 and 3 – 6
** – significant increase /$p \leq 0.05$/

Table 2. Concentrations of Cytochrome p450, Caspase-3 and sVEGFR in testes of rats

Drug/Extract →	**Normal Saline only Group 1**	**$CdCl_2$ only Group 2**	**$CdCl_2$-exposure + MO11 post-treated Group 3**	**$CdCl_2$-exposure + MO11 + MS06 post-treated Group 4**	**Olive Oil only Group 5**	**$CdCl_2$-exposure + Doxorubicin post-treated Group 6**
Cytochrome p450 (ng/ml)	**465.12±20.80	181.52±7.20	**378.45±1.76	**396.32±2.08	**349.12±7.77	**352.32±0.92
p–value	p<0.01		p<0.01	p<0.01	p<0.01	p<0.01
Caspase-3 (ng/ml)	*125.00±0.63	252.50±3.13	*137.50±0.72	*87.19±0.74	*176.67±6.14	*209.69±5.94
p–value	p<0.01		p<0.01	p<0.01	p<0.01	p<0.01
sVEGFR (ng/ml)	*28.75±0.42	49.72±2.65	*20.00±1.67	*15.00±0.59	*34.72±1.00	43.89±1.21
p–value	p<0.01		p<0.01	p<0.01	p<0.01	0.25

p – value at $p \leq 0.05$: Group 2 versus Groups 1 and 3 – 6
** – significant increase /$p \leq 0.05$/
* – significant decrease /$p \leq 0.05$/

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Retrobulbar Pleomorphic Adenoma of Ectopic Lacrimal Gland – Case Report

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Retrobulbar ectopic lacrimal gland is a rare choristoma with a risk of neoplastic transformation. This report describes a case of a 56-year-old female presented with left eye proptosis, paradoxal sensation, and paresthesia on the left side of the face along with persistent headache and decreasement of vision. Magnetic resonance imaging revealed a formation in the left retrobulbar area. An excision biopsy was performed and a fragmented mass, away from the lacrimal fossa, was removed. The microscopical evaluation of the specimen showed features of pleomorphic adenoma.

Key words: retrobulbar, pleomorphic adenoma, lacrimal gland

Introduction

Choristomas are congenital lesions where histologically normal tissues with little or no growth potential are present at abnormal locations [1]. Lacrimal gland choristomas are benign lesions formed by normal lacrimal gland tissue present outside the lacrimal fossa [3]. We report a case of unilateral proptosis with diminished vision and paradoxical sensation, secondary to retrobulbar pleomorphic adenoma arising from the ectopic lacrimal gland.

Case report

A 56-year-old female complained of pain in the left eye, persistent headache, and diminished vision. The examination revealed proptosis of the left eye with paradoxal sensation in the area. Magnetic resonance found a left-situated retrobulbar mass with well-circumscribed borders. Consultation with a neurosurgeon was performed and the neurological status assessment reported amblyopia of the left eye, paresis of nervus facialis, and nervus oculomotorius on the same side with clinical signs of increased intracranial pressure. Based on these observations, the patient was admitted into neurosurgery for an operation.

There were no data for biopsy evaluation, before the operation. The formation was excised and sent for a frozen section evaluation. Grossly the specimen was fragmented with greyish-pinkish color and soft consistency. The rapid microscopical analysis suggested a wide spectrum of differential diagnoses with a final word on the permanent slide.

The biopsy specimen was fixed in formalin and embedded in paraffin, and 5-µm-thick tissue sections were used for staining with hematoxylin and eosin. Written informed consent was obtained from the patient.

Macroscopically, the specimen showed a fragmented mass measuring 1 cm in diameter, with a soft consistency. The microscopical evaluation showed tumor composed of tubules and cysts lined by epithelial cells and surrounded by myoepithelial cells with myxoid stromal component (**Figs**. **1, 2**). The full excision of the tumor can not be assessed, because of the lack of a capsule.

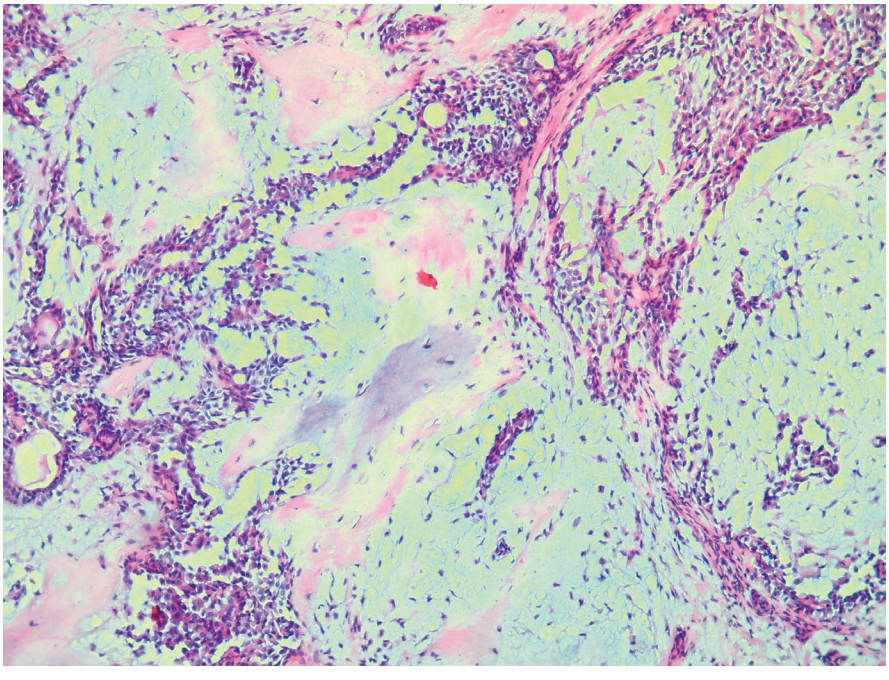

Fig. 1. Lesion characterized by tubules and cysts lined by epithelial cells and surrounded by myoepithelial cells with myxoid stromal component. HE, ×50.

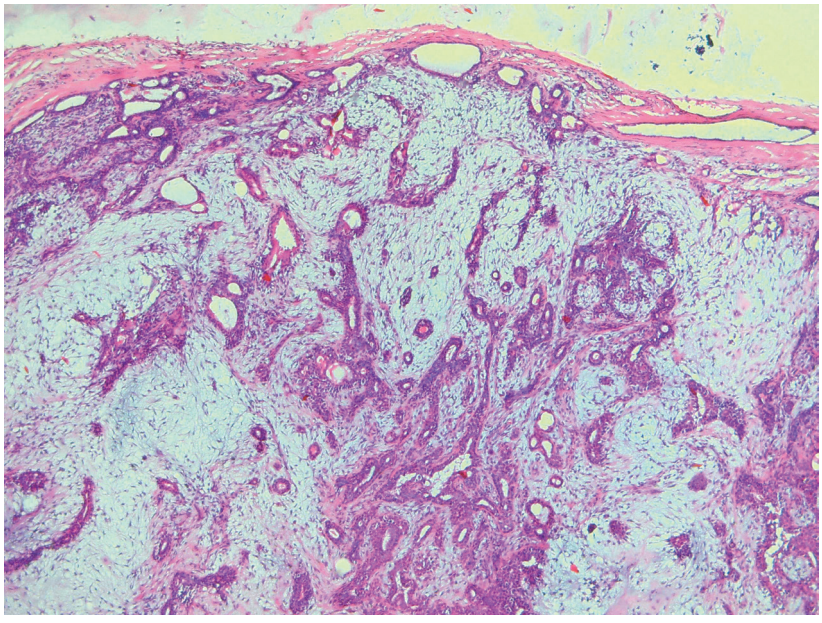

Fig. 2. Tumor, presented by tubules and cysts. HE, ×200.

Discussion

The secretory lacrimal apparatus is composed of the main lacrimal gland located in the orbital lacrimal fossa, with its palpebral lobe at the temporal side of the superior fornix, and two further sets of accessory lacrimal glands, the glands of Krause and the glands of Wolfring (or Ciaccio). Lacrimal gland tissue located at any other site is considered ectopic [2]. Ectopic lacrimal gland tissue is found most commonly in the bulbar conjunctiva and unusually in the retrobulbar region. It can also be observed in the caruncle, outer canthus, lower lid, and intraocular regions. Pleomorphic adenoma is the most common epithelial tumor of the lacrimal gland and has a high tendency to occur in the orbital lobe of the lacrimal gland and rarely occurs in the accessory lacrimal gland. In the literature, six patients have been reported for pleomorphic adenoma arising from the ectopic lacrimal gland [4]. In our patient, pleomorphic adenoma originated from an ectopic lacrimal gland located in the retrobulbar region, causing significant displacement and compression of the globe, visual dysfunction, and symptoms of increased intracranial pressure. Pleomorphic adenoma presents an excellent prognosis when the lesion is completely excised [6]. The difficulty with calling the final diagnosis on frozen section evaluation in our case came from the extensive differential diagnosis and absence of previous histopathological assessment. A wide variety of processes can produce space-occupying lesions in and around the orbit. These include benign neoplasms, malignant neoplasms, vascular lesions, inflammatory disease, congenital lesions, and infection, among other causes [5].

Conclusion

Even it is rare, retrobulbar pleomorphic adenoma arising from ectopic lacrimal gland should be considered in the differential diagnosis in retro-orbital tumors.

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Morphological Aspects on Heart and Main Blood Vessels of Black Sea Turbot (Psetta maxima) – Corrosion Cast and Morphological Studies

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Abstract

The turbot (*Psetta maxima*) is one the most valuable and economically important species for the Black Sea countries. The purpose of the current study was to investigate the cardiovascular system of this species with both corrosion cast and routine histology methods on 12 sacrificed turbots (6 males and 6 females) obtained from a hauls. After that fish were sacrificed and the blood vessels were filled through the heart with self-curing castable resin Duracryl® Plus U. Apart, materials from some parts of vasculary system for histology examination were taken and fixed in 10% neutral formalin solution. Based on the corrosion cast and histological techniques we described luminal structure and size of the aorta in turbot.

Key words: Black Sea Turbot, Cardiovascular system, Corrosion cast, Histology

Introduction

In the Black Sea, the major target demersal fish species with commercial importance are – turbot (*Psetta maxima*) and gobies (*Gobiidae*) with by-catch of thornback ray [9]. The turbot is intended mainly for human consumption and the scientific value of this species increased during the last decade [3]. Annual turbot stock assessments for scientific aims are made at the Bulgarian Black Sea coast [6,10]. Despite the increased

scientific interest there is still missing information about anatomy and morphology of turbot, except for one report about eye structure [4] and morphological of tongue [5].

The lack of data on the detailed morphological structure of the cardiovascular system of turbot gave us reason to undertake the present study in order to describe its cardiovascular system using a corrosion technique and hematoxylin-eosin histological method.

Material and methods

Sample collection

According to the Directive 2010/63/EU and respective the national program for data collection in the field of fisheries and aquaculture, especially assessment of the stock of turbot in the Bulgarian waters of the Black Sea twelve sexually mature turbot (6 males and 6 females) with body weight 2900-3200 g were captured by gillnets in the period from mid-March to late April. The main fishing areas covered the shelf area at depths from 15 to 90 m.

Corrosion cast

The blood vessels were washed with distilled water until leakage of the residual blood via the heart chamber. Two syringes were mounted on the heart chamber for the introduction of 100 ml of Duracryl® Plus U cold-curing resin (Spofa Dental, Czech Republic) solution (proportion powder : liquid, 1:3) using a special device described previously [8]. After that, the filled specimens were left at room temperature for 24 hours to complete the resin polymerization. Then the fish were placed into 5% Potassium hydroxide for 10 days at 45^{o}C for removing the soft tissue. Finally, the corrosion specimens were washed with slow running water with some detergent to remove the remaining fat.

Histological examination

Tissue piesces from the ventral aortic wall were fixed by immersion in 10% buffered neutral formalin for 24 hours at room temperature and processed for paraffin embedding. Sections of 4-5 μm thick were stained with haematoxylin and eosin (H&E) and examined by light microscopy, using a describet method in specialized literature [1].

Result and Discussion

For the first time the replicas from turbot's heart chamber and initial vessels – aorta and its branches and histological structure of the aorta as well as, are described The corrosion castings of the gill arches showed an extremely accurate three-dimensional image (**Fig. 1**).

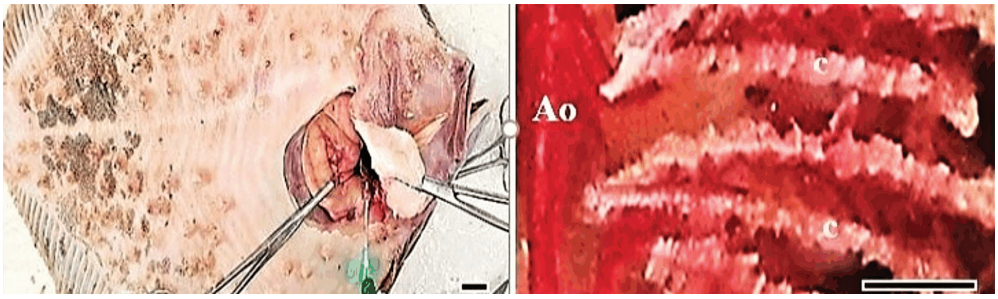

Fig. 1. Fulfillment of the circulatory system through the heart chamber (left). Corrosion cast from a gill arch on a turbot (right). **Ao –** Aorta, **c** – capillaries. Bar = 2 cm.

For notice is that, the weakly left-right asymmetry of the gills was found. The blood vessels, which came from the branchiostegal rays, were located in the distal part of the light elongated bones – ceratobranchial, as mentioned [11]. These findings gave reason to presume that the blood circulation in turbot is similar to other fish species and consists of a single circuit, as the deoxygenated blood from the heart is carried through the ventral aorta to the gills. Oxygenation process takes place in the lamellae and from them through dorsal aorta and secondary arteries distributed to the whole body of fish. The size of the middle segment of the aorta shows higher values of the lumen (caliber) in females (×= 0.90 mm) compared to males (×= 0.79 mm) (**Table 1)**. Relationship of the gender with size of aorta was also tracked with respect to depth but no significant correlation was detected.

Table 1. The lumen size of the middle segment of aorta in both gender of Black Sea turbot

	15-20 m	**50-60 m**	**80-90 m**
Male turbot, mm	0.72	0.84	0.79
Female turbot, mm	0.97	0.93	0.86

It was also found that the histological structure of turbot vessels was similar to vertebrates. The results of histological analyses exhibited that in the Black Sea turbot (*Psetta maxima*) three layers of the vessel wall were differentiated (**Fig. 2**). The results of the study were consistent with previous studies that described the same histological organization of blood vessel walls in fish [7], but they are in little contrast to data of [2] according to which the media of ventral aorta consists mainly of elastic fibers, as smooth muscle cells were also observed in its middle shell.

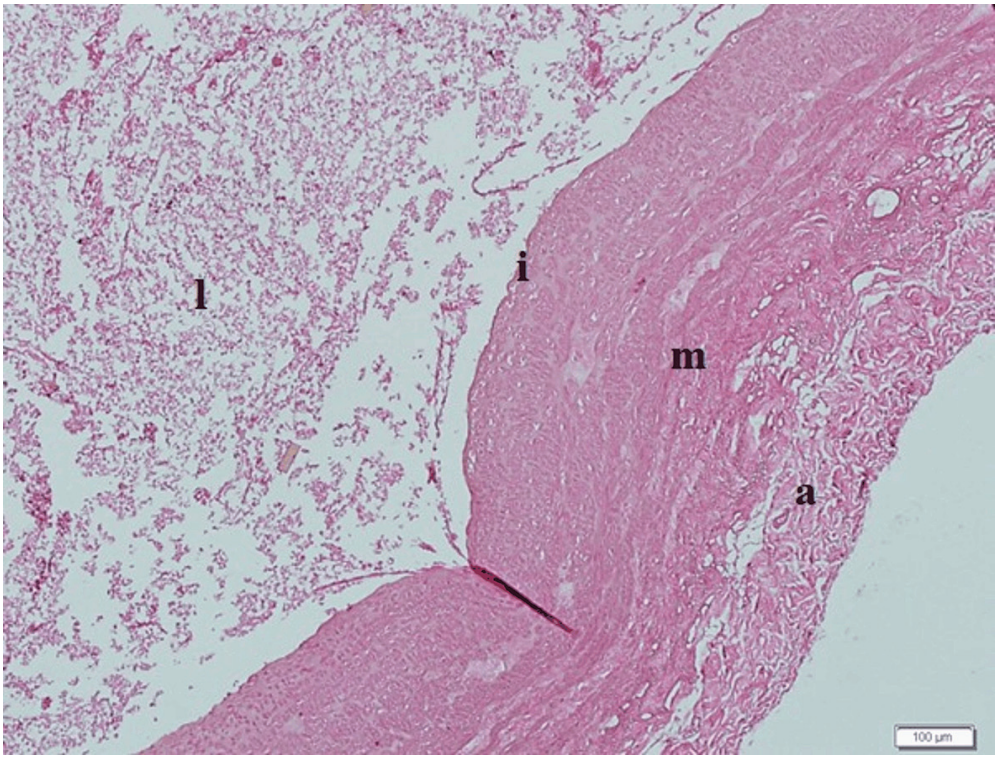

Fig. 2. Histological section of wall‘s segment of ventral aorta: **i** – intima, **m** – media, **a** – adventitia; **l** – lumen. Bar = 100 µm

Conclusion

As far as we know this is the first study which described the circulatory system of Black Sea turbot by the corrosion method. The data obtained add the knowledge about fish morphology for cardiovascular system in Black sea inhabitants and they could be useful for further morphological and pathological studies.

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Review Articles

The Complex Role and Implications of VEGF-A on Cardiac and Renal Physiology and Pathology with Special Focus on Hypertensive Injury – a Critical Review

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Vascular endothelial growth factor (VEGF) is a signaling protein essential for angiogenesis. Despite vigorous research in the field for several decades, the exact role of VEGF in the sophisticated regulatory mechanisms of cardiac and renal homeostasis still remains to be fully elucidated. Recent studies have reported that the expression of VEGF in the heart and kidneys changes with age, which leads to modifications in the microvasculature and age-related remodeling of the myocardium and renal parenchyma. Furthermore, literature data suggest that the levels of VEGF are altered in response to hypertensive injury, which plays a crucial role in the pathogenesis and progression of multiple cardiac and renal pathologies. Therefore, this review strives to assess the accessible literature and provide clarity on the role of VEGF in the complex signaling cascades responsible for maintaining cardiac and renal homeostasis both under physiological and pathological conditions.

Key words: VEGF, heart, kidney, aging, hypertension

Introduction

Vascular endothelial growth factor (VEGF) is an endogenous peptide essential for the formation of blood vessels, i.e. angiogenesis. In humans, five subtypes of VEGF have been isolated. VEGF-A, VEGF-B, and the placental growth factor (PGF) are mainly responsible for forming new blood vessels. In contrast, VEGF-C and VEGF-D are primarily associated with the formation of lymph vessels [15]. The members of

the VEGF family accomplish their role via binding to specific receptors, known as VEGF receptors (VEGFR). There are three subtypes of the VEGFR, all members of the tyrosine kinase superfamily of receptors. VEGFR1 and VEGFR2 are the primary receptor subtypes predominantly expressed by endothelial cells (EC), macrophages, monocytes, and hematopoietic cells [18, 42]. VEGFR1 and VEGFR2 predominantly bind with high affinity VEGF-A, VEGF-B and PGF, thus regulating the formation of new blood vessels [18]. VEGFR3 is mainly expressed in the EC of lymph vessels and binds with high affinity VEGF-C and VEGF-D, thus playing a pivotal role in the angiogenesis of lymph vessels [7].

The VEGF/VEGFR system plays a crucial role in maintaining homeostasis. VEGF is mainly expressed by EC, as well as cardiomyocytes [4]. In addition there is evidence throughout the literature, linking VEGF expression and arterial hypertension [13]. Both the heart and kidneys are target organs for hypertensive injury, due to their rich vasculature. This makes them the perfect target organs for research on the role of VEGF/VEGFR in the pathophysiology of different cardiovascular diseases (CVD) and renal diseases [36].

This review strives to offer a comprehensive assessment of recent studies exploring the physiological and pathological role of VEGF. In particular, we aim at providing clarity on the role of the VEGF/VEGFR system in maintaining cardiac and renal homeostasis over the course of physiological aging and in the setting of various pathologies.

Expression of VEGF in the heart and kidney

VEGF expression in the heart has recently been mapped via immunohistochemistry by Stanchev et al. [46] and Iliev et al. [22]. VEGF immunoreactivity was predominantly registered in the cardiomyocytes' cytoplasm, the walls of capillaries of various size and the perivascular zones. VEGF expression was stronger in the left ventricle (LV) in comparison to the right ventricle (RV) [22, 46]. Such deviations in the distribution of VEGF correlate with the higher workload and oxygen demands of the LV [13]. An alternative method of detection of VEGF in the heart is the use of immunofluorescence. Through that methodology, Stanchev et al. revealed that VEGF expression was observed in the perinuclear and perivascular zones of cardiac muscle cells. The immunofluorescent reaction showed a similar pattern in both ventricles and was stronger in the LV [46]. Similar results were reported by Iliev et al. in another immunofluorescent study on normal rat hearts [22]. VEGF-A exerts its function on cardiomyocytes via the VEGFR1 and VEGFR2 receptors. Moreover, cardiomyocytes are not only target cells for the function of VEGF, but they can also produce VEGF [4].

Expression of VEGF in the kidney has been described in the visceral epithelial cells of Bowman's capsule (podocytes), distal tubules and collecting ducts and less often in proximal tubules [1, 35]. VEGFR-2 is the receptor most often found in the kidney, on the membrane of preglomerular and glomerular endothelial cells, as well as on endothelial cells in blood vessels surrounding the proximal and distal tubules and collecting ducts. VEGFR-2 has also been observed on the membrane of cortical fibroblasts, interstitial cells in the medulla and mesangial cells [1, 35, 55]. According to Stanchev et al., in the renal cortex (RC), VEGF immunoreactivity is observed

in the visceral layer of Bowman's capsule and the epithelial cells of proximal and distal tubular segments. On the other hand, scarce to no staining was observed in the glomerular capillary tufts. The staining in the renal medulla (RM) was most prominent in Henle's collecting ducts and loop [46].

Role of VEGF in the heart and kidney

The exact physiological role of VEGF in the heart after embryological development is yet unclear. Giordano et al. performed a heart-specific VEGF knockout – using genetic engineering techniques, they developed mice with cardiomyocyte-specific deletion of the third exon of the VEGF coding gene. The results were mice with lower body weight, the density of the wall of their hearts was significantly lower, and their hearts were dilated, hypovascularized and with contractile dysfunction. Moreover, the number of coronary microvessels in the hearts of these gene-altered mice was lower [17]. Several studies suggest that cardiomyocytes secrete VEGF in response to different stimuli as per se: hypoxia [30], IL-1β [38], stretching [56, 31], gp130 [16], etc. Karpanen et al. performed a study on the effects of VEGF-B on the heart. They used genetically altered mice with overexpression of VEGF-B via alpha myosin heavy chain promoter. The study revealed a scarce angiogenetic effect of VEGF-B, but surprisingly the gene-modified mice developed cardiac hypertrophy with lower blood pressure and heart rate. These results were explained by altered lipid metabolism leading to mitochondrial morphology changes, enlargement of the cardiomyocytes and development of cardiomyopathy. Thus was concluded that VEGF-B played a crucial role in lipid metabolism in cardiomyocytes [27].

VEGF-A plays an important role in several aspects of the normal renal anatomy and physiology, particularly in glomerular capillary formation and repair and in the maintenance of the fenestrated endothelium of glomerular capillaries [55]. In addition, VEGF-A takes part in the proliferation and apoptosis of tubular epithelial cells [39]. Although the role of VEGF in renal pathology and physiology has been the focus of many studies, results are controversial. Some studies have shown that inhibition of VEGF does not lead to significant alterations in the glomerular filtration barrier [25]. Baderca et al. reported a negative expression of VEGF in the renal corpuscles of the normal renal parenchyma, suggesting that it is not normally present under physiological conditions [2]. Others have reported on potential renoprotective effects under pathological conditions [32]. VEGF is responsible for glomerular and tubular proliferation and hypertrophy in response to nephron reduction and thus, any subsequent decrease in VEGF levels may lead to the development of glomerulosclerosis and tubulointerstitial fibrosis in the remnant kidney. Furthermore, VEGF has been suggested as a major modulator of glomerular recovery in proliferative glomerulonephritis. Last but not least, glomerular and tubulointerstitial repair in pathological conditions such as thrombotic microangiopathy and cyclosporin nephrotoxicity may also be VEGF-dependent [43].

VEGF expression in the heart and kidney during physiological aging

Aging is a physiological process driven by a plethora of factors, such as genetics, environment, gender, nutrition, etc. As a result, old age is considered a significant risk factor for the development and deterioration of CVD [8]. Age-related remodeling of the myocardium is characterized by cardiac hypertrophy, due to an increase in the volume of the cardiomyocytes mainly in the LV, in contrast to the RV, where hypertrophy is not as significant [34]. Moreover, older age exacerbates the energy supply and depletion of angiogenesis in the heart [14]. Despite older age being a significant risk factor, the main emphasis of research has been on the role of VEGF in the pathogenesis and progression of CVD, and only a few studies have focused on the expression of VEGF during physiological aging. Several studies have shown decreased angiogenesis activity with age, which leads to impaired revascularization of ischemic tissues, thus hindering the recovery ability of the myocardium [40]. Capillary density (CD) is a histomorphometric marker of myocardial perfusion, which was found to decrease with age in both ventricles [21]. Iliev et al. reported a statistically significant elevation in VEGF expression with age progression in both ventricles, predominantly in the LV. Furthermore, a statistically significant positive correlation was reported between VEGF expression and CD in both ventricles with age progression [22].

According to literature data, renal aging has been associated with the development of glomerulosclerosis, loss of tubules and development of interstitial fibrosis [10, 57]. Normally, kidneys are organs rich in vasculature due to their physiological demands. Decrease in CD is pivotal for the physiological and morphological changes which occur with age and might be paramount for the development of chronic kidney disease [6]. Undoubtedly, VEGF plays a central role in the maintenance of the sophisticated regulation of renal homeostasis, since both podocytes and tubular EC produce VEGF. Furthermore, both EC and podocytes express VEGFR1 and VEGF2, which further highlights the complexity of renal vascular maintenance and underlines the possible role of VEGF in renal aging [6]. In addition compared VEGF expression in hypoxia in old and young rat kidney and reported a statistically significant decrease of VEGF-A, VEGF-B and VEGFR-2 in the old rat kidney [41]. Because of the vital role which VEGF has in the renal vascular repair after acute kidney injury, it is likely that reduced VEGF expression in the aging rat kidney contributes to repair defects. The decrease in VEGF expression results in an increased expression of thrombospondin-1 (TSP-1). TSP-1 is an antagonist of the VEGF. The imbalance between pro- and antiangiogenic factors could be an explanation of the age-dependent progressive rarefaction of peritubular capillaries and the deficiency of adequate oxygen supply and vascular remodeling during renal repair [26]. The recent study of Iliev et al. demonstrated a decrease in the expression of VEGF in older versus younger Wistar rats in both the RC and RM. Comparing the two age groups, a statistically significant decrease in capillary density was also reported. It has been demonstrated that tubulointerstitial fibrosis, one of the hallmarks of renal aging, is accelerated by the loss of peritubular capillary density [51, 54]. The data of Iliev et al. [22] showed a positive correlation between the decreased expression of VEGF and the lower capillary density which confirmed earlier literature data of Kang et al. [26]. Podocytes and tubular epithelial cells are also subject to age-related alterations, but a possible link between them and the parallel decrease of VEGF and capillary density has not been fully explored [5, 47]. It is likely that podocytes and

tubular epithelial cells, as primary sources of renal VEGF, fail to produce sufficient levels, which leads to an impaired vasculogenesis and reduced capillary density and in turn – to tubulointerstitial fibrosis.

Role of VEGF in cardiac and renal pathology

The role of VEGF in different cardiac pathologies has been the subject of intense research over the last few decades, although it is yet unclear and debatable. Multiple studies have suggested that VEGF plays a key role in the pathogenesis of several CVD, such as arterial hypertension (AH) and heart failure (HF) [13, 46, 53].

AH is among the leading health problems and poses a major risk factor for stroke, myocardial infarction, and HF [36, 37, 48]. AH initially leads to compensatory myocardial hypertrophy as an adaptive response to the higher workload demands. Another compensatory mechanism during the adaptive phase is the elevated angiogenesis manifested with higher CD. An intriguing detail is that the deterioration of AH is accompanied by a significant decrease in CD [21]. Furthermore, the progression of AH leads to the depletion of the compensatory mechanisms, and they can no longer reduce the discrepancy between the enlarged cardiac volume and the decreased CD [13, 52]. Moreover, with time this adaptive hypertrophy advances to HF, which can be explained by structural damage to the membranes of the cardiomyocytes due to physical overstretching on the one hand and in response to reactive oxygen radicals, pathological cytokines and endothelial damage [3, 44]. Despite vigorous research in the last few decades, the etiology of hypertension is not yet completely known. VEGF, in particular, is of utmost significance for the compensatory mechanisms during the progression of AH [53]. Jesmin et al. performed a comparison study on the age-related level of expression of VEGF in the hearts of spontaneously hypertensive rats (SHR), stroke-prone spontaneously hypertensive rats (SHRSP), and a control group of Wistar-Kyoto rats (WKY). Their study found no age-related changes in VEGF expression in the LV of WHY; in the SHR group, VEGF expression in the LV was increased in 6-week-old animals and then decreased with age; contrariwise, VEGF expression in the LV of the SHRSP group was significantly higher in the 6 and 20-week-olds, thus confirming the age-related increase in the expression of VEGF in the LV of SHRSP. Furthermore, Jesmin et al. reported that VEGF expression in the LV was significantly decreased in 40-week-old SHR and SHRSP [23]. The exact role of VEGF in the pathogenesis, development, and advancement of hypertension is not yet completely identified. However, several studies indicate that pressure overload increases VEGF expression during compensatory hypertrophy. VEGF is paramount for angiogenesis in the hypertrophied myocardium, and its levels increase in correlation with the hypertension stage [13, 23]. Stanchev et al., in their recent study, demonstrated a statistically significant depletion of VEGF expression in both ventricles, predominantly in the LV. Furthermore, they reported a statistically significant positive correlation between VEGF expression and CD in both ventricles with the progression of AH. Depleting these critical vascular compensatory mechanisms is key in the deterioration of AH to HF. [46].

Due to the fact that the kidneys are highly vascularized organs, they are also target for hypertensive injury [36]. The balance of proangiogenic and antiangiogenic

factors is essential for the maintenance of renal vasculature [50]. Despite various previous studies the exact role of VEGF in renal pathology is yet unclear. One study implied that a higher expression of VEGF in SHR compared to normotensive animals might participate in a renoprotective mechanism under hypertensive conditions. Moreover, the inhibition of VEGF leads to glomerular sclerosis and alterations in the podocytes, which are also seen in the hypertensive kidney [1]. The renoprotective effect of VEGF in SHR was recently studied in detail by Liu et al. [33]. The authors reported a reduction in the infiltration of inflammatory cells in the tubulointerstitium and preservation of the structural morphology of the glomerular filtration barrier, the endothelial fenestrations and podocyte foot processes in particular. An earlier study by Kelly et al. [28] established a link between nephron injury, VEGF expression and renal microvasculature changes. The study found that nephron reduction was initially compensated through proliferation of peritubular and glomerular endothelial cells, which was then followed by a loss of peritubular and glomerular capillaries along with a decrease in the expression of VEGF. Dimke et al. [11] highlighted the key significance of VEGF for the maintenance of renal microvasculature. The authors discovered that a specific deletion of VEGF in the renal tubules is associated with disruption of the peritubular capillaries and decreased capillary density. VEGF apparently mediates the hypertrophy of the remaining functional glomeruli in kidney injury which takes place in the early stage of glomerular sclerosis. As kidney damage progresses, the glomerular capillary tufts are subjected to glomerular shrinkage, which first reduces their size back to the initial one, before a progressive decrease in the size of the glomeruli is observed in the late stage of glomerular sclerosis. In addition, more data have supported the role of peritubular capillary rarefaction in the development of hypertensive nephrosclerosis and shown that a correlation exists with the severity of tubulointerstitial injury [29]. Recently, Stanchev et al. [46] demonstrated a decrease in VEGF immunohistochemical expression in the renal cortex and medulla in SHR with the progression of hypertension-induced kidney injury, which was also accompanied by a statistically significant decrease in capillary density. In their previous work, the authors reported a significant increase in two parameters of kidney injury – glomerular sclerosis index and tubulointestinal damage index – in 12-month-old SHR compared to 6-month-old [46]. As suggested earlier, this altered expression of VEGF could be among the triggers for the development of hypertension-induced renal damage.

Conclusion

In conclusion, multiple literature data suggest that the VEGF/VEGFR system plays an essential role in the maintenance of cardiac and renal homeostasis. The results discussed in this review highlight the pivotal role of the changes in the expression of VEGF which take place in conjunction with the decrease in CD during physiological aging. The elevated VEGF expression in the myocardium strives to compensate for the continuously decreasing CD. On the other hand, in the kidney VEGF depletion mirrors the imminent decline in CD. Under pathological conditions, the alterations in VEGF expression are more straightforward, manifesting with perpetual drastic depletion of VEGF along with the diminishment of CD. Such findings further underscore the critical role of the VEGF/VEGFR system in the pathogenesis and deterioration of AH to HF.

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Spermatozoa under Oxidative Stress: Risk or Benefit?

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Modern exploration of the etiology of many pathological processes focuses on oxidative stress as the culprit. This is especially studied in the context of reproduction namely spermatozoa and male infertility. With this review we aim to descriptively summarize the contemporary notion that reproductive inability in the male can stem, among other things, from oxidative stress and its interplay with other pathologies. Discussing the generation of free radicals, their sources and impact on the cellular morphology and physiology of sperm cells and their fertilizing capacity a detailed picture of current literature is provided. Approaches for identification and evaluation of oxidative stress is also considered in term of male reproduction and fertility.

Key words: oxidative stress, sperm, ROS, RNS, infertility

Free radicals and oxidative stress

Oxidative stress stems from an impaired balance between reactive molecules and cellular antioxidant systems. Most often oxidative damage is caused by free radicals – reactive oxygen (ROS) or nitrogen species (RNS) although other groups of radical molecules have also been studied. Free radicals can carry a positive, negative or a neutral charge depending on how they entered the reactive state – through gain or loss of an electron [56]:

$$A - 1e^- \rightarrow A^{\bullet +};$$
$$B + 1e^- \rightarrow B^{\bullet -}.$$

They are capable to react with proteins, membranes, and devastatingly with DNA [46].

Among the most extensively studied free radicals are ROS. They are a product in the course of aerobic metabolism and the flow of electrons during cellular respiration. Examples of reactive oxygen species are the superoxide anion ($O_2^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2) (**Fig. 1**). They are generated when electrons flee

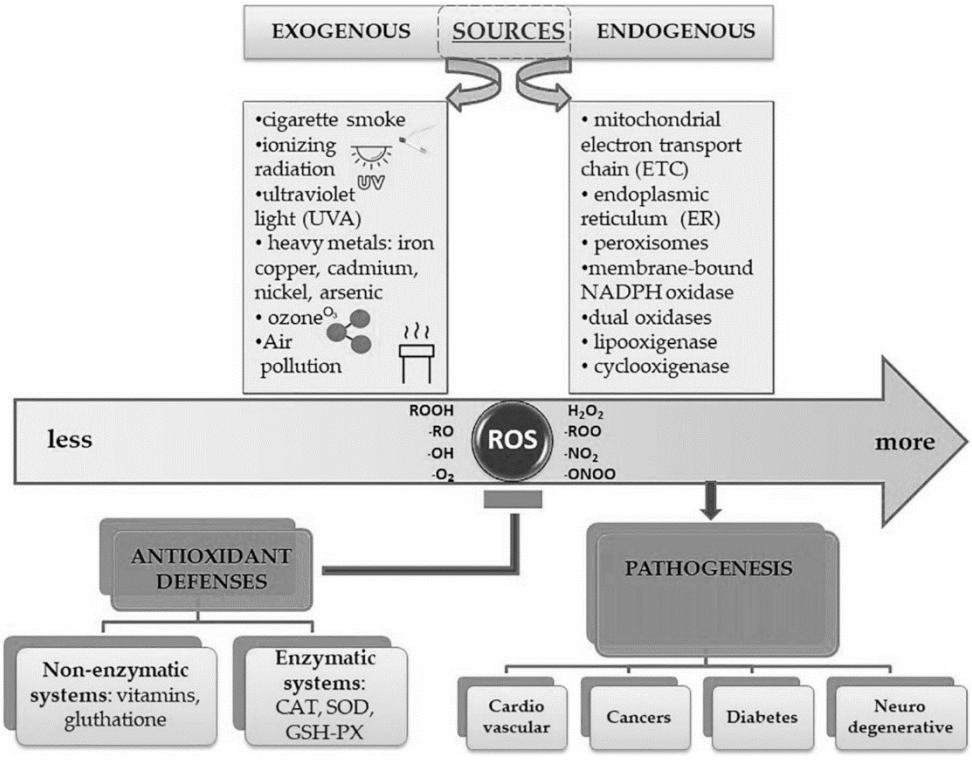

Fig. 1 Systematic comparison between the types of free radicals and their origin, followed by antioxidant defenses and pathologies [48].

the electron transport chain, under conditions of hypoxia or they can be generated by enzymes such as xanthine oxidase, NADPH oxidase. Apart from the mitochondrion they are abundant in the endoplasmic reticulum [13].

Another major group of damaging radicals are RNS. Here the reactive molecules are derived from nitrogen, and they are intrinsically connected to ROS. For instance, the reaction between the superoxide anion and nitric oxide (NO) gives one of the most typical representatives peroxynitrite ($ONOO^{\bullet}$) which can later undergo additional reactions to produce nitrogen dioxide ($NO_2^{\bullet}$) and nitric radicals. Similar to ROS, RNS can attack proteins, lipids and DNA which is described as nitrosative stress. This type of stress can be detrimental because it interferes with NO which has crucial role in the proper function of blood vessels including testicular blood flow.

Of course, these molecules are not accidentally generated in the cell – they serve a purpose. Free radicals are observed in multiple life forms such as animals, plants, aerobic bacteria, and fungi [45]. They can be utilized by the immune system where macrophages can produce them when infections are present in order to eliminate the pathogen [14]. Additionally, free radicals can exhibit anti-cancer properties and some chemotherapy drugs use this property [28]. They also have the capacity to affect the expression of certain genes and even induce epigenetic modifications [53].

When there is an overabundance of free radicals without their particular need cellular structures are in danger. Failing to address this in the long-term can cause premature aging, neurodegenerative diseases, cancer, infertility [8, 11, 40, 44]. Cells have the capacity to reduce the amount of free radicals. Part of its defense system are the enzymes catalase, superoxide dismutase (SOD), and glutathione peroxidase. These enzymes overlook the breakdown of H_2O_2, dismutation of $O_2^{\bullet}$, and protect lipids respectfully. Non-enzymatic antioxidants have been identified such as vitamin C, E, glutathione, and various exogenous molecules [38]. It can be deduced that ROS/RNS pose a threat to the cell when antioxidant defense systems are incapable of eliminating them.

Oxidative stress in sperm

Three main parts are structurally differentiated in sperm cells: the head, midpiece, and tail, each with specific features and functions. The sperm head is composed of the nucleus, acrosome, and postacrosomal region. The midpiece of spermatozoa is a unique region containing mitochondrial helix that provides energy necessary for the movement of the tail and hence for sperm motility. The sperm cell tail is a long, slender structure that pushes sperm towards the egg (**Fig. 2**) [31]. The structure and function of human spermatozoa are critical for successful fertilization and abnormalities in their structure leads to infertility.

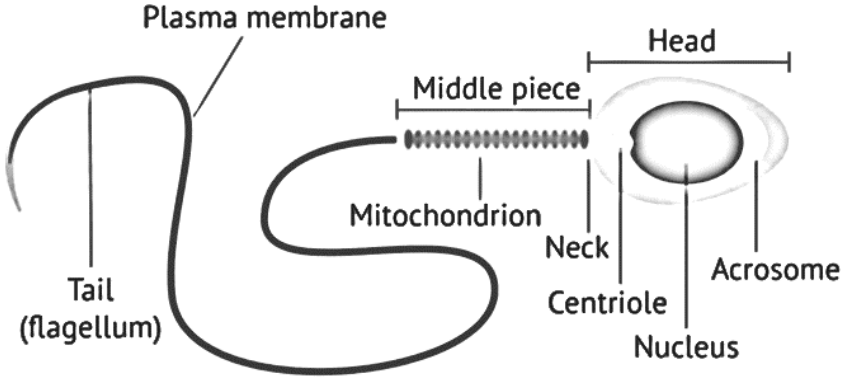

Fig. 2. Simple structure of typical human mature sperm [14].

While free radicals can be located throughout the sperm cell, they are mostly found in the midpiece region, specifically in the mitochondria which are the place where the cellular respiration cascade is located. Oxygen is utilized to produce energy (ATP – adenosine triphosphate) and inevitably this generates ROS as a byproduct, damaging the cells, in particular the mitochondria. Additionally, the midpiece of sperm cell is also where the majority of the cell's antioxidant defense systems operate, including enzymes such as SOD, catalase, and glutathione peroxidase as well as non-enzymatic antioxidants.

It is known that the volume of the mitochondria plays a key role in the availability of ATP hence the sperm performance. Measuring the oxygen consumption can provide an understanding of its metabolic state. It seems, increased oxygen consumption equals an abundance of ROS/RNS. This however, is not inherently bad as some authors reported that the most fertile ejaculates may have high amounts of free radicals [24]. According to other investigations significantly higher levels of ROS are found in the semen of infertile patients as compared to fertile men. Therefore, low and controlled (physiological levels) concentrations of ROS may play an important role in normal physiological processes in sperm such as capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion in order to ensure appropriate fertilization [4]. This may point to a beneficial aspect of free radicals as a product of intense mitochondrial activity.

Oxidative and nitrosative stress can exert their detrimental effects far beyond the mitochondria in the midpiece. Both types of stresses can react with plasma membrane which is exceptionally rich in polyunsaturated fatty acids [17]. High levels of ROS in sperm can cause loss of up to 60% of the fatty acids, hence affecting membrane fluidity, membrane-bound receptors and enzymes that are associated with abnormal fertilization [6]. Lipid peroxidation (LPO) is an autocatalytic process involving three main steps resulting in formation of end products, namely nonreactive malondialdehyde and 4-hydroxynonenal (4-HNE), which are disastrous for the genome and proteome as they can cause double-stranded DNA breaks [58]. Ultimately, the process of fertilization is affected negatively because a damaged membrane is incapable of proper sperm-oocyte fusion [27]. Parallel to impaired fusion, the motility of sperm can also be affected which makes passage through the cervical mucus impossible. One suggested mechanism is that ROS/RNS can inhibit the proper phosphorylation of axonemal proteins and render sperm immobile. This process can be reversed by treating ejaculate with antioxidants [7].

Despite its tight packing and relative resistance to oxidative stress DNA in spermatozoa is still considered a susceptible target. During the process of spermiogenesis, chromatin undergoes a series of modifications where histones are replaced with transitional proteins and finally with protamines. They condense DNA to form the principal unit of sperm chromatin called toroid which undergo further tightening by disulfide cross-linking to make DNA extraordinarily resistant to oxidative stress [61]. However, if the compactization is not optimal, the ROS can attack and cause base-free sites, deletions, frame-shift mutations, DNA cross-links, and chromosomal rearrangements. Damaged DNA was found in human testicular, epididymal and ejaculated spermatozoa [34]. Single or double stranded DNA breaks can be visualized by TUNEL and Comet Assay and they mark differences in reproductive capacity between fertile and infertile men [61]. Reparation of double stranded breaks is notoriously hard in the Y chromosome and this responsibility falls upon the oocyte [16]. Prolonged damage to DNA could result in meiotic sex chromosome inactivation (MSCI) where transcription is notably suppressed [37]. Y-bearing spermatogonia can be a target of mutations in the euchromatic Y region (Yq11), known as the azoospermia factor, resulting in complete absence of spermatozoa and infertility [50]. In case of unsubstantial DNA damage, spermatozoa are capable for self-repair mechanism and eventually maintain their fertilizing ability. However, oocytes are also capable of repairing damaged sperm [7]. DNA damage contributes to sperm apoptosis, poor fertilization rate, and high frequency of miscarriage and offspring morbidity. In

humans, 80% of DNA damage is of paternal origin and it is suggested to be a result of unsuccessful apoptosis [4].

Apoptosis is crucial for having healthy sperm and it is the quality control regulator in spermatogenesis, in particular germ cell to Sertoli cell ratio. Two main mechanisms are known to be responsible for initiation of germ cell apoptosis – Fas membrane mechanism of Fas ligand (FasL) and Fas receptor (FasR) and mitochondrial mechanism of Bcl-2 family. Similar to developing germ cells, spermatozoa can undergo apoptosis and they express apoptotic markers such Fas, Bcl-xl and p53. Sertoli cells express FasL, which after binding FasR on the germ cell membrane initiates apoptosis [25, 42] and that mechanism is probably involved in Sertoli cell control of germ cell/spermatozoa number. In transgenic models the overexpression of Bcl-2 or Bcl-xl leads to an abundance of spermatogonia and infertility and knockout of Bcl-xl causes a lack of spermatogonia [36, 43]. Knockout of p53 results in increased number of defective sperm due to suppression of germ cell apoptosis. A balance between pro and anti-apoptotic events is crucial for maintenance of adequate number of spermatozoa. The presence of ROS/RNS in semen due to the various stressor factors, cause mitochondrial damage and DNA fragmentation of sperm. Injured mitochondria leak cytochrome C, caspase 3, and 9 in seminal plasma that along with the broken DNA results in apoptosis [52, 57].

Sources of oxidative stress in sperm

Reactive oxygen species originate from various endogenous and exogenous sources. Endogenous free radicals generated by enzymes are just as harmful as exogenous ones. However, if an individual maintains a healthy lifestyle their generation and neutralization should be at equilibrium. Environmental stressors pose a much larger threat due to their potential to induce oxidative and nitrosative stress. In the course of modern daily life, the presence of an exogenous stressor is more frequent than others and alcohol is the most notable. According to the World Health Organization (WHO) 15+ year old Europeans consume at least 10 liters of pure alcohol per year and alcohol consumption has been increasing [62]. Ethanol metabolism has detrimental effect on whole organism and particularly on the male reproductive system. Excessive alcohol consumption is associated with reduced percentage of normal sperm in men with asthenozoospermia. Acetaldehyde, a product of ethanol metabolism interferes negatively with proteins, lipids and DNA generating oxidative and genotoxic stress that results in double stranded DNA breaks [4, 22].

Another distinct and documented inducer of free radicals in seminal fluid is cigarette smoke containing more than 4000 chemical compounds for some of which have shown to cause an imbalance between antioxidant system and ROS in semen of smokers. Decreased levels of antioxidant such as vitamin E and C was found in seminal plasma in smokers. Apart from being a complete carcinogen cigarette smoke worsens almost every aspect of semen such as DNA integrity, sperm motility, sperm count associated with increased seminal leukocyte concentration and levels of ROS [39].

Chemicals can become into contact with an organism both industrially and domestically. Exposure to well known toxicants such as heavy metal ions – mercury, cadmium, lead, and manganese is a serious threat leading to reduced sperm quality,

sperm volume and count associated with increased presence of reactive species [29]. Other exogenous factors such as industrial endocrine disrupting chemicals, can also contribute to an increased level of reactive species, in particular generation of ROS coupled with reduction of glutathione and induction of germ cell apoptosis [33]. Endocrine disruptors are widely spread throughout the environment and there are multiple ways for human exposure and penetration into the body. They are present in everyday items such as plastic containers, self-care products, water, fabrics, and food through pesticides, etc. In particular, phthalates were shown to affect spermatogenesis and induce DNA damage. It should be taken into account that concomitant sources of ROS are possible such as smoking, alcohol consumption and intake of multiple disruptors [54, 55].

Radiation, ionizing and non-ionizing, represents an emerging threat to male fertility and oxidative stress generation appears to be one of its damaging mechanism of action. Humans are not usually exposed to high levels of ionizing radiation (IR) in their everyday life and such incidences are often occupational or in a clinical setting. IR includes gamma rays and X-rays and it attacks primarily DNA which leads to two types of damage [12]. Direct action on the structure of DNA is resulting in damage of the sugar backbone or nitrogenous bases or double-stranded DNA breakage [59]. Indirectly, extortionately high levels of ROS, mainly hydroxyl radical are generated in the cytoplasm by water radiolysis resulting in impaired lysosomes and membrane damage [18]. Even at low doses, IR can cause reduced sperm count and sperm motility [60]. Mitotic dividing germ cells (spermatogonia) are the most vulnerable germ cell type, whereas primary spermatocytes during meiosis are somewhat protected as in that time frame proteins facilitating DNA repair are present and highly active [15].

Non-ionizing radiation (NIR) is equivalently studied for its negative effect on spermatozoa. Modern life introduces microwaves like radio-frequency electromagnetic radiation (RF-EMF) by laptops, cell phones, microwave ovens, security scanners at levels hardly found in nature. Free radicals appear to be the culprit behind the damaging effect of nonionizing radiation [26]. In an organism NIR can exercise a thermal effect where its energy is converted to heat or a non-thermal effect where electric and magnetic fields can alter skin and cell membrane permeability. *In vitro* exposure of human spermatozoa to electromagnetic radiation can worsen sperm motility, viability and sperm concentration depending on the duration of exposure and these changes are associated with elevated ROS production in the mitochondria and DNA fragmentation [32]. Additionally, kept in proximity to Wi-Fi connected laptops men can exhibit lower sperm count [35]. The thermal effect can raise the temperature in the scrotum and testes causing raised levels of ROS and induce germ cell apoptosis [49].

ROS found in seminal plasma originates from exogenous sources (mentioned above) as well as various endogenous ones. Leukocytes - mainly neutrophils as well as macrophages and defective spermatozoa are considered the main endogenous sources of ROS [4]. Abnormal sperm morphology manifested by excessive residual cytoplasm around midpiece is associated with high amount of ROS affecting sperm motility. It is well known that sperm, while in the testes are protected from immune system via the blood-testis barrier. Once they enter epididymis and move along the duct, the sperm are protected by antioxidant enzymes secreted by the epididymal epithelium into the lumen. Once ejaculation occurs, while located in the urethra sperm might come into contact with activated phagocytic leukocytes producing free radicals as

a result of an infection. Inflammatory process affecting prostate or seminal vesicles such as prostatitis can trigger peroxidase-positive leucocytes and they can produce exorbitant level of ROS. This condition is described as leukocytospermia and often requires pharmacotherapy [23]. In *in vitro* fertilization (IVF) procedures application of antioxidants in washing suspension is important to maintain sperm functional in case of leukocyte contamination [21].

As a result of such inflammation an increase in proinflammatory cytokines, such as interleukin (IL)-8 occurs in tandem with a decrease in the enzymatic antioxidant SOD that leads to production of high levels of ROS. Correlation between impaired sperm function and seminal plasma with elevated levels of ROS, TNF-α (Tumor Necrosis Factor), IL-6 and IL-8 was found to result in an increased LPO of sperm membrane [3].

Increase in free radicals is involved in varicocele - one of the major factors contributing to male infertility. This pathology is characterized by abnormally high venous dilation in the testes and around the spermatic cord responsible for blood pooling and hence local heat excess causing oxidative stress on sperm. The level of ROS is positively correlated with the grade of varicocele. In addition, in men with varicocele positive relationship between ROS and germ cell apoptosis was revealed and both are negatively correlated with sperm concentration [49].

Evaluation and preventions of oxidative stress

To evaluate oxidative stress in the male reproductive system first it needs to be identified and then quantified. Reactive oxygen species was suggested to account for 30% – 80% of pathology in infertile men [2]. The main tool for diagnosis of different cases of male infertility is routine semen analysis based on sperm count/concentration, sperm motility, viability and morphology. Numerous studies point toward a negative correlation between ROS and semen parameters although there is still lack of evidence of an interdependence between increased ROS levels and pregnancy outcomes [2, 9, 10, 19]. A reduction in semen parameters is more frequently found in men with oxidative stress (OS) and asthenozoospermia is suggested as a surrogate marker for OS. Another marker is hyperviscosity of seminal plasma associated with increased levels of MDA and impaired antioxidant status. Urobacteria infections that affect prostate and seminal vesicles can also contribute to increased seminal plasma viscosity and an increase in ROS production. The presence of a large number of round cells imply possible oxidative stress caused by leukocytospermia or immature spermatozoa. To distinguish leukocytes from germ cells a peroxidase test is required, CD45 (leukocyte common antigen) immunostaining or measurement of seminal elastase. Visualization of excessive residual cytoplasm in abnormal sperm is indicative for high levels of ROS.

A significant number of methodologies to measure the ROS levels in semen can be applied however few of them are clinically relevant taking into consideration cost and patient convenience. Direct assay of oxidative stress is applied to assess the amount of oxidation in sperm membrane by measurement of MDA via the thiobarbituric acid assay. This is one of the oldest and most widely used method demonstrating that MDA is associated with decreased sperm motility and sperm-oocyte fusion. For indirect measurement of OS a chemiluminescence method is applied by incubating semen plasma with luminol in luminometer [1]. The number of free radicals produced

is measured as relative light units/s/10^6 sperm. This method allows measurement of both intracellular and extracellular ROS. Based on chemiluminescence, attempts have been made to determine a reference range of ROS in human sperm in the context of different pathologies. They present a quick and easy approach that can be incorporated in standard laboratory practice [30].

Apart from being cost consuming, luminol can also be used for indirect measurement of the total antioxidant capacity of the seminal plasma and for analyzing the balance between ROS and the antioxidant protection of sperm. An assay that has gained popularity due to its cost-effectiveness is nitroblue tetrazolium (NBT) that measures intracellular ROS concentration. This is a light microscopy method that provides information on the source(s) of ROS and accurately predicts whether ROS have been produced by spermatozoa or leukocytes. Therefore, application of relevant tests for measurement of ROS has clinical importance as they can help to identify subgroups of infertile patients suffering from oxidative stress that may be treated with antioxidant supplementation.

Having in mind innate OS prevention mechanisms, it should be expected that spermatozoa can be protected from OS by the endogenous antioxidants present in the seminal plasma including the enzymes catalase and SOD as well as non-enzymatic compounds – vitamins C and E and carotenoids. Spermatozoa also contain the antioxidants lactoferrin and coenzyme Q10 [5]. Another mechanism to prevent OS involved prostasomes that are extracellular vesicles secreted by the prostate. Fusion of prostasomes with the sperm plasma membrane is required for regulation of different aspects of sperm function, such as motility and capacitation. The presence of prostasomes in the seminal plasma results in a decreased ability of neutrophils to produce ROS.

Should one decide to take action against free radicals in the body, effort should obviously be directed to lifestyle changes such as reducing alcohol consumption, cessation of smoking, balanced diet, exercise, and reducing exogenous sources of oxidative stress in general. Advances in pharmacology, nutraceuticals, and aging research have pinpointed numerous molecules that work to quench free radicals. For example, supplementation with antioxidants can be considered as a precaution from oxidative stress. Based on their mechanism of action they can be divided into two types: (1) preventive antioxidants which prevent the formation of ROS – metal chelators or binding proteins (lactoferrin and transferrin); (2) scavenging antioxidants which remove ROS that is already present - vitamins C and E [51]. Another example is application of approved drug metformin [20], quercetin, lycopene, various flavonoids and many others [41]. Within IVF techniques protection of semen is considered within the window between taking a sample and fertilization. During the stage of liquid cooled storage of sperm in the range 4°C – 25°C and the subsequent freezing both enzymatic and non-enzymatic antioxidants can be added such as melatonin, vitamin E, catalase [51].

Conclusion

Based on the depth of current scientific biomedical research in terms of oxidative stress in sperm cells it's reasonable to consider that oxidative stress causes harm rather than benefit. Despite beneficial biological function in both germ and somatic cells, the free

radicals remain a damaging stressor. The prognosis however, is positive since effort to prevent oxidative damage and its concomitant conditions consolidates finance and intellectual work in this direction. Knowing that education and prevention are cheaper and easier than diagnosis and treatment, a campaign to acknowledge the problem is an approach that can be implemented while waiting for the pharmaceutical industry to solve the problem. In cases where oxidative stress is a byproduct of exposure to environmental pollutants, their source should be limited. In addition, changes in lifestyle and use of balanced diet is another measure to manage oxidative stress. Despite the well documented sources of ROS and their effects maintenance of a balance between reactive species and antioxidant systems should be the goal in the strategy to protect sperm function and male fertility.

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Mechanisms of Action of Heavy Metals, Related with Abnormal Protein and Enzyme Activity in Male Infertility Aspect

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Abstract

There is the main agreement that proteins are key targets for heavy metals. Apart from the oxidative stress (OS) pathway, the harmful effects of heavy metal ions are also represented by different modes of interaction with protein molecules. For example, by changing the functions of proteins (such as displacement of the main metal ions in enzymes and metalloproteins/MT, or oxidation of amino acids in side residues of peptide molecules), including their attachment to free functional groups (thiol, carboxyl or other groups). In addition, heavy metals can interfere with the synthesis and spatial structuring of forming proteins (by inhibiting the processes of protein folding or refolding upon transfer through cell membranes, or denaturation), causing aggregation of nascent proteins in living cells. The current review aims to discuss some of the possible biochemical and physiological mechanisms related to the protein/enzyme structure and functional activity through which metals influence or contribute to the disruption of male reproductive processes.

Key words: heavy metals, peptide molecules, protein/enzyme structure and functions, protein folding, oxidative stress, male infertility

Introduction

Proteins are the most varying molecules in the living systems, involved in all cellular processes. Each of them performs a specific biological role. Their importance in the preservation and realization of genetic information determines them among the main substances in living matter, together with nucleic acids, lipids, and carbohydrates.

About their important biological role, the abnormalities in their structure and functions underline many diseases, some of which could be fatal or connected with reproductive problems. The spatial structure of the protein molecule (three-dimensional or 3D structure) is related to the formation of the active and allosteric centers that determine the chemical reactivity and biological function of proteins [31]. It is known that for the normal catalytic activity of many enzymes is necessary metal ions for the correct folding of the peptide chain and formation of their active center. Disturbed homeostasis of the main metal ions - zinc, iron, copper, and others could be the reason for the misfolding and aggregation of proteins. Structurally changed proteins are already cytotoxic, as they can participate in the course of pathological processes in cells [28, 81, 85]. For instance, different variations are possible in the structure of the proteins' diluted forms (in which they are normally present in the living cells), as they are subjected to various effects by different other substances (from microelements to bio-active substances). At all stages of structural and functional organization, the protein molecules could be influenced by changes in pH, temperature, pressure, the concentration of certain substances (heavy metals, detergents, etc.), or ionizing radiation. Proteins subjected to similar change undergo various levels of denaturation. When homeostasis is restored, the reverse renaturation process is possible, in which the native physiologically active conformation of the protein molecule is restored [9]. In the living cell, this process is helped by other additional proteins, which are known as chaperons and chaperonins. In this aspect, metal toxicity can occur in two main directions: one is related to the inhibition and/or blocking of the physiological activity of specific, naturally folded proteins/enzymes (associated with increased free radicals or OS generation), and the other is aimed at structural changes and damages in the protein molecules involved in vital cellular processes (formation of cell complexes/organelles, metabolism, DNA synthesis, cell division or proliferation, etc.). Here we can add a third direction of terminal toxicity of metals (which is a consequence of the first two), associated with the initiation of cellular death processes – apoptosis or necrosis. Besides the OS pathway, the detrimental effects of heavy metal ions are also represented through various mechanisms of interaction, for instance, by displacing essential metal ions in enzymes or metalloproteins (MPs) or by modification of some amino acids (oxidation of amino acid residues), including their binding to free functional groups (thiol, carboxyl, etc.) in the peptide molecules [54]. MTs, like glutathione (GSH), have an important protective role against metal toxicity (metal detoxification) and OS and are involved in the regulation of the balance between Zn and Cu [78]. MT biosynthesis depends on the presence of both essential trace elements and amino acids histidine and cysteine. Their production increases several times during OS to protect cells against cytotoxicity and DNA damage. This process could be induced by appropriate agents or factors, such as different hormones, medicaments, alcohols, or other biologically active substances [86]. Advanced studies have revealed an additional mode of metal action that targets both naturally folded proteins/enzymes and non-folded proteins [42, 81]. For instance, *in vitro* experiments have shown that Pb and Cd, as well as As and Hg suppress the proteins' folding [75]. Heavy metal ions proved to inhibit very efficiently the spontaneous refolding of chemically denatured proteins by forming high-affinity multidentate complexes with thiol and other functional groups. Furthermore, the last is just as effective in the inhibition of chaperone-assisted refolding of chemically or thermally denatured proteins [32]. In the living cells, chaperons ensure the return of

the protein conformation after damage, as well as the creation and/or degradation of protein complex structures. Most of the chaperons belong to the group of heat shock proteins (HSP) and the increased environmental temperature has been assessed as a significant increase in their intracellular concentrations. From a medical point of view, both denaturation and renaturation of the proteins are pivotal in the processes of immune protection. The non-specific immune response of the organism is expressed with increasing body temperature, and in this way is possible denaturation of the proteins in the composition of the virus particles, which causes the degradation of the last. Presently, there is ample evidence that metals may increase the tendency to aggregate disease-related proteins and promote the progression of some neurodegenerative diseases [3, 28]. During evolution, mechanisms are formed to control the quality of proteins, which protect cells against the harmful accumulation of protein aggregates. The malfunction of these quality-control systems may result in disease or cell death [32, 82]. **Figure 1** suggests some heavy metal-induced pathological mechanisms of cell damage and reprotoxicity, respectively.

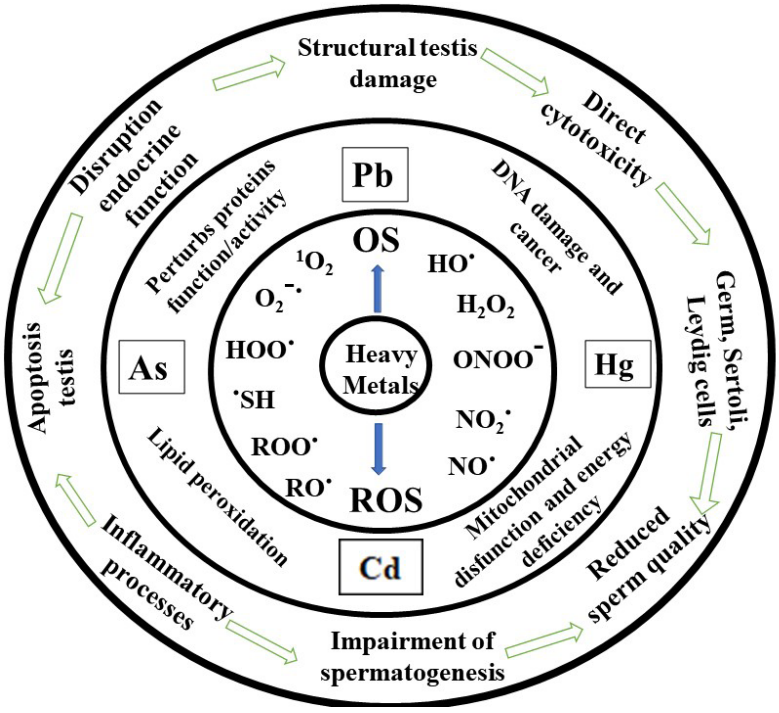

Fig. 1. Schematic review of the proposed pathogenic mechanisms of metal-induced cellular damage and reprotoxicity.

The effect of heavy metals on proteins can significantly affect protein homeostasis and cell viability. Independently of the mechanisms of influence of these metals on many proteins, which are of key importance in the germ cells development (such as β-tubulin, thioredoxin reductase (TrxR), insulin-like growth factors/IGFs, EGF, mitochondrial

enzymes, transporters, etc.), leading to the appearance of cytotoxicity, inflammatory processes and damage in the testes, serious disturbances in the endocrine functions and spermatogenesis, with a reduction in sperm quality (**Fig. 1**). Our previous reviews of heavy metals bring together abundant literature proving their negative impact on the male reproductive system and general human health [38-40]. Despite this, the mechanisms of the harmful effects of these elements on the male reproductive tract and fertility are not yet sufficiently elucidated. The purpose of the current review is to be discussed some of the possible biochemical and physiological mechanisms related to the protein/enzyme structure and functional activity, by which metals influence or contribute to the disruption of the male reproductive processes.

Arsenic toxicity. Arsenic can alter the functioning of about 200 proteins/enzymes, most notably those involved in cellular energy pathways and DNA replication and repair [70]. This metal affects the mitochondrial enzymes and interrupts the production of energy [91]. Arsenic through various mechanisms damages cellular respiration and, accordingly, to reduced ATP formation in cells [29]. Both As^{3+} and As^{5+} inhibit the activity of many enzymes involved in cellular metabolic pathways as the processes of glycolysis or gluconeogenesis, the citric acid cycle, and lipid oxidation [37, 40]. Arsenate (iAsV) can replace phosphate in several biochemical reactions, while arsenite (iAsIII) and the organic (trivalent-methylated) arsenicals react with SH-groups in proteins and inhibit their activity. As (III) interacts with many proteins and is supposed to interfere with their activity, e.g., binding to β-tubulin inhibits its polymerization [47]. Probably, in the same way, arsenic trioxide (ATO, As_2O_3) inhibits mammalian thioredoxin reductase (TrxR) by direct binding to the thiol groups of the enzyme. Inhibition of TrxR leads to thioredoxin oxidation, which is one of the main SH-dependent electron donor cellular systems, thereby affecting the cellular redox environment, as well as a wide range of cellular processes [58]. Arsenic can directly modulate the activity of key enzymes and hormones, causing hormonal dysregulation and impaired androgens (testosterone) production [40, 44]. It was found also that arsenic increased the expression of some enzymes and proteins such as selenoproteins (e.g., glutathione peroxidase 4 and selenoprotein P), 11β–hydroxysteroid dehydrogenase (HSD11B1), nuclear autoantigenic sperm protein (NASP), and calcium-binding and spermatid-specific protein 1 (CABS1), while others decreased critically, e.g., scaffolding factor B1 (SAFB1), transcriptional intermediary factor 1β (TIF1β), retinol-binding protein 1 (RBP1), DnaJ homolog subfamily A member 1 (DNAJA1), Y-box binding protein 3 (YBX3), and allopregnanolone, which causes abnormal spermatogenesis in the testes due to germ cell deficiency and low testosterone levels [36]. Of all the selenoproteins, glutathione peroxidase 4 (GPX4) and selenoprotein P (SELENOP) have the most significant role in male reproductive functions [6,19,23,69,73]. Moreover, GPX6 is the lone exception and a selenoprotein in men [19]. *GPX4* is distinctly expressed in testes and has both an antioxidant as well as a structural role [23]. It is believed that in the early stages of spermatogenesis, GPX4 protects developing germ cells from DNA damage caused by oxidative stress, but in the later phase ensures the integrity of the middle part of the sperm, becoming a structural component of the mitochondrial membrane, enveloping the flagellum, which is the basis of the stability and motility of sperm [6,74]. It has been also found that in humans GPX4 is abundantly distributed in late spermatocytes, and spermatids, and localized in the sperm midpiece, particularly

in the mitochondria [41]. SELENOP serves as a transport protein for Se and is also expressed in vesicle-like structures in the basal region of the Sertoli cells (SCs), and also *Selenop* mRNA was expressed in Leydig cells (LCs) of rats [66]. In addition, selenogins (including GPX1 and GPX3) are found in the epididymal epithelial and sperm [65]. Decreased levels and/or inactivation of these proteins be able to lead to severe disorders of spermatogenesis.

Lead toxicity. Literature data show that lead has significant effects on various vital cellular processes such as folding and maturation of proteins, enzyme regulation, ion transport, internal and intercellular signaling, cell adhesion, apoptosis, neurotransmitters release, and others [24]. These effects are related to the ionic mechanism of action of lead, with its ability to replace other bivalent cations such as Mg^{2+}, Fe^{2+}, Ca^{2+}, and Zn^{2+} (which act as cofactors), and monovalent cations such as Na^{+}, despite their more difficult replacement [22, 57]. The interaction between Pb and Na also seriously impairs the normal functioning of the sodium-dependent processes in cells [11]. The effect of Pb on the concentration of Na^{+} can lead to the disruption of important biological processes, such as the generation of action potentials in the excitatory tissues for the cell to cell communication, including the uptake of neurotransmitters (choline, dopamine, and GABA) and regulation of uptake and calcium retention by synaptosomes [11]. After the replacement of Ca^{2+}, Pb could cross the blood-brain barrier, as also through the blood-testis barrier (BTB). Pb^{2+}, even at very low (picomolar) concentrations, replaces Ca^{2+}, thereby affecting key neurotransmitters like protein kinase C, which regulates long-term neural excitation and memory storage [23]. The mechanisms of Pb-induced toxicity in testes also include changes in zinc bioavailability as a result of the displacement of Zn in MT molecules, leading to interference in calcium-mediated processes, involving disruption of BTB in the area of adhesion junctions. In addition, Pb interferes with the normal metabolism of Ca in cells and causes it to accumulate in them. Pb is considered a calcium mimic and may affect a variety of systems in the organism [12]. For instance, the interference of Pb in multiple isoforms of calcium and potassium channels in human testes and sperm may be involved in the early events of acrosomal reactions [7]. Another main reason for the toxicity of Pb is its interference in the activity of various enzymes, as it binds to the SH-groups contained in them. The influence of lead, in the reproductive organs of rats, have been reduced the activities of some enzymes such as alkaline phosphatase and sodium-potassium ATPase [4, 83]. Also, the decreased activity of other enzymes, such as δ-aminolevulinic acid dehydratase (ALAD, an indicator of long-term lead exposure), which are associated with decreased seminal plasma zinc levels, demonstrates the adverse effects of lead on prostate function [60].

Lead alters blood vessel permeability and collagen synthesis [62]. Specific targets of Pb include inhibition of enzymes involved in heme production, possibly due to its accumulation in erythrocytes, and induction of inflammation in vascular endothelial cells [83]. Pb inhibits ALAD and causes an increased concentration of the substrate aminolevulinic acid (ALA, the first compound in the porphyrin synthesis and heme synthesis, respectively) in the blood, which leads to oxidation of hemoglobin and directly causes hemolysis of red blood cells (RBC) together with the generation of hydroxyl radicals [1,67]. Pb also inhibits the enzyme ferrochelatase, which catalyzes the binding of protoporphyrin and Fe^{2+} (necessary for heme formation), and thus

leads to disruptions in heme synthesis and production [45, 75]. Pb also interferes with enzymes that maintain cellular membrane integrity or aid in vitamin D synthesis and DNA transcription [87]. Along with these toxic effects, lead can cause excessive production of inflammatory proteins and the development of an inflammatory process in the testicular tissue and accessorial germ glands.

Mercury toxicity. Different forms of mercury (Hg^{2+}, CH3Hg/MeHg) have shown higher affinity to SH-groups (of cysteine/Cys residues) compared to Cd, As, and Pb, which suggests higher toxicity of Hg to thiol reactivity, causing enzyme inactivation [2, 18, 43, 56, 88]. Hg-Cys conjugation mediates many toxic effects on various endogenous/exogenous peptide molecules, potentially altering their normal biological function. For example, the inactivation of manganese superoxide dismutase/Mn-SOD, arginase I, sorbitol dehydrogenase, δ-aminolevulinate dehydratase, etc. [2]. Many studies have demonstrated the impact of MeHg on enzyme activity by covalent modifications of thiol-containing biomolecules, known as "S-mercuration" [88]. On the other hand, pathways of MeHg transport through the cell membrane may involve mimicking the amino acid methionine by the sodium-independent exchanger large neutral amino acid transporter (LAT-1) [18]. LAT-1 is found in the brain, testes, and placenta, and mediates the transport of large neutral amino acids (such as tyrosine) and thyroid hormones (triiodothyronine) through the cell membrane [25]. There is evidence that glutathione peroxidase 3 (GPX3), selenoprotein P, albumin, and hemoglobin are the primary Hg-binding molecules/ligands after Hg exposure *in vitro* (100-1000 mg/l Hg) and *in vivo* (139.7-778.4 mg/l HgCl2) [56]. Transferrin, as well as ApoE, ApoA-I, and ApoA-IV have also appeared to bind to Hg at increased concentrations of $HgCl_2$ *in vitro*. Transferrin (synthesized by SCs) is involved in the transport of iron ions (iron shuttle system) necessary for the normal development of germ cells, and inactivation of this transporter may be the cause of impaired spermatogenesis. The levels of seminal transferrin, are proportional to sperm production in humans and may be an effective indicator of Sertoli cell function [80]. Additionally, albumin, cysteine, selenocysteine, GSH, hemoglobin, as well as MT, are major binding sites for Hg *in vivo*, and except for the last two ligands, both Hg^{2+} and CH_3Hg^+ bind through Cys thiols or selenol (–SeH) groups of selenocysteine - the active centers of selenoproteins [43]. Other Cys-rich proteins like G-protein-coupled receptors-targeting proteins (interleukin-8, somatostatin, oxytocin), enzyme-coupled receptor-targeting proteins (insulin-like growth factors, epidermal growth factor), extracellular enzyme inhibitors and antimicrobial peptides could also be targets for Hg [53]. For instance, the insulin growth factor family (insulin, insulin-like growth factors - IGF1 and IGF2, and their insulin receptors/IGF1R), provide essential signals for the control of growth, metabolism, and reproductive functions (during embryogenesis, SC proliferation, germ cell proliferation/differentiation and steroidogenesis) [14,30]. In the testis, IGFs act in an autocrine-paracrine manner [30], and IGF1R mediates the effects of follicle-stimulating hormone (FSH) via the PI3K/AKT pathway [14].

Mercury may also influence the cofactors, including by binding to thiol groups of coenzyme A (CoA) with the formation of the Hg-CoA complex, leading to altering mitochondrial β-oxidation and enzyme reactions disruption [27]. CoA is a metabolite of pantothenic acid/Vit. B5, which is essential for many key cellular processes, including energy production, and lipid and amino acid metabolism [79].

In experiments with mice, enzyme acyl-coenzyme A (CoA) synthetase (ACSL) 6 preferentially converts long-chain polyunsaturated fatty acids/LCPUFAs into LCPUFA-CoA, thus contributing to the local accumulation of LCPUFA-containing phospholipids in spermatids, which is important about the normal spermatogenesis [76]. The involvement of the Hg-SH interaction in altering the function of the Na^+-K^+-ATPase ion channel, which appears to be a potential target of Hg toxicity, has been demonstrated [50]. Na^+-K^+-ATPase is a ubiquitous plasma membrane enzyme that uses ATP hydrolysis to regulate cellular Na^+ and K^+ levels and fluid volume. This enzyme is also proved in the seminiferous and epididymal epithelium of rats of various ages, and it has been associated particularly with the borders of SCs (on the apical and lateral SC membrane and of junctional specializations), and this distribution continues until spermatids present in the epithelium. Furthermore, Na^+-K^+-ATPase is found in the excurrent and efferent ducts in the testes of immature and mature rats [13]. As the Na^+-K^+-ATPase-bound Hg(II) ions cause a decreased activity of mitochondrial NADH-O_2 oxidase accompanied by F1FO-ATPase/F-ATPase activation, the thiol-dependent mechanism could also confirm the link between Hg exposure and mitochondrial dysfunction [63]. This defines Na^+-K^+-ATPase as a potential biomarker for male infertility [55]. Thiol-dependent inactivation may also be involved in the Hg-induced reduction of different Ca^{2+}-ATPase isoforms [61]. Ion homeostasis is determined by Na^+-K^+-ATPase, and Ca^{2+}-ATPase, which are the integral enzymes found within the plasma membrane of most cells, including sperm [84]. A considerable variety of tissue-specific functions of the plasma membrane Ca^{2+}-ATPase (PMCA) [77] (and of different tissue-specific subtypes/isoforms) associated with the regulation of normal physiological cell functions have been assessed. This enzyme has been established to be localized in sperm flagella membranes [89] and interacts with the sperm-activating and attracting factor/SAAF, which amplifies the ATPase activity of PMCA. The impairment of Na^+ K^+-ATPase, PMCA4, and their isoforms has been proven to lead to decreased sperm motility. Ca^{2+}-ATPase has also been proven as a calcium pump, which is responsible for the support of Ca homeostasis and in the initiation of spermatozoa motility and acrosome reaction [55]. Hg exposure is also proved to inhibit the action and/or activation of other important ion channels as transient receptor potential channels (TRPCs) by Hg ions binding to their extracellular Cys residues [72]. TRPCs are integral membrane proteins, performing the role of membrane ion channels, important in the mediation of spermatozoa Ca^{2+} transport, thus regulating Ca homeostasis and supporting vital sperm functions (motility, chemotaxis, thermotaxis, capacitation, acrosome reaction, etc.) [52]. In the spermatozoa of patients with asthenozoospermia (associated with varicocele), altered activity of the TRPC5 was found, accompanied by reduced SOD activity and cellular motility [90].

Cadmium toxicity. Cadmium could also bind to glutamate, histidine, and aspartate ligands, thus leading to iron deficiency [16]. On the other hand, Cd may displace Zn and Ca^{2+} from metalloproteins and Zn finger proteins (ZNFs) [21, 33]. ZNFs, similarly to MPs, are a numerous group with very diverse functions. They can interact with DNA, RNA, and other key molecules, thus influencing the regulation of many cellular processes as for instance, gene transcription, translation, DNA repair, mRNA trafficking, signal transduction, cytoskeleton organization,

epithelial development, cellular adhesion, protein folding, chromatin remodeling and numerous other vital processes [15, 52]. These data suggest important biological roles of the ZNFs in the development of the organism under normal physiological and pathological conditions. The damages caused by Cd are mainly due to its interference with Zn-mediated metabolic processes in cells, probably by molecular mimicry of Zn [12]. It has also been found that Cd and Zn are specifically coordinated with cysteine residues and that each MPs molecule can bind up to 7 Cd atoms instead of Zn [68]. Cd has been found to transit easily into the sperm nucleus, adhering tightly to the free SH groups in the protamines by displacing or competing with Zn which is normally bound to Cys residues. Cd bound in this way prevents the formation of normal disulfide bonds between protamines during the final phase of gamete maturation. The formed Cd-SH bonds are very stable and prevent the necessary decondensation of chromatin immediately after fertilization [37]. Cd also inhibits *in vitro* human thiol transferases (thioredoxin reductase, thioredoxin, glutathione reductase), again by binding to Cys residues in their active sites, causing cellular damage [17]. Furthermore, Cd has been shown to cause DNA damage by influencing the DNA mismatch repair system that is, by inhibiting the ATPase activity of the Msh2p-Msh6p complex. It is, however, not known whether Cd binds to a specific site or displaces a critical Zn ion [5, 46]. According to other data, there is evidence that Cd mediates functional changes in ion transport or ion channels associated with reproductive toxicity in men. Variations in genes, coding proteins containing plasma membrane ion channels and transporters, could affect the sensitivity to Cd. While these proteins normally regulate Ca^{2+} flow, other cations, such as Cd and Pb, have also been shown to use them [48]. For instance, L-type voltage-dependent ion channels (L-VDCC) usually provide cellular access for Ca, and the ion selectivity is determined by binding sites in the pore area of the channel [35]. Several L-VDCC isoforms exist with variations in their performing units, one of which, a1C, is testes specific [26]. According to Benoff et al. (2005), two-thirds of men with varicocele contained a splice variant in the L-VDCC a1C region (responsible for ion channel activation) and at the same time significantly higher levels of Cd in the testes than men without variations in the range of L-VDCC a1C [8]. These data suggested the possibility men with Cd-channel variants are at increased risk for severe varicocele and infertility, associated with the higher Cd levels of the testes.

It is assumed that the basic mechanism of the toxic effect of Cd on the reproductive system of mammalians is due to morphological changes and dysfunction in the blood vessels of the testis and epididymis, which makes them more permeable [59]. Cd produces these effects by causing damage to the vascular endothelium integrity of the testicular capillaries and venules, including the BTB [64]. Cd has been shown to induce changes in the expression and function of vascular endothelial cadherin (VE-cadherin), which is a calcium-dependent cell adhesion molecule, involved in the reorganization of the actin cytoskeleton, and cell-cell contacts [49]. Another molecule, ZIP8 (a specific metal ion transporter), has also been identified to enhance Cd uptake by vascular endothelial cells in the testes of mice, and its expression supports Cd-induced testicular damage [10, 34]. In this way, Cd can cause specific injury to the internal spermatic artery, its testicular and epididymal branches, as well as the pampiniform plexus. This mechanism may also include the cytotoxic effect of Cd on vascular smooth muscle cells (VSMCs), which are involved in pathological

changes occurring in the vessel wall, especially when the metal accumulates in these cells. VSMCs perform a variety of physiological functions (both contractile and synthetic) that are characterized by changes in the morphology, proliferation, rate of migration, and expression of various marker proteins [20, 71] relevant to the normal development of the germ cells.

Conclusion

The current review combines the literature data about the mechanisms of influence of metal ions on the protein molecules, but several concrete mechanisms are particularly underlined: the metal ions either replace Zn and other essential metal ions in metal-dependent proteins or bind to free thiol and other functional groups of certain native proteins. These mechanisms not only affect individual proteins but also lead to the formation and accumulation of toxic protein aggregates in the cells. Possible consequences of protein folding inhibition by heavy metal ions could be expressed in deficiencies in the amounts of the affected proteins, in disruption of the normal activity of enzymes, as well as of the normal process of DNA transcription, in the initiation of mutations, in imitation of hormones, thus disrupting the endocrine and reproductive system, and leading to male infertility. In such conditions the homeostasis of the cellular proteins could be affected, including some key proteins, participating in many subtle long-term changes in the germ cells during spermatogenesis. Another main mode of action of the heavy metals is by their participation in different ion mechanisms, mainly by mimicry and interference with essential ions such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+} (co-factors), injuring in this way the normal functions of ion-dependent processes in the cells. So, the toxic action of heavy metals decreases the activity of the enzymes mitochondrial oxidases (which are thiol-dependent), which could lead to mitochondrial dysfunction and ATP-ase (energy) deficiency. Studies on the induced by heavy metals mechanisms leading to cytotoxic effects in the organism are crucial not only for the development of appropriate therapeutic strategies but also for clarifying the pathological process diagnosis.

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Original Articles

Morphometric Study of Scapula and Related Surgical Importance of Suprascapular Notch among West Coastal Population of South India

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Morphometric measurements of suprascapular notch give us a better understanding of predisposing factors of the compression of suprascapular nerve. The objectives were to correlate the variations in morphometric features of the scapula and suprascapularnotch. Across sectional study design was carried out with 150 dry adult human scapulae of unknown sex (70 right and 80 left). Morphometry of the scapula, maximum depth (MD) and superior transverse diameter (STD) of suprascapular notch were measured. Scapular index and infraspinous index were calculated. The indices show that the right scapula is shorter and broader than that of left scapula. There was no statistically significant difference between the anthropometric measurements of the suprascapular notch between right and left side ($p>0.05$).The present study has provided a database on the morphometric variables of the scapula and suprascapular notch in the South Indian population.

Keywords: Suprascapular notch; Suprascapular nerve; Scapula morphometry; Scapular Index, Infraspinous index

Introduction

The scapula is a triangular flat bone which has three borders, two processes and two surfaces that lies overlying the 2nd to 7th ribs on the posterolateral aspect of the thorax. The scapula has a spine which subdivides the posterior surface into a supraspinous fossa and infraspinous fossa. The concave anterior surface has a subscapular fossa [7]. The suprascapular notch is seen as a depression on the superior border of the scapula which is converted into a foramen by the superior transverse scapular ligament. The suprascapular nerve and vein crosses below the ligament while the suprascapular artery crosses above the ligament to reach the anterior surface [1, 5]. The morphology and morphometry of the scapula has clinical and anthropological importance and will help the surgeons in the field of prosthesis and shoulder girdle arthroplasty. The study of dimensions of the suprascapular notch can provide with a guide way for landmark for suprascapular nerve block and to correlate suprascapular nerve entrapment syndrome [10,11]. Variations in the morphology of suprascapular notch have been identified as one of the causes of suprascapular nerve neuropathy by suprascapular nerve entrapment [2,6,11,14,16]. Morphometric measurements of suprascapular notch give us a better understanding of predisposing factors which cause compression of the suprascapular nerve. Many researchers had attempted to classify suprascapular notch and the most popular classification is by Rengachary et al. [14].

The anthropometric data obtained from different geographical locations may vary due to racial, genetic and geographical factors across populations. Upon the back drop of paucity of data in west coastal population of South India, this study was proposed with an aim to generate reference data for both clinical and research purposes in this region. The study objectives were to correlate the variations in morphometric features of the scapula and suprascapular notch. We also focused to calculate scapular index and infraspinous index of human dry scapulae of South Indian population.

Material and Methods

Across sectional study was carried out with one hundred and fifty (150) dry adult human scapulae of unknown sex (70 right and 80 left). All ethical principles for human research were followed and ethical approval was obtained from the Institutional Ethics Committee of the medical college from where data were collected. The inclusion criteria were the human scapulae which are completely ossified and with no deformity. Scapulae with any deformities and pathologies with broken notches were excluded.

The measurements were taken with a digital Vernier caliper (Vernier Caliper with Fine Adjustment, Yuzuki Company, India), with precision of 0-600 mm/24 inch. The following variables were studied and documented. Maximum length and breadth of scapula, length and width of scapular spine, length of supraspinous line and infraspinous line, Maximum distance, length and breadth between/ of acromion and coracoid process, glenoid fossa and axial border were measured. The dry weight of the scapula was noted down with a paediatric weighing scale **(Fig. 1).**

For suprascapular notch dimensions and morphometric analysis the maximum depth of suprascapular notch (MD) and superior transverse diameter (STD) of suprascapular notch were measured. The indices namely scapular index, infraspinous index were calculated.

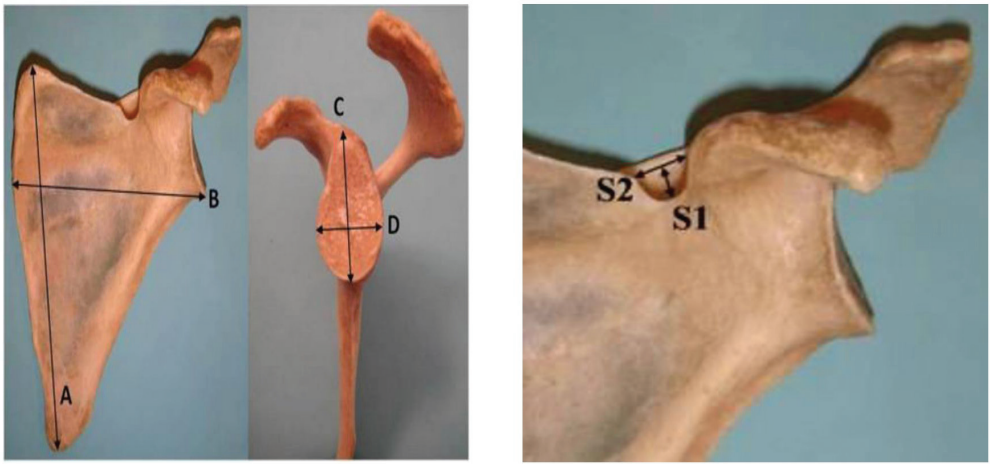

Fig. 1

The STD was measured as the maximum distance between most superior edges of suprascapular notch (SSN).The maximum depth was measured as the maximum value of the longitudinal measurements taken in the vertical plane from an imaginary line between the superior corners of the notch to the deepest point of the suprascapular notch. Scapular index was calculated toexpress scapular breadth as percentage of scapular height. Lengthier scapulas had increased scapular index and vice versa. Infraspinous Index was calculated by scapular breadth X100/ Infraspinous height. Infraspinous index expresses scapular breadth as percentage of infraspinous height, broader scapulas had increased infraspinous and vice versa indicates a narrower scapula. Details were photographed, recorded and analyzed statistically. The measurements above were taken for three repetitions and the average was recorded to avoid any possible measuring error. Rengachary *et al* (1979) [14] method of classification of suprascapular notch was used in the present study. It is as follows, Type I–the entire superior border of the scapula shows a wide depression from the medial superior angle to the base of coracoid process; Type II – a wide and blunt V-shaped notch; Type III–asymmetrical U-shaped notch; Type IV–a small, V-shaped notch; Type V –the medial part of the ligament being ossified; and Type VI – Ligament completely ossified and forming a foramen.

Statistical analysis was done by using the Statistical Package for Social Sciences (SPSS) version 22.0 (SPSS Inc., Chicago Illinois, USA). The mean and standard deviation were noted down. Comparison of means for the right and left sides of scapula was done with Z test. Frequencies of the various shapes were obtained while chi-square was used to compare for both sides. Difference was noted down as statistically significant if the $p< 0.05$.

Results

Scapular index were calculated for scapulas of both right and left side. The mean of right scapular index was higher than left scapular index (**Table 1, Fig. 2**). Infraspinous index was found higher in right sides scapulas. The mean of right infraspinous index was 96.98mmand left was 96.38mm (**Fig. 3**).The scapular index and infraspinous index shows that the right scapula is shorter and broader than that of left scapula.

Table 1. Difference between Scapular Index and Infra Spinous Index (Right and Left)

Details of measurements	Right scapular Index(mm)	Left scapular Index(mm)	Right Infra Spinous Index(mm)	Left Infra Spinous Index(mm)
Samples	70	80	70	80
Mean	74.82	72.41	96.98	96.38
Std. deviation	6.052	5.893	9.54	10.05

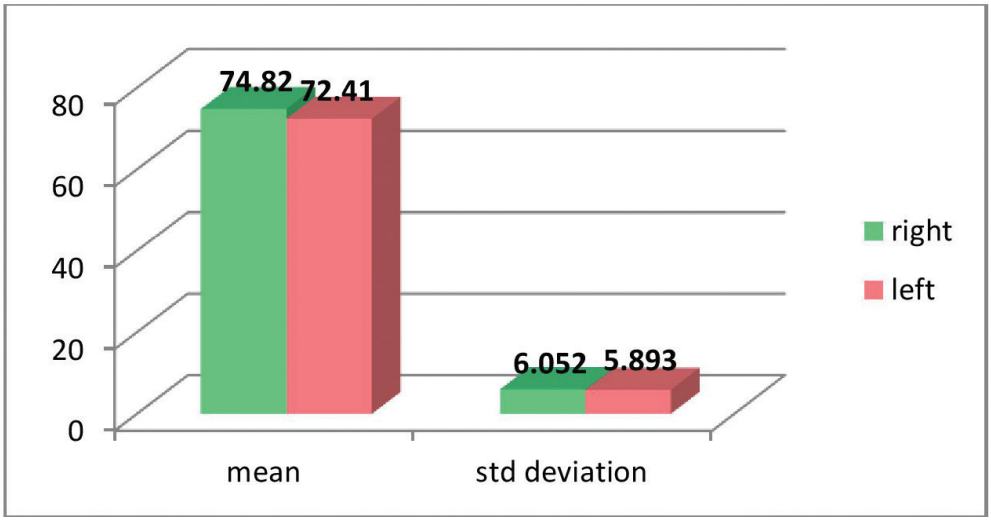

Fig. 2

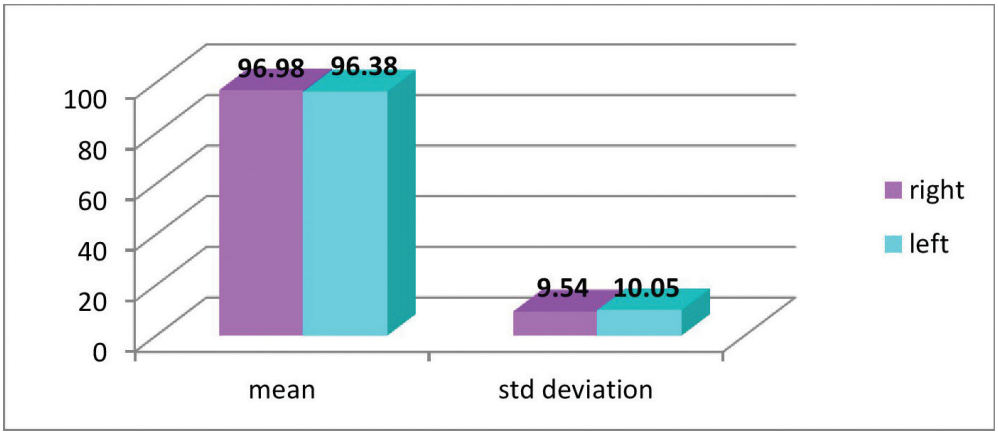

Fig. 3

In the scapulae with longer superior transverse diameter (STD), the morphological length and width of scapula, maximum length of the coracoid process, width of the glenoid cavity were higher than in the bones with longer maximal depth (MD) i.e; (STD>MD). In scapulae with (MD>STD)length of scapular spine, maximal width of scapular spine, length of acromion, length of the glenoid cavity were higher than in the scapulae with longer STD (**Table 2**).

Table 2. Measurements and indices of the scapulae and suprascapular notch

Measurements and indices of scapula(mm) Mean(mm)	**Scapulae with longer maximal depth(MD>STD):R-L**		**Scapulae with longer superior transverse diameter(STD>MD):R-L**			
	Standard deviation	**Min-Max(mm)**	**Mean (mm)**	**Standard deviation**	**Min-Max(mm)**	
1.MORPHOLOGICAL LENGTH	138.83	11.96	109-165	142.67	12.43	103-170
2.MORPHOLOGICAL WIDTH	100.11	7.75	80-114	103.33	6.51	81-116
3.PROJECTION LENGTH OF SCAPULAR SPINE	131.71	9.923	103-151	131.11	9.78	112-152
4.MAXIMAL WIDTH OF SCAPULAR SPINE	44.05	4.00	36-53	42.68	4.1	33-53
5.LENGTH OF ACROMION	49.79	6.86	33-60	48.96	7.19	32-84
6.MAXIMAL LENGTH OF THE CORACOID PROCESS	43.27	5.05	31-53	44.55	4.75	32-57
7.LENGTH OF THE GLENOID CAVITY	35.44	4.00	28-47	35.31	4.30	20-45
8.WIDTH OF GLENOID CAVITY	24.53	2.89	19-31	25.14	3.44	18-40
9.WIDTH-LENGTH INDEX(%)	62.4	4.5	59.4-71.5	63.8	4.6	59.3-72.6
10.GLENOID CAVITY INDEX(%)	73.4	6.2	58.3-84.1	74.1	6.8	59.6-85.3

There was no statistically significant difference between the anthropometric measurements of the group with higher MD and the group with higher STD of the suprascapular notch ($p>0.05$). The correlation between morphometric features of the

scapula and the dimensions of the suprascapular notch (depth) and superior transverse diameter (STD) is analyzed by pearson correlation indexes which are explained in **Table 3**. We found no statistically significant correlation between the depth and STD of the suprascapular notch and the major dimensions of the scapula and glenoid fossa.

In the present study, Type III SSN was the most frequent type of suprascapular notch (63%) followed by Type II observed in 34% and Type VI seen in 3%of the scapulas. Descriptive analysis of the dimensions of the suprascapular notch types according to Rengachary *et al* (1979) [14] classification have been mentioned in **Table 4**.

Table 3. Correlation indexes between the dimensions of the scapula and the dimensions of the scapular notch

Particulars	A	B	C	D	E
MD.SN Pearson's correlation	0.111	0.001	-0.07	0.043	-0.01
Sig.(2-tailed)	0.17	0.98	0.38	0.59	0.92
STD.SN Pearson's correlation	0.034	0.07	-0.07	0.001	0.12
Sig.(2-tailed)	0.67	0.37	0.35	0.99	0.11

*statistically significant ($p<0.05$) correlation indexes
Abbreviations: MD. SN – Maximum depth of suprascapular notch, STD.SN-Superior transverse diameter of scapular notch,A- Maximum length of scapula, B-Maximum breadth of scapula, C – Maximum length of glenoid fossa, D – Maximum breadth of glenoid fossa, E – Length of axial border

Table 4. Descriptive analysis of the dimensions of the suprascapular notch types according to Rengachary *et al* (1979) [13] classification

Type		Mean (cm)	Std. deviation	Minimum (cm)	Maximum (cm)	Median (cm)
III	DSN	0.74	0.23	0.21	1.62	0.71
	WSN	0.91	0.25	0.41	1.72	0.91
II	DSN	0.54	0.12	0.23	1.02	0.53
	WSN	1.23	0.37	0.52	3.12	1.21
VI	DSN	0.90	0.3	0.22	1.92	0.91
	WSN	0.64	0.23	0.42	1.12	0.62

Abbreviations: DSN – depth of the suprascapular notch, WSN – width of the suprascapular notch

Discussion

The present study correlates the variations in morphometric features of the scapula and suprascapular notch (SSN) of dry human scapulae of the Southern India. The variables of consideration included the superior transverse diameter (STD), the maximal depth (MD) and the shape of the SSN. The result showed that there is no significant difference in the values of STD and MD between the right and left SSN. Manikum *et al* (2015) reported that the right MD was significantly deeper in right than that of the left, while there is no significant difference in the STD [6]. Jezierski *et al* noted that the suprascapular notch was significantly wider and shallower on right side [4].

The STD in the present study was a little narrower (1.285 cm) compared to studies of Manikum *et al* (2015) of South Africa (1.39 cm) while the MD in the present study was deeper (0.997 cm) than the comparative study (0.68 cm) [6]. The size and dimensions of the SSN has been considered as a possible factor for suprascapular nerve entrapment as SSN is the frequent site for nerve compression [1, 2, 11]. However, compression of the suprascapular nerve may also occur at the base of the scapular spine [1].

Suprascapular nerve entrapment syndrome is characterized by pain on the posterolateral aspect of the shoulder, weakness of the arm, difficulty in external rotation and abduction movements resulting from paresis and atrophy of the infraspinatus and supraspinatus muscles [5]. Flower and Garson measured the mean scapular index of Europeans and Negroids. Scapular index of European population was 65.91mm and that of Negroid samples was 68.16 mm. Negroes had shorter scapula than that of Europeans. In present study there is an increased mean dimensions for right and left scapular index which itself is an indicator for shorter scapulae. This suggests that scapulae of South Indian population are shorter than European and Negroid populations [3, 8, 11]. This indicates that a geographical variation does exist between anthropometric measurements of bones among different populations in various countries.

In the present study, the mean of right infraspinous index was 96.98 mm and left was 96.38 mm. This shows that right scapula is slightly broader than the left scapula. Flower and Garson reported infraspinous index of Europeans were 87.79 mm and that of Negroid population as 93.88 mm. Scapulae of Negroes were shorter and broader than that of Europeans. Similarly, the present study results suggest that scapulae of South Indian population are broader and shorter than European and Negroid population. These differences may be due to the difference in general small built and stature of South Indian population when compared to other study population [3].

In the present study, classification of SSN was based on most popular and verified classification system. In the present study, Type III SSN was the most frequent type of suprascapular notch with 63% of cases. This is similar to the findings in various studies with a frequency of 45%, 52%, 66.9% in Europe, Asia and in Africa [5, 6, 8,12]. However, few authors reported type III as the third frequent type [15, 17]. Type VI SSN involves ossification of the superior transverse scapular ligament. In the present study, type VI SSN was found (3%).The shape of the SSN is important in the aetiopathogenesis of suprascapular nerve entrapment as it has been hypothesized that 'V' shaped narrower notch is more likely to cause nerve entrapment than 'U' type broader notch [11]. Narrow notch has been found in patients with the suprascapular neuropathy [1, 10]. Polguj et al. [9] reported the existence of a direct correlation between the scapular length and

the suprascapular notch depth (R=0.265) and an inverse correlation between the ratio length/width of the scapular body and the suprascapular notch depth (R=−0.327). But, present study reports no correlation between the above mentioned parameters.

The study of dimensions of suprascapular notch will help the anatomists, radiologists, neurosurgeons and orthopaedic surgeons for a better understanding, diagnosis and management of suprascapular nerve entrapment syndrome correlating with the specific type of suprascapular notch. Surgical removal of the ossified ligament is a treatment option in the management of suprascapular nerve entrapment syndrome in patients with complete ossification of the superior transverse scapular ligament [13].

Conclusion

The morphometric variables of the scapula and suprascapular notch of the study samples had decreased dimensions when compared to recent data for the scapular dimensions of from other continents. The present study has provided a database on the morphometric variables of the scapula and suprascapular notch in the South Indian population. Morphometric variables are the most important factor affecting the procedural outcomes and management of suprascapular nerve entrapment syndrome. However, this warrants further investigation with larger multi centric study involving different ethnic groups from the country.

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Demographic Specifics of Skeletal Population from Early Bronze Age Necropolis of Bereketska Mogila - Preliminary Results

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On a preliminary state of investigation, skeletal remains from 36 structures from Early Bronze Age necropolis of Bereketska mogila are examined. Skeletal remains from at least 75 individuals are recognized. Being demographically unrepresentative for mortality and life expectancy in child ages the material provided opportunities for reconstruction of some demographic features of adult population.

Key words: Early Bronze Age skeletal population, paleodemography

Introduction. Archaeological situation and dating

Bereketska mogila site is situated near the South-West outskirts of the contemporary town Stara Zagora. It is a multilayer settlement, which, with satellite settlement structures around the tell, presents cultures from a vast chronological limits of Praehistory from the Neolithic to the Early Bronze Age up to the Iron Age [6, 7, 9]. The unearthed necropolis is situated in East direction according to the mound and presents structures mostly from the Early Bronze Age, nevertheless reduced number of graves date from the Late Eneolithic period (from the Kodzhadermen-Karanovo VI- Gumelnitsa culture) [9]. The present study concentrates on the Early Bronze age series.

Material and Methods

The present study concentrates on the series from necropolis from Bereketska mogila, preserved in the National Anthropological Museum in the Institute of Experimental Morphology, Pathology and Anthropology. The material is obtained during the excavations held in 1970s [6, 7, 9] and consequently transferred for investigation

in the Department of Anthropology. Many of the graves from the necropolis, 12 (10 from Bronze Age), are registered being disturbed from later field works and they do not present skeletal remains, or present only singular bone fragment, which is not preserved for anthropological investigation. These are Bronze Age burials N 7, 11, 22, 24, 28, 29, 36, 38, 41 and 45 [9]. Other, 10 graves, also present disturbed fragmented, incomplete skeletons with no anatomical position of bones, also interpreted as a result of later field works. These are graves N 4, 5, 10, 14, 16, 19, 30, 49, 55 and 77. Most of them are noted in the field documentation as containing incomplete, fragmented skulls [9]. The skeletal remains from these graves also are not committed for preservation in the National Anthropological Museum. The preserved material presents high degree of fragmentation and destruction, which obstacles the anthropological investigation.

At the preliminary stage, the investigation aims recognition of number of individuals in the skeletal remains from each grave and age and sex identification of individuals. It is achieved at first after the detected graves with multiple burials on field [9]. In the materials from some of the studied complexes are detected bones from more different skeletons than registered on field. This is achieved after registered duplication of bones and bone locations on fragments, detection of bones from skeletons, which present individuals with different anthropological features as sex, stage of development, anatomical specifics or anthropological type. Many complexes present skeletal remains from more than one individual **(Table 1).**

The age and sex of the individuals are reconstructed after classical methods of assessing morphological features of skeleton of both sexes and stages of development, maturation and aging. For individuals in childhood and juveniles the age at death is ascertained after methods for assessment the dental development [15, 18], the timings of epiphyseal fusion as summarized by Schwartz [14], Alekseev [2] and Bass [4] and lengths of long bones compared to the tables of Maresh [11]. In single case of bone from a newborn, its fetal development is assessed after the length of radius by the methods of Fazekas and Kosa [8]. In adults age is ascertained after stages of cranial sutures obliteration after Olivier-Simpson methods [3], simphyseal surface relief after Todd's scale [13] and auricular surface relief after Lovejoy et al. [10]. Sexual dimorphism is assessed after the features of preserved fragments from pelvic bones based on the methods, summarized in Acsádi and Nemeskéri [1] and of cranial fragments as summarized in Walrath et al. [16]. After results of osteometry study performed after the standard methods, the measurements are correlated to tables of mean values for both sexes summarized in Bass [4] and Alekseev [2]. In complex of features used for sex determination priority is given to the data obtained in the investigation of the pelvic bones.

In analysis of sex and age distribution at first step, the identified individuals are distributed in the qualitative age groups and both sexes **(Table 2)**. At the next stage of analysis, in order to reconstruct mortality and survival conditions in the population are used methods of paleodemography as defined in Acsádi and Nemeskéri [1]. High level of fragmentation of the material obstacles the precise determination of the age in 5-year age intervals. In overcoming this situation, the analysis is proceeded in 10-year age intervals **(Table 3)**.

In the investigated material are analyzed also skeletal remains from two complexes dated in the Late Eneolithic, graves N 64 and 69. Results for age and sex of the individuals **(Table 1)** are excluded from the statistical analysis, performed for Early Bronze Age

complexes. Position of skeleton in the Grave No 35 [9] presents a deviation from the one, characteristic for the burial ritual in the necropolis, most pronounced in the upper limbs, and results for age and sex of this individual **(Table 1)** are also excluded from the demographic analysis of the series. Similar position is registered in grave No 34 [9], material from which was not available for anthropological investigation.

Results and Discussion

The investigation recognized in the bone material many complexes, which contained skeletal remains from more than one individual. In some cases, different skeletons are recognized on field, in other – there is no documentation for recognition during excavation of ascertained individual in the studied material. Anthropological investigation ascertained 18 complexes with multiple (double and more) burials (47.37 % from investigated complexes) and 20 singular burials (52.63 %). Complexes, for which only field archaeological data are available, also present multiple burials (one, from grave No 19 being quadruple) [9]. From these published complexes the proportion of multiple vs. singular burials is 2:11. Lack of anthropological investigation of the material from these complexes and their high destruction makes this proportion unsure.

Specific for the demographic distribution of the skeletal population is the highly reduced number of children in the first age group 0-7 years **(Tables 1-2, Fig. 1)**. The identified individuals under 7 years at death are ten (13.89%), from graves N 1, 20, 25, 31, 39, 50, 58, 59, 72 and 74. In all these cases, excluding grave No 20, these individuals are found in complexes with burials of more than one body, laid simultaneously. Similar situation is reported in the publication of the necropolis for grave No 19 with no anthropological investigation [9]. In most cases individuals from this age group (0-7 years) are found with skeletal remains from adults, in graves with double and multiple burials – graves No 25, 31, 39, 72, 74 **(Table 1)**. In grave No 1 an individual at the age ca. 4 years at death is found in a double burial with an individual at age of about 7 years of age. Only in one case, grave No 20, is identified a single burial of an individual at about 6 years of age. Under one year of age are identified only two individuals – from graves No 50 and 72. In both graves are found skeletons from multiple burials, in grave No 50 are recognized skeletal remains from five individuals and from grave N 72 – two individuals. Both infants are identified after singular bone fragments. In grave No 50 is found a preserved right radius, which presents a length close to the mean value of full term newborn at 40 weeks of gestational development. The presence of this fragment in the grave may be explained as clue for the hypothesis that in the grave had been buried a pregnant female. The fragment from grave No 72 does not allow measurement, but may be associated with a newborn or a breast fed baby. Both graves contain female skeletons, the one from grave No 50 at the age of 40-50 years and the one from grave No 72 – at 35/40 up to 45 years. As in most skeletal populations the relative number of individuals in the age group of Infants II is reduced in comparison to the juvenile and adult ages.

As in many paleopopulations male to female ratio in the studied skeletal population (28:21) presents a predominance of male sex with 57.14 to 42.86 %. Specific for the age-sex distribution of the studied skeletal population is the relatively equal representation of specific age groups in both sexes **(Fig. 2)**.

Nevertheless relatively similar distribution by age groups in males and females, after the analysis in more precise age intervals of 10 years, a higher mortality and lower survival in females in relation to males is visible in paleodemographic indices **(Table 3)**.

The investigated series is one of the few known from the period in Bulgaria. The other site, which presents graves from the people from the settlements on the tells in the Thrace region is Yunatsite. Here are studied intramural grave complexes, which mostly present remains of small children [5, 12]. The demographic distribution is the opposite of the observed in the necropolis of Bereketska mogila, where in general a lack of individuals under one year of age is ascertained, with two exceptions, one explained with possible burial of a pregnant woman, or case of death during childbirth and no singular burials of individuals under 6-7 years of age are detected. The comparison between both sites could be interpreted as a confirmation of the hypothesis for specific ritual rules in populations from the Early Bronze Age in Thrace, which prevented burials of small children in the regular necropolis and their deposition in settlements.

The other materials from the period of the Early Bronze Age from Bulgaria, those from the Pit-grave culture, also present small representation of child ages in necropolises. Published materials are mostly from the dispersal of the culture in the regions North from Stara planina mountain with one exception of necropolis near Boyanovo, Yambol district, which is in the area of the Thracian Plain [17, 13]. In sex distribution of identified from these sites a higher prevalence of male sex in comparison to the observed in population from Bereketska mogila is visible. The studied Pit-grave complexes present also higher mortality in females at younger ages, respectively lower life expectancy by them, than ascertained for the population from Bereketska mogila.

Conclusions

Obtained results for age and sex distribution of identified individuals from studied series suppose a situation of unfavorable conditions for survival in the population. This assumption is supported from high incidents of simultaneous burials in the grave complexes. Different ages and sex of buried in these graves suppose dispersal of infectious diseases as a possible cause of death of the individuals in these complexes.

The specific age distribution can be explained with deposition of deceased in early age in distant area from the necropolis, possibly in the settlement. Some uncertainty in this conclusion cannot be excluded, as material from some graves remains unavailable for anthropological study. Some of these complexes present high level of destruction of skeletal material.

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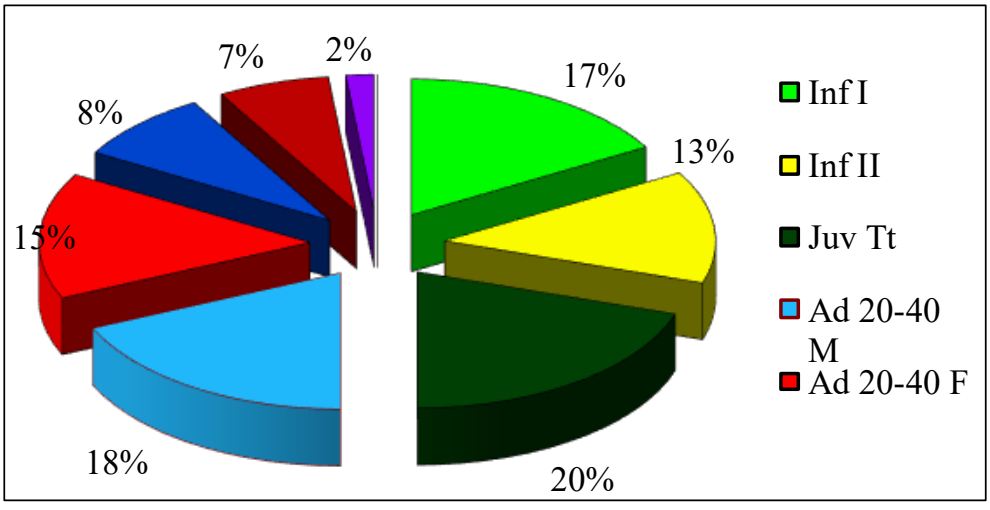

Fig. 1. Age and sex distribution of studied material.

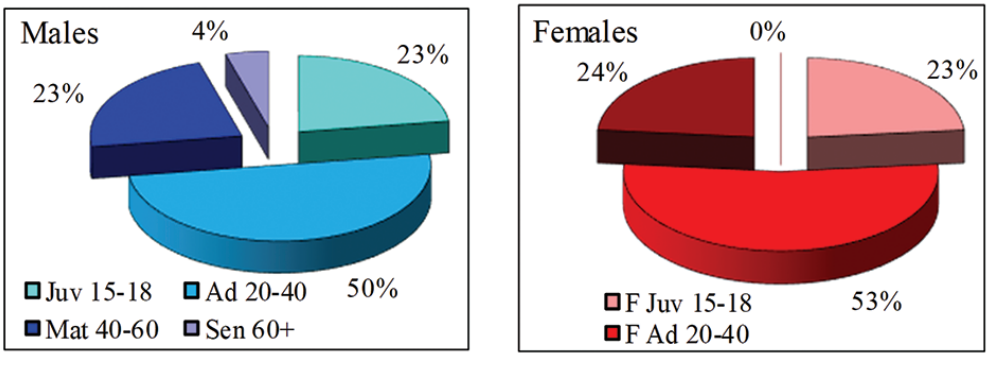

Fig. 2. Age distribution of identified individuals in both sexes.

Table 1. Individual identification of studied skeletal remains. y. – age in years; * – individual identified in laboratory analysis of the skeletal remains; S - individual identified in laboratory analysis after a single bone fragment; 0- sex unidentifiable; ? – features for sex identification are controversial and some are assessed with priority; M/F – features for sex identification are controversial and none can be assessed with priority; 30+ – over the specified age/20+ – individual with completed skeletal development.

Gr. N	Sex	Age y.	Gr. N	Sex	Age	Gr. N	Sex	Age
1	0	4	40	F	18/20-25	61	F?	15-18
1A*	0	7	46	M?	14-16	62	M?	14-16
2	M?	40/45-50	47	M	17-18	64	M?	30-40
2A*	F	20+	48	F	30-50	65	M?	40-60
2B*	F	20-30	50A	F	40-50	65A*	F?	30-50
6	0	15-18	50B	M	20+	66	F?	20-30
8	F?	16-18/19	50C	M	20+	68	M?	40-50
15	M	25-30	50S	0	0	67	M	25-30
18	F	20-25	50E*	0	5/6-10/11	69	M	25/30-35
18A*	0	12-14	51A	M	30-40	70	0	14-18?
18B*	0	10-11	51B	F?	20-25	71A	M	16-18
20	0	~6	51C	M	30-40	71B	F	17-18
25	M	30-40	51D*	M	50-60+	71C*	M	18/20-25
25A*	F	36-39	54A	M?	25/30-40	72A	F	35/40-45
25B*	0	6-7	54B	M	60-70	72B	0	0-1
25C*	0	10-11	54C*	F	20-25	73A	M	16/18-20
32	F	20+	54D*	F	40-45	73B	M	18/20-25
32A*	M	20+	56	M/F	30-40/45	74	M?	20+
31	M	50-60	57	M?	20+	74A*	0	20+
31A*	0	7-8	58	F?	20+	74X*	0	5-7
31S	0	14+	58A	0	~2	76A*	0	12-13
31S	0	2-6	58A*	M?	31-35?	76B*	F	20-30
35	M	30-35/40	58Б	0	~2=58A	76C*	M?	25/30-35
37	M	18/20-25	59A	0	7-8			
39	M	20+	59B	0	3?			
39A*	F	20+						
39B*	F?	14-16						
39X	0	<7						

Tabl. 2. Individual identification of studied skeletal remains

	Inf		Juv			Ad			Mat			Sen	Tt	Ad+			
	I	II	M	F	Tt	M	F		M	F		M		M	F	Tt	Tt*
N	10	8	5	4	12	11	9	20	5	4	9	1	60	6	4	12	72
%	17	13	8	6	20	18	15	28	8	7	15	2					
%*	14	11			17			28			13	1	83			17	

Table 3. Paleodemographic indexes for first 10-years age intervals for anthropological groups of *InfansII-Juvenis*, *Adultus*, *Maturus* and *Senilis* for both sexes and total population. Distribution of identified skeletons. $D_{(x)}$ by age intervals; $d_{(x)}$ – relative number of dead by age intervals; $l_{(x)}$ – relative number of survived by age intervals; $q_{(x)}$ – risk of death by age intervals; $e_{(x)}$ – mean life expectancy by age intervals; $a_{(x)}$ – mean life span by age intervals; M – males; F – females; Tt – total population; * – in the number of identified are added individuals with no sex identification in the age group.

Age	$D_{(x)}$ (M)	$d_{(x)}$ (M)	$l_{(x)}$ (M)	$q_{(x)}$ (M)	$e_{(x)}$ (M)	$a_{(x)}$ (M)
10\19	5	22.73	100	0.23	21.82	36.82
20\29	7	31.82	77.27	0.41	16.76	41.76
40\49	3	13.64	27.27	0.50	11.67	56.67
60\69	1	4.55	4.55	1.00	5.00	70.00
	22					
Age	$D_{(x)}$ (F)	$d_{(x)}$ (F)	$l_{(x)}$ (F)	$q_{(x)}$ (F)	$e_{(x)}$ (F)	$a_{(x)}$ (F)
10\19	3	18.75	100	0.1875	18.75	33.75
20\29	7	43.75	81.25	0.5385	11.923	36.923
40\49	3	18.75	18.75	1	5	50
	16					
Age	$D_{(x)}$ (Tt)	$d_{(x)}$ (Tt)	$l_{(x)}$ (Tt)	$q_{(x)}$ (Tt)	$e_{(x)}$ (Tt)	$a_{(x)}$ (Tt)
10\19*	15	33.33	100	0.33	18.11	33.11
20\29	14	31.11	66.67	0.47	14.67	39.67
40\49	6	13.33	20.00	0.67	9.44	54.44
60\69	1	2.22	2.22	1.00	5.00	70.00
	45					

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Alterations in Masseter Muscle Tones after Treatment with Different Obturators

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Surgical treatment of maxillary cancer causes serious disorders in the chewing and eating functions of patients. As a result of the concomitant radiotherapy, the tone of masticatory muscles is changed which restricts mouth opening and mandibular mobility. The aim of the study is to analyse the alterations in m. masseter after maxillary resection and prosthetic treatment with different obturators. Electromyography is used to record the changes in muscle tones and action potentials regarding the muscles contractions of the resected and healthy sides. Research findings demonstrate normal action potentials with 350-500 μV and 150-300 μV amplitudes on the healthy side and the operation area, respectively, after the end of treatment. It is shown that the prosthetic treatment after maxillary resection improves the condition of masticatory muscles regardless the type of applied obturators. Irrespectively of the treatment method, the amplitude values of m.masseter on the resected side remain lower in comparison to the normal one.

Key words: m. masseter, maxillary resection, maxillary defect, obturator, electromyography.

Introduction

The electromyography (EMG) is a contemporary method for registration and evaluation of muscle activity with broad application in medicine [7]. In dentistry, EMG is used for activity assessment of masticatory muscles while treating and diagnosing some parafunctions and different diseases of temporomandibular joint [11]. Some authors define the application of this method as a golden standard in bruxism diagnosis and treatment [17, 10]. The examination of muscle activity of m.masseter is successfully used in diagnosing and treating patients with different types of orofacial pain [12]. Previous findings demonstrate the relationship between hyperactivity of masticatory muscles and pain in temporomandibular joint [8].

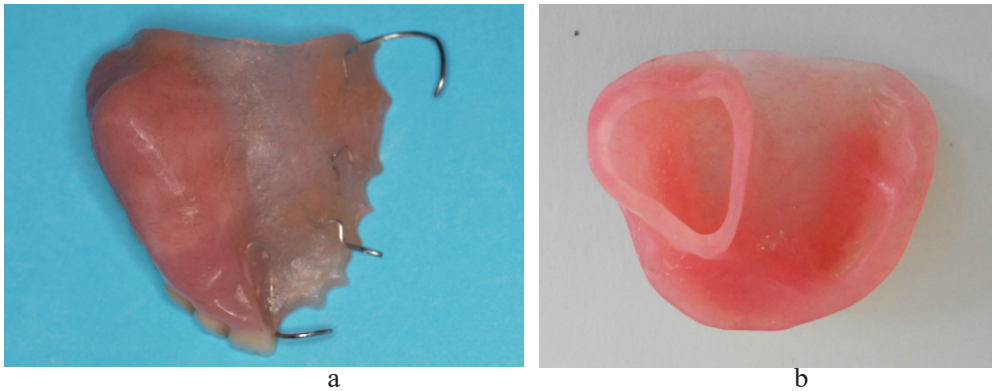
The available literature provides anecdotal evidence regarding the alterations in m. masseter after maxillary resection and treatment with obturator, despite its role as the main masticatory muscle [9]. An electromyographic study among six patients with unilateral defects and preserved partial dentition, who have hollow-bulb and buccal flange obturators, shows better recovery of masticatory function when treatment is based on the latter type of dentures [6]. Comparative studies about these two types of obturators confirm the above-mentioned advantages when speech recovery and comfort of patients are taken into consideration [4]. Other studies indicate that prosthetic treatment after maxillary resection has positive impact on the masticatory muscles on the resected side; thereby, contributing to the recovery of the muscle tone to identical levels seen on the healthy side [2]. The results regarding patients with mandible resection are the contrary – they experience reduced muscle activity after treatment [5].

Research findings show that prosthetic treatment with partial or complete dentures improves EMG activity of the masseter muscles [15, 19]. According to some studies, the recovery of the muscle tone of masticatory muscles depends on denture's type and used materials [20]. It is found that the most commonly used acrylic dentures improve EMG activity of m.masseter which in turn facilitates chewing [3]. Such alterations in m. masseter and m. temporalis are not found in the cases of treatment with occlusal splints made of acrylic resin [1, 16]. The application of dentures with a silicone base leads to an increase of masseter and temporal muscles activity, as well as improved chewing effectiveness [14, 13]. Electromyographic activity of masticatory muscles also depends on the correct tongue positioning along the oral cavity floor. The latter requires the development of a barrier isolating the nasal cavity during the obturator treatment [18].

Materials and methods

A two-channel 16-bit EMG devise Нейро-МВП-Микро (Neurosoft, Russia) is used for the purpose of the study. The devise measured the action potentials of m. masseter among six patients with hollow-bulb and buccal flange obturators (**Fig. 1 a, b**). Each patient had a defect limited to the midline, as well as a preserved partial dentition – characteristics allowing for a treatment with the two types of obturators. Only six patients, who were equally distributed in two groups, took part in this study due to the inclusion criteria, specifics of the disease and its relatively low frequency. The prosthetic treatment of each patient took place four months after surgery in order to complete the healing process in the defect, as well as overcome the trismus caused by the radiotherapy. After adjustments and articulations of the dentures over a period of one month, their stability and tightness were examined. During this period, minor corrections were performed in some areas in order to cope with decubitus wounds and pain.

The EMG examination took place after a monthly period of adaptation and six months after the resection. Superficial bipolar silver electrodes were used and attached to the m. masseter on the healthy and resected sides in order to measure the action potentials at rest and during muscle activity.



a b

Fig. 1. Hollow-bulb (a) and buccal flange (b) obturators

Results

The electromyographic tests of m. masseter on the healthy side of all patients showed no spontaneous activity at rest. Normal action potentials with amplitude of 350-500 μV were recorded during muscle contraction (**Fig. 2**).

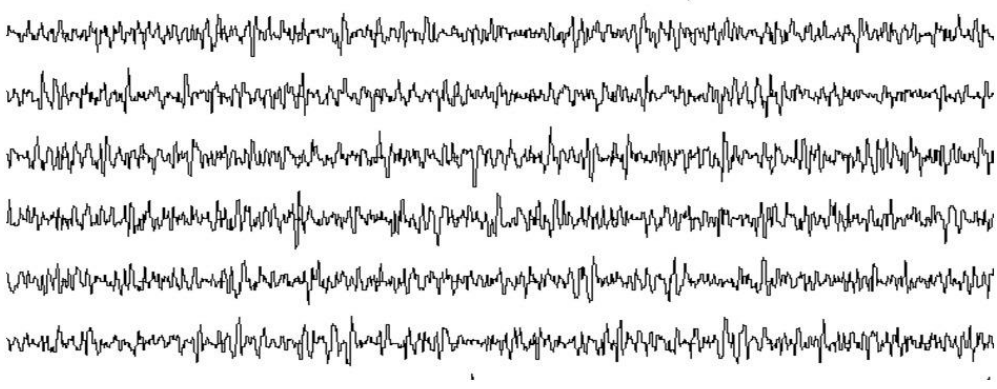


Fig. 2. EMG potentials during contraction of m.masseter on the healthy side

The results from the examination of patients with buccal flange obturators showed single fibrillar potentials at rest and low amplitude action potentials within the range of 150-300 mV during muscle contractions (**Fig. 3**).

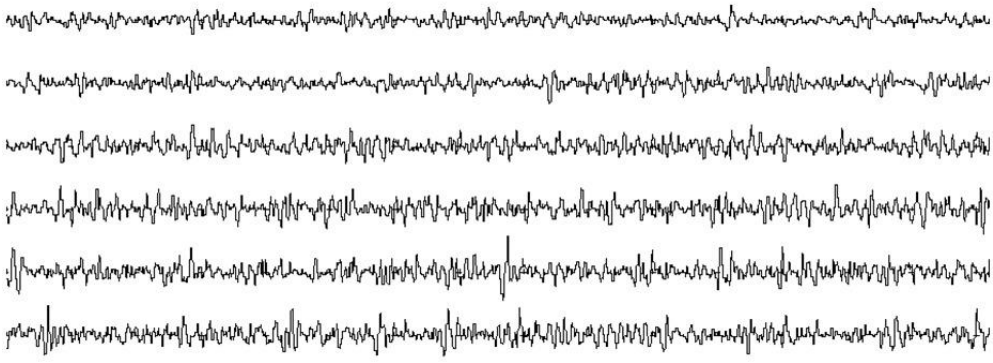


Fig. 3. EMG potentials of m. masseter on the resected side of a patient with a buccal flange obturator

The results of patients with hollow-bulb obturators reported no spontaneous activity at rest with single fibrillar potentials. During contraction of m. masseter were registered action potentials within the range of 150-300 μV were registered during contractions of m. masseter (**Fig. 4**).

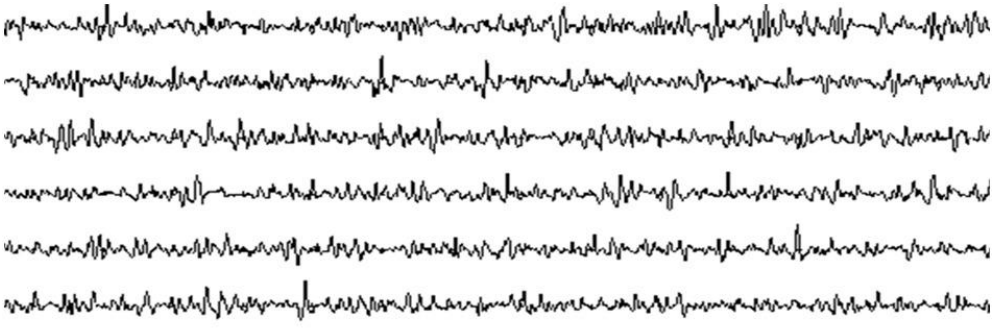


Fig. 4. EMG potentials of m.masseter on the resected side of a patient with a hollow-bulb obturator

Discussion

The results from the EMG examination delivered an objective assessment of m. masseter condition of the healthy and resected sides. Regardless the conducted radiotherapy and subsequent trismus, there were no alterations in m. masseter tone, while action potentials during contractions remained normal. Changes were observed in the muscles on the resected side where lower action potentials from 150 to 300 μV were reported in both groups. The amplitude values were measured as a result of conducted prosthetic treatment. They were close to the minimum values on the healthy

side. Research findings confirmed the conclusions from other studies suggesting that prosthetic treatment with definitive obturators improved the tone of m. masseter; thereby, contributing to the achievement of almost normal amplitude values [2].

The study demonstrated that obturator's type and design did not influence treatment results, as action potentials in both groups remained within the range of 150–300 μV. Previous findings from identical studies among patients with similar defects suggesting better results from the application of buccal flange obturators were not confirmed [6].

Research findings demonstrated an increase of m. masseter's action potentials on the resected side after treatment. The measured outcomes are comparable to the results achieved from treatment with partial or complete dentures [15, 19]. The idea that acrylic resins provide stable transmission of the masticatory pressure and improve masticatory tone, was confirmed [3]. The durability of the material facilitated the creation of a stable barrier between oral and nasal cavities in order to achieve a proper tongue positioning – a reliable approach for the reduction of muscle activity [18].

Conclusions

The prosthetic treatment after maxillary resection improves the condition of masticatory muscles regardless the type of applied obturators. Irrespectively of the treatment method, the amplitude values of m.masseter on the resected side remain lower in comparison to the normal one.

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The Arteries, Veins and Nerves in the Antebrachium of the Brown Bear (Ursus arctos)

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The aim of the study is to establish the arterial and venous vessels and the nerves of the forelimb in the Brown bear through macro anatomical dissection and contrast radiography on six thoracic limbs. The vessels and nerves of the target area were dissected and photographed on the previously detached from the body limbs. For the use of contrast radiography barium sulfate solution was introduced through the brachial artery and medio-lateral and craniocaudal images of the forearm were taken. The main arterial and venous vessels, together with the nerve supply were established and subsequently compared with those of the dog and cat. A comparison with the human was also conducted due to the development of the pentadactyl plantigrade limb in these mammals.

Key words: brown bear, artery, vein, nerve, antebrachium

Introduction

Most of the studies of the Brown bear (*Ursus arctos*) are focused on their habitat and geographic expansion [33, 15], their conservation [31, 32] and reproduction [12, 31]. Morpho functional and histological studies of the Brown bear stomach and adrenal glands were conducted in the recent years [25, 26]. From an anatomical perspective the brown bear's musculoskeletal system is broadly researched [21, 7, 17]. Sasaki et al. [24] study the muscles of the hindlimbs of the Malayan sun bear (*Helarctos malayanus*), the Polar bear (*Ursus maritimus*), the Brown bear and the Giant panda (*Ailuropoda melanoleuca*) in relation of their climbing ability. Amaike et al. [1] compared the mobility of the forearm skeleton in the Asiatic (*Ursus thibetanus*), Brown, and Polar bear.

As part of the Caniformia suborder of the Carnivora, the Brown bear brings all the characteristics of their representatives [14, 30]. Among this order the vessels and nerves of the dog (*Canis lupus familiaris*) and the cat (*Felis catus*) have been the most researched [16, 20, 29, 11]. Davis [6] established a resemblance between the blood vessels of the forelimb of the Red panda (*Ailurus fulgens*) and the ursids.

In dogs and cats, the main arterial vessel supplying the antebrachium is the median artery, accompanied by the median vein and nerve, positioned deep between the forearm muscles. Superficially are the antebrachial superficial cranial artery, together with the cephalic vein and the superficial branch of the radial nerve [13, 27]. The cephalic vein is the preferred vessel for blood collecting [4, 2]. Study on the Andean bear (*Tremarctos ornatus*) and Asiatic black bear shows that 10 cm proximal to the carpal joint is most suitable for blood collection by the cephalic vein [19]. Branches of the ulnar nerve are also responsible for the innervation of this region [13].

In our previous study the vessels and nerves of the Brown bear's crus were established and was concluded that their branching patterns bring similarities to those of the cat, dog and human. In this study therefore we examined the arterial and venous vessels, together with the nerves of the forearm of the Brown bear.

Materials and methods

The limbs from four brown bears (two male and two female) were included in this study. The cadavers originate from the Dancing bears park in Belitsa, Bulgaria. The limbs were detached from the body by incising the skin and the muscles. Macro anatomical dissection of the arteries, veins, and nerves of the antebrachium was conducted with subsequent photographing with Lumix DC FZ82 (Panasonic, Japan). For the use of the contrast radiographic study barium sulfate – $BaSO_4$ solution (Milve AD, Bulgaria) prepared according to the manufacturer's instructions was introduced through the brachial artery with 20 ml syringe. The amount of the solution was 50 ml. Images in craniocaudal, and mediolateral projections were made on Eickemeyer® Vet, model E 7239X radiograph. The nomenclature was acquired from Nomina Anatomica Veterinaria 6th edition [24].

Results and Discussion

The cephalic vein (*v. cephalica*) at the antebrachial region of the Brown bear was found on the cranial surface of *m. extensor carpi radialis longus* and *brevis*, as the one in the cat, starting from the radial vein (*v. radialis*), and reaching the angle of the elbow joint (**Fig. 1**), as the one in the dog and cat [13, 28]. At the level of the distal antebrachium receives *v.cephalica accessoria*. The cephalic vein was also established on the x-ray image in mediolateral projection (**Fig. 2**). The cranial superficial antebrachial artery (*a. antebrachialis superficialis cranialis*) was visualized as a trunk that shortly divides into lateral and medial branches. These two vessels accompany the antebrachial part of the cephalic vein on its lateral and medial side (**Fig. 1**). The two arterial branches were flanked laterally and medially accordingly by *r. radialis superficialis – ramus lateralis* and *n. radialis superficialis – ramus medialis* (**Fig. 1**). The separation of *a. antebrachialis superficialis cranialis* and *n. radialis* in the Brown bear was at the level of the proximal antebrachium, unlike dogs, where these divisions are found at the level of the elbow joint. The view of the cephalic vein, bordered medially and laterally by the described arteries and nerves confirmed the described pattern in dog [13].

V. mediana cubiti was found at the flexor surface of the elbow joint passing obliquely on the distal end *m. biceps brachii*, connecting the *v. brachialis superfiialis* and *v. cephalica* as we described it in our previous research of the arteries and veins of the elbow in dogs [22, 8]. The median cubital vein was accompanied by the anastomosis between the superficial brachial artery and the collateral radial artery (**Fig. 3**) as the vessels described in dogs and cats [13, 27]. An anastomotic branch between the cephalic and the median cubital vein was detected (**Fig. 1**).

A. interossea communis was established on the level of the proximal part of the pronator quadratus muscle passing through *spatium interosseum antebrachii* and dividing in cranial and caudal branches (**Fig. 4**), like the described vessel in the dog [13, 27], but different from cat, where the cranial and caudal interosseous arteries arise separately from the brachial artery [27, 5]. The cranial interosseous artery was presented as the smaller vessel and coursing on the cranial aspect of *membrana interossea antebrachii* in the deep surface of the proximal end the *m. extensor carpi ulnaris* and the lateral and common digital extensor, confirming the literature data for dogs [13, 27]. On the x-ray image in cranio-caudal projection the ulnar artery (*a. ulnaris*) was found as a branch from the common interosseous artery (**Fig. 2**), like in dogs [13] but unlike cats, where it is a branch of the caudal interosseous artery [27, 5]. On the other hand, during the macro anatomical dissection it was also detected as a branch from the median artery (**Fig. 4**).

The median artery (*a. mediana*) and vein (*v. mediana*) were found between the antebrachial muscles (**Fig. 4**). The median artery was the largest arterial vessel of the forearm as the one in the dog [13]. *A. radialis* was established as a branch of *a. mediana* at the level of the distal radius seen on the dissected (**Fig. 4**) and radiographic image (**Fig. 2**) which is different from the one in the dog and cat, where *a. radialis* starts just proximal to the middle of the forearm [13, 27]. After the arising of the radial artery, the median artery continued as a thin vessel, which joined the palmar arch (*arcus palmaris superficialis*) confirming the described in dogs [13, 10], but unlike cats, where the median artery does not join the arch [9]. The palmar arch and *rete carpi dorsalis* formed by the radial and the caudal interosseous arteries in dogs [13, 10] and cats [9], were established by the present study as formed by the radial and the ulnar arteries similar to the human [3]. Between the dorsal branch of *v. ulnaris* and *v. cephalica accessoria* a venous arch was formed, from which begin the four dorsal common veins, which as branching is specific only to the bear and is absent in dogs, cats, and human. The dorsal branches of *a. radialis* and *a. ulnaris* form a deep arch, from which begin the four dorsal metacarpal arteries, which resemble the blood supply to the human arm [3]. The superficial palmar arch in the brown bear was formed by the palmar branch of *a. radialis*, the thin *a. mediana* and the palmar branch of the ulnar artery, while in the dog and the cat the last artery is substituted with *a. interossea caudalis.*

A division of *n. medianus* into lateral and medial branch (**Fig. 5**) was established like the cat [23], but at a different level – at the distal antebrachium in the Brown bear, while in cats is at the level of the carpus [27]. This separation of the median nerve is different from the branching pattern in dogs, where it divides in three (*n. digitalis palmaris I abaxialis*, *n. digitalis palmarlis communis I* and *n. digitalis palmarlis communis II*), described by Hermanson et al. [13], or human – in proper palmar digital branch and common palmar digital branch [3].

The ulnar nerve (*n. ulnaris*) and its division into palmar (*r. palmaris*) and dorsal (*r. dorsalis*) branch was observed (**Fig. 6**) in the level of the proximal quarter of the radius and ulna, unlike dogs and cats, where this is found in the middle of the antebrachium [13, 27].

The innervation of the forearm is given also by the *n. cutaneus antebrachii cranialis, n. cutaneus antebrachii caudalis, n. cutaneus antebrachii lateralis* and *n. cutaneus antebrachii medialis* (**Fig. 7**). The dorsal abaxial digital nerve for the first digit of the Brown bear was established as a branch of the *n. cutaneus antebrachii medialis* (from *n. musculocutaneus*), which is different from the innervation of this digit in the dog and cat, where it comes from *ramus medialis* of the superficial branch of the radial nerve [13, 27], or human, where it is given by *ramus superficialis* of the radial nerve [3]. *N. cutaneus antebrachii medialis* in dogs reaches the distal part of the antebrachium [13].

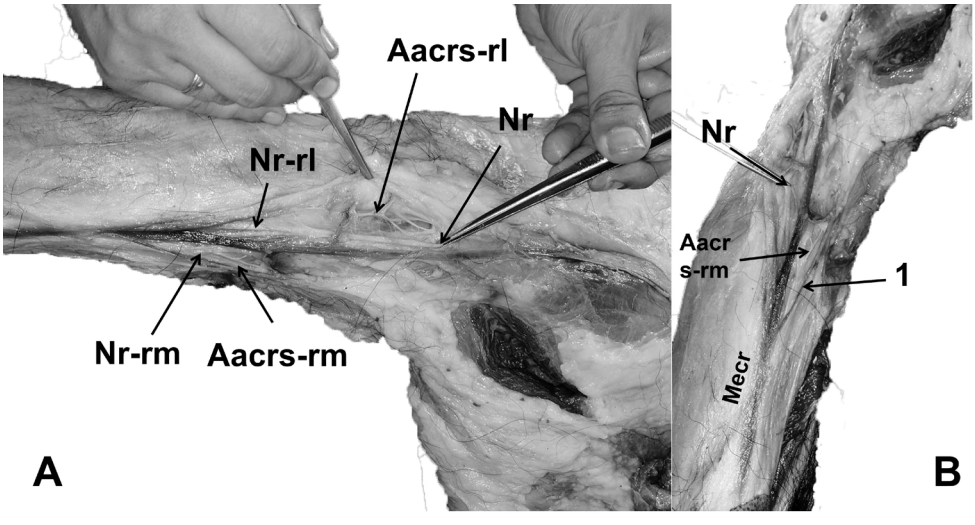

Fig. 1. Dissection appearance of the thoracic limb of the Brown bear; A – lateral view; B – dorsal view; Nr – n. radialis, Nr-rl – lateral branch of n. radialis, Nr-rm – medial branch of n. radialis, Aacrs-rm – medial branch of a. antebrachialis cranialis superficialis, Aacrs-rl – lateral branch of a. antebrachialis cranialis superficialis, Mecr – m. extensor carpi radialis; 1 – anastomotic branch between the cephalic and the median cubital vein

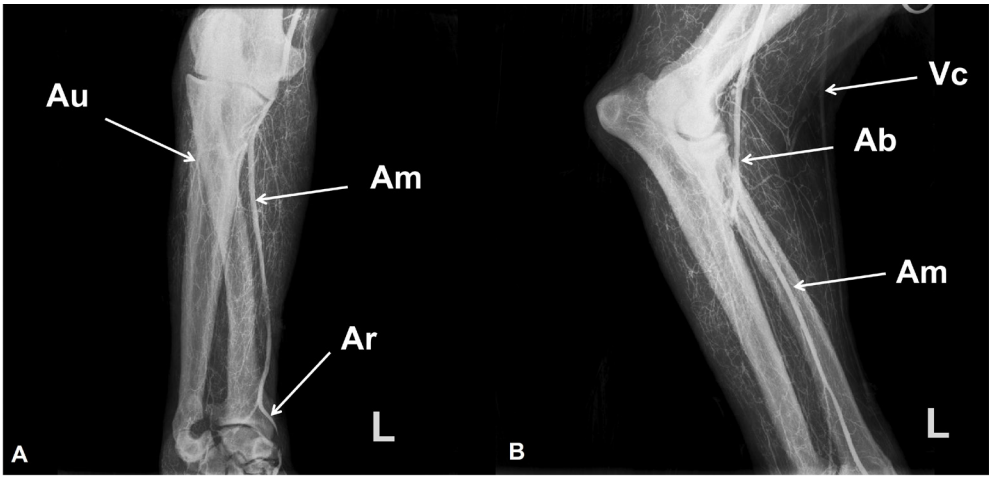

Fig. 2. Radiagraphic image of the Brown bear thoracic limb; A – cranio-caudal projection; B – medio-lateral projection; Au – a. ulnaris, Am – a. mediana, Ar – a. radialis; Vc – v. cephalica, Ab – a. brachialis

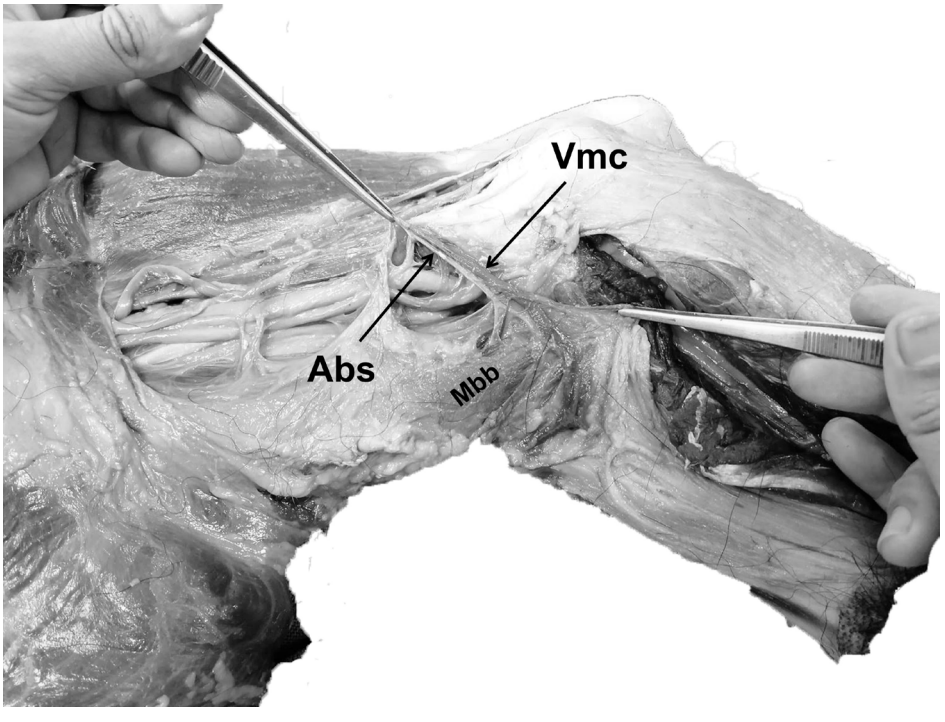

Fig. 3. Dissection appearance of the thoracic limb of the Brown bear, elbow level; Abs – a. brachialis superficialis; Vmc – v. mediana cubiti, Mbb – m. biceps brachii

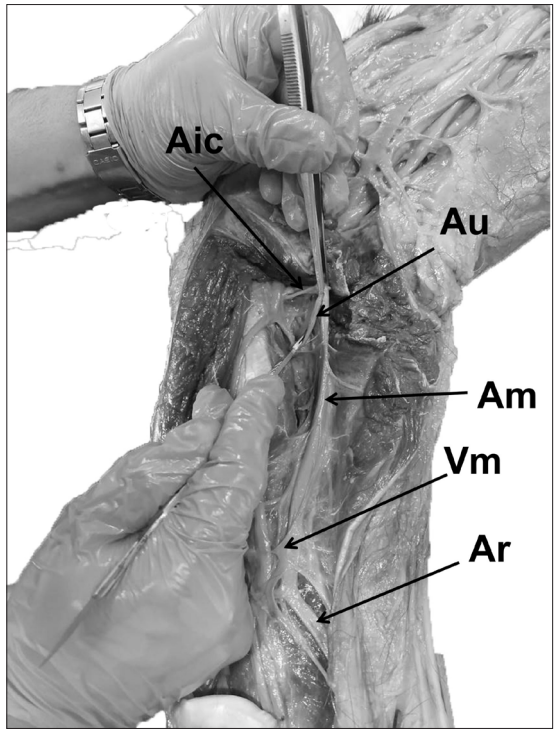

Fig. 4. Dissection appearance of the thoracic limb of the Brown bear, level of the antebrachium, medial view; Aic – a. interossea communis, Au – a. ulnaris, Am – a. mediana, Vm – v. mediana, Ar – a. radialis

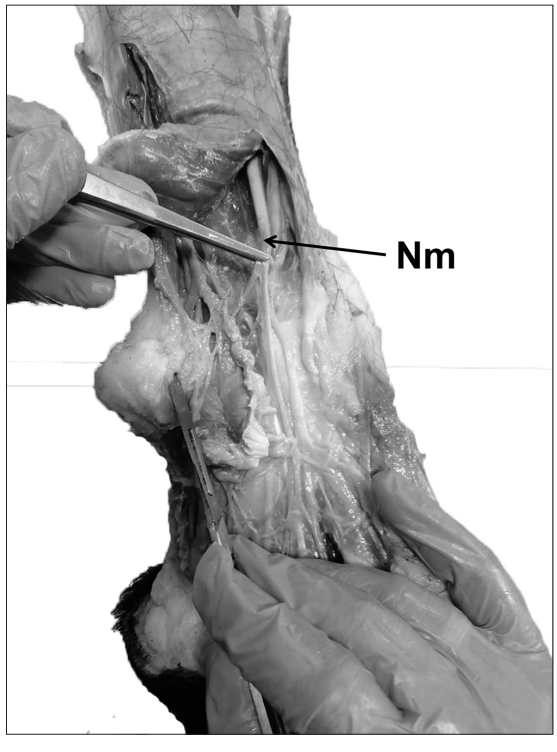

Fig. 5. Dissection appearance of the thoracic limb of the Brown bear, level of the carpus, dorsal view; Nm – n. medianus

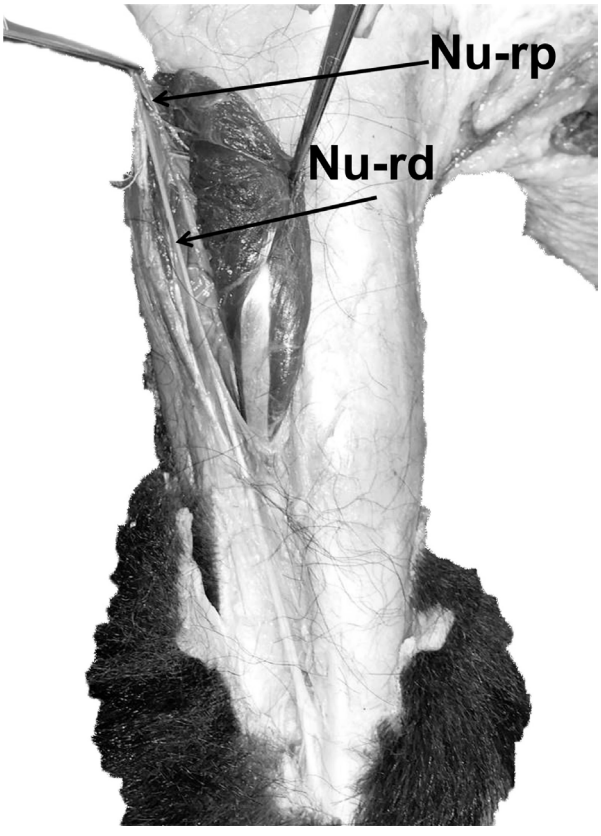

Fig. 6. Dissection appearance of the thoracic limb of the Brown bear, level of the antebrachium, lateral view; Nu-rp – palmar branch of the ulnar nerve; Nu-rd – dorsal branch of the ulnar nerve

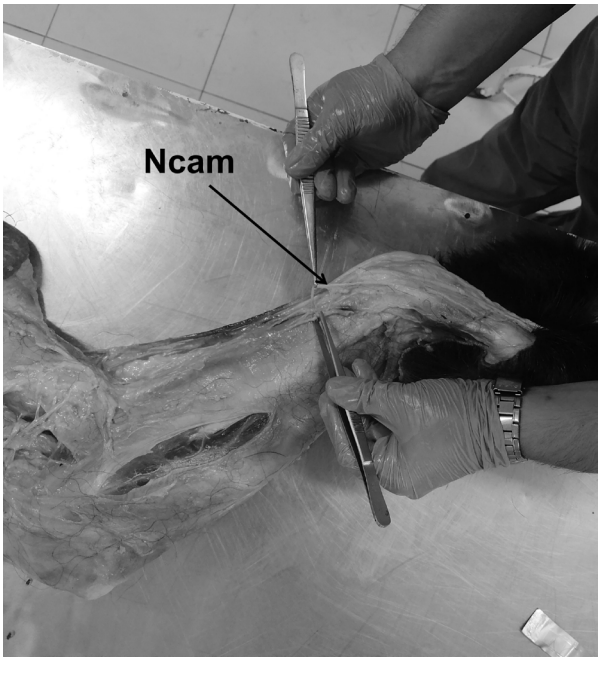

Fig. 7. Dissection appearance of the thoracic limb of the Brown bear, medial view; Ncam – n. cunateus antebrachia medialis

Conclusion

To our knowledge this is the first study to describe the arteries, veins, and nerves of the Brown bear's forearm. From the conducted anatomical dissections and radiographic investigations, we can conclude that their blood vessels and nerves bring characteristics not only of the different representatives from the Carnivora order, but also of the human.

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Strongyloides sp. Infection in a Brown Capuchin (Sapajus apella L.): Case Report

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Strongyloids are widespread helminths commonly causing chronic, asymptomatic infections, which under certain circumstances can became severe, even fatal. The diverse clinical picture accompanying acute cases is often confusing in the search for a diagnosis. The aim of this work is to document the case of strongyloid infection in a monkey from the Sofia Zoo and supply morphometric data on its causative agent. Strongyloid eggs and rhabditiform larvae were found in feces of a brown capuchin. The eggs were 48-56 µm in length, and 25-27 µm in width, and the larval sizes were 495-598 µm and 19-24 µm respectively. We assume they were of the species Strongyloides fuelleborni, although the diagnosis is not definitive. Morphometric data regarding found eggs and larvae could be useful basis for future research, and combined with molecular analyses, would provide reliable diagnostic tools in the field of strongyloid infections.

Key words: brown capuchin, soil-transmitted helminths, *Strongyloides fuelleborni,* morphometric data

Introduction

The genus *Strongyloides* (Nematoda: Rhabditida) consists of widespread helminths with complicated life cycle. Strongyloids are facultative parasites, can also develop as free-living organisms depending on environmental conditions: Only the female individuals parasitize, they are localized in the small intestines of the hosts, where release eggs. Eggs or first stage larvae (L1) are released into the external environment with feces. There, for a few days, L1 develop to rhabditiform larvae, which under unfavorable conditions (low temperature, humidity and oxygen concentration) turn into invasive filariform larvae. Filariform larvae penetrate their hosts by the oral or percutaneous route, migrate to the lungs, where molt and differentiate into male and female individuals. After copulation, the males die, and the females, passing through the trachea and pharynx, are swallowed and end up in the small intestine. Under favorable conditions of the external environment, rhabditiform larvae do not become

filariform, but give a free-living generation of male and female individuals. After their copulation, the females release embryonated eggs, which further develop depending on external conditions as parasitic or free-living generation [8]. Due to these peculiarities, strongyloid infections cannot be directly transmitted between hosts [7]. They are among the so-called soil-transmitted infections [4], their source is the environment (soil, water, and food) contaminated with parasitic forms.

Strongyloids are about 50 species and can infect most of the vertebrates around the world, including humans [15]. Strongyloidosis in humans is commonly chronic, asymptomatic infection, but a change in immune status of the hosts can lead to an increase in parasite burden, hyper-infection syndrome, dissemination, and even death [11]. In animals, the disease occurs acutely mainly at a young age, and the symptoms are diverse and related to the ways of penetration and migration of the parasites in the body: they generally are characterized by diarrhoea, vomiting, malabsorption, and bronchopneumonia [2, 9]. In adult individuals or in weak infections, it is often asymptomatic or accompanied only by decreased appetite, skin eczemas, weight loss or growth retardation [8]. With asymptomatic and chronic course, strongyloidosis remains undiagnosed, and the diverse clinical picture accompanying acute cases is often confused diagnosis and unrecognition of the disease.

In order to succeed in the fight against parasitoses, special attention must be paid to their accurate diagnosis, to develop adequate guidelines for epidemiological studies and to obtain reliable data that enable health services to determine current ways to prevent and control them [13]. Accumulation of basic knowledge regarding all aspects of different parasitic diseases would contribute to this. In connection with the above, the aim of the present work was set, namely to document the case of strongyloid infection in a brown capuchin and supply morphometric data on its causative agent in present materials.

Materials and Methods

It concerns an 8-year-old female brown capuchin, born and kept in the Sofia Zoo. Visible weakening of the capuchin in the winter of 2022, followed by the medical check-up has revealed unclear organism inflammation. That was the reason why zoo officials approached us with a request for parasitological tests of the animal. Fecal sample of the monkey was evaluated for presence of parasites by the common flotation, sedimentation and Baermann techniques [5]. Imaging and measurement of parasite forms were performed using a Motic Images Plus 3.0 camera connected to an Amplival microscope, with accompanying software.

Results and Discussion

Examination of fresh fecal samples (same day of collection) revealed presence of nematode eggs, and 72 hours later nematode larvae were observed. Eggs were elongated elliptical in shape, 48-56 μm in length, 25-27 μm in width, with a thin smooth wall, contained a fully developed motile larvae (**Fig. 1**).

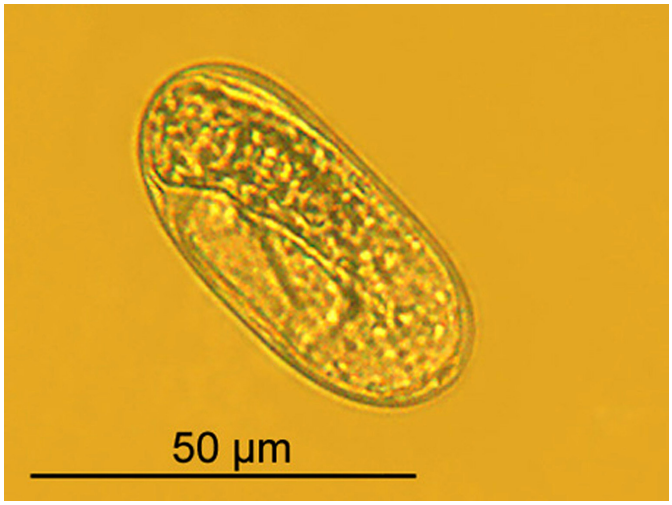

Fig. 1. *Strongyloides* sp. egg found in feces of a brown capuchin

Larvae were without sheath, 495-598 µm in length and 19-24 µm in width, with three small lips around the mouth opening and pointed tail (**Fig. 2**).

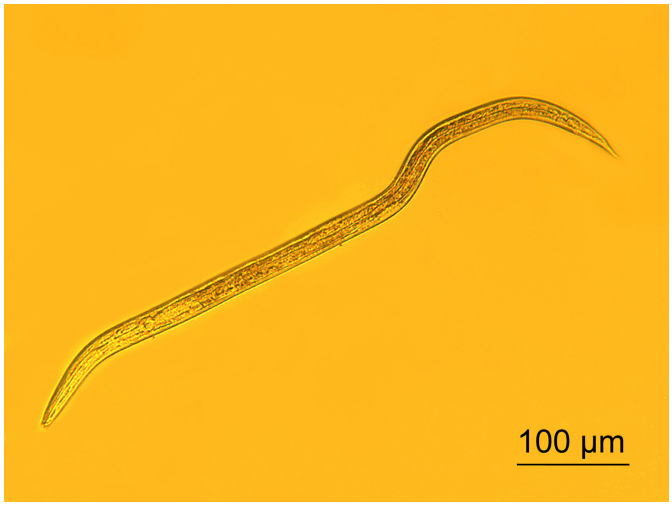

Fig. 2. Rhabditiform larva found in feces of a brown capuchin

Oesophagus of the larvae was 126-146 µm in length and 12-13 µm in width, rhabditiform, with club-shaped anterior portion, post-median constriction and posterior bulbus (**Fig. 3**). Genital primordium was situated between the second and third part of the body, and anus – at 44 – 50 µm from the tail end (**Fig. 4**).

The morphometric characteristics of the observed eggs and larvae gave reason to consider that it was a strongyloid infection. *Strongyloides* spp. are generally host-specific [14]. Among the species isolated from primates, *Strongyloides stercoralis* (Bavay, 1876), *Strongyloides fuelleborni* von Linstow, 1905, and *Strongyloides fuelleborni kellyi* (Viney et al., 1991) are found in Old World monkeys, apes, and/

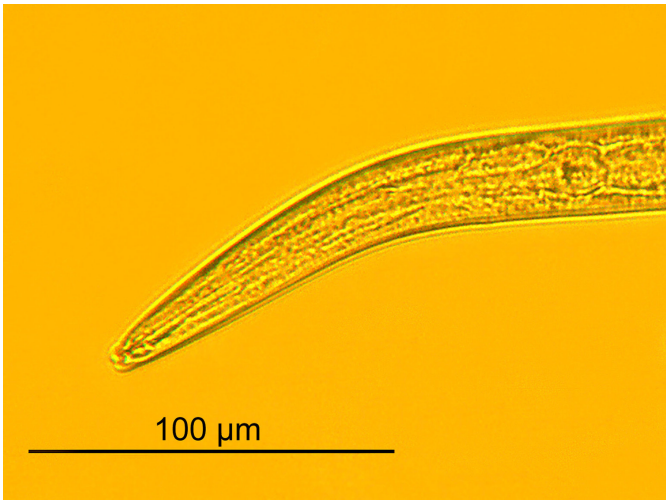

Fig. 3. Rhabditiform larva found in feces of a brown capuchin – anterior end

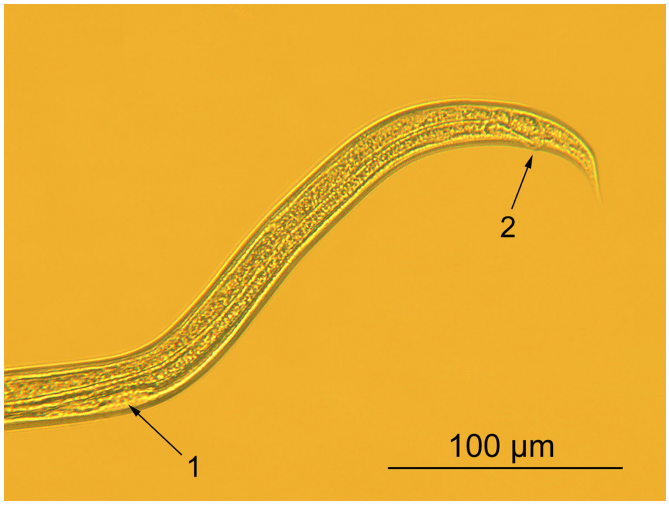

Fig. 4. Rhabditiform larva found in feces of a brown capuchin – posterior end

or humans, while *Strongyloides cebus* Darling, 1911, is considered the only natural species found in New World monkeys [10]. The possibility that the parasites found in the present case were *S. stercoralis* was excluded due to the peculiarities of the life cycle of the species: the eggs hatch in the host intestine and only larvae passed out in the feces [9], and in the present case we found both eggs and larvae. The probability that the parasites are of the species *S. f. kellyi* also was rejected, as this species so far has been found only in humans from New Guinea [1, 3]. *Strongyloides fuelleborni* normally is a parasite of non-human primates in Africa and Asia and of humans in Africa [1]. It was also reported in zoo primates from Europe [6]. In this connection, we assumed that the strongyloides in the present case were most likely of the *S. fuelleborni* species. However, the fact that the monkey, the object of the study, lives in a zoo, where the probability for exchange of parasites between different species of animals is greater

[12] assumes that the found parasites could also be from another strongyloid species. Such, for example, could be *Strongyloides westeri*, which despite its specificity to ungulates has zoonotic potential [5].

Based on the established diagnosis, the capuchin was treated for two consecutive days with Ivermectin - orally at a dose of 0.200 mg/kg, as well as with anti-inflammatory drugs. The treatment carried out visibly improved the condition of the animal. Twenty days after treatment, control fecal samples were examined and no parasites were detected.

Conclusion

Strongyloid eggs and rhabditiform larvae found in the feces of a brown capuchin from Sofia Zoo are most likely of the species *S. fuelleborni*. However, in this case diagnosis based on microscopic examination and host specificity alone cannot be definitive. Morphometric data regarding found strongyloid eggs and larvae would be a useful basis for future research, and combined with molecular analyses, would provide reliable diagnostic tools in the field of these infections.

Acknowledgements: This work was done on a voluntary basis, according to an Agreement for Collaboration between the Institute of Experimental Morphology, Pathology and Anthropology with Museum - Bulgarian Academy of Sciences and the Sofia Zoo.

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Unilateral Pectoralis Quartus Muscle – A Case Report

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Historically, numerous variations of thoracic wall muscles have been reported in literature. In addition to the classically described pectoralis major and pectoralis minor muscles, supernumerary muscles such as the pectoralis quartus muscle are sometimes registered. During a routine educational dissection of a 72-year-old Caucasian male cadaver, a unilateral accessory muscle was encountered on the left anterior thoracic wall, which showed the morphological characteristics of pectoralis quartus. We believe that knowledge of such anatomical variations is important for surgical interventions in the axillary region, as the presence of pectoralis quartus may affect axillary lymphadenectomy by causing limitations to the surgical field, and thus leading to dissection of the lymph nodes at a lower level.

Key words: pectoralis quartus, accessory thoracic muscle, pectoralis major, anatomical variation, axillary lymphadenectomy

Introduction

The pectoral region classically contains the pectoralis major (PMa) and pectoralis minor (PMi) muscles, which partake in upper limb movements. In literature, throughout the years, many different variations in the anterior thoracic wall have been described. The pectoralis quartus (PQ), when present, runs parallel to the lateral border of the PMa and over the brachial neurovascular bundle [5]. Failure to recognise PQ by the surgeon could lead to transposition of the medial border of the axillary lymphadenectomy's surgical field laterally and downwards, which could lead to dissection of the lymph nodes at a lower level [12].

Material and Methods

The hereby reported variant muscle was found during a routine educational dissection of a 72-year-old Caucasian male cadaver, embalmed in formalin. All the materials were available at the Department of Anatomy, Histology, and Embryology of the Medical University - Sofia.

Case report

The dissection was carried out in the left pectoral region and left axillary fossa. The overlying skin was removed and dissection through the subcutaneous fat tissue was carried out in order to demonstrate the superficial neurovascular structures. After the removal of the subcutaneous fat tissue and the superficial fascia of the region, the superficial layer of the pectoral fascia was encountered. It laid superficial not only to the PMa, but also to a supernumerary muscle slip which, based on its morphological characteristics, was determined to be PQ (**Fig.1a**). It appeared to originate by a thin aponeurosis from the anterior layer of the rectus sheath, overlying the external oblique muscle in the region of the sixth and seventh costochondral junctions. Its fibres ran superolaterally, parallel to the inferior border of PMa and completely separated from the latter. The aberrant muscle joined the lower portion of the tendon of PMa (**Fig. 1b**), and thus attached distally to the lateral lip of the intertubercular groove. It measured 16 cm in length and 3 cm in width. Thin nerve fibers from the medial pectoral nerve were identified to supply the PQ.

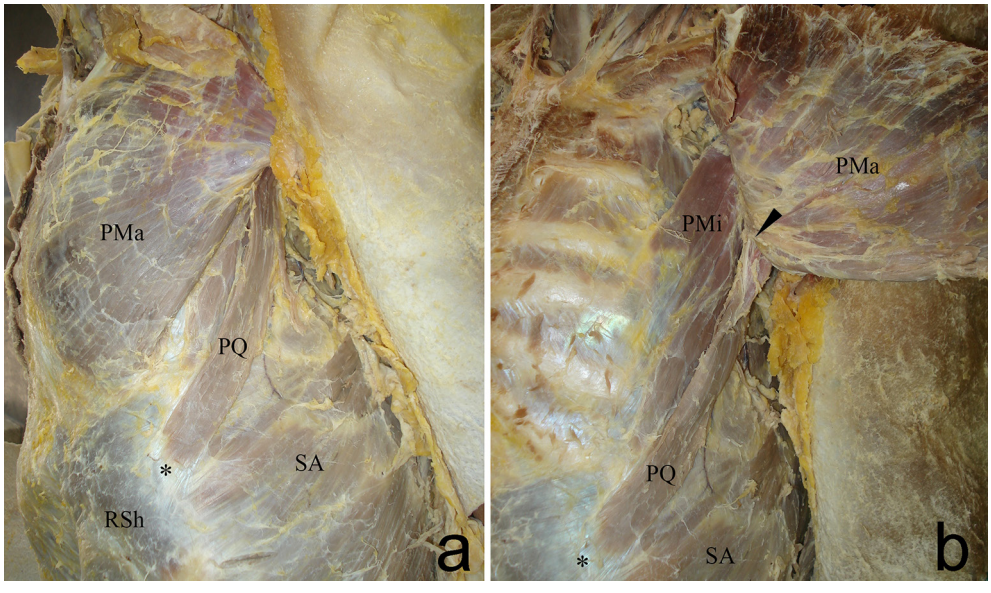

Fig. 1a, b. Photograph of the described dissection in the left anterior thoracic wall, taken after removal of the superficial layer of the pectoral fascia **(a)**, and after detachment of the PMa from its origin **(b).** The aponeurotic origin of the PQ is marked with an asterisk. Distally PQ joins the PMa's tendon as they enter the axillary fossa (arrowhead). PMa – pectoralis major; PMi – pectoralis minor; PQ – pectoralis quartus; SA – serratus anterior; RSh – rectus sheath.

Discussion

PQ was first described in the XIXth century and a prevalence of 11-16% of the dissected cadavers was reported [4]. More recent studies indicate that the PQ is much rarer, with frequency of 2.8% [8]. To our knowledge, PQ has been described in literature only once intraoperatively [12], which could be attributed to a more thorough dissection during anatomical cadaveric studies and the fact that considering its low prevalence, surgeons might not recognise PQ, even if the muscle is present.

The origin (thoracic attachment) of the PQ is fairly constant, as most case reports describe the muscle's origin from the costochondral junction of the 5th and 6th ribs [5]. There have been reports, such as ours, of PQ originating from the anterior wall of the rectus sheath [4, 10]. The muscle insertion (upper limb attachment), however, shows much more variability. Usually, the PQ runs superolaterally from its origin, crosses the axilla anteriorly and has been reported to attach to the PMa fascia [6], the lateral lip of the intertubercular groove and the tendon of the short head of biceps brachii [1] or the coracobrachialis fascia [10,11].

Other supernumerary muscles, originating from the anterior thoracic wall and crossing the axilla anteriorly have been described previously, namely the chondrofascialis and the chondroepitrochlearis. Those muscle variants, however, attach to the medial part of the brachial fascia and the medial epicondyle of the humerus, respectively [2]. There have been reports that when PQ is found in co-existence with Langer's axillary arc, the two of them have a common distal attachment via a shared tendon [4].

The PQ is innervated by the medial pectoral nerve, as most case reports suggest [5], although there have been reports of the PQ receiving innervation from the IVth intercostal nerve [1]. In 1889, Birmingham describes the different theories about the embryological origin of PQ and its homology in other species. According to him, the assumption that the PQ is a derivative of the panniculus is wrong. Consequently, in terms of embryologic development, he defines it as a segmented portion of the PMa. His claims stem from the fact that in terms of origin, insertion and innervation, PQ corresponds to PMa [3]. As far as comparative anatomy is concerned, PQ has been reported in common chimpanzees *(Pan troglodytes)*, where it shows similar morphological characteristics of the human's PQ [9].

The clinical significance of PQ is debatable. To the best of our knowledge, there has been a single report of the variant muscle intraoperatively, and even though the PQ limited the surgical field, the result was satisfactory [12]. A PQ has also been reported during a mammographic screening, and although the authors suggest that it would not raise suspicions about neoplasia, they state that knowledge of such anatomical variations is important to avoid additional examinations [7]. It could be possible that due to PQ's course through the axilla, the muscle may compress the neurovascular structures of the brachial region, thus leading to the corresponding pathology.

Conclusions

The hereby presented case is peculiar, as it shows an anatomical variation, rarely described in most anatomy textbooks. Knowledge of such structures, that pass over the

axilla, is important for surgeons, as they might alter the operating field. In our case, the PQ's lower margin passed significantly lower than that of the PMa, which might lead to confusion of the surgeon, if not recognised.

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Review Articles

Stroke – Some Risk Factors, Neuropathological Aspects and Neurorehabilitation Approaches

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In many cases the stroke is fatal, in other patients various types and degrees of disability are available, most often affecting the cognitive, motor and perceptive functions. Some vascular and circulation diversions concerning the function of the central nervous system may result in parenchymal lesions that could be hemorrhagic, ischemic or mixed. Modifiable risk factors are hypertonia, metabolic syndrome, changes in blood coagulation status, etc. Non-modifiable are age, gender, race and genetic predisposition. Consequences of the stroke could affect different motor and/or cognitive functions. Stroke rehabilitation includes any procedure that aims to facilitate the optimal functioning of individuals experiencing or likely to have post-stroke difficulties in interacting with their environment. Neurorehabilitation after stroke is based on neurorecovery, neuroplasticity and neuroregeneration. The main goals are directed to prevention and reduction the severity of stroke, death risk and long-term consequences. Development of interdisciplinary social programs is necessary. Elimination of the risk factors would be the best prophylactic.

Key words: stroke, neuropathological aspects, risk factors, cognitive rehabilitation, robotic rehabilitation

Introduction

According to statistics, every minute one person in the European Union suffers a stroke. Strokes can be fatal or debilitating, causing a change in the life of not only the patient but also their loved ones. The number of ischemic strokes in Bulgaria is

around 40,000 – 50,000 cases per year and 3,500 patients with non-traumatic cerebral hemorrhages (parenchymal and subarachnoid) [19, 22]. In the recent decades, health systems worldwide have faced increasing challenges:

– increase in healthcare costs;
– shortage and uneven distribution of health professionals at all levels;
– inequality in terms of access to health care;
– aging of the population, which leads to an increase in chronic diseases and polymorbidity.

Four over-arching targets for 2030 have been identified according to "Stroke Action Plan for Europe" (SAP-E) [28]: Creation of a national plan covering the entire chain of stroke care – from primary prevention to life after stroke; Creation and implementation of a national strategy to encourage the population for a healthy lifestyle; Over 90% of stroke patients receive prompt and adequate treatment in a specialized hospital unit that has specialists and equipment; To reduce the number of strokes by 10% [28].

Some risk factors for stroke

Risk factors are divided into two large groups; one group is called non-modifiable which reflects factors without significant difference. These are: age, gender, race and genetic predisposition [19, 22, 29].

The other group are the so-called modifiable risk factors. They can also be divided into two groups – one group are those with known and proven impact and are called "well-documented". The other group are the factors we called "less well documented". It is a combination of several facts that have a great cumulative weight for the occurrence of a stroke. These include: hypertonia, metabolic syndrome, alcohol abuse, hyperhomocysteinemia, hyperuricemia, drug abuse, changes in blood coagulation status, long-term and uncontrolled use of oral contraceptives, severe migraine, obstructive sleep apnea, depression.

Obstructive sleep apnea has recently taken an increasingly prominent place in risk factor commentary and is even expected to move from the group of less well-documented to well-documented risk factors. It has been shown that during some part of the night, the blood in these people is not sufficiently enriched with oxygen. This has an impact on its coagulation qualities and on the oxygen saturation of the neurons, and they need two things – oxygen and glucose, supplied only by the circulating arterial blood [19, 22, 29]. Another recent focus is elevated blood levels of uric acid, which could be related to both increased production and impaired excretion by the kidneys. In the recent years, a group of strokes has been distinguished, called "wake-up strokes", in which we do not have clarity when the initial time of symptom onset is. This limits us in the possibility of carrying out a specific treatment – venous thrombolysis. The main limitation for carrying out this treatment is time – up to four and a half hours from the first signs, or this is the so-called "therapeutic window".

Viruses, including SARS-CoV-2/COVID-19, and other infectious microbes have also been associated with an increased risk for stroke through direct or indirect influence due to long-term complications [22, 33].

Some neuropathological aspects in stroke

Strokes are most often due to thrombus formation, atherosclerosis and hemorrhage (**Fig. 1**, [11]; **Fig. 2**, [14]). Cerebral thrombosis and atherosclerosis are the main cause for ischemic stroke, which accounts for more than 80% of all strokes. Thrombolysis and thrombectomy **(Fig. 3)** [34] may significantly improve functional outcomes after ischemic stroke when performed within the first hours of the onset of symptoms. The type and size of infarcts are often associated with different hemodynamic patterns **(Fig. 4)** [15]. The consequences depend on which part of the brain is affected, the severity and duration of ischemia. The hippocampus, neocortex, striatum and the cerebellar cortex are the brain regions with the highest ischemic vulnerability [15].

Stroke neuropathology is associated with brain swelling, edema, blood-brain barrier breakdown and inflammation. The most sensitive cell types within the brain are the neurons, followed by oligodendrocytes, astrocytes and vascular cells [15]. Histopathological characteristics of the infarct brain areas include neuronal necrosis, reactive astrogliosis and activated microglia [10]. Endothelial dysfunction (including tight junction disruption and loss of barrier properties), contributes to parenchymal damage and neurological deficits [2]. Pathophysiological disturbances of microcirculation in stroke disrupt the neuron-microvascular interactions in the "neurovascular unit", which consists of microvessel components (endothelial cells, basal lamina matrix, astrocytic endfeet, pericytes), astrocytes, the nearby neurons and their axons and supporting cells (microglia and oligodendrocytes) [15]. Targeting the "neurovascular unit" may have beneficial effect against deleterious outcomes following an ischemic stroke.

Strategies for prevention of stroke

Primary prevention of stroke includes prevention of atherosclerosis in general, because it is a major risk factor for damage to cerebral vessels. Changing people's behavior and completely eliminating risk factors such as smoking, unhealthy eating, alcohol use, etc., would be the best choice to reduce the risk of the diseases associated with them. However, experience shows that this is not possible in most cases. As we already mentioned, these risk factors are often in combination and their negative effect aggregates. When we discuss physical activity, we mean that it should be regular, non-exhausting and systematic. Therefore applications for smartphones have been developed that allow a person to plan his route by walking 10 000 steps a day which has a beneficial effect on the overall physiology of the body and on brain function.

Recognizing transient ischemic attacks is the moment when we must act actively. We must assess the patient's condition and focus on what exactly needs to be added to the therapy, what to change in his/her diet and lifestyle, so that a second similar or more severe incident does not occur - that is known as personalized medicine. This action of ours is called secondary prevention. The development of telemedicine at the present time is of great benefit to us [19,22].

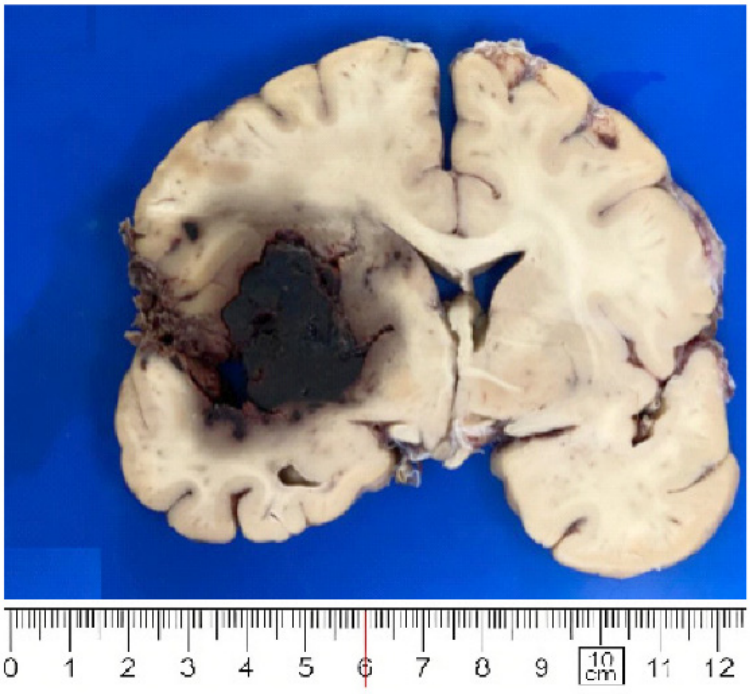

Fig.1. Large hemorrhage in basal ganglia [11].

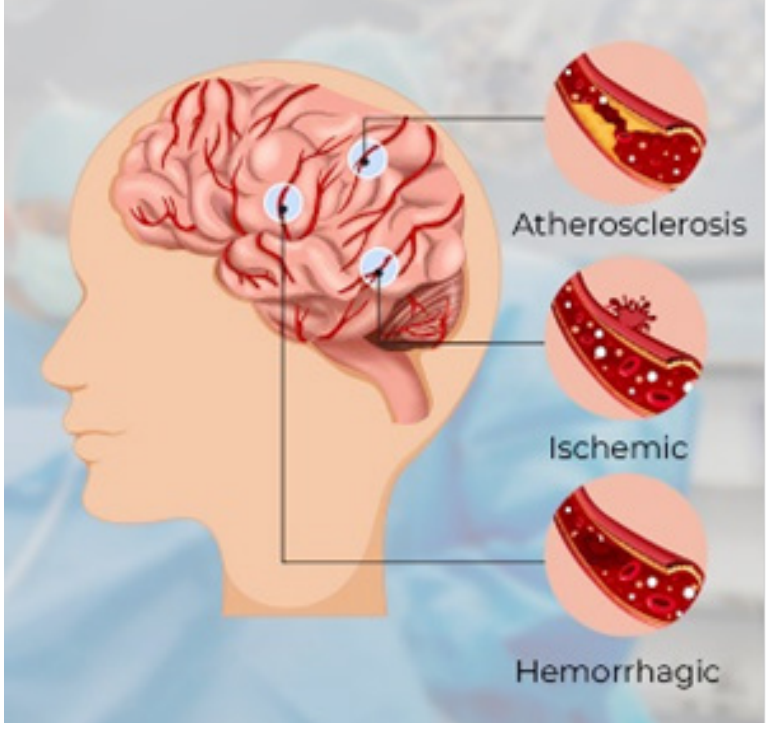

Fig. 2. Types of stroke [14].

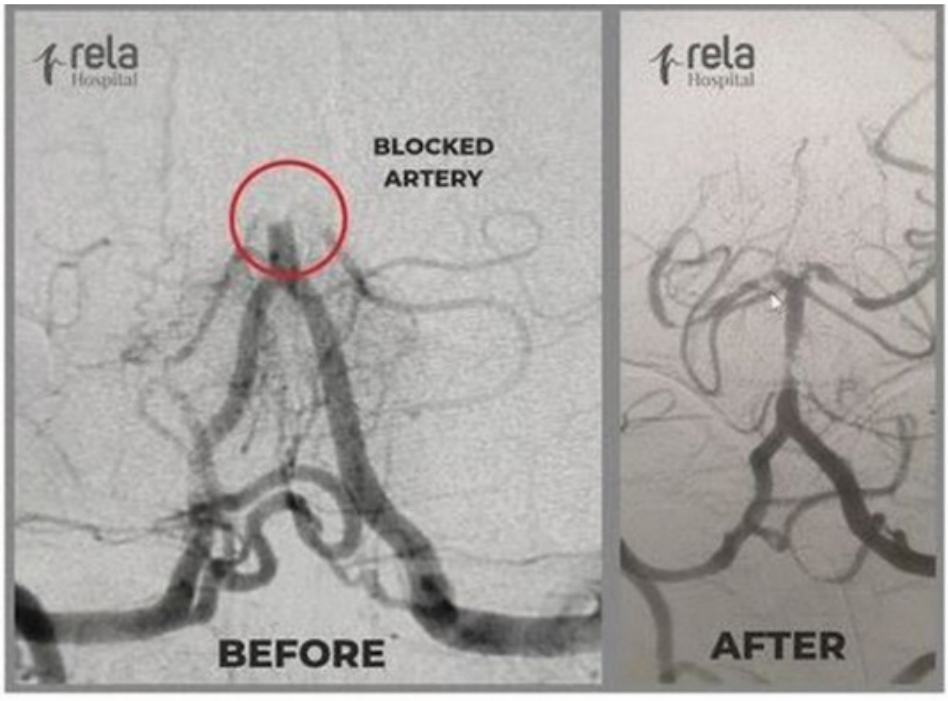

Fig. 3. Mechanical Thrombectomy [34].

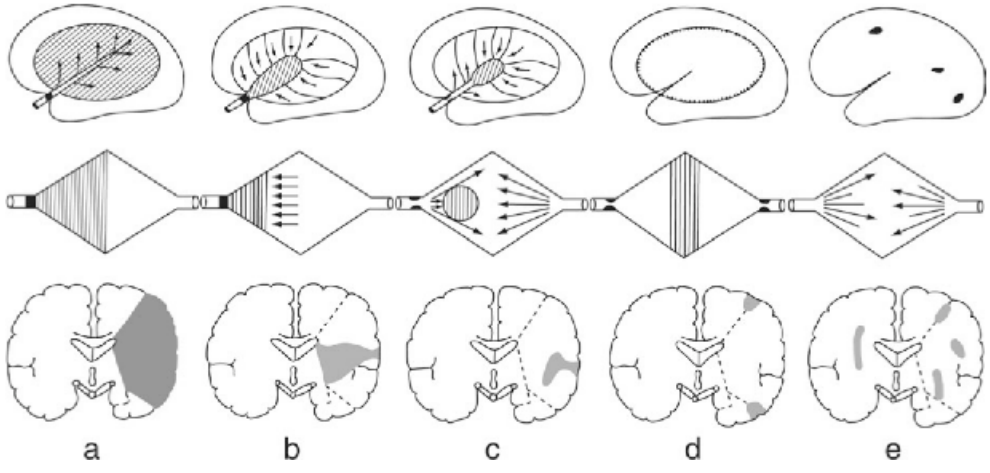

Fig. 4. Various types and sizes of infarcts due to different hemodynamic patterns: a. Total territorial infarct due to defective collateral supply. b. Core infarct, meningeal anastomosis supply peripheral zones. c. Territorial infarct in center of supply area, due to branch occlusion. d. Borderzone infarction in watershed areas due to stenotic lesions in arteries supplying neighboring areas. e. Lacunar infarctions due to small-vessel disease [15].

Neurorehabilitation after stroke

The pharmacologic intervention for early motor rehabilitation after acute ischemic stroke should always be complement (not compete with) neurorehabilitation programs and the initiation of treatment should occur within 7-day window after stroke to enhance endogenous plasticity, consistent with the definitions and shared vision for new standards of stroke recovery research [19, 30].

Stroke rehabilitation includes any procedure that aims to facilitate the optimal functioning of individuals experiencing or likely to have post-stroke difficulties in interacting with their environment. Neurorecovery is a dynamic and multifactorial process and occurs most actively during the first 30 days after stroke onset [22, 24]. Neuroplasticity is a process of biological support for brain recovery, including all mechanisms of neuronal reorganization, including synaptogenesis, dendritic growth, axonal sprouting, the establishment of new anatomical pathways with similar functions to damaged ones, activation of functional but silent synapses, and cell genesis [25]. These metabolic, inflammatory and genetic processes occur in a specific temporal sequence, depending on the time elapsed since the onset of the stroke.

A thorough understanding of the sequence and interrelationships between these processes is vital, as various pharmacological or non-pharmacological therapies have the potential to reduce disability only if applied at the right time. Pharmacological intervention can overcome inhibitory mechanisms and stimulate neuroplasticity in many ways ranging from behavior to gene expression [13, 26]. Neuronal plasticity is at the core of functional recovery after stroke, and it is important to develop robust strategies to facilitate this process in order to offer the best treatment for stroke patients.

In the last years the information, received by the people all over the world by Internet, including the approach to a social net in each separate moment, has significantly increased. By taking in consideration the recommendations of the World Neurology Federation, World Stroke Federation, etc., the mobile application Stroke Riskometer has been developed. Its main goal is related with possibility about a better determination of the individual risk of eventual stroke for a 5-10 years period, which could give directions about decrease and prevention of this risk.

The advantages of computer programs for cognitive rehabilitation compared to classical methods are systematization of the process and the levels of difficulty, fast feedback, the possibility of quantitative assessment and analysis and application in home conditions [6];

– training and recovery of daily routine activities of the patient, which is important to recover up to a year after the stroke;

– although the evidence for the benefit of rehabilitative interventions after the first year of illness is scarce, it is important to know that improvement can continue long after the stroke and patient needs vary over time. Therefore, it is never too late for rehabilitation. If this is not possible, an adequate alternative should be provided for the continued recovery of the patient.

Cognitive rehabilitation. Studies show that up to 13% of patients have mild cognitive impairment before the onset of stroke, and 10 to 15% have pre-existing dementia. After the first cerebrovascular accident, approximately 30% have varying

degrees of cognitive impairment, and 10% have a more severe form of cognitive impairment – dementia. Over 30% of patients develop dementia as a result of multiple recurrent lacunar strokes [4, 5, 21, 26].

Cognitive neurorehabilitation has 4 main areas of application [17, 18, 23]:

1. Training and guidance – with a focus on developing self-awareness and insight into the problem. Through specialized training, the patient with brain damage aims to become aware of existing problems. This is done through accessible explanations of brain functions, brain areas potentially affected and their effect on daily activities. Awareness of the problem leads to a higher degree of adaptation and adjustment.

2. Learning process - with a recovery focus. The learning process aims to restore cognitive skills that have been lost or impaired as a result of the brain injury. All competencies are included here with the idea of automating actions. Neuronal plasticity enables the creation of new anatomical and functional connections and pathways to redirect information around damaged areas. Through the specific cognitive abilities, the natural processes of neuroregeneration and the set of skills built in the course of training are stimulated.

3. Strategically oriented training - with a focus on compensating the problem during a difficult recovery process. When cognitive skills cannot be restored, training in balancing logistic models is switched. They most often use additional aids such as electronic devices, diaries, notebooks, clocks or alarms. Their correct use creates an opportunity for alternative problem solving and the patient can preserve his independent functioning and quality of life, without an increased level of anxiety and/or depression.

4. Training for functional activities - with a focus on improvement in real life. The ultimate goal of all cognitive rehabilitation programs is better and independent functioning. Emphasis is placed on the performance of tasks of varying difficulty and thus working on a certain cognitive ability, that is, training to perform routine daily activities also contributes to the recovery of premorbid cognitive capacity.

Transcranial magnetic stimulation. Neuronal reorganization after stroke has been observed in functional imaging studies that provide information on changes in stimulus or task-induced activation patterns in the affected and contralateral brain hemispheres [7, 31, 32]. The finding that modifications in network connectivity are relevant to neurological dysfunction has inspired the concept of "correcting" dysfunctional network architecture by using noninvasive brain stimulation techniques such as repetitive transcranial magnetic stimulation (rTMS) [3, 16].

Mirror therapy. One of the most severe syndromes after a stroke is the "paretic arm". The motor representation of the cortex is impaired in function and due to the lack of movements in the corresponding hand, as sensory deficits limit cortical activation [1]. Rehabilitation strategies should be intensive, repetitive, specifically oriented and directed at endogenous neuroprotective and restorative processes (integral neurogenesis) after stroke. The concept of mirror therapy has a neurophysiological basis. The mirror is placed in the patient's midsagittal plane, presenting the participant with the mirror image of their unaffected arm. By moving the nonparetic upper limb, visual image inversion elicits lateralizing cortical activation [12, 13]. The involvement of

different cortical areas in the processes that mediate recovery and the exact mechanism of mirror therapy are still not fully understood. Its effects are often associated with "mirror neurons" in monkey and human premotor areas activated during observation of corresponding movements [8, 9, 29].

Virtual reality. Virtual reality involves specific software-generated actions that are performed by the patient and facilitate the improvement of motor and cognitive impairments. An important advantage is the availability of immediate feedback for the implemented activities of the participant. At the beginning of the implementation and implementation processes, virtual reality was used to test the one-dimensional side of vascular lesions, but over time the experimental paradigm shifted to multimodal peer manifestations and procedures.

Mirror box illusion. The Mirror Box Illusion is one of the most famous cognitive mirror illusions based on visual-motor conflict. While one hand is behind the mirror, hidden from view, participants move the other hand in front of a parasagittal mirror. Possible perceptions are projected onto the hidden limb with the characteristic of symmetrical bimanual movements and other deceptive kinesthetic (kinesthesia - a type of joint-muscle sensation related to displacement in space) and motor effects. Thus, observing one's own actions in the mirror increases excitability in the motor cortex ipsilateral to the moving hand [25].

Rubber hand illusion. Another crossover method is the Rubber Hand Illusion – it consists of moving a brush with soft hair (or a feather) on one of the subject's hands (hidden and invisible to the observer), while at the same time the researcher moves a similar object on an artificial rubber hand located in place of the hidden one. In this way, a sense of ownership of the rubber hand is created in the patient [7]. The principles used are [7, 20]: an increase in proprioceptive function, an altered volume of movements performed with the stimulated arm, and a stronger connection between body representation and the multimodal integration of touch, proprioception, and visual stimuli.

Robotic Therapies (RT)

– Functional electrostimulator (Foot Drop System - FDS) – is an innovative system with many sensors for measuring movement in the paretic limb. The device adapts to the patient – walking speed, foot length, walking symmetry. In the walking phase, the device generates pulses and stimulates dorsiflexion at the required moment. Similar is the interactive hand rehabilitation system, through which passive and active assisted rehabilitation, active assistance and interactive games are carried out [27].

– LEXO® systems enable training towards a physiological walking pattern that, through specific training, strongly engages the patient (initial contact, stance phase, swing phase).

– Lokomat® is another leader in robotic medical devices that support brain function in neurorehabilitation through state-of-the-art gait training - especially for patients with severe and moderate disabilities.

– The SafeGait 360° Balance and Mobility Trainer® is a ceiling mounted dynamic

body support and fall protection system. It provides a safe and effective therapy session for people with various disabilities who regain walking, improve their muscle strength and overcome balance problems. SafeGait tracks the patient's movements 2,500 times per second. The state-of-the-art device slides along a monorail, moves and protects patients during their daily therapeutic activities. The system stabilizes the participant's position and ensures constant tension on the support band. In this way, a safe environment and high individual motivation are created, necessary for conducting early and intensive therapy. All performance parameters are documented, including time, distance, repetitions, falls prevented, average body weight support and speed.

– RecoveriX stroke therapy is the first rehabilitation system based on a brain-computer interface that links mental activities (especially movement imagination) with real-time visual and tactile feedback i. e. with motor functions. Patients are instructed to imagine movement of an upper or lower limb. Once the RecoveriX system successfully recognizes the motor imagery, virtual reality and functional electrical stimulation are activated. Patients report feeling warmth in their extremities and a parallel improvement in memory, concentration and language functions.

– The Stiwell system focuses on motor learning in the following known aspects: active activities, goal-oriented and randomized actions, varied exercise options, "basic" daily activities, optimal feedback, task repetition, and maintenance of motivation. The recommended frequency of therapy is 5 exercises of 30-45 minutes per week.

– Biofeedback. Movement disorders can be treated with biofeedback. An electromedical device communicates the movements in person using visual and audio signals and the patient becomes aware and understands their movements. The result is relearning of the motor act and/or conscious maintenance of tension in the affected muscles in existing paralysis. Symptom-oriented biofeedback training is an effective tool and can be combined with muscle-controlled electrotherapy. It is used in the course of rehabilitation or in the form of controlled therapy in outpatient settings [16].

There is significant potential to reduce the severity of stroke, including its long-term consequences. This requires joint and coordinated actions of health professionals from different specialties (mainly neurologists, but also interventional radiologists, physiotherapists, etc.), the pharmaceutical industry, the ministries of health and social policy, other government bodies and non-governmental organizations. It is extremely important to raise the level of literacy of the population regarding the early recognition of the signs of a stroke.

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