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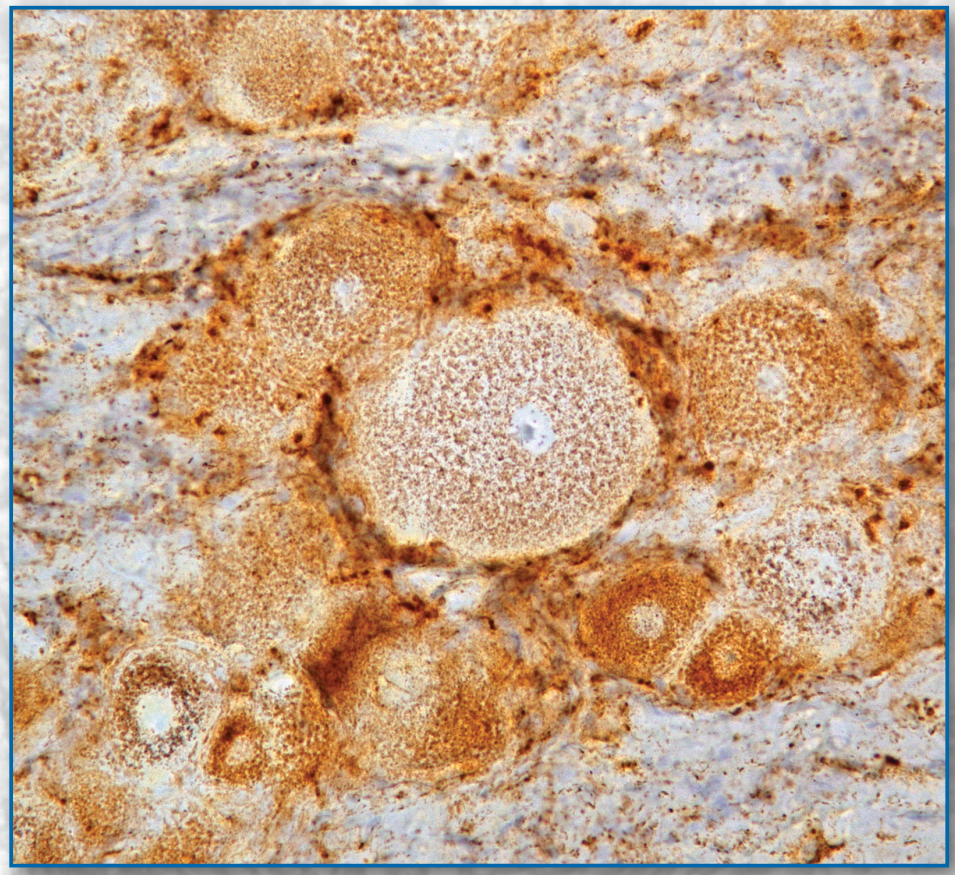

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Morphology

Novel Substrates for Determination of the Fibroblast Activation Protein-α Activity

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Fibroblast activation protein-α (FAP- α) is a membrane-associated serine protease of the S9b family of post-proline cleaving enzymes. It is usually expressed in reactive stromal fibroblasts in many types of diseases connected with extensive pathological alterations of the connective tissue like arthritis, fibroses, carcinomas and sarcomas. That is why the enzyme is considered a valuable marker for those entities. Design and development of specific FAP-α substrates are rather challenging due to the enzyme's structural similarity with the other proline-specific enzymes. In this paper we present the design of three novel substrates for the determination of FAP-α activity as well as the assessment of their efficacy and specificity. According to the obtained results, one of the newly developed substrates has a potential to be used as a highly specific substrate for FAP-α.

Key words: fibroblast activation protein-α, molecular modeling, enzyme substrate, substrate specificity

Introduction

Fibroblast activation protein-α (FAP-α; EC 3.4.21.B28) is a membrane-bound post-proline cleaving serine protease. It represents a 97 kDa glycoprotein existing as 170 kDa homodimer in its native form [15]. The enzyme hydrolyzes polypeptide substrates possessing Pro in P_1 position. It can act both as exo- and endopeptidase but is more efficient as an endopeptidase [14]. Some of the well-known enzyme`s natural substrates are collagen type I, neuropeptide Y, B-type natriuretic peptide, substance P and peptide YY [9]. FAP-α is involved in normal processes like tissue remodeling during the embry-

onic development, wound healing, etc. However, normal adult human and mammalian tissues do not express FAP-α [18]. Otherwise, the enzyme is highly induced in many diseases such as rheumatoid arthritis and osteoarthritis, liver and pulmonary fibrosis and in cancer [reviewed in 11]. It is expressed mostly by reactive stromal fibroblasts but has also been found in certain types of tumor cells [5, 11]. Many studies have shown that FAP-α participates in the mechanisms of tumor growth, angiogenesis and inhibition of the antitumor immune response [12, 17]. Studies on the enzyme activity in norm and pathology need the application of highly selective substrates [6]. However, the design of FAP-α specific substrates is very difficult due to its close structural similarity with the other proline-specific enzymes [reviewed in 5].

In the present paper we describe the design and development of three novel substrates intended for the biochemical assays of FAP-α activity in tissue homogenates and/or cell lysates. Additionally, we present the assessment of these substrates' efficacy and selectivity.

Materials and Methods

Molecular modeling. Using the crystal structure of human FAP-α (Protein Data Bank ID: 1Z68), obtained by Aertgeerts et al. [1], we modeled the structure of the enzyme-substrate complex with isonicotinoyl-D-Ala-Pro-4-nitroanilide by Dreiding forcefield method [13].

FAP-α substrates. We synthesized, purified (recrystallization, high performance liquid chromatography) and analyzed (nuclear magnetic resonance, mass spectrometry) the following substrates: β-Ala-D-Ala-Pro-4-nitroanilide (AAP), β-Ala-Nle-Pro-4-nitroanilide (ANP) and isonicotinoyl-D-Ala-Pro-4-nitroanilide (IAP). The synthetic methods, substrates purification and spectral analyses will soon be published elsewhere.

Cell culturing. Three permanent cell lines were used – MCF-10A (normal immortalized human epithelial cells from mammary gland), MCF-7 (human tumor cells obtained from mammary gland carcinoma of low invasiveness) and MDA-MB-231 (human tumor cells from mammary gland carcinoma of high invasiveness). The cancer cells were cultured in 75 cm^2 tissue culture flasks in Dulbecco's Modified Eagle's Medium – high glucose 4.5‰ (DMEM), supplemented with 10% fetal calf serum and antibiotics in usual concentrations. Normal cells were cultivated in the same conditions but with the addition of 20 mg/l human epidermal growth factor (EGF), 0.5 mg/l hydrocortisone, 0.1 mg/l cholera toxin and 10 mg/l insulin. Cell cultures were maintained at 37.5 °C in a humidified atmosphere and 5% CO_2 until 95% confluence was achieved.

Biochemical assays. For the estimation of FAP-α activity towards different substrates, aliquots of human recombinant FAP-α (Enzo Life Sciences, Inc.) were incubated with 0.1 mM of the respective substrate in 0.1 M phosphate buffer (pH 7.4), containing 0.1 M NaCl and 1 mM EDTA at 37 °C. Enzyme assays were carried out in 96-well plates. Absorption of the samples at 405 nm was measured every 4 minutes on multifunctional spectrofluorimeter Varioscan. The results were statistically estimated by regression analysis and curves showing the time-dependence of the adsorption at 405 nm were built by means of EnzFitter V2. In the cases of non-linear correlation, the enzyme activity was determined from the initial rate of the reaction.

The cells were harvested by a rubber policeman and homogenized using homogenizer MSE (England) in 5 ml 0.1 M phosphate buffer (pH 7.4) with 0.1 M NaCl and 1 mM EDTA. After a spectrophotometric measurement of the protein amount [2], the samples were incubated with the above FAP-α substrates (0.1 mM) in the same buffer at 37 ºC. The enzyme reactions in the samples were followed and analysed as above.

Results and Discussion

In cancer, FAP-α is usually expressed by cancer-associated fibroblasts where it takes part in the hydrolyzes of collagen type I thus opening free spaces for tumor invasion and blood vessels formation, as well as in the inhibition of the immune system's antitumor activity [reviewed in 4, 6 and 11]. However, several studies have shown that in some types of tumors FAP-α can be a tumor suppressor, e.g. in melanomas and in non-small cells lung carcinomas [reviewed in 4 and 18]. Thus, the marker role of FAP-α needs to be clarified separately in the oncological diseases of different origin. For this purpose, highly specific substrates are required to determine the activity of the enzyme. Unfortunately, the design of selective substrates for FAP-α is a difficult task due to its close structural similarity with other proline-specific proteases. For example, FAP-α and dipeptidyl peptidase IV (DPPIV) have 50% identity in the entire amino acids sequence and 70% in the catalytic domain [3] (**Fig. 1**).

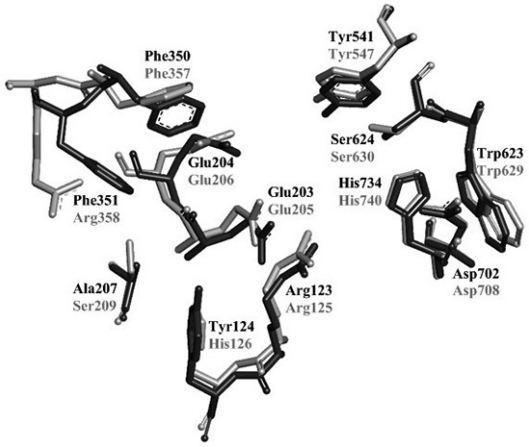

Fig. 1. Structural similarity between active centers of FAP-α (bolt) and DPPIV (pale). In the right site of the scheme, the catalytic triads of both enzymes are seen to coincide almost perfectly - Ser624, Asp702, His734 (FAP-α) and Ser630, Asp708, His740 (DPPIV). Tyr124 of FAP-α is very important for the substrates orientation. In DPPIV molecule, His126 is present in this position

The careful view of the entire active centers of the two enzymes shows that in FAP-α it is covered up mostly by non-polar amino acids, whereas in DPPIV it contains extra polar amino acids (not shown here). This fact explains why FAP-α is more efficient as an endopeptidase while DPPIV represents a typical exopeptidase. That is why, NH_2-Gly-Pro-based synthetic substrates commonly used to determine DPPIV and DPPIV-like enzymes' activities (DPP 8 and 9) are useless for the analyses of FAP-α activity. Further on, a commercial substrate for FAP-α is available – Z-Gly-Pro-7-amido-4-methylcoumarine. However, it is known to be cleaved also by other prolyl oligopeptidases (POPs).

Recently, several selective inhibitors (1, 2) and a selective substrate (3) for FAP-α were reported [7, 10, and 16] (**Fig. 2**).

All the above selective for FAP-α compounds contain isonicotinic or isoquinolinic acid residues connected with D-Ala.

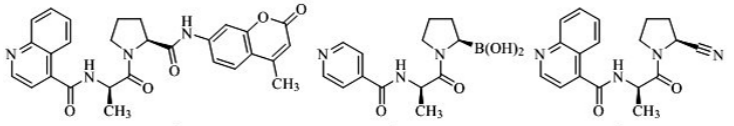

Fig. 2. FAP-α selective inhibitors N-(pyridine-4-carbonyl)-D-Ala-boroPro (1), N-(quinoline-4-carbonyl)-D-Ala-2-cyanopyrrolidine (2), and the selective substrate N-(quinoline-4-carbonyl)-D-Ala-Pro-4-methyl-7-coumaryl amide (3)

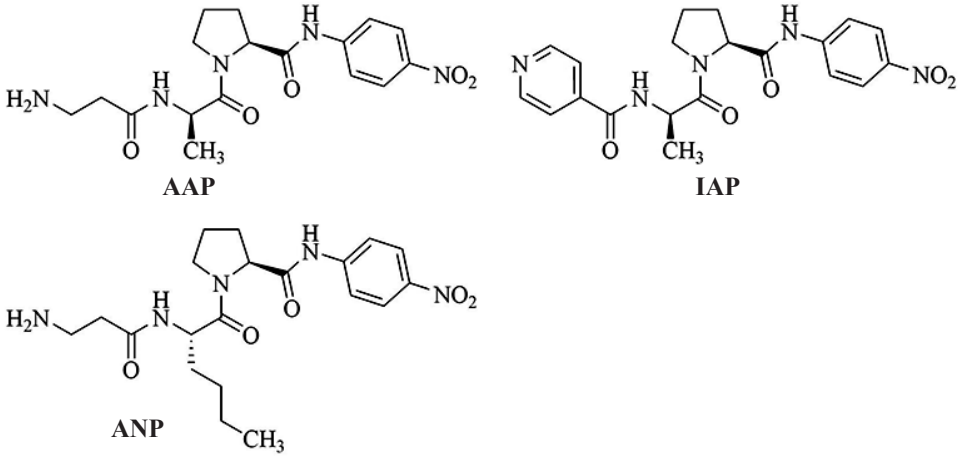

Fig. 3. Chromogenic substrates for FAP-α designed and synthesized by us: β-Ala-D-Ala-Pro-4-nitroanilide (AAP), isonicotinoyl-D-Ala-Pro-4-nitroanilide (IAP), and β-Ala-Nle-Pro-4-nitroanilide (ANP)

Based on the above studies, we designed and synthesized three novel chromogenic FAP-α substrates intended for biochemical assays of the enzyme activity (**Fig. 3**). Those substrates are as follows: isonicotinoyl-D-Ala-Pro-4-nitroanilide (IAP), which possesses the same N-(pyridine-4-carbonyl)-D-Ala - sequence as the compounds specific for FAP-α and listed above; β-Ala-D-Ala-Pro-4-nitroanilide (AAP) with a β-Ala- flexible moiety resembling the structure of isonicotinic acid and β-Ala-Nle-Pro-4-nitroanilide (ANP), possessing Nle at P_2 position which non-polar side-chain is expected to fit in the non-polar active center of FAP-α.

Molecular modeling showed that the cyclic nitrogen of the isonicotinic acid forms a hydrogen bond with the OH-group of Tyr124, thus stabilizing the enzyme-substrate complex and properly orientating the C=O-group of scissile bond towards the catalytic serine (**Fig. 4**).

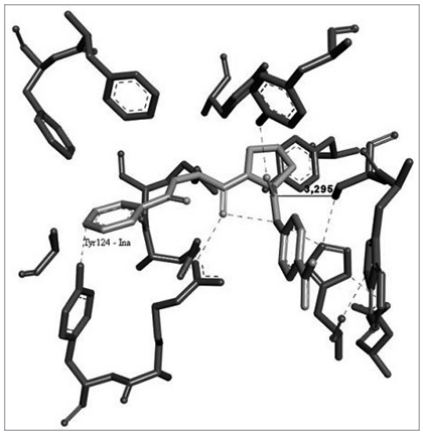

Fig. 4. Binding of the substrate isonicotinoyl-D-Ala-Pro-4-nitroanilide in the active center of FAP-α. The nitrogen atom of the cycle of isonicotinic acid forms a hydrogen bond with the OH-group of Tyr124

The principle of biochemical analyses of FAP-α activity is as follows: The enzyme cleaves the amide bond at the proline carboxyl group to liberate 4-nitroaniline, which is of yellow color and has maximum absorption at 405 nm. The enzyme activity can be estimated by the quantity of 4-nitroaniline liberated per minute per 1 mg protein at 37 ºC.

First, we studied the efficacy of every substrate to be hydrolyzed by the human recombinant FAP-α at the optimal conditions for the enzyme action (pH 7.4, 37 ºC in the presence of NaCl and EDTA). The results are given in **Fig.5**.

The results showed that FAP-α hydrolyzes all the three substrates but ANP is the most efficient, whereas IAP has the lowest efficacy. These outcomes are logical since Nle has a long non-polar side-chain which fits precisely in the enzyme active center. On the other hand, low efficacy of the substrates can be compensated by a

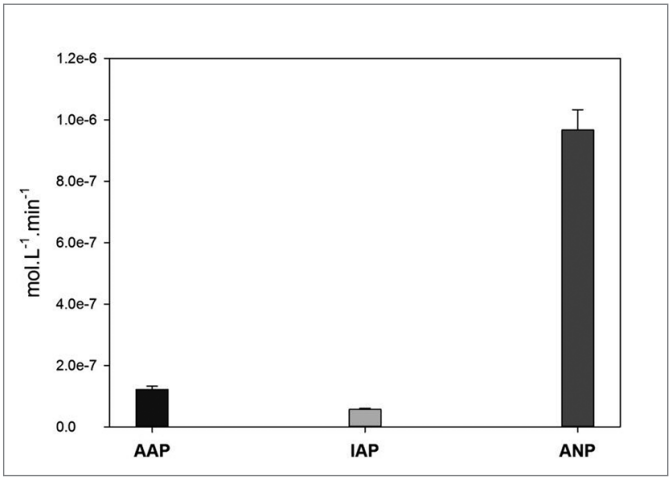

Fig. 5. Estimation of the efficacy of the newly synthesized FAP-α substrates to be cleaved by the enzyme

prolonged incubation, whereas their specificity may be crucial for the precise determination of the enzyme activity especially in heterogeneous mixtures like tissue homogenates or cell lysates.

Further, we tested the selectivity of our substrates in cell lysates using three cell lines: MCF-10A (normal immortalized human epithelial cells from mammary gland), MCF-7 (human tumor cells obtained from mammary gland carcinoma of low invasiveness), and MDA-MB-231 (human tumor cells from mammary gland carcinoma of high invasiveness). While MCF-10A has a low FAP-α activity, MCF-7 and MDA-MB-231 are usually used as negative controls since they are known to lack any enzyme activity [see e.g. 8]. According to the results (**Fig. 6**), the most efficient substrate – ANP has the lowest specificity towards FAP-α. Obviously, it is cleaved by a number of POPs and shows similar results in the three cell lines.

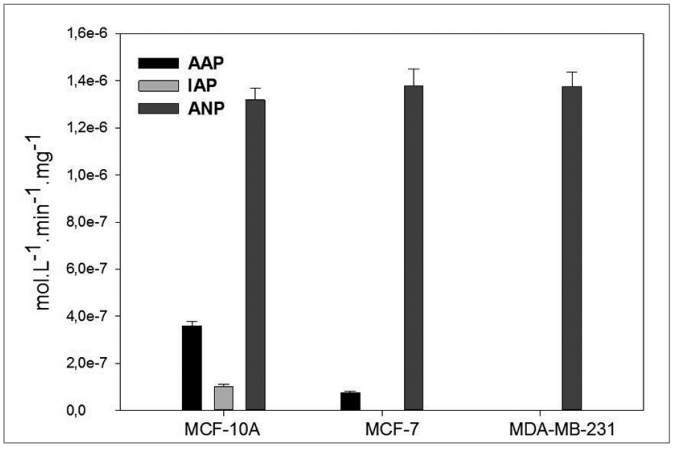

Fig. 6. Estimation of the specificity of the newly developed FAP-α substrates

The AAP substrate is more specific for it shows no FAP-α activity in MDA-MB-231. However, it demonstrates a cross-reactivity with some POP(s) in MCF-7 cell line. Finally, IAP can be considered as a highly specific substrate for the enzyme in this experiment.

Conclusion

We designed and synthesized three novel substrates for the determination of FAP-α activity – AAP, IAP and ANP. The last substrate is quickly and efficiently hydrolyzed by the enzyme and can be a useful tool to study the activity of isolated and purified FAP-α. Additionally, IAP although having a low efficacy to be cleaved by FAP-α, can be considered a highly selective substrate and can be valuable for the specific determination of the enzyme activity in heterogeneous mixtures such as tissue homogenates, plasma samples or cell lysates.

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Comparative Evaluation of the Effect of Sodium Nitrite on Reproductive Organ Weights and Sperm Count in Rats and Mice

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Sodium nitrite ($NaNO_2$) is a water soluble compound, well-known as a principal food preservative and colorant in the food industry. Besides the variety of industrial and medicinal applications, toxicity to humans and animals is well documented after nitrite overexposure. In the testis changes in hormonal profile and vascularisation have been reported. The current study aimed comparative assessment of early effects of acute $NaNO_2$ treatment on reproductive organ weights and indices in tandem with sperm count in rats and mice. Spermatozoa were isolated from both vas deferens and counted. An increase in testis weight and gonado-somatic index was found in tandem with reduction in epididymis weight and sperm count in both species following acute $NaNO_2$ treatment. Our comparative analysis on macro parameters of rat and mouse reproductive organs (testis and epididymis) and sperm counts suggest that mice are more vulnerable to the exposure to $NaNO_2$ than rats.

Key words: sodium nitrite, hypoxia, sperm count

Introduction

Sodium nitrite ($NaNO_2$) is an inorganic salt with various applications. It is widely used in the food industry as color fixative and preservative of fish and meat products (E250). It acts as a flavor-enhancer and retards rancidity by preventing fat oxidation. It also inhibits the growth of micro-organisms as *Clostridium botulinum* spores. Sodium nitrite is used for dye synthesis, manufacture of rubber chemicals and nitroso compounds and has several other industrial purposes. Medicinally, it is used for vasodilation, bronchodilation and as antidote for cyanide poisoning [3]. However, industrialization and uncontrolled use of nitrate/nitrite salts has increased human exposure to high levels of $NaNO_2$ which can act as a pro-oxidant and pro-carcinogen. Exposure to nitrite mainly occurs through the oral route. Nitrite taken through contaminated drinking water or food, primarily affects the gastrointestinal tract and small intestine [3]. Acute exposure to high levels of nitrite has been reported to cause death, mainly due to methemoglobinemia [4]. Chronic exposure to

lower doses of nitrite causes adverse health effects, which includes birth defects, respiratory tract ailments, damage to nervous system and paralysis. Prolonged exposure to nitrite can also cause carcinogenicity and mutagenicity [3]. Oxidative damage is considered to be one of the main mechanism by which nitrite exerts its toxicity. There is evidence of developmental and reproductive toxicity of $NaNO_2$ in experimental animal studies. In the testis changes in hormonal profile [6] and vascularisation have been reported [5]. Data about its influence on reproductive system are controversial. The current study aimed comparative assessment of the early effects of acute $NaNO_2$ treatment on reproductive organ weights and indices and sperm count in rats and mice.

Materials and Methods

The experiments were carried out on four-month-old male Wistar rats and two-month-old male ICR mice. Animals were maintained in the institute's animal house in standard hard bottom polypropylene cages at 23 ºC ± 2 ºC and 12:12 h light-dark cycle with free access to laboratory chow and tap water throughout the study. Sodium nitrite was injected intraperitoneally at a single dose of 50 $mg.kg^{-1}$ body weight for rats and 120 $mg.kg^{-1}$ body weight for mice. Treated animals were sacrificed at different time intervals following the administration (1h, 5h, 1d, 2d and 5d) under light diethyl ether anesthesia. The control animals were injected with distilled water. Testes and epididymides were sampled, weighed and their indices were calculated (organ weight to body weight ratio). Spermatozoa were isolated from both vas deferenses and counted using Buerker's chamber. Data were statistically processed using Student's t-test. The animal experiments were performed in accordance with the animal protection guidelines approved by the Ethics Committee for Experimental Animal Use at IEMPAM, BAS.

Results

Testis weight of rats was in normal range with slightly lower values at the 1st and the 5th hour, as well as on the first day after sodium nitrite administration. On the 2nd and the 5th day, this parameter reached control values (**Fig. 1**). Gonado-somatic index revealed more convincing tendency of significant increase by 13% compared to the control at the first hours (1h, 5h) and on the 5th day after treatment (**Fig. 1**).

In mice, there was slight and insignificant elevation by 7-10% in testis weight at the first hours (1h, 5h) and on the 1st and the 5th day after treatment (**Fig. 1**). This finding corresponded to significant elevation of mice gonado-somatic index by 20% - 35% at the same time points as in rats (**Fig. 1**).

Epididymis weight of rats was insignificantly lower at the 1st hour, followed by significant decrease by 20% at the 5th hour, compared to the control. Epididymis weight of mice revealed clear tendency of reduction in all time points investigated and the values were reduced by 30-50% from the control value (**Fig. 2**). Corresponding to these findings are the data on epididymis weight/body weight index in mice that revealed marked decrease by 35-40% compared to control value (data not shown).

Reduction in rat sperm count was observed in all the treatment groups that reached significance at most of the time points – by 30% at the 1st hour, by 47% at the 5th hour, by 28% on the 2nd day, and 22% on the 5th compared to the control (**Fig. 2**).

In mice we found reduction in sperm count in most of the treated groups with statistical significance on 2nd day after treatment (60% lower than the control value) (**Fig. 2**). At the 5th hour after treatment we estimated a significant elevation of the sperm count (30% higher compared to control).

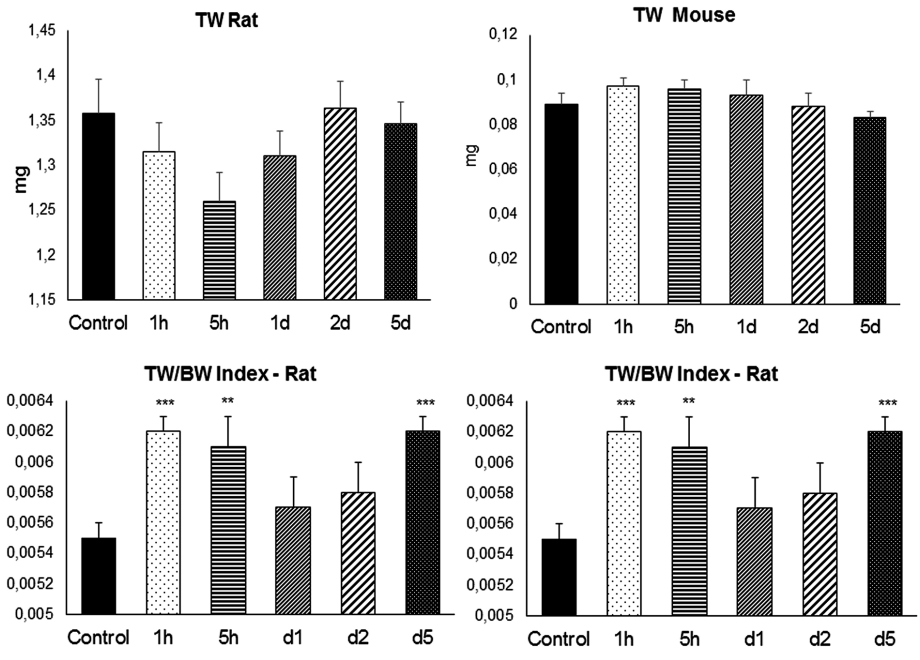

Fig. 1. Changes in testis weight (TW) and in gonado-somatic index (ratio of testis/body weight) at different time intervals following $NaNO_2$ treatment in rats and mice. Data represent mean value ± SE (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

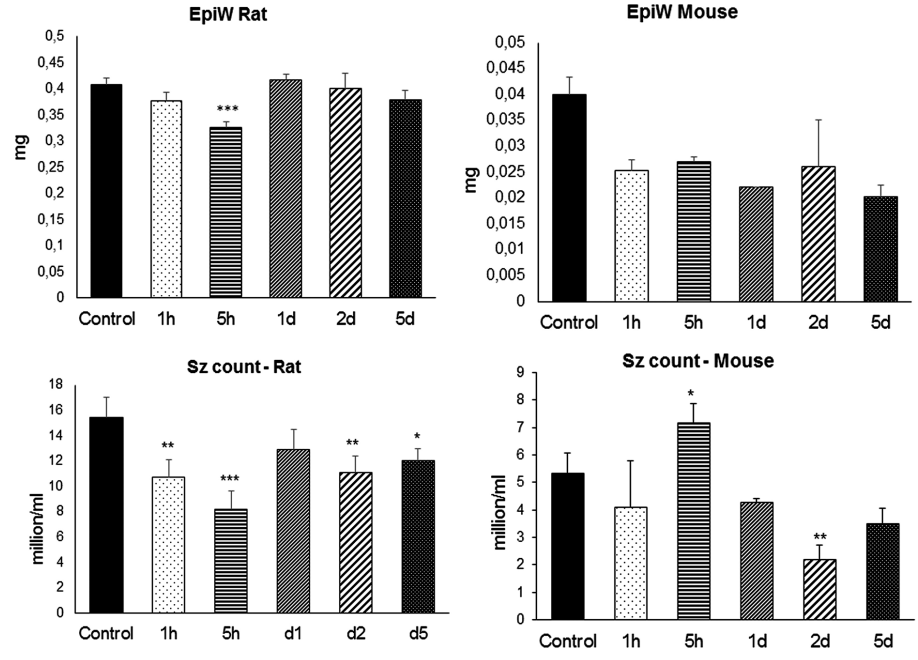

Fig. 2. Changes in epididymal weight and in sperm count at different time intervals following $NaNO_2$ treatment in rats and mice. Data represent mean value ± SE (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Sz - spermatozoa

Discussion

The toxic effect of nitrates and nitrites are well documented in mammalians, including impairment of reproductive function and hepatotoxicity. The major acute toxic effect of sodium nitrite intoxication is methemoglobinemia [10]. The over dosage of $NaNO_2$ leads to the accumulation of excess methemoglobin in the blood, which does not bind oxygen strongly, thus causing hemic hypoxia. The rate of methemoglobin formation varies between species, as well as with age of the organism, and the reaction is reversible. According to literature data, LD50 values of 85-220 mg of sodium nitrite per kilogram of body weight have been reported for mice and rats [13]. Because of the wide range of LD50 values and the species-specific rate of methemoglobin formation, we conducted experiments with graded doses of $NaNO_2$ in order to determine the optimal dose that induces acute toxicity and methemoglobinemia in both species without animal loss. At 50 mg of sodium nitrite per kilogram of body weight for the rats and 120 mg of sodium nitrite per kilogram of body weight for the mice the methemoglobin reached peak levels one hour after treatment (41%). Most of the mammals have little tolerance to hypoxia, and their response involves the activation of regulatory mechanisms at systemic, tissue, and cellular levels. Hypoxia has a decisive impact in different molecular pathways, which modulate several cellular functions, such as proliferation, apoptosis, angiogenesis, pH balance, and anaerobic glycolysis. The susceptibility of the mammalian testis to low oxygen pressure or content is a causative factor in some forms of male infertility [12]. In animal models (rat, mouse, guinea-pig, rabbit, monkey, sheep), it has been shown that hypobaric hypoxia induces partially reversible quantitative changes such as decrease in semen volume, sperm count and sperm motility [9]. Literature data for the effect of sodium nitrite-induced hemic hypoxia on the male reproductive system are controversial and uncompleted [8].

Our quantitative results in mice are more consistent than in rat. There was an increase in mouse testis weight up to 10% and in gonado-somatic index by 20-35% from the corresponding controls. In contrast, epididymis weight and ratio epididymis weight/body weight of mice were greatly reduced by 30-50%. With one exception at the 5^{th} hour after treatment, the mouse sperm count was decreased by 20-60% compared to control value. A possible explanation for elevation of testis weight and gonado-somatic index could be inflammatory process, based on data by Alyoussef et al. [1, 2] about enhanced gene and protein expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) after exposure to sodium nitrite. The authors have been reported elevation in testis weight and gonado-somatic index, as well. The elevation in testis weight and gonado-somatic index in tandem with reduction of epididymis weight and sperm count in our study on mice and rats could be interpreted as a result from retention of seminiferous tubules fluid together with sperms released into the tubular lumen. These findings might be a consequence form compromised hypothalamus-hypophysis-gonadal axis, in particular decreased serum testosterone levels associated with increased serum LH, FSH concentrations [1, 5]. Alternatively, prolonged residence of sperms in rete testis cannot be excluded.

We have established decrease in sperm count in all experimental groups in both species – in mice by 20-60% and in rat by 20-50% from corresponding controls. Our data are in agreement with the results by Alyoussef et al. [1, 2] who have demonstrated almost 50% reduction in the sperm count in tandem with increase in testis weight and gonado-somatic index after sodium nitrite administration. They also found significantly elevated levels of caspase-3, caspase-8 and caspase-9 activity associated with significant increase of cytotoxicity. Increased oxidative stress by $NaNO_2$ was believed to produce oxidation and damage to DNA leading to germ cell apoptosis. Our previous

histological findings on sodium nitrite-treated rats revealed destructive and degenerative changes in rat testis after sodium nitrite treatment [7]. We have found disorganized seminiferous tubules and sloughs of undifferentiated germ cells into the luminal area in some experimental animals. In some tubules the lumen has not be seen. Blood vessels with larger diameter were more frequently found compared to the control [7]. Tissue hypoxia of the male reproductive system with subsequent atrophy of germinal epithelium was associated with arrest of primate spermatogenesis [11].

Local changes in testicles exposed to hypoxia involved neovascularization and an increase in temperature as reported by Farias et al. [5]. Hyperthermia is well known to affect spermatogenesis leading to infertility. In this respect, the response of the testis to hypoxia (in particular, sodium nitrite-induced hemic hypoxia), could resemble other hyperthermia-related pathologies, such as varicocele and cryptorchidism.

In conclusion, our comparative analysis on macro parameters of rat and mouse reproductive organs (testis and epididymis) and sperm counts suggests that mice are more vulnerable to the exposure to $NaNO_2$ than rats.

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Lifestyle and Environmental Factors Affecting Fertility in Men

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Quality indicators of sperm studies have deteriorated in all industrialized countries in the world, prompting scientists to seek the reasons for these bad results. Till now lifestyle has been overlooked as a factor, but today more and more attention is paid to it as a reason for the poor results. It was impossible using the uncontested statistical data to prove which of the environmental factors and the way of the daily life of modern man affect his health and in particular the male reproductive function. But looking at the recent past and taking into account the changes that occur in the last 100 years, we cannot fail to take account of changes in way of eating, use of hormones, chemicals, antibiotics in the processing of food, lifestyle and more accurate the sedentary life compared to the time spent outdoors performing active physical activity, stress that we are subjected daily, affects human health and in particular the quality of sperm parameters.

Key words: infertility men, semen analysis, sperm morphology, lifestyle

Introduction

Investigations that follow sterility in men over a long period and comparing sperm indicators has reported deterioration in quality indicators in all industrialized countries in the world for years. Different factors have been the cause of a decrease in sperm count. In recent years, the tendency to reduce sperm concentration (> 20 mil ≥ 30% m1 WHO) has increased, another trend observed in their morphology, more than 4-14% can be defined as normozoospermia using Kruger's strict criteria [6] and WHO [16].

In the last two decades, a series of reports have been published that take into account the global decline in the quality of the ejaculate - the quantity, motility and sperm morphology [7, 12, 13]. One of Geoffroy-Siraudin's latest series of reports [3] traces a period of 20 years (1988-2007), the sperm count analyzes trends for the progressive reduction of sperm concentration (1.5% per year), the total number of sperm (1.6% per year), general mobility (0.4-5.5% per year) and normal morphology (2.2% per year). All scientific teams declare that there is no change in the methodology of work for the

reporting period, and that andrological laboratories are working in accordance with the WHO standards.

Different factors have been seen as the cause of a decrease in sperm count. The authors highlight factors such as lifestyle, smoking [4], alcohol, drug abuse, aging [10], and others. Apparently, several factors may be responsible for the condition of male sterility and it is difficult to make a meaningful assessment of their impact. Spermatogenesis started in puberty, and since it is a highly vulnerable process, all harmful actions will have adverse effects on its quality.

The aim of the present study is to relate the deteriorating sperm parameters such as concentration, motility and morphology to the lifestyle and environmental factors such as medicines, anabolic substances, drugs, alcohol, smoking and heat exposure.

Materials and Methods

This study was done in accordance with the ethical standards of the Medical University of Plovdiv/Bulgaria (resolution of the University Ethic Committee No P-1166/15.04.2016). Each patient completes a set of documents in a dossier as required - informed consent and poll. The survey was conducted among 80 men of average age 34.9 years (20-51 years) from families with long (primary or secondary) infertility from the town of Plovdiv and its region. Sperm study was obtained by masturbation, after 3-5 days sexual abstinence, in a sterile container and it was stored at room temperature 18-20 °C. Qualitative and quantitative research was carried out to determine the ejaculate volume, sperm concentration, total sperm count, assessment of mobility and morphology applying strict criteria of Kruger [6]. Concentration, motility and morphology were analyzed using the Computer Aided Sperm Analysis (CASA) to provide an objective assessment of sperm fertilization. The evaluation of the results was carried out according to the criteria of the WHO [16]. All data were processed with statistical program SPSS 19.0.

Results

Our study on infertile patients found significant deviations from the WHO standards and they are illustrated in Figs. 1 and 2. After evaluation of sperm motility, we found that 34.13% are normally motile, 8.79% are weakly motile, and 57.07% are fixed (immotile). According to WHO, the criteria for fertility recommended minimum is 40% of sperm to be progressively motile (fast and slow linear translational motions) (**Fig. 1)**.

Examining the morphology of the sperm we found 96.66 % pathological forms of which 58.89% showed head defect, 32.77% - head and neck defect, and 4.99% - neck defect. Only 3.34% are with normal morphology (**Fig. 2**).

Morphological analysis classifies sperm as normal only if the shape and size of sperm segments (head, midpiece and tail) fall within defined parameters. Strict criteria by Kruger [6], illustrated in **Fig. 3**, were applied for sperm evaluation, according to which a spermatozoon is normal if it has an oval head, 4.0-5.0 μm long and 2.5-3.5 μm wide, measured with an ocular micrometer. The length-to-width ratio should be 1.50-1.75. A normal spermatozoon has a well-defined acrosome that covers 40-70% of the head. The midpiece is thin, less than 1 μm wide, about 1.5 times longer than the head. Cytoplasmic droplets, if present, should not be larger than half of the head width. The tail is thin, uniform, uncoiled and about 45 μm long. According to this classification system, all borderline forms are considered as abnormal. In case normal forms accor-

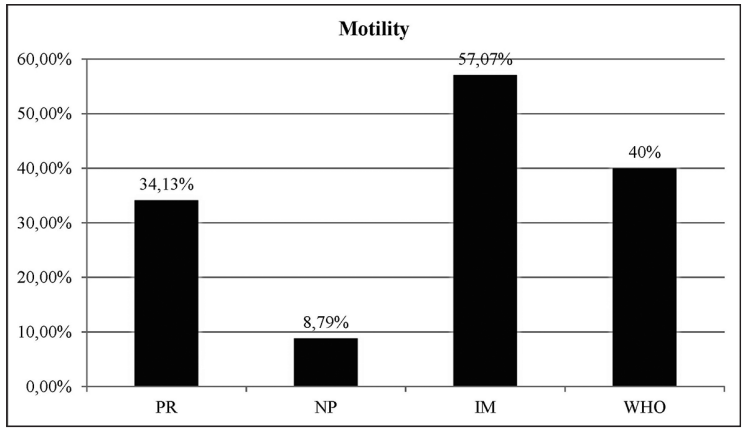

Fig. 1. Progressive (PR), non-progressive (NP), immotile (IM) and WHO

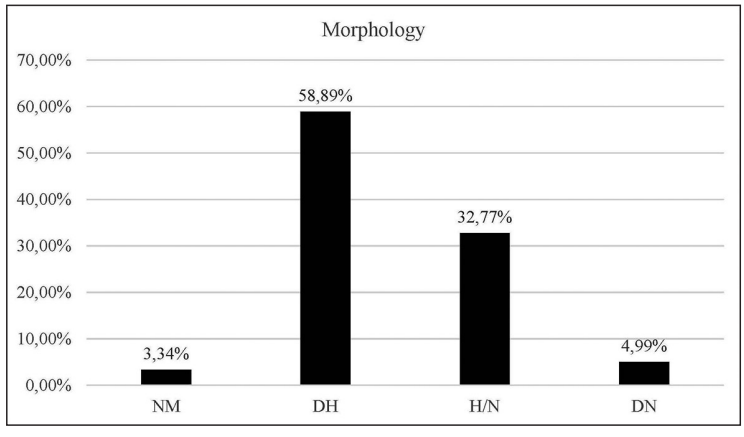

Fig. 2. Normal morphology (NM), pathological forms – defect head (DH), head and neck (HN), defect neck (DN)

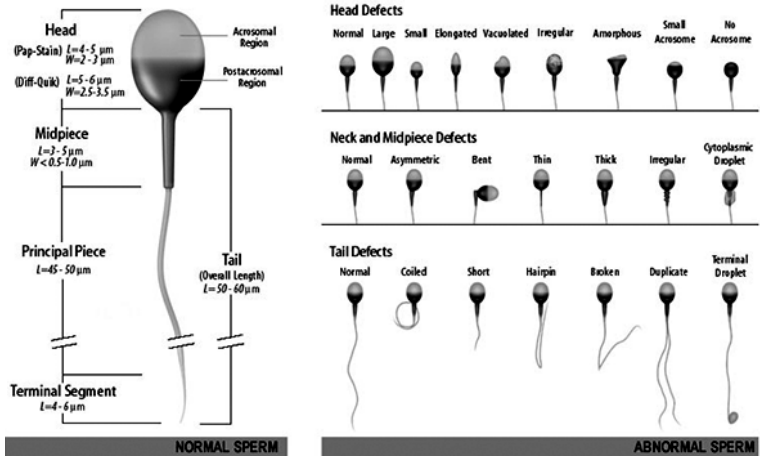

Fig. 3. Kruger's morphological criteria for evaluation of the sperms

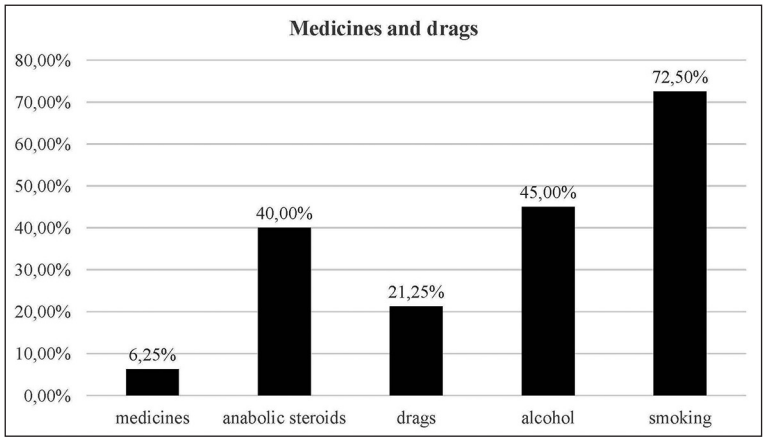

Fig. 4. Results of patient survey 1

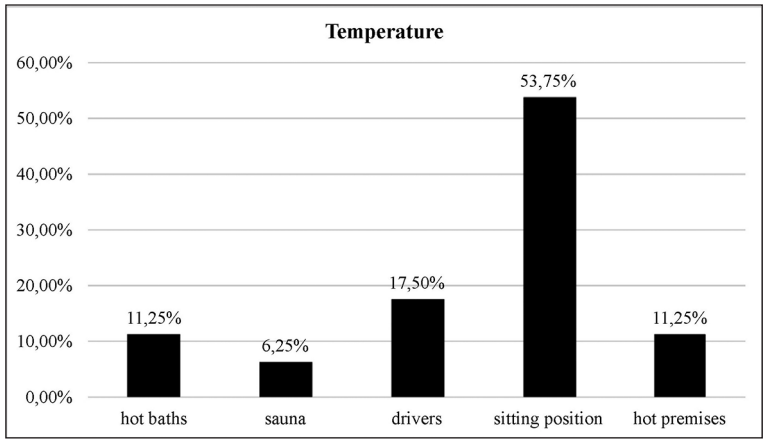

Fig. 5. Results of patient survey 2

ding to Kruger's criteria exceed 15% after counting 200 sperm the sample is considered as normal.

The increasing percentage of infertile men motivates us to make a poll among men in the town of Plovdiv and its region, in which we try to identify the causes leading to this severe problem, which raises epidemic proportions.

Figs. 4 and 5 show some of the answers to the questionnaire. On the question "Do you take medications and other chemical substances?" (**Fig. 4**), 6.25% of respondents answered that they take medications on a daily basis, 40% take anabolic substances, 21.25% take or have taken opiates, 45% drink alcohol every day, and 72.50% of them are smokers. The results obtained are more than 100% because some of the questions gave more than one answer and others were unanswered.

On the question "Causes leading to elevated temperature in the small pelvis?" (**Fig. 5**) – 11.25% of respondents answered that they like to take a continuous hot shower, 6.25% often use sauna, 17.50% are drivers, 53.75% are sitting on all working day, and 11.25% work in hotspots.

Discussion

Over the last decades, the efforts have been directed to attempts of identifying the causes leading to a steady decrease in the number and quality of human sperm. Drastic reduction in reproductive performance led to lowering of several benchmarks, determined by the WHO, most recently in 2010. Moreover, very small percentage of men managed to cover these standards and this is illustrated by the results shown in Figs. 1 and 2. Thinking of the causes of these negative effects, daily routines become more and more important, such as long-term hot showers, use of the sauna, time spent sitting, the number of smoked cigarettes, use of anabolic drugs, medicines, opiates and alcohol.

In warm-blooded animals, the evolutionary solution is to have the testes outside the body where the temperature is 3-4 °C lower than the body temperature (37.6 °C, which is harmful for spermatogenesis) [8, 11]. Another key element in providing the testicle cooling is the presence of the pampiniform plexus which cools the arterial blood entering the testis. The functioning of this plexus is of importance for normal testicular function. Therefore, occupations associated with long-standing sitting, such as drivers, programmers, clerks, workers in premises with increased temperature, etc. could cause deterioration of sperm counts and quality. The reason for these results is a slowing of blood flow in the small pelvis resulting in a rise in temperature [8, 14].

In experimental mouse models, it was found that immersion of the testes for 30 min in hot water at 42 °C for a period of 30 days induces infertility with impairment of sperm quality by 44.9% due to germ cell apoptosis, resulting in decreased fertility *in vitro* compared to controls and decreased in vivo quality of embryos [9]. It is suggested that short immersion of the testes in hot water cause thermal damage to DNA and proteins in germ cells [15]. Within the years, a gradual increase was reported in the use of anabolic substances, opiates, especially so-called mild opiates such as marijuana, and the many synthetic analogues most commonly combined with alcohol and cigarettes. In our current study these findings are clearly demonstrated.

A large study conducted in Denmark among 1221 young men aged 18-28 (in the period 2008-2012) found a decrease in the concentration, the total number and percentage of sperm with normal morphology between 33% and 59% depending on the amount of alcohol intake and combination with marijuana more than once a week [5]. Numerous studies reported an increase in the percentage of spermatozoa with morphological defects, fragmentation of the DNA and decreased viability in cases of idiopathic infertility and aging [1, 2, 4].

Conclusion

The reasons leading to disruption of fertility in men are complex. More attention is needed to the increasing etiological impact of lifestyle factors. Establishing the cause of infertility is not a simple task and requires comprehensive and multifactor analysis of all possible influences.

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Morphometric Features of Three Lungworms in Materials from Wild Boars from Bulgaria

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A necropsy of wild boars from some regions of Southwest Bulgaria showed an infection with three lungworm species from family Metastrongylidae - *Metastrongylus pudentotectus, M. salmi* and *M. elongatus*. The collected parasites were used for a morphometric description of the species. The results were compared with the existing ones in the literature. It was established that as a whole the morphometric features of the species correspond to such described by other authors in materials from Bulgaria and abroad.

Key words: Metastrongylus elongates, Metastrongylus pudentotectus, Metastrongylus salmi, wild boars, morphometric description

Introduction

Lungworms in wild boars are nematode parasites from family Metastrongylidae. They are believed to be the most important parasites influencing the health status of the wild boar [12]. The infection with metastrongylids leads to verminous pneumonia and secondary disorders in the hosts, loss of weight, abortion and higher mortality, especially in young animals. Because of their importance, many studies on different aspects of the induced by them illness have been performed [2, 3, 4, 8, 10, 13]. The systematic position of these parasites, however, is still unclarified. Kontrimavichus et al. [9] include in Metastrongylidae family two subfamilies – Heterostrongylinae (parasites on marsupials and insectivores) and Metastrongylinae. These authors [9] associate only one genus with subfamily Metastrongylinae – *Metastrongylus* in which include 7 species (*M. madagascariensis*, *M. pudendotectus, M. asymmetricus, M. salmi, M. confusus, M. elongatus, M. tschiauricus*) infected domestic swine and wild boars. Anderson et al. [1], however, believe that the above system is in error and Metastrongylidae should be restricted to *Metastrongylus* of swine, the genus being characterized by a pair of large, lateral trilobed labia, thick-shelled sculptured eggs, an atypical bursa and earthworm intermediate hosts. It is necessary to collect great quantity of information in order to solve

such kind of contradictions, in particular information about morphology of the parasites in materials from different hosts around the world. During a study on the etiology of helminthoses in wild boar in Bulgaria we have established an infection with three lung nematode species - *Metastrongylus pudendotectus, M. salmi* and *M. elongatus*. In connection with the above mentioned we set a goal to supply morphometric descriptions of these species in our materials.

Materials and Methods

The internal organs of 6 wild boars that were hunted during the shooting season in 2016 in three regions of Southwest Bulgaria were helminthologically necropsied. The discovered helminths were collected in a saline solution and after cleaning they were stored in 70% ethyl alcohol. Some of the helminths were enlightened with an alcohol-glycerol sequence so that the taxonomic important features could be easier observed. After that the helminths were included in gelatin-glycerin and the edges of cover glasses were coated with Canadian balm for longer storage. The measurements were performed with the help of the classical parasitological methods or after shooting of the separate structures of the parasites with a Web camera Logitech 4000, which was attached to the "Amplival" microscope, and their measuring with the picture analyzing computer program Image-Pro Plus-Version 6. Pictures were taken using a light microscope Leica DM5000 B, supplied with a camera and software (Leica Application Suite LAS v. 3.1). The obtained data were statistically analyzed according to Georgieva et al. [6]. Measurements in the tables are in μm.

Results and Discussion

We found metastrongylids in the lungs of 4 wild boars. They were *Metastrongylus pudentotectus, M. salmi* and *M. elongatus*. Further down data about their morphological and metrical features are supplied.

***Metastrongylus* spp. – general features**

Metastrongylids have a thread-like form and whitish color. The mouth opening is encircled with two trilobed lips; the oesophagus is slowly widening distally (**Fig. 1a**). The males have a small bursa copulatrix with poorly-developed and sometimes hard visible rays. The spicules have spongiform, comb-like structure. The females have a prevulvar cuticular swelling (valve). The eggs in the vagina are oval and contain fully developed larvae.

***M. pudentotectus* Vostokov, 1905**

Male. Bursa copulatrix (**Fig. 1b**): The dorsal ray is short and consists of two branches that look like a pair of pincers. The exterodorsal rays are detached, thin and short. The lateral rays come from one trunk. The anterolateral ray is the longest. It is the first that separates from the trunk, after that gradually becomes narrow and ends with roundness. The medio- and posterolateral rays remain connected longer. The mediolateral ray is wider and longer than posterolateral one and ends with an enlargement. The posterolateral ray also ends with an enlargement. The ventral rays are wide and begin from one trunk. The spicules are long and thin. Their wings are narrow and do not reach to the end of the stem. The distal end of the spicule stems is bifurcated and it looks like an anchor (**Fig. 1c**). The gubernaculum is small and looks like a shield (**Fig. 1d**).

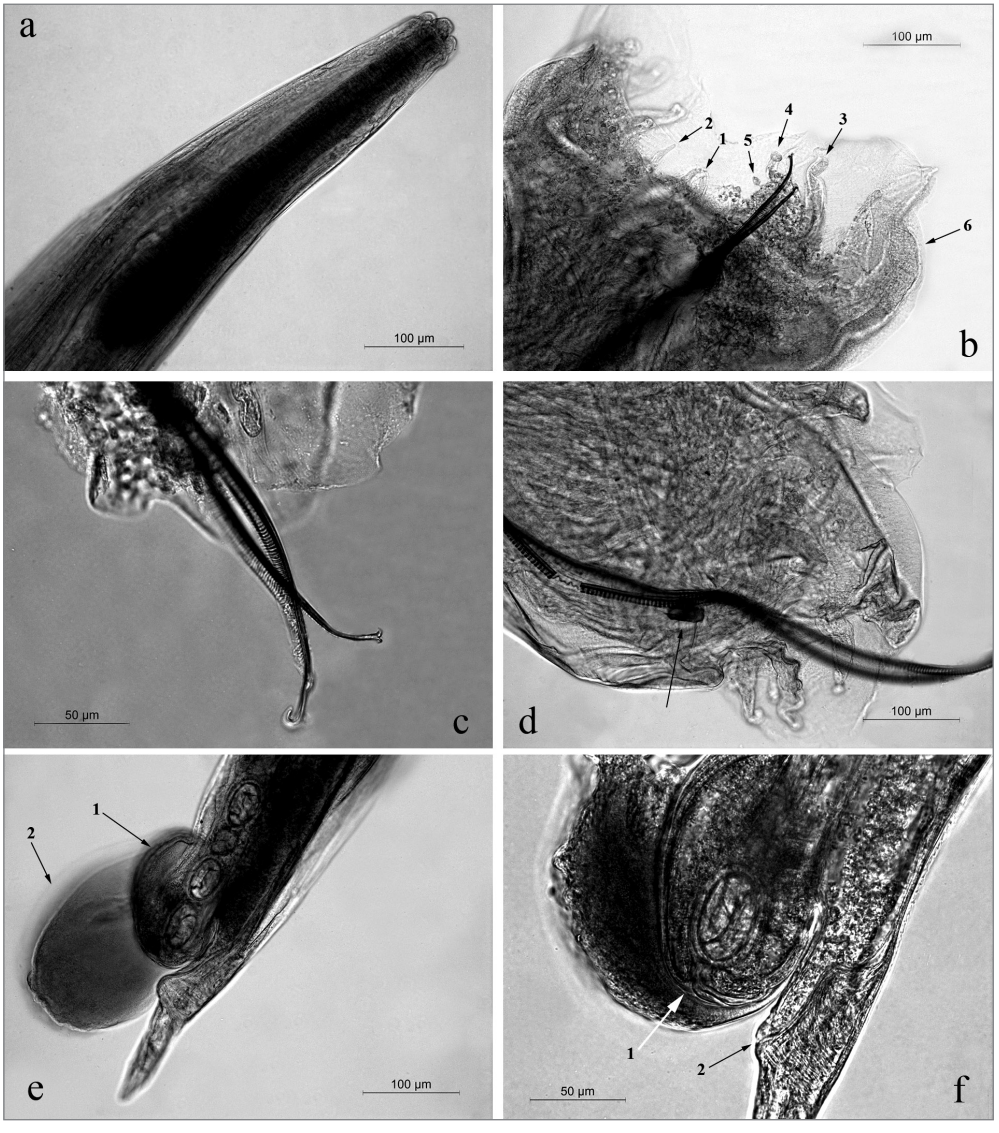

Fig. 1. Morphologic features of *Metastrongylus pudentotectus* in materials from wild boars from Bulgaria: a) anterior end; b) bursa copulatrix: 1- dorsal ray, 2 - exterodorsal ray; 3 - anterolateral ray, 4 - mediolateral ray, 5 - posterolateral ray, 6 - ventral rays; c) distal spicule end; d) gubernaculum; e) posterior end of female: 1- prevulvar cuticular valve, 2 - cuticular dilatation; f) posterior end of female: 1- vulva, 2 - anus

Female. The vagina is situated parallel to the body. The prevulvar cuticular valve is well-developed and surrounded by spherical cuticular dilatation (**Fig. 1e**). The vulva is opened at the distal end of the valve (**Fig. 1f**). The metric data about this species in our materials as well as those available in the literature are given in **Table 1**. Some of our metric data are outside the limits given by Kontrimavichus et al. [9], who have summarized the literature data up to that point. Our specimens have shorter oesophagus and gubernaculum.

Table 1. Metric data of *Metastrongylus pudentotectus* by different authors

Structure	Kontrimavichus et al., 1976	Gasso et al., 2014	Our data
Body length ♂ (in mm)	14.5-19.25	-	16.5 ± 0.14 (14-21)
Body width in the end of oesophagus ♂	-	-	108 ± 8,7 (90-126)
Oesophagus length ♂	414-520	-	408 ± 15 (385-462)
Max. oesophagus width ♂	-	-	64 ± 5.5 (58-83)
Spicule length	1310-1650	1380 ± 70 (1300-1500)	1500 ± 120 (1300- 1700)
Gubernaculum length	43-55	34 ± 5.55 (27.5-45)	39.7 ± 2.8 (31-46)
Body length ♀ (in mm)	21.5-40		23 ± 0.16 (19-29)
Body width in the end of oesophagus ♀	-	-	142 ± 11 (103-174)
Oesophagus length ♀	540-630	-	454 ± 10.8 (423-486)
Max. oesophagus width ♀	76-108	-	82.5 ± 7.2 (68-104)
Tail length ♀	-	119 ± 14.99 (100-150)	189 ± 8.4 (157-217)
Length of prevulvar cuticular dilatation	-	262.75 ± 42.07 (200-350)	186 ± 9 (165-207)
Width of prevulvar cuticular dilatation	-	254.5 ±44.05 (200-330)	160 ± 11.4 (139-204)
Length of prevulvar cuticular valve	-	165.25 ± 33.36 (87.5 – 200)	147 ± 6.4 (125-164)
Width of prevulvar cuticular valve	-	87.5± 22.21 (50-137.5)	80 ± 4.6 (65-94)
Egg length	60-64	56 ± 2.93 (50-60)	56.3 ± 2.8 (50-67)
Egg width	43-45	43.5 ± 6.26 (37.5-55)	35.8 ± 2.8 (30-47)

The mean length of the spicules measured by us is in the upper limit pointed in previous study in materials from wild boars from Bulgaria [11]. There is also a certain difference with the data provided by Gasso et al. [5] in materials from wild boars from Spain and Poland. The gubernaculum and the tail of the females are shorter in their specimens, whereas the prevulvar cuticular valve, cuticular dilatation and width of the eggs are shorter in our specimens.

***M. salmi* Gedoelst, 1923.**

Male. Bursa copulatrix (**Fig. 2a**): The dorsal ray is short and bifurcated, with a form of pincers. The exterodorsal rays are detached, comparatively thin and short. The lateral rays come from one trunk. The anterolateral one is the longest and widest. Gradually it is narrowed and ends with a major rough thickening which is directed towards the ventral rays. The mediolateral ray is vastly shorter. It also ends with a thickening, which is turned to the posterolateral ray. The posterolateral ray is short – only one third

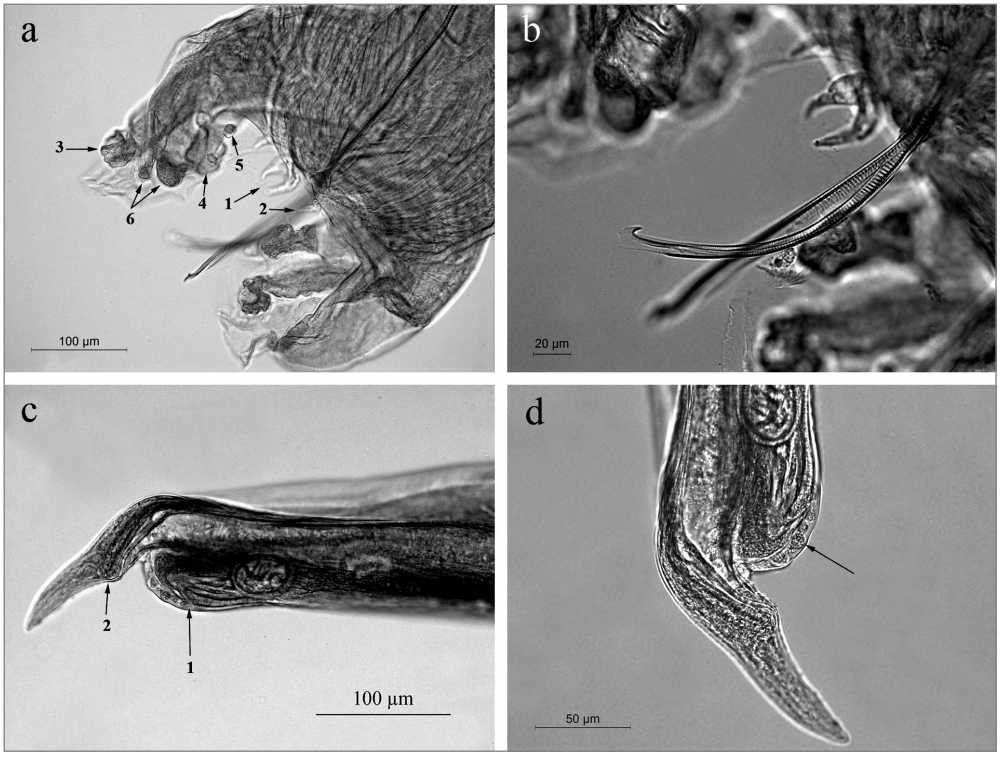

Fig. 2. Morphologic features of *Metastrongylus salmi* in materials from wild boars from Bulgaria. a) Bursa copulatrix: 1- dorsal ray, 2 - exterodorsal ray; 3 - anterolateral ray, 4 -mediolateral ray, 5 - posterolateral ray, 6 - ventral rays; b) distal spicule end; c) posterior end of female: 1- vulva, 2 - anus; d) posterior end of female: marginal sickle-like line of light-refracting formations

of the length of mediolateral ray, it begins from the base of mediolateral ray, can hardly be observed and ends with a thickening too. The ventral rays begin from one trunk. One of them has an obtuse end and another ends with a big thickening. The spicules end with a single growth with a form of hook (**Fig. 2b**). The gubernaculum is small and hard to be observed.

Female. The vagina is parallel to the body to the middle of the valve, after which it turns perpendicular towards the cuticle and passes into the vulva that opens on the free side of the valve (**Fig. 2c**). In the distal end of the cuticular valve a marginal sickle-like line can be observed, which consists of large light-refracting formations (**Fig. 2d**). The tail has a cone-like form. The metric data about this species in our materials as well as those available in the literature are given in **Table 2**.

It is clear from the table that our results correspond with those by Hollo [7] in materials from Hungary. On the other hand, it is visible that our mean values for most structures are either lower or around the lower limits of those pointed by Kontrimavichus et al. [9], and Gasso et al. [5]. An exception of this relation is the gubernaculum – in our measurements its mean size is 26.4 µm and according to Kontrimavichus et al. [9] it is 22 µm. The small size of the gubernaculum makes its observation difficult. Perhaps, this is the reason because of which why gubernaculum has not been established by Gasso et al. [5] in this species.

Table 2. Metric data of *Metastrongylus salmi* by different authors

Structure	Kontrimavichus et al., 1976	Hollo, 1965	Gasso et al., 2014	Our data
Body length ♂ (in mm)	14-17	11-15	-	14.3 ± 0.12 (11.6-18)
Body width in the end of oesophagus ♂	120-160	-	-	98 ± 5 (87-108)
Oesophagus length ♂	396-468	-	-	367 ± 14 (332-408)
Max. oesophagus width ♂	100	-	-	60.7 ± 3 (56-70)
Spicule length	2120-2370	1900 -3460	2120±220 (1600–2400)	2290 ± 80 (2150-2450)
Gubernaculum length	22	-	No gubernaculum	26.4±3.1 (23-29)
Body length ♀ (in mm)	39-40	25-39	-	29.5 ± 0.26 (25.2-42.8)
Body width in the end of oesophagus ♀	-	-	-	136 ± 19 (90-179)
Oesophagus length ♀	594-638	-	-	454 ± 4.6 (407-486)
Max. oesophagus width ♀	-	-	-	77.8 ± 6.8 (64-98)
Distance vulva- tail tip	-	-	-	90 ± 5.8 (78-105)
Distance anus-tail tip	-	71.3-108.5	71 ± 9.59 (62.5–92.5)	71 ± 4.4 (54-80)
Length of prevulvar cuticular valve	-	-	102.92±17.78 (75–125)	72.7 ± 6.8 (60-93)
Width of prevulvar cuticular valve	-	-	58.5 ± 10.35 (50-82.5)	61 ± 5.6 (47-78)
Egg length	82	46.5-52.7	51.75 (47.5 -60)	48.9± 2 (46-58)
Egg width	42	31-37.2	36.26 (35-37.5)	31.1± 2 (28-36)

Metastrongylus elongatus Gmelin, 1790

Male. We found only one male specimen. The bursa copulatrix is small with hardly distinguishable parts (**Fig. 3a**). The distal parts of the spicules were cut, the length of the rest ones was 4.4 mm. There is no gubernaculum.

Female. The prevulvar cuticular valve is oval. The vagina goes parallel to the body, its distal part in the valve makes a light S-form curve and is opened in the vulva near the anus (**Fig. 3b**). The metric data about this species in our materials as well as those available in the literature are given in **Table 3**. The sizes of our specimens are lower or around the lower limit of those pointed by Kontrimavichus et al. [9]. Our metric data are within the limits given by Hollo [7], and Gasso et al. [5] with one exception: the width of eggs that is smaller in our specimens. The specimens measured in present study are smaller than those from other populations of the species from Bulgaria [11].

Table 3. Metric data of *Metastrongylus elongatus* by different authors

Structure	Kontrimavichus et al., 1976	Hollo, 1965	Mutafova, 2005	Gasso et al., 2014	Our data
Body length ♂ (in mm)	14 - 19	11-20	16.4 (14-18)	-	11
Body width in the end of oesophagus ♂	104 - 160	-	148 (117-169)	-	86
Oesophagus length ♂	414 - 504	-	490 (465-504)	-	415
Max. oesophagus width ♂	-	-	-	-	62
Spicule length	3870 - 5530	3710-5360	3600-3800	4200 ± 210 (3900-4500)	> 4400
Body length ♀ (in mm)	28 - 48.5	23-48	37.38 (29-43)	-	33.1 ± 0. (29.2-36.2)
Body width in the end of oesophagus ♀	-	-	-	-	163 ± 16 (131-180)
Oesophagus length ♀	576 - 774	-	593 (465-625)	-	510.6 ± 44 (431-581)
Max. oesophagus width ♀	-	-	-	-	82.6 ± 8.4 (71-99)
Distance vulva-tail tip	-	-	-	-	87.2 ± 20.8 (60-109)
Distance anus-tail tip	-	75-117	-	71.75 ± 9.59 (57.5 - 87.5)	74.5 ± 5.5 (68-82)
Length of prevulvar cuticular valve	-	-	-	100.25±24.09 (77.5 - 160)	93.7 ± 16 (76-119)
Width of prevulvar cuticular valve	-	-	-	50 ± 11.67 (35 - 70)	63 ± 4.7 (54-69)
Egg length	40	46,5-52.7	-	53 ± 2.84 (47.5-57.5)	47.5± 1.1 (46- 49)
Egg width	32-44	34.1-43.4	-	36.5 ± 1.75 (35-40)	29.5±1.8 (27-32)

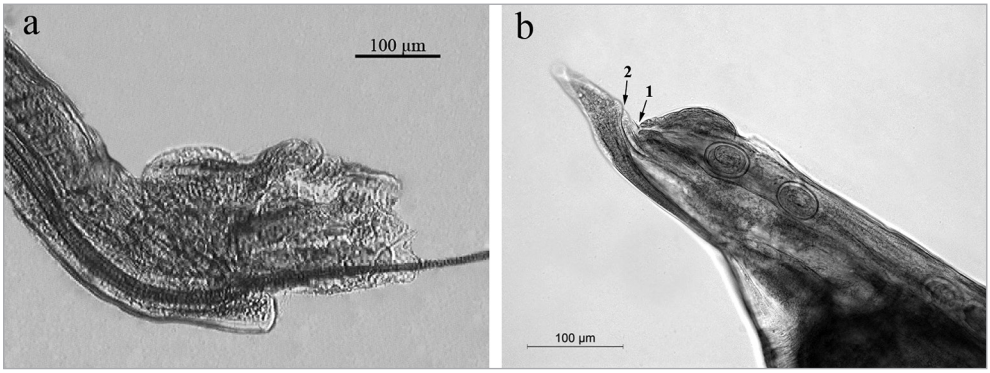

Fig. 3. Morphologic features of *Metastrongylus elongatus* in materials from wild boars from Bulgaria. a) bursa copulatrix; b) posterior end of female: 1 - vulva, 2 - anus

Conclusion

As a whole, morphological features of the described in the present work three metastrongylid species correspond to those pointed in previous descriptions by other authors in materials from domestic pigs and wild boars from different parts of the world. Variation regarding the metric characteristic is observed. It could be due to differences in methods of preparation and observation of the helminths as well as to the peculiarities of the separate parasite populations from different parts of the world.

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Morphometric Features of *Oesophagostomum dentatum, O. quadrispinulatum* and *Ascarops strongylina* in Materials from Wild Boars from Bulgaria

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A necropsy of wild boars from three regions of Southwest Bulgaria showed an infection with two oesophagostomid species (*Oesophagostomum dentatum* and *O. quadrispinulatum*) and one gastric spirurid (*Ascarops strongylina*). The collected parasites were used for a morphometric description of the species. The results were compared with the existing ones in the literature. It was established that the morphological features of the species from wild boars were the same as those from domestic pigs given by other authors. Diversity in the metric data was observed. However, metric features specific for the species were within the limits given by other authors.

Key words: Oesophagostomum dentatum, Oesophagostomum quadrispinulatum, Ascarops strongylina, wild boars, morphometric description

Introduction

Pigs are even-toed ungulate animals from the family Suidae. They include the domestic pig and a number of other wild species, distributed all over the world. The European wild boar (*Sus scrofa scrofa* L.) is one of the most important game species in Europe, with populations that are increasing continuously [5]. The species can be found everywhere in Bulgaria and it is important from an ecological point of view. The wild boar is also important for the game-breeding and the hunting reserve. As omnivorous animals wild boars are susceptible to invasion by numerous species of parasites [18]. The most infected organs are lungs (with metastrongylids), stomach (with strongylids, spirurids), small intestines (with ascarids, acanthocephalids), caecum and colon (with trichurids, oesophagostomids), and liver (with trematodes).

Many authors in Bulgaria have studied the helminthofauna of the domestic pig [1, 2, 3, 6, 7, 9, 10, 20]. The studies for the wild boar are less [8, 11, 12, 14, 15]. During our recent studies in this aspect we have established two species of oesophagostomids (*Oesophagostomum dentatum, O. quadrispinulatum*) and one gastric spirurid (*Ascarops strongylina*) parasitizing wild boars. The available literature data on the morphology of these species are based only on materials from domestic pigs (*Sus scrofa domestica* L.) [3, 13, 16, 17, 19]. There is, however, no such data in the literature based on materials derived from wild boars. Therefore, the aim of the present study was to fill up this gap with providing morphometrical data on these three parasites in materials from wild boars spread on the territory of Bulgaria.

Materials and Methods

The internal organs of 6 wild boars that were hunted during the shooting season in 2016 from three regions of Southwest Bulgaria were helminthologically necropsied. The discovered helminths were collected in a saline solution and after cleaning they were stored in 70% ethyl alcohol. Part of the helminths were enlightened in alcohol-glycerol sequence or with lactophenol, so that the taxonomic important features could be easier observed. After that the helminths were included in gelatin-glycerin and the edges of cover glasses were coated with Canadian balm for longer storage. The measurements were made with the help of classical parasitological methods or after shooting of separate structures of parasites with a Web camera Logitech 4000, which was attached to the "Amplival" microscope, and their measuring with the picture analyzing computer program Image-Pro Plus-Version 6. Pictures were taken using a light microscope Leica DM5000 B, supplied with a camera and software (Leica Application Suite LAS v. 3.1). The obtained data were statistically analyzed according to Georgieva et al. [4]. Measurements in the tables are in μm.

Results and Discussion

We found two species of *Oesophagostomum* genus in the large intestines of two wild boars – *O. dentatum* and *O. quadrispinulatum*. In the stomach of one wild boar we found the gastric spirurid *Ascarops strongylina*. Further down data about their morphological and metrical features are supplied.

Oesophagostomum spp. – general features

Oesophagostomids were white colored with a spindle shape. The two species had on their cranial end a vesicular cuticular dilatation. The dilatation begins from the buccal capsule and distally is restricted with a transversal cuticular line (**Fig. 1a**). The buccal capsule is formed around the mouth opening. It is relatively small, wider than longer and has two leaf crowns that consist of sharp lamellae (**Fig. 1a**). The outer crown is bigger and its sharp lamellae are leaf-shaped. The inner crown is poorly developed and has numerous small lamellae. The oesophagus begins after the buccal capsule, in its second half it is dilated and assumes a bulb-like form.

The male specimens have well-developed bursa copulatrix, genital cone, equally long spicules and spade-shaped gubernaculum which is pointed distally. The female oesophagostomids possess well-developed ovejector. The vulva opens in the distal end of the body, before anus.

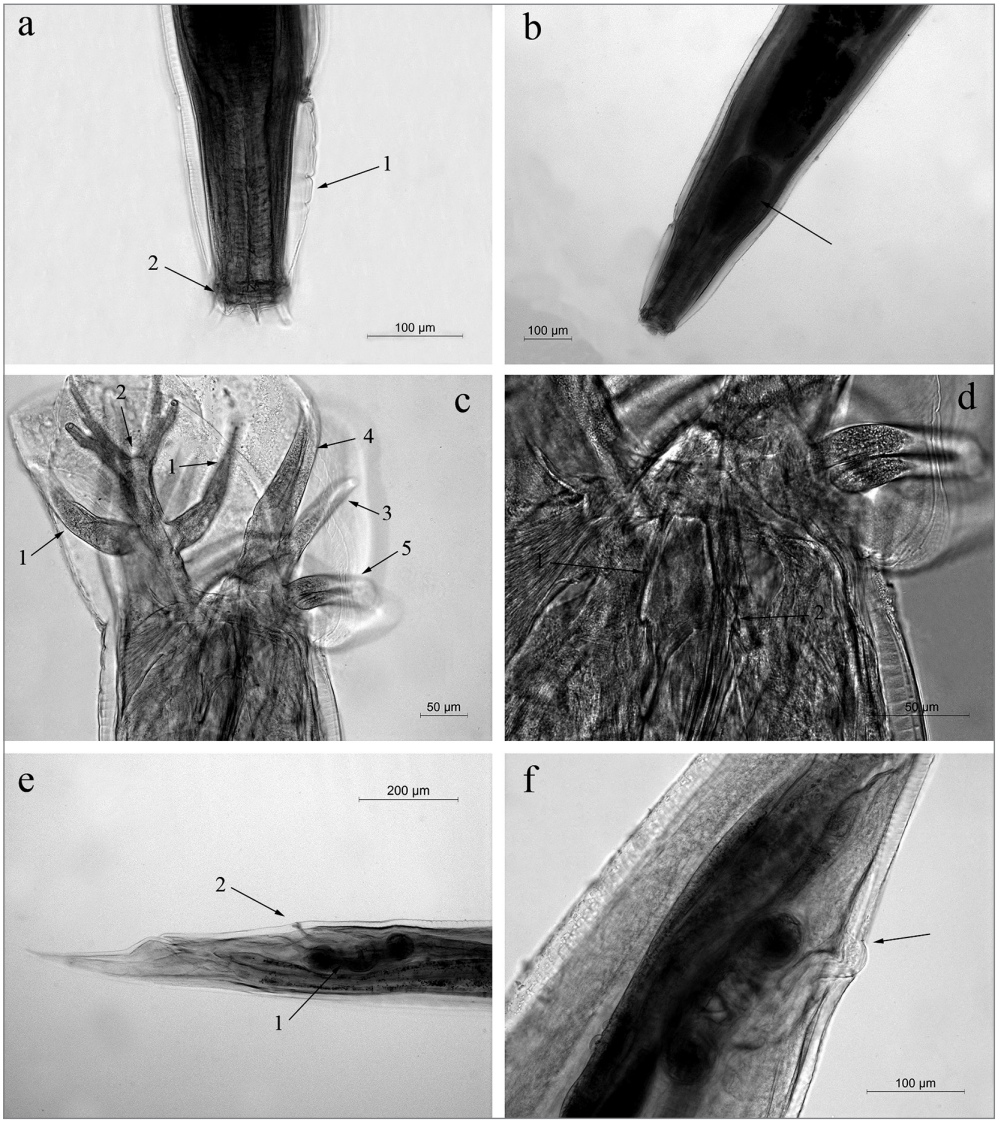

Fig. 1. Morphologic features of *Oesophagostomum dentatum* in materials from wild boars from Bulgaria. a) anterior end: 1- vesicular cuticular dilatation, 2 - buccal capsule; b) oesophagus; c) bursa copulatrix: 1 - exterodorsal rays, 2 - dorsal ray, 3 - anterolateral ray, 4 - medio- and posterolateral rays, 5 - ventral rays; d) posterior end of male: 1 - gubernaculum, 2 - distal spicule end; e) Posterior end of female: 1- ovijector, 2 - vulva; f) opening of the vulva

O. dentatum (Rudolphi, 1803), Molin, 186

The buccal capsule has a cylindrical form (**Fig. 1a**). The first half of the oesophagus has the same width and the second half is widened like a bulb (**Fig. 1b**). **Male.** The bursa copulatrix is massive with three well-developed parts (**Fig. 1c**). The dorsal ray and exterodorsal rays come from one wide trunk. First from the trunk are separated the exterodorsal rays. They do not reach the bursal edge. The trunk continues in the dorsal

Table 1. Metric data on *O. dentatum* by different authors

Structure	Ozerskaya 1930	Georgiev & Kamburov, 1977	Poelvoorde (1978)	Our data
Body length ♂ (in mm)	8.829	-	7.7 – 8.74	9.46± 0.28 (8.5-10)
Body width in the end of oesophagus ♂	-	-	-	218 ± 14 (184-255)
Oesophagus length ♂	-	-	-	397 ± 10.4 (364-428)
Max. oesophagus width ♂	-	-	-	110 ± 7,6 (81-128)
Spicule length	896-937	1030-1205	1004-1043	1135 ± 30 (1070-1250)
Gubernaculum length	101-122	92-138	-	127.7 ± 6.6 (111-148)
Body length ♀ (in mm)	7.5-13.4	-	8.72 - 10.83	11.5 ± 0.6 (9-12.4)
Body width in the end of oesophagus ♀	-	-	-	233.5 ± 14 (182-260)
Oesophagus length ♀	-	-	-	426.6 ± 10.2 (406-451)
Max. oesophagus width ♀	-	-	-	115.7 ± 5.8 (101-126)
Vulva - anus	315-366	265-393	-	347 ± 15.8 (280-370)
Anus - tail tip	255-265	241-324	313	293 ± 7.4 (270-310)

ray, which later is divided into two branches. At a half of their length those branches are also divided into one shorter lateral branch and one longer medial branch. Only the medial ones reach the bursal edge. The lateral rays also come out from one trunk. The anterolateral ray is the first separated from the trunk and it is the shortest from the lateral rays. The other two lateral rays are differentiated a little after that and stay adhering to each other until their end. The mediolateral ray is a little shorter than the posterolateral one. The ventral rays have the same length and also begin from one trunk. These rays differentiated early but stay stuck together for all of their length. The spicules are equal in length. Their proximal end is round and thickened and distal end is sharped (**Fig. 1d**). The gubernaculum looks like a spade directed distally (**Fig. 1d**). **Female.** The tail has a form of prolonged cone (**Fig. 1e**). The ovejector is chitinized and composed of two round parts and a channel system, which passes into the vulva (**Fig. 1e, f**). The metric data for this species in our materials together with the measurements from other authors that were available in the literature are shown in **Table 1**.

When we have compared our data from those by Ozerskaya [16] and Poelvoorde [17] who have used materials from domestic pigs, it can be seen that our male specimens as a whole are bigger. The mean values for the body length, spicules and gubernaculum exceed the maximal values pointed by those authors. The same is assigned to the distance between the anus and tail tip in females, measured by Ozerskaya [16]. However, Poelvoorde [17] has measured a longer distance between the anus and tail tip

than us. Our metric data for this species are completely overlapped with those pointed by Georgiev and Kamburov [3] in materials from domestic pigs from four regions of Bulgaria, including the Sofia region.

O. quadrispinulatum Marcone, 1901

The buccal capsule (**Fig. 2a**) has a form of a truncated cone with the basis directed at the oesophagus. The oesophagus (**Fig. 2a**) has two dilatations: one small round widening at the beginning and a bulbous widening in its second half. The oesophagus canal is surrounded by a row of round light-refracting cells.

Male. The bursa copulatrix, spicules and gubernaculums have the same morphology as *O. dentatum* (**Fig. 2 b, c**). The genital cone is well-developed and there are some papillae on its surface (**Fig. 2c**). **Female.** The tail is thin and styliform. The ovejector is similar to this of *O. dentatum* (**Fig. 2d**). Our metric data and the available literature data on this species are shown in **Table 2**.

In Bulgaria *O. quadrispinulatum* has been reported by Georgiev and Kamburov [3]. They have found it in domestic pigs from 4 regions, including the Sofia region, where our materials from wild boars originated too. The metric data from those authors almost entirely corresponds with our data. There is a lack of correspondence only about the length of females. It is about 1 mm higher than that from our materials. Our data also

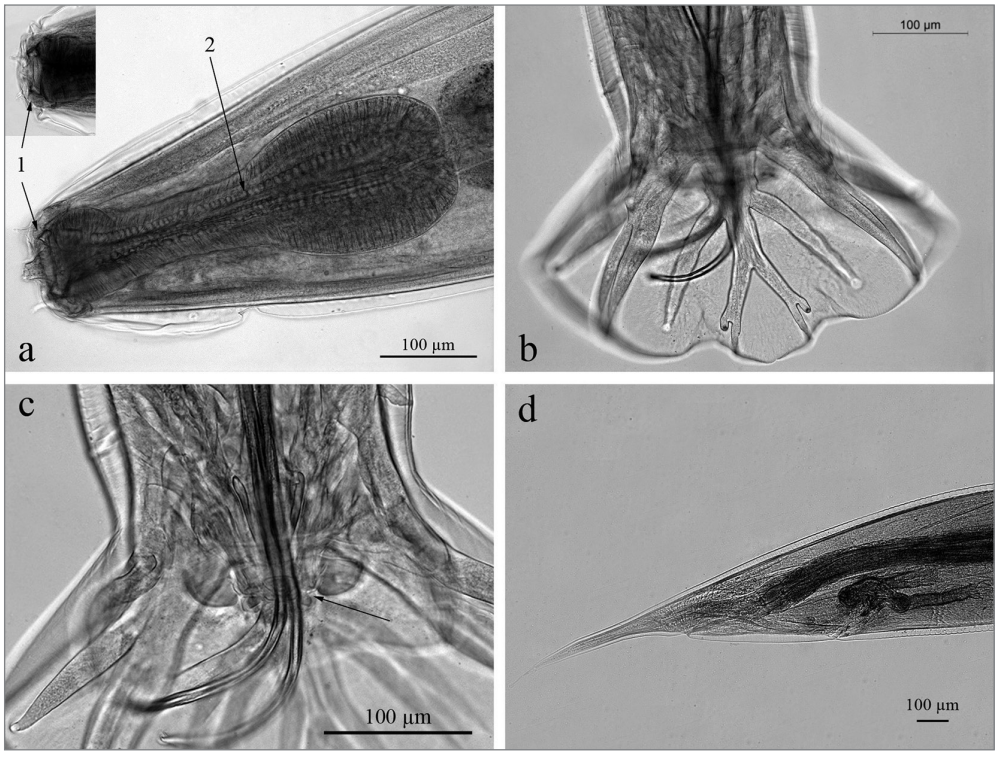

Fig. 2. Morphologic features of *Oesophagostomum quadrispinulatum* in materials from wild boars from Bulgaria. a) Anterior end: 1- buccal capsule, 2 - oesophagus; b) posterior end of male; c) genital cone; d) posterior end of female

Table 2. Metric data on *O. quadrispinulatum* by different authors

Structure	Mapelstone, 1930	Georgiev & Kamburov, 1977	Our data
Body length ♂ (in mm)	6.56-8.85	7.8-8.8	8.13 ± 0.53 (6.8-9)
Body width in the end of oesophagus ♂	–	–	217 ± 15.7 (180-243)
Oesophagus length ♂	–	380-420	402 ± 13 (371-420)
Max. oesophagus width ♂	–	–	115 ± 7.7 (105-125)
Spicule length	780-880	765-955	843 ± 63 (760-990)
Gubernaculum length	104-120	85-128	105.6 ± 5.6 (93-115)
Body length ♀ (in mm)	8.33-10.36	9.6 - 12.1	9.93 ± 0.62 (8-11.1)
Body width in the end of oesophagus ♀	–	–	266.8 ± 24.8 (210-330)
Oesophagus length ♀	–	426 - 462	439.8 ± 8 (416-456)
Max. oesophagus width ♀	–	–	133 ± 5.6 (118-156)
Vulva – anus	380-520	400 - 552	445.4 ± 19.2 (400-500)
Anus – tail tip	360-370	458 - 588	530.1 ± 22 (460-600)

correspond to those pointed by Mapelstone [13] in materials from domestic swine from Bengal. There is only one exception concerning the distance between the anus and tail tip in females, which is longer in our specimens.

The two oesophagostomid species are distinguished by some metric and morphological features. *O. dentatum* has a longer body than *O. quadrispinulatum*. The spicules of *O. dentatum* are longer too. The proportion between the thin and leaf-shaped parts of the gubernaculum in *O. dentatum* is 1:1. In *O. quadrispinulatum* this ratio is 1:2. *O. quadrispinulatum* specimens have longer distances between the vulva and anus and between the anus and tail tip than these of *O. dentatum*. The oesophagus of *O. dentatum* usually has not light-refracting structures or these structures can be found only in its beginning. The oesophagus of *O. quadrispinulatum* has a small, but clearly visible second dilatation right after the buccal capsule.

Ascarops strongylina Rudolphi, 1819

This is a brown nematode with fusiform form. It has one narrow lateral cuticular wing (**Fig. 3a**). From the mouth opening begins the pharynx, which is consisted of spiral rings (**Fig. 3b**). The oesophagus has two parts – the anterior part is muscular and short and posterior is longer and glandular.

Male. There is no bursa copulatrix. The posterior end is curved ventrally. Two asymmetric, transversally furrowed wings are visible on it, as one is wider than another (**Fig. 3c, d**). Around the cloaca are several pairs of genital papillae. The spicules are different in length and form. The one spicule is long and thin. Its distal end is lancet-shaped (**Fig. 4a**).

The second spicule is shorter and wider, its proximal end is thickened and distal end looks like an arrow-head (**Fig. 4b, c**). The gubernaculum is situated around the cloaca and looks like an elongated lamella (**Fig. 4d**). **Female.** The vagina is tubular and

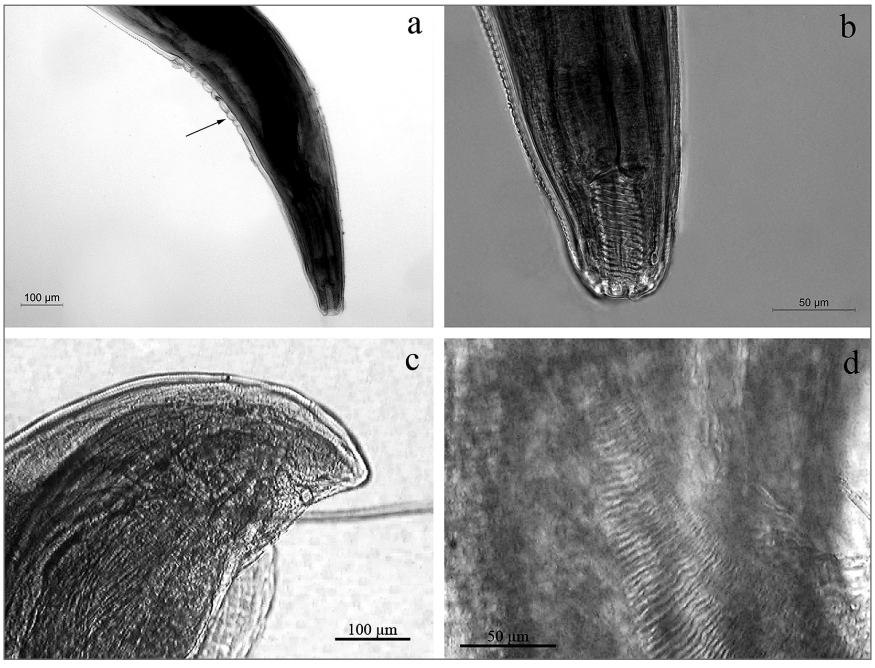

Fig. 3. Morphologic features of *Ascarops strongylina* in materials from wild boars from Bulgaria. a) anterior end – lateral cuticular wing; b) anterior end – pharynx; c) posterior end of male; d) cuticular wing

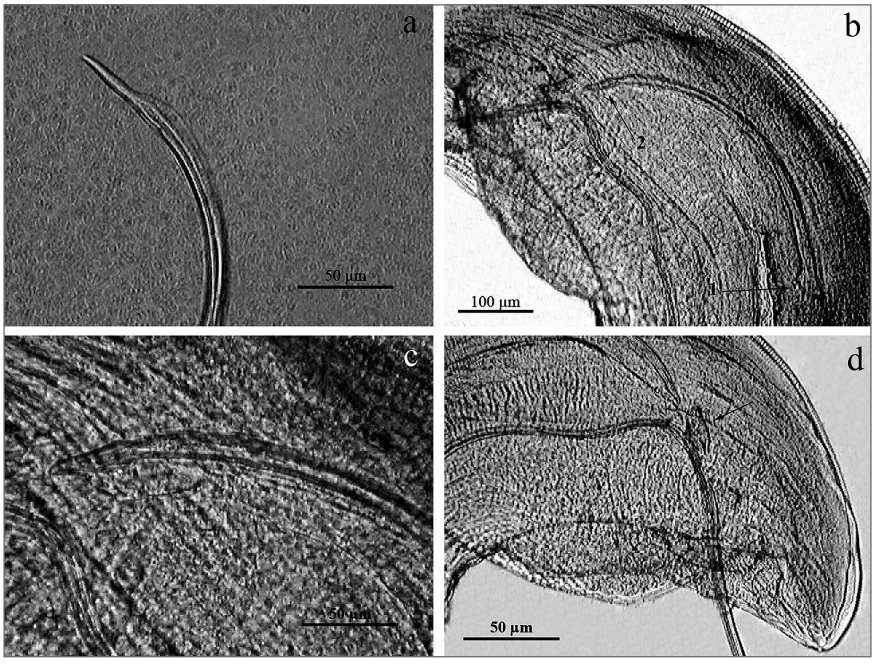

Fig. 4. Morphologic features of *Ascarops strongylina* in materials from wild boars from Bulgaria. a) distal end of big spicule; b) small spicule: 1 – proximal end, 2 – distal end; c) distal end of small spicule; d) gubernaculum

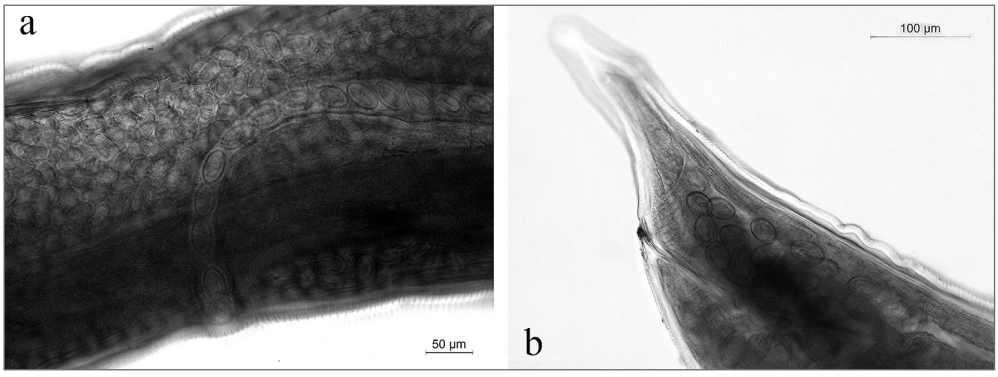

Fig. 5. Morphologic features of *Ascarops strongylina* in materials from wild boars from Bulgaria. a) vagina and vulva; b) posterior end of female

Table 3. Metric data on *Ascarops strongylina* by different authors

Structure	Shmytova, 1963	Our data
Body length ♂ (in mm)	10.1-14.9	12
Body width in the end of oesophagus ♂	270 - 360	330
Anterior end - beginning of lateral wing ♂	210 - 290	211
Length of the pharynx ♂	80-100	86
Width of the pharynx ♂	30	32
Number of pharynx's spiral rings in ♂	14-18	17
Length of muscular oesophagus ♂	300-400	350
Length of glandular oesophagus ♂	2340-3700	3200
Oesophagus width ♂	-	119
Small spicule length	500-600	575
Big spicule length	2300-2900	2100
Gubernaculum length	66-80	55
Gubernaculum width	50-69	38
Width of the bigger caudal wing ♂	200-230	290
Cloaca – tail tip	-	253
Body length ♀ (in mm)	12.4-23.7	16.52 ± 2.76 (14-20)
Body width in the end of oesophagus ♀	230 - 430	357.8 ± 37.9 (300-382)
Anterior end - beginning of lateral wing ♀	230 - 340	340 ± 67 (288-420)
Length of the pharynx ♀	90-105	83 ± 8.2 (74-92)
Width of the pharynx ♀	30-40	31 ± 1.8 (29-33)
Number of pharynx's spiral rings in ♀	14-18	15-16
Length of muscular oesophagus ♀	290-540	359 ± 12.1 (345-370)
Length of glandular oesophagus ♀	2730-3800	3360 ± 400 (2900-3800)
Oesophagus width ♀	-	124 ± 13.6 (108-137)
Anterior length - vulva	6800-1110	8120 ± 1340 (6800-9400)
Anus - tail tip	230-300	232 ± 5.9 (219-240)

situated in the middle of the body. At first it goes parallel to the body, afterwards turns perpendicular and opens in the vulva (**Fig. 5a**). The eggs in the vagina are embryonated. The body's caudal end is straight and the tail is short with a rounded tip (**Fig. 5b**).

We compared our metric data for *A. strongylina* with those from description by Shmytova [19]. Comparative data are shown in **Table 3**.

Our results about most of the measurements correspond with hers. There are few differences in the females: the lateral cuticular wing of specimens from our materials starts backwards, the pharynx is shorter and its width is in the lower limits pointed by Shmytova [19]. More important differences are observed in the male sexual structures. Our male specimen has shorter big spicule and gubernaculum, whereas its bigger cuticular wing on the caudal end is wider.

Conclusion

The morphological features of the established by us species *O. dentatum, O. quadrispinulatum* and *A. strongylina* in wild boars from Bulgaria completely correspond to those described by other authors in materials from domestic pigs. Diversity in the metric features is observed, which could be due to differences in methods of preparation and observation of the helminths as well as to the peculiarities of the separate parasite populations from different parts of the world. The measurements about taxonomic important features, however, are in the limits, which have been pointed by other authors.

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Primary Apocrine Carcinoma of the Skin – a Case Report

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Sweat gland skin carcinomas are extremely rare. The differentiation between apocrine and eccrine neoplasms is very difficult, since composite adnexal tumors may often be encountered. We present a case of 72-year-old male with a slow-growing painless erythematous-livid nodule on the frontal scalp. According to the medical record, the lesion evolved on the site of a pre-existing cylindroma, excised 2 years before. Diagnostic punch biopsy showed solid apocrine carcinoma. Wide excision with en-bloc lymph node dissection was recommended. A dose of alertness, together with a thorough review of the clinical, histology and immuno-histochemical features of this very rare skin adnexal tumor is, herein, given.

Key words: sweat gland tumors, apocrine carcinoma, histogenesis

Introduction

Cutaneous apocrine gland carcinoma is a rare form of sweat gland neoplasm with distinct cytological appearance. No more than 100 cases have been described worldwide. Most common localization is axilla, followed by scalp, nipple, trunk, anogenital region, wrist, fingertip, foot, toe, lip, ear, chest and eyelid [14]. Usually, the cells show abundant eosinophilic cytoplasm and eccentric, basally located nuclei. Luminal cells decapitation secretion is the outstanding histological feature. Most tumors present periodic acid Schiff (PAS) positivity, and iron pigment. The luminal cells are reactive to low molecular weight keratin and androgenic receptor and do not express oestrogen receptors [6]. Those characteristics facilitate the proper assessment of suspicious lesions.

Case report

A 72-year-old man presented with a slow-growing soft painless erythematous-livid nodule with a diameter of 3 cm, located on the frontal scalp. The tumor mass was fixed to the skin and slightly ulcerative on the lower aspect. No palpable regional lymph nodes were identified. Other physical examination did not show any abnormalities. According

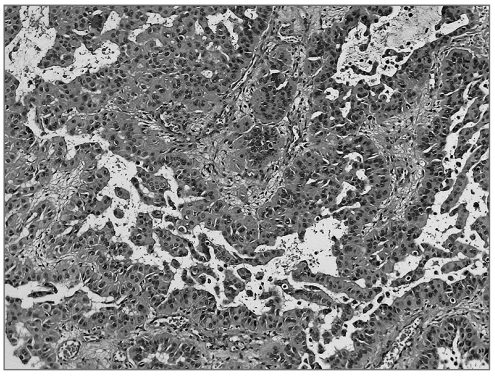

Fig. 1. Subcutaneous infiltrative tumor represented by un-uniform nests of tubular structures, covered by atypical cells with eosinophilic cytoplasm and central decapitation secretion (HE, × 100)

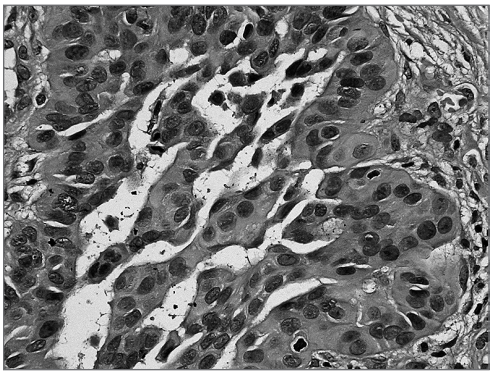

Fig. 2. Hyperchromic nuclei and frequent mitotic figures (HE, × 400)

to the medical record, this lesion evolved on the site of a pre-existing tumor, histologically verified as cylindroma, excised 2 years before. Unfortunately, no skin specimen was available for revision. Diagnostic punch biopsy was performed. Histological analysis showed invasive tumor, located on the border of the reticular dermis and subcutis, forming various-shaped nests of tubular structures, some of which were related to the follicular infundibulum (**Fig. 1**). The cells had eosinophilic cytoplasms and showed central apocrine secretion. Hyperchromic nuclei with frequent mitotic figures were also evident (**Fig. 2**). The immunohistochemical analysis showed positivity for cytokeratin 7 (70%) and cytokeratin 20 (30%). No expression of oestrogen receptor was found. The diagnosis of primary skin apocrine gland carcinoma was concluded. Wide excision with en-bloc lymph node dissection was recommended.

Discussion

A malignant counterpart of most benign sweat gland lesions have been described. Although very rare, those carcinomas deserve special attention because of their high recurrence rate and metastatic potential [1]. Features, suggestive for neoplastic degeneration, include architectural asymmetry, infiltrative borders, irregular arrangement of the nests, nuclear atypia, and increased mitotic activity. The presented case showed clear-cut cytological atypia and architectural dis-cohesiveness [11]. The striking feature, which gives the ground for concluding an apocrine origin, was the typical decapitation secretion. This finding was extremely interesting and did not correspond to the identification of pre-existing cylindroma.

Tumors of pure apocrine origin appear to be much less common than those of eccrine differentiation [4]. The very few cases described to date showed male predominance and old age of onset (average of 57.9 years). The tumors cannot be differentiated by virtue of localization. Moreover, many sweat gland tumors traditionally assumed to have an eccrine origin are now recognized to have apocrine analogues [10]. These examples encompass hidrocystoma, poroma, cylindroma, spiradenoma and chondroid syringoma.

Cylindromas usually affect adult females. Most common localization is head and neck, particularly scalp and face. They can be sporadic and present as a solitary lesion,

or represent multiple coalescing mass defined as turban tumor [12]. Histologically, cylindroma is easily recognized by non-encapsulated variable-sized islands and nodules of epithelial cells with peripheral hyalinised material, arranged in a "jigsaw puzzle". Some tumors may resemble spiradenoma, suggesting a continuous morphological spectrum of a single entity, or a transitional from one lesion to the other.

No apocrine secretion has been described in cylindromas and their malignant counterparts. Therefore, we may speculate that our case may have been an unrecognized apocrine gland carcinoma a priori. On the other hand, according to the histogenetic theory, multiple loss mutations of chromosome 16 q 12-13, essentially important for development of various adnexal skin tumors, may lead to stem cell degeneration towards apocrine gland neoplasm [3, 4, 7]. Apart from this hypothesis, a possible association of apocrine carcinoma and benign sweat gland tumors may also arise [5, 8]. Such co-morbidity seems to be not so rare, having in mind 5 cases of Japanese patients, Ogata et. al. [9] reported to have axillar apocrine carcinoma in association with apocrine hyperplasia and apocrine adenoma, and two cases of perianal apocrine tubulo-papillary hidradenoma and ductal carcinoma [8].

Standard treatment options for cutaneous apocrine carcinoma include wide excision with 2-3 cm margins [13]. Sentinel lymph node dissection prove to be beneficial, as almost 90% of cases return with recurrence in less than 5 years. Adjuvant radiotherapy is not recommended with the exception of unoperable cases.

The prognosis depends on tumor size, degree of histological differentiation, vascular invasion. Various literature sources pointed out a 10-year-recurrence rate up to 50% and fatal outcome between 20-40% [2, 8, 9, 13].

Conclusions

Apocrine carcinoma of the skin is a rare adnexal neoplasm, presenting as tender slow-growing dermal or subcutaneous nodule, located on sites, rich in apocrine glands. Histogenesis remains controversial. Sometimes stereotypical presentation may hardly be differentiated by other types of sweat gland tumors and primary breast or gastrointestinal adenocarcinomas. Due to its aggressive course and high recurrent rate, cutaneous apocrine carcinoma represents a diagnostic challenge and extreme therapeutic provocation.

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HIV-Associated Sarcoma Kaposi in an Athlete

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We present a case of a 26-year-old homosexual male, an athlete, diagnosed with a HIV-associated Kaposi's sarcoma. We want to point that HIV is worldwide infection, also seen in Bulgaria and to stress that Kaposi's sarcoma is one of the leading sigh of AIDS.

Key words: Kaposi's sarcoma, HIV- infection

Introduction

The acquired immunodeficiency syndrome (AIDS) affects vast groups of individuals around the world. AIDS is a disease of the human immune system, caused by the human immunodeficiency virus (HIV). The illness decreases the effectiveness of the immune system, which leads to opportunistic infections. People with AIDS are also in higher risk of developing different types of cancer, such as Kaposi's sarcoma and lymphomas. Other symptoms of the disease include fever, sweating, swollen glands, fatigue and weight loss [4, 5, 7].

AIDS has been discovered as an illness for the first time in 1981 in New York. AIDS is one of the most dangerous viral diseases known to humankind. Nowadays, it is the leading cause of death for active-age individuals. Over 33 million people around the world carry the HIV virus; every 12 seconds a person gets infected, and every 16 seconds someone dies from AIDS. In Bulgaria, the number of people carrying the virus is 4 thousand, 3 thousand of which live unaware of the fact. Half of the infected in the country are under 30 years old [10].

Kaposi described in 1872 a case as a malignant multifocal neoplastic process, arising from the endothelium of the vascular and the lymph vessels [1, 3, 4]. In about 30% of the patients suffering from AIDS, Kaposi sarcoma is an initial dermal manifestation of the disease. In these cases, the dermal lesions are different from the classic Kaposi sarcoma type. They are smaller in size, localized mainly in the upper part of the torso and the oral mucosa, and the progress is faster. Internal organs are affected in 75% of the cases [1, 2, 3]

Stages of the HIV infection: The *first stage*, or the primary HIV infection, also known as an "acute retroviral syndrome", develops in the first few weeks after con-

tracting the HIV virus. It is characterised by symptoms similar to those of the flu or the infectious mononucleosis that often go away after a couple of weeks [2]. The *second stage* is known as the "clinical latency" one, when the HIV virus progressively destroys its CD4 T-lymphocytes, while the patient shows no symptoms. This condition can remain for an average period of 8 to 10 years [1]. The *third stage* occurs when the immune system is so badly damaged, that it starts developing opportunistic infections. This exact stage is called AIDS. "A person with AIDS" refers to an individual infected with the HIV virus, whose blood contains less than 200 CD4 T-lymphocytes per microliter, and who suffers from one or more of 26 given conditions. For people with AIDS the opportunistic infections are often extremely grave and life-threatening [10]. The worldwide spread of the HIV pandemic is taking alarming proportions. The number of people living with HIV around the globe is constantly growing, as well as the death rate among them. The lack of a cure for the disease is beneficial for its spread. There is a need of serious prevention measures to control the epidemy.

Transmission of the HIV virus: HIV is transmitted through sexual contact, parenteral transfusion of blood and blood components, contamination of wounds or the mucous with infected blood or from a mother with HIV to her new-born [6, 8, 9].

Case report

We present a case of a 26-year-old homosexual male, who is an active athlete. The patient has visited a training camp in the Netherlands in 2013, where he had a couple of sexual encounters without using prevention methods. He was diagnosed as HIV(+) in 2014 in Sofia, with initial symptoms of swollen inguinal lymph nodes. He lost 8 kilograms for 6 months. Pathologic changes in the derma occurred with livid erythematous macules and plaques on the hard palate mucous membrane.

No abnormal digressions in routine paraclinical test results have been observed. Reported lower levels of CD4 + 133kn/µl. Serological detection of syphilis scored negative. The histopathological examination of a biopsy sample from the hard palate revealed vascular formations with predominance of endothelial cells and spindle-shaped cell formations with vascular slits, vasodilatation with extravasates and inflammatory perivasal dermal infiltrate- Sarcoma Kaposi (**Fig. 1** and **Fig. 2**).

The patient is a male of apparent age higher than the actual, asthenic habitus, pale skin and mucous. He has a rhythmic cardiac activity, AP – 110/70, cardiac frequency – 83 beats/minute, clean vesical breathing, painless abdominal palpation and no swelling of the joints. Body weight – 55kg. BMI -15.

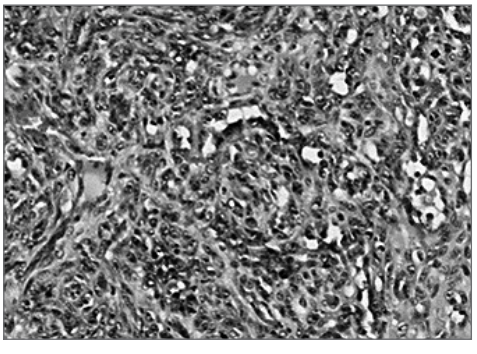

Fig. 1. Vascular formations with predominance of endothelial cells and spindle-shaped cell formations with vascular slits (HE, × 200)

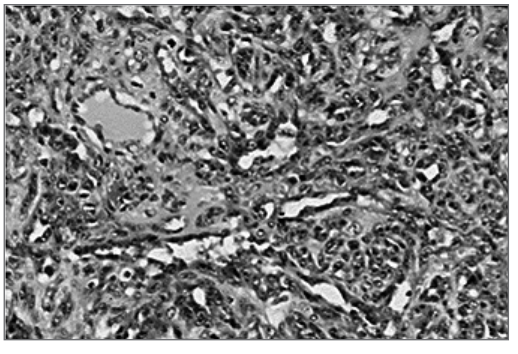

Fig. 2. Vasodilatation with extravasates and inflammatory perivasal dermal infiltrate (HE, × 200)

Discussion

International Health Organisation Criteria for clinically-manifested HIV infections in adults:

1. Major feature: Sarcoma Kaposi; Candidiasis; Pneumonia (Pneumocystis carinii); Retinitis (Cytomegalovirus); Encephalitis;
2. Characteristic features: Hair Leucoplakia; Herpes zoster; B-cell lymphoma; Tuberculosis;
3. Disease-related features: Genital ulcers; Loss of weight; Lymphonodulomegaly; Diarrhea; Cough.

According to the criteria of the International Health Organisation for clinically manifested HIV infections in adults, there were two major features present in our patient - Kaposi sarcoma and candidiasis. Some of other HIV-related characteristics include B-cell lymphoma; while three disease-related symptoms are manifested by genital ulcers, loss of weight and lymphadenomegaly.

It is agreed that a person has a clinically-manifested HIV infection if clinical testing detects:

1. At least one major feature;
2. At least two characteristic features;
3. One characteristic and at least two related features;
4. Two related features along with a HIV positive test.

Our patient has been diagnosed with one major feature - Sarcoma Kaposi and two disease-related features (loss of weight and lymphonodulomegaly).

Diagnosis: On the basis of data from the history, clinical presentation and laboratory findings, it was concluded that the patient had HIV-associated Kaposi sarcoma.

In conclusion, the patient we presented has a HIV-associated Kaposi sarcoma.

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Serum IgG Antibodies to GM1 and GD1a Gangliosides in a Patient with Relapsing-Remitting Multiple Sclerosis under Treatment with Glatiramer Acetate. A 15-year Longitudinal Study

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Multiple sclerosis (MS) is a coplex and heterogeneous, most likely autoimmune, demyelinating disease of the central nervous system (CNS). The IgG antibodies can serve as biomarkers indicating nervous system chronic dysfunction. Titers of the serum IgG anti-GM1 antibodies are associated as potential biomarkers with the diagnosis of demyelination whilst the serum IgG anti-GD1a antibodies are associated with neurodegeneration and acute motor axonal neuropathy. This study presents the case of a patient with 20 years relapsing – remitting MS (RRMS) who is under treatment with the immunomodulator Glatiramer acetate (GA) for 15 years. During these years the patient has had pregnancy, child-birth, post-partum and long periods of remission. Hormones produced during pregnancy, could reverse some of the neurological damages, associated with MS. Our long-term study showed that the patient responds very well to treatment with GA. She has a family, a child, career and chance for a normal life. Our immunological methods demonstrated lack of demyelination and evident neuroprotection.

Key words: relapsing-remitting multiple sclerosis (RRMS), glatiramer acetate (GA), serum IgG anti-GD1a and anti-GM1 antibodies, ELISA

Introduction

Recently it has become clear that multiple sclerosis (MS) is a common immune-mediated neurodegenerative disease of the central nervous system (CNS) [3]. Neurodegeneration develops in association with inflammation and demyelination [18]. The multifocal nature of the disease is characterized by heterogenous genetic background and immunopathogenetic subtypes, two clinical disease courses - attack and progredient, functional damages (sensorimotor, cerebellar, visual, cognitive, neuropsychiatric), and

un-predictable therapeutic effects. Therefore, IgG anti-GM1 and anti-GD1a antibodies can serve as biomarkers, suggesting nervous system dysfunction [2].

During the last decade enormous efforts have been made to discover biological markers of neuronal damage, capable to predict the disease course and effective response to therapy [4]. Currently the disease prognosis is based on clinical information (relapse rate and disability scales) and diagnostic tests (brain MRI or the presence of oligoclonal bands in the cerebrospinal fluid). However, the ability of neurologists to make an accurate prognosis is very limited, based on such information, a situation perceived by patients as one of their biggest concerns [17].

Gangliosides are a family of acidic glycosphingolipids highly concentrated in the nervous system where they represent about 10% of the total lipid content. These molecules are found mainly in the neurons, but also occur in smaller concentrations in other cell types [8]. The ganglioside spectra of normal blood plasma are remarkably stable, but show pronounced changes in pathological conditions [12]. The main gangliosides in the human central nervous system myelin are monosialogangliosides GM1 [16]. GD1a is one of the major CNS neuronal ganglioside fractions. In our previous studies, a considerable increase of serum GD1a ganglioside was determined in MS - neurodegenerative multifactor disorder with an autoimmune component [11]. The finding of anti-ganglioside antibodies in inflammatory demyelination in the CNS may identify avenues for research into pathogenesis.

Autoantibodies against GD1a gangliosides are associated with acute motor axonal and acute motor-sensory axonal neuropathy [15]. Antibodies to gangliosides have been detected in the sera of MS patients, due to damage of the blood-brain barrier (BBB). High titers of IgG anti-GM1 antibodies were associated with demyelination, whereas high titers of IgG anti-GD1a antibodies with neurodegeneration, respectively [12].

In the current investigation the clinical significance of serum IgG anti-GM1 and anti-GD1a antibodies, estimated by enzyme-linked immunosorbent assay (ELISA) in patient sera, is presented. The patient is a 42 years old woman with relapsing-remitting multiple sclerosis (RRMS). She has been treated with corticosteroids and interferons since the onset of the disease. The patient is under influence of various medications for a total of 20 years. For 15 years she has been under treatment with immunomodulator glatiramer acetate (GA).

Glatiramer acetate (licensed in 1996) is a random chain (polymer, synthetic tetrapeptide) of amino acids - Glutamic acid, Lysine, Alanine and Tyrosine (hence GLATiramer). It is synthesized in solution from these amino acids in a ratio of approximately 5 parts Alanine to 3 parts of Lysine, 1.5 of Glutamic acid and 1 - of Tyrosine, using N-carboxyamino acid anhydride. The substance was originally designed to mimic a protein in myelin, called myelin basic protein (MBP), with the intention of inducing experimental autoimmune encephalomyelitis (EAE - an animal model of MS). Quite to the contrary it was found to suppress the disease and as a result it came to be tested in human MS. For this reason it was originally believed to act as a decoy by drawing the immune system's attack away from the myelin. Nowadays, the researchers are no longer at all sure how it works. There is some evidence that it converts the body's immune response from type Th1 to Th2, promotes suppressor T-cells or acts as an altered peptide ligand. GA is self-administered by daily sub-cutaneous injections. The recommended dosage is 20 mg/day administered subcutaneously. The sites for injection include the arms, abdomen, hips, and thighs [10].

Although the clinical definition of MS requires two or more episodes of symptoms and signs, GA is approved for treatment after single episodes. It is also used to treat RRMS. The most common side effects of GA are redness, pain, swelling, itching, or a lump at the site of injection, flushing, rash, shortness of breath, and chest pain. These

reactions are usually mild and seldom require professional treatment. A permanent indentation under the skin at the injection site may occur due to a local destruction of fat tissue. The treatment and management of MS should be targeted toward relieving symptoms of the disease, treating acute exacerbations, shortening the duration of an acute relapse, reduction of relapses frequency, and prevention of disease progression. Drugs approved for use in MS that reduce the frequency of exacerbations or slow disability progression, are referred to as disease-modifying drugs (DMDs). These DMDs can be further classified as immunomodulating (or receptor-modulating), or immunosuppressives [10,13]. Reports which support the role of T-regulatory cells but not in an exclusive fashion in the therapeutic effect of GA have been published [1]. GA is an expensive product fully subsidized by the Health Insurance Fund.

Materials and Methods

Sera were obtained from a 42-year-old woman with clinically defined MS. The disease was established in a patient aged 22 (in 1995-96) with a confirmed clinical diagnosis of MS, relapsing-remitting variant, satisfying McDonald MRI criteria, with assigned level 1.5-2 Kurtzkye Disability. In the beginning, the patient was treated with corticosteroids (in February 2002), and then - with interferons, in particular Betaferon (from 2002 to 2004). She had no contraindications for therapy with GA and since 2005 she started a treatment with GA. During the last 15 years, the treatment was with GA, having a distinctly positive effect. She became pregnant at the age of 34 (during 2007). From conception until child-birth (2008), she had an interruption in her usual immunomodulatory therapy which was eventually restarted 2 weeks post-partum with GA, Milgamma N and Nivaline. Prenatal and postnatal development of her child was normal. During the pregnancy and one month after that, the drug intake has been discontinued. In 2009, a hospitalization due to double vision and weakness in her left leg was required. From 2005 onwards the patient has been in a remission except two or three weak exacerbations. She has currently double-dose injections every other day. MRI, performed four times during 20 years, confirmed the clinical diagnosis of MS. MRI have been conducted under the Health Insurance Fund. The different types of antibodies appear at various stages of the disease. For example IgM antibodies are reported in acute cases, while IgG antibodies titers are found in long-term illnesses.

There is growing evidence suggesting that hormones, including sex hormones, can affect but also can be affected by the immune system. It is known that hormonal changes during pregnancy promote increased oligodendrocyte production in the maternal CNS. The hormone prolactin regulates oligodendrocyte precursor proliferation and mimics the regenerative effects of pregnancy. What's unique about prolactin is that it promotes the formation of new oligodendrocytes – cells that produce myelin. Gregg et al. [6,7] suggest that prolactin may be used as a potential therapeutic agent for MS. A hormone produced during pregnancy could reverse some of the neurological damages, associated with MS. This finding could help to explain why women with MS suffer fewer symptoms during pregnancy. The authors assume that rising levels of the hormone prolactin, which promotes breast development and milk production, might have a protective effect and might be used to treat people with MS. Progesterone immunomodulatory effects differ from those of estrogens and androgens. Higher progesterone levels during the pregnancy may suppress disease activity in MS. During late pregnancy (third trimester) there is a decrease in MS disease activity due to the protective effect of testosterone [5].

Serum IgG anti-GM1 and anti-GD1a antibodies were estimated by the enzyme-linked immunosorbent assay (ELISA). This ELISA protocol was performed according

to Ravindranath and Muthugounder [14] and the optical density (OD) was read spectrometrically at 490 nm on an ELISA reader (TECAN, Sunrise TM, Austria). The patients were considered strongly positive only if the mean OD of their sera exceeded 2 ± SD (standard deviation) of the healthy controls. Determinations were carried out in triplicate [12].

Results and Discussion

For many years we perform various investigations by applying ELISA technique. We conclude that neurodegeneration and demyelination can be presented with numerical values of IgG titers of anti-GMl and anti-GD1a ganglioside antibodies. However, we use IgG class antibodies because they detect the existence of a chronic process. In humans, gangliosides induces an IgG independent T-cell response.

The aim of MS treatment is to prevent demyelination and to reduce axonal loss. In the current investigation, the clinical studies were focused on the titers of sera IgG antibodies to GM1 and GD1a gangliosides in a patient with RRMS under GA treatment during this (2002 – 2017) - 15-year long period (**Table 1**).

Moreover, our previous findings [9, 11, 12, 20] of significantly elevated titers of serum IgG antibodies to GM1 and GD1a gangliosides of the same patient have suggested immune-mediated demyelination and neurodegeneration as underlying pathogenetic phenomena in MS. Unchanged IgG anti-gangliosides antibodies titers during long-term disease period (in comparison with healthy subjects) support the concept of beneficial effect of GA treatment on disease progression, provided that it is taken continuously for many years. Currently, the intake injection to our patient is 40 mg/48 hours.

The patient follows recommendations for lifestyle and food intake according to the current clinical research [19]. Wekerle has specifically discussed the ignition of brain autoimmunity by the seemingly healthy gut flora. The same author examined whether

Table 1. Estimation of titers of IgG antibodies to GM1 and GD1a anti-gangliosides antibodies in the serum of a RRMS patient before and during the treatment with Glatiramer acetate

Years / Titers	2002 A	2004 B	2005 C	2007 D	2008 E	2008 F	2008 G	2014 H	2016 I
IgG anti- GM1 antibodies	+ +	+	+	-	-	-	-	-	-
IgG anti-GD1a antibodies	+ +	+	+	-	-	-	-	-	-
Healthy Subjects	-	-	-	-	-	-	-	-	-

A - in relapse - serum was obtained before injection of corticosteroids, because the treatment close the BBB; B - in the beginning of interferons treatment;

C - in the beginning of the GA treatment, before pregnancy; D - 8 months into pregnancy;

E - 10 days after child-birth; F - 3 months postpartum during a neuroprotective treated relapse;

G - 7 months after child-birth; H - during a long remission; I - during a long remission;

his experimental observations can be extended to clinical brain autoimmunity, the most pertinent to human MS [19].

These findings are in full agreement with our studies [9, 11, 12, 20], which have demonstrated a considerable increase of anti-GM1 and anti-GD1a gangliosides antibodies in patients sera, connected with the neuronal damage in the neurodegenerative diseases exacerbations.

Conclusion

1) It is recommended the GA admission should not be interrupted. 2) GA should be admitted for long period of time. 3) The normal serum IgG titers to GM1 suggest lack of immune-mediated demyelination. 4) The normal IgG titers to GD1a are most probably due to the lack of immune-mediated neurodegeneration due to the long-term, continuous therapy. 5) Pregnancy has a beneficial influence on the course of disease progression. 6) Our case demonstrated that even though some relapses may appeare during treatment, long-term GA has a beneficial effect. 7) Our previous studies demonstrated significantly elevated serum IgG titers to GM1 and GD1a in patients with RRMS without long-time GA treatment.

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Anthroplogy and Anatomy

Prevalence of Underweight and Overweight among Preschool Children from Sofia Assessed through Different International References

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The aim is to describe the frequency of categories nutritional status in Bulgarian preschool children according to different classification systems based on body mass index.

Results: The overall prevalence of overweight including obesity in boys is 7.8%, 9.3%, 12,0% according to WHO, IOTF, BG respectively. In girls, overweight including obesity varied from 9.4% (WHO), 10.6% (IOTF), and 13.4% (BG-references). The overall frequencies of moderate and severe thinness in boys are 14.2% and 2.0% (IOTF), and 12.2 % and 5,1 % (BG-reference). In girls the moderate thinness frequency is 9.10% and 7.0% assessed by IOTF and BG values respectively and girls classified as severe thin are 3.4% (IOTF) and 6.7 % (BG-reference). The total prevalence of thinness according to WHO criteria is 0.7%.

Conclusion: We found significant differences in estimated frequencies of thinness and overweight using the three selected criteria. In the absence of a global definition, we need both national and international growth references.

Key words: underweight, overweight, thinness, preschool children, body mass index, international body mass index references

Introduction

Excess body weight is the sixth most important risk factor, contributing to the overall burden of disease worldwide, with 10% of children now classified as overweight [2]. Childhood overweight, including obesity is recognised as a serious public health problem and is subject of many publications. Data about underweight (thinness), on other hand, are scarce and unsufficient in the literarture. Thinness also entails health

consequences: it lowers immunity, decreases the general efficiency of the system and increases the risk of chronic diseases, so it should not be neglected in surveys concerning the health status in children. The preschool age is an immensely significant period of time for the subsequent stages of life [10] and is one of the critical periods for the development of obesity [23], however, it is one of the least studied childhood periods.

The aim of the study is to describe the frequency of obesity, overweight and thinness by gender and age in 3-6-year-old Bulgarian children according to different classification systems based on Body Mass Index (BMI).

There is general consensus that studies of childhood obesity prevalence should be reported according to different international references, which makes the results comparable at international level, and should be complemented with national references or additional references such as the CDC [16, 3]. Therefore, we report the prevalence of the categories Nutritional Status (NS) according to three classification systems - the International Obesity Task Force reference (IOTF-reference), the World Health Organisation growth standard (WHO-reference) deffinitions and in addition we use Bulgarian reference values (BG-reference).

Materials and Methods

The study is carried out in 15 randomly selected kindergartens on the territory of Sofia - the capital of Bulgaria, throughout the period 2014-1015. The study is cross-sectional and includes 870 healthy children from 3 to 6 years of age, who attend the selected institutions. The sample includes 451 boys (51.8%) and 419 girls (48.2%) with balanced number of children in all age and sex classes (≈ 100). Anthropometric characteristics (height, weight and BMI) of the sample are presented according to gender and age in **Table 2**.

The research is performed with the approval of Bulgarian Ministry of Health and of Health department and Education department of the Sofia Municipality. Undersigned informed consent form is obtained from the parents/caregivers of participants prior the implementation of the study procedures. The study is conducted according to the Helsinki Declaration in its latest version from 2013 year.

Measurements

All anthropometric measurements are taken by a trained team according to the standardized procedures [11, 22]. Body weight is measured by body fat monitor (Tanita BF666, Japan) to the nearest 50g. Height is measured by anthropometer (GPM Antropologishe Instrumente, Switzerland) to the nearest 0.5 cm. Anthropometric measurements are performed in the morning in lightly dressed children, without shoes. BMI is calculated as weight (kg)/height (m)^2.

Three BMI references are used in order to assess the children's NS: 1 - IOTF-reference (2000 [5], 2007 [6]); 2 - WHO - reference (2006 [21], 2007 [19]), and 3 - Bulgarian reference values. The Bulgarian reference values are gender and age specific percentile values (< P5 (severe thinness); P5-P15 (moderate thinness); > P85 (overweight); > P95 (obesity)) elaborated on the basis of a data gathered in previous representative study of 3-6 years old children conducted in Sofia (2004-2005) by the first author of the current publication (Y. Zhecheva, 2008 [25]). The percentiles are the same recommended and used by the WHO [8] and CDC [4] as a cut-off values for the assessment of different categories NS.

The BMI cut-offs used to define overweight, obesity, moderate and severe thinness according to the three classification systems are presented in **Table 1.**

Table 1. Cut-offs for BMI used to define categories nutritional status in children according to various references

Nutritional status categories	BMI-cut-offs		
	WHO	IOTF***	BG-reference
Severe thinness	< -3SD	BMI < 17 kg/m^2	< P5
Moderate thinness	< -2 SD	BMI 17 to 18.5 kg/m^2	P5-P15
Overweight	>+1 SD*	BMI 25-30 kg/m^2	> P85
Obesity	>+2SD**	BMI > 30 kg/m^2	> P95

*equivalent to BMI 25 kg/m^2 at 19 years; **equivalent to BMI 30 kg/m^2 at 19 years
***IOTF child cut-offs correspond to the cut-offs of a BMI of 17, 18.5, 25 and 30 at age 18 years

Table 1a. Bulgarian BMI cut-off values for defining thinness, overweight and obesity by sex and age

Age (years)	Severe thinness	Thinness	Overweight	Obesity
Boys				
3	< 13.75	13.75-14.44	17.05-18.34	> 18.35
4	< 13.45	13.45-14.44	17.05-18.24	> 18.25
5	< 13.95	13.95-14.34	16.85-18.04	> 18.05
6	< 13.65	13.65-14.44	17.65-20.44	> 20.45
Girls				
3	< 14.05	14.05-14.44	16.95-18.04	>18.05
4	< 13.35	13.35-14.14	17.65-18.14	> 18.15
5	< 13.75	13.75-14.04	16.35-17.94	> 17.95
6	< 13.25	13.25-13.94	18.05-19.54	> 19.55

Statistics

Statistical analysis is performed using SPSS v.16 software. Mean and standard deviation for each variable are calculated. The statistical signifficance between age- and gender groups is evaluated using t-test of Student. Comparisons of the frequencies between age classes or between genders are performed using tests for nonparametric comparisons - z-test and chi-square on p-level 0.05.

Results and Discussion

The present study provides information about the current NS of Bulgarian preschool children aged 3 to 6 years. The assessment of NS according to both international criteria and the Bulgarian cut-offs gives us the possibility to determine which of the internationally applied criteria gives closer results to the results estimated using BG-references.

Anthropometric characteristics (height, weight and BMI) of the sample are presented according to gender and age in **Table 2**.

Frequencies of different categories NS, estimated according to the three references, are presented in **Table 3**.

Table 2. Statistical data about investigated anthropometric features by age and sex

	Boys				Girls			
Age (years)	3	4	5	6	3	4	5	6
n	103	100	128	120	102	100	111	106
Height (cm)								
Mean	99.8*	106.7‡	113.9‡	121.5*‡	98.4	106.5‡	113.1‡	119.4‡
Std. Deviation	4.7	5.2	5.2	5.7	4.9	5.4	5.0	4.8
Minimum	89.6	95.7	101.8	106.5	84.5	93.0	101.5	106.3
Maximum	111.6	123.5	126,4	135.9	110.4	118.5	125.1	130.8
Weight (kg)								
Mean	15.8*	18.0‡	20.4‡	23.3*‡	15.1	17.7‡	20.1‡	22.4‡
Std. Deviation	1.9	3.6	3.2	4.0	2.0	2.6	3.7	3.1
Minimum	12.5	12.9	14.8	15.9	10.5	12.3	13.3	16.8
Maximum	21.3	45.2	30.5	50.4	21.4	26.4	40.0	32.2
BMI (kg/m^2)								
Mean	15.8	15.7	15.6	15.7	15.6	15.6	15.6	15.6
Std. Deviation	1.2	1.9	1.5	1.7	1.2	1.4	2.0	1.6
Minimum	13.7	13.3	12.7	13.0	13.5	12.1	12.4	12.6
Maximum	21.2	29.6	20.1	27.3	19.9	20.2	25.6	19.7

*Statistical significant sexual differences, $p \leq 0.05$; ‡ Statistical significant age differences, $p \leq 0.05$

Table 3. Frequencies of obesity, overweight and thinness (moderate and severe) at different ages in girls and boys according to the WHO, IOTF and Bulgarian referrence values

	Boys				Girls				Both sexes
Age (years)	3	4	5	6	3	4	5	6	3-6
n	103	100	127	120	102	99	110	106	867
	%	%	%	%	%	%	%	%	%
Overweight									
BG reference	11.7	8.0	10.2	5.0‡	13.7	1.0*	12.6	8.5‡	8.9
IOTF	7.8	8.0	10.2	5.8‡	2.9	6.0	12.6	11.3*‡	8.2
WHO	2.9	3.0	4.7	10.8	2.0	3.0	9.0	18.9	6.9
Obesity									
BG reference	2.9	4.0	5.5	0.8‡	2.0	4.0	9.9*	0.9‡	3.8
IOTF	1.0	1.0	2.3	0.8	1.0	3.0	3.6	0.9	1.7
WHO	1.0	1.0	3.1	3.3	0.0	0.0	2.7	0.9	1.6
Moderate thiness									
BG reference	12.6	19.0	6.2	12.5	7.8	9.0	1.8	9.4	9.7

Table 3 – continued

IOTF	15.5	17.0	13.4	11.7	14.7	9.1	7.3	5.7	11.8
WHO	0.0	0.0	0.8	0.8	0.0	2.0	0.9	0.9	0.7
Severe Thiness									
BG reference	1.0	1.0	10.9	5.8	8.8	5.0	9.9	2.8	5.9
IOTF	1.0	3.0	3.2	0.8	2.9	6.1	2.7	1.9	2.7
WHO	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

* – Statistical significant sexual differences, $p \leq 0.05$; ‡ - Statistical significant age differences, $p \leq 0.05$

Overweight

The overall prevalence of overweight including obesity in boys is 7.8%, 9.3%, 12.0% according to WHO, IOTF, BG respectively; the obesity prevalence varied between 2.2% assessed by WHO standards, 1.3% using IOTF, and 3.3% according to BG-reference (**Fig. 1**).

In 3-6 years old girls, overweight including obesity varied from 9.4% using WHO, 10.6% using IOTF, and 13.4% according to BG-references, and obesity alone: 1.0%, 2.2%, 4.3% (WHO, IOTF, BG respectively) (**Fig. 2**).

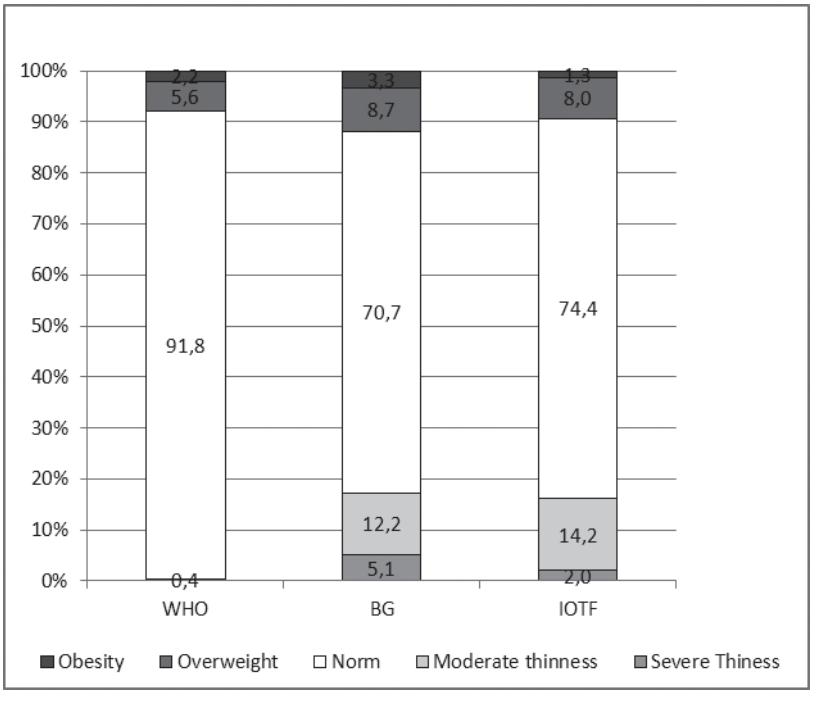

Fig. 1. Frequencies of different categories NS in 3-6 years old boys assessed on the basis of three references

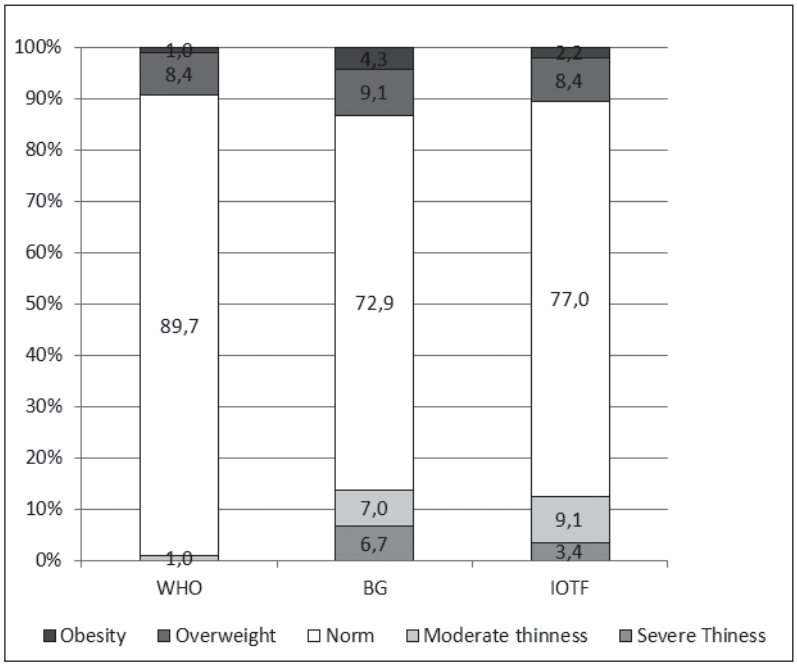

Fig. 2. Frequencies of different categories NS in 3-6 years old girs assessed on the basis of three references

As a rule, most of the studies in children and adolescents report higher estimates for overweight and obesity using WHO reference values than these of IOTF [15, 17, 3, 1]. Our results show that overall WHO generated lower overweight (including obesity) prevalence compared to IOTF criteria and both international criteria estimate lower frequencies compared to Bulgarian reference values.

The results established in 5 years old Chech girls are similar to our data; 3,4% were classified as overweight using the WHO reference values versus 15.3% using the IOTF definition. Authors explain that the result is due to the choice of cut-offs and to the different criteria used to select the sample for the IOTF-reference and that of the WHO standards for children aged 0 to 5 years (sample including individuals who were breastfed for at least 6 months and came from socioeconomics conditions that were favorable for healthy growth) [12].

The difference between estimations with the three criteria is more evident in obesity rates compared to overweight. In a study conducted in Spain, for example, the prevalence of obesity from 2 to 24 years was two to three times higher using the Spanish reference population cut-offs than using the IOTF cut-offs, while the rates of overweight were similar [18].

Whatever reference is used, the number of children classified as overweight or obese is approximately equal in both sexes and sexual differences do not reach statistical significance with some exceptions - at the age of 4 and 5 using the BG-reference and in 6 years old children using IOTF. 4 years old overweight boys are considerably more (8.0%) than the overweight girls (1.0%) at the same age**.** The prevalence of obesity at five years of age is significantly lower in boys than in girls. At the age of 6 IOTF classified significantly more girls in the category overweight.

Sexual dimorphism in the prevalence of overweight or obesity is not apparent during the investigated period, but overall a slightly higher prevalence in girls is observed according to IOTF and BG-references. Using the WHO, the prevalence of overweight is also higher in girls, while the obesity is more common for boys. The observed differences are consistent with previous and recent studies [23, 22, 14, 3] as more boys than girls are classified as overweight or obese according to WHO, whereas the reference values published by Cole and Lobstein [7] show a higher prevalence in girls.

Significant variation in the overweight and obesity frequencies with age are established using the three different criteria. According to the BG-reference, the frequency of overweight girls decreases and increases successively between 3 and 5 years of age, reaching its lowest value at the age of 4 (1.0%). Between 5 and 6 years of age, according to BG- and IOTF-reference, a decrease in categories "overweight" and "obesity" is observed in both sexes with statistical significance in both categories using BG-reference and only in overweight boys according to IOTF.

At the age of 6 the frequency of obese boys and girls reaches its lowest values according to BG and IOTF-references (0.8% in boys; 0.9% in girls). Using the IOTF and BG values the frequency of obesity is highest in 5 years old boys and girls. The highest value of overweight frequency assessed by IOTF is also observed at this age, but according to BG-reference overweight boys and girls are most frequent at the age of 3.

Despite the change in the prevalence of overweight and obesity with age is not consistent using these two references, the trend for decreasing frequencies of overweight (more evident in boys) and obesity (more evident in girls) at the end of investigated period is observed.

An opposite trend is shown by the WHO estimations: the frequency of boys and girls categorized as overweight at the age of 6 has doubled compared to 5 years old, but the increasing is significant only in girls (from 9.0% to 18.9%). The obesity frequency increases with age in boys, reaching its highest values at 6 years of age, while in girls the prevalence of obesity is highest at the age of 5.

Thinness

Comparing the estimates for NS categories of the three criteria, the greatest discrepancies are observed in thinness.

The overall frequencies of moderate and severe thinness ***in boys*** are 14.2% and 2.0% using IOTF, and 12.2 % and 5.1 % according to BG values. In 3-6 years old ***girls*** the moderate thinness frequency is 9.10% and 7.0% assessed by IOTF and BG values respectively and ***girls*** classified as severe thin are 3.4% (IOTF) and 6.7 % (BG-reference). The total prevalence of thinness is smallest according to WHO criteria – 0.7%.

The estimated frequencies of thinness (moderate and severe) using IOTF and BG references are relatively equal reaching up to 20.0% in boys (4 year olds) and 17.0-18.0% in girls (4 year olds), while according to the WHO criteria the prevalence is most often under 1.0 % in the separate age and gender groups. According to the WHO not a single boy or girl is categorized as severe thin, while according to IOTF and Bulgarian references, severe thinness is presented in all age groups with total frequencies for boys and girls of 2.7% and 5.9% according to IOTF and BG references respectively (**Table 3**).

According to our findings, it could be suggested that the prevalence of thinness in Bulgarian preschool children is underestimated when using the WHO criteria. A study of Russian preschoolers report two times lower thinness frequencies assessed by the WHO references compared to the CDC estimations. [14] The probable reason for this result could be sought again in the specificity of the sample used for elaborating the growth standards of WHO for 0-5 years old children.

Gender differences in the thinness categories are more obvious compared to the overweight categories. Significant gender differences in the frequencies assessed by the Bulgarian reference values are observed: at the age of 3 - girls categorized as severe thin (8.8%) are considerably more than boys (1.0%); at 4 and 5 years of age the moderate thinness frequency is much higher in boys. Categorised by IOTF, 4 and 6 years old boys with moderate thinness are significantly more than girls. Compared to BG-reference, the values recommended by IOTF determine higher frequency of moderate thinness and lower frequency of severe thinness.

As being established in other studies, the moderate thinness occurs more often than severe thinness in both sexes [24] with predominance of boys with moderate thinness, while severe thinness is more common among girls [13]. According to WHO criteria, moderate thinness is more frequent in girls.

The thinness frequencies differ significantly with age only by using BG-reference values. The variations are not consistent but are similar in both sexes - decreasing of the frequency of moderate thinness between 4 and 5 years of age (from 19.0 to 6.2% - boys ($p \leq 0.05$)); from 9 to 1.8% - girls ($p \leq 0.05$)) accompanied with increasing in severe thinness (from 1.0 to 10.9% - boys; from 5.0 to 9.9% - girls). During the following age period the changes are opposite - severe thinness decreasing (significantly only in girls) and moderate thinness increasing significantly in both sexes. Using IOTF reference values the thinness is more common among 3 and 4 years old boys and girls and decreases insignificantly with age, more pronounced in girls. Both criteria show a decreasing trend in thinness with age more pronounced in girls, and more evident when using IOTF references. Our results are consistent with a study in children and adolescents in Netherlands where it is established that thinness is occurring most often in children aged 2-5 years. [9]

At the age of 6, as a result of decreasing frequencies of the unhealthy weight categories – overweight, obesity and underweight, the percentage of boys and girls with healthy NS is highest using the IOTF and BG criteria. In contrast, according to the WHO reference values, the relative share of boys and girls with healthy NS decreasing successively with age.

Conclusions

We found significant differences in estimated frequencies of thinness and overweight using the three selected criteria, two international and one Bulgarian.

Based on the results of the current study we could conclude that compared to WHO standards, the results of the frequency of different categories NS estimated by IOTF-references are closer to these estimated by BG-references. Therefore we consider that for international comparisons the cut-offs of Cole are more appropriate for use. For the identifying of population groups with higher health risk concerning NS and for planning preventive care services and evaluating the impact of policy initiatives on national and regional level the use of national references is more appropriate and relevant.

In the absence of a global definition, we need both national and international growth references.

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Asymmetry of Lean Body Mass Accumulation in 12-year-old Tennis Players. (Preliminary Results)

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Tennis is a sport characterized by high physical activity and frequently repeated motions, especially for the dominant upper limb. This creates differences between upper limbs and lead to an asymmetric distribution of muscle mass and unbalanced muscle tonus.

The aim of the study is to estimate the degree of muscle mass asymmetry between the dominant and non-dominant limbs in young Bulgarian tennis players, using multi-frequency bioelectrical impedance measurements. The study sample includes 14 male tennis players and 11 school children aged 12 years. Segmental analysis of body composition was done by bioelectrical impedance analyzer (model: InBody 170). The athletes have a larger muscle mass of the dominant upper limb compared to the non-dominant. The non-athlete boys are characterized with lower asymmetry coefficient level of the upper limbs' muscle mass compared to the tennis players ($p < 0.05$). The significant relationship between asymmetry coefficients of the upper limbs, mean age and years of training experience in tennis players are not found.

Key words: asymmetry, bioelectrical impedance, muscle mass, body composition, Bulgarian tennis players

Introduction

Both genetic potential and optimal environmental factors contribute to achieving a high level of sport performance. Therefore the interest of scientists to anthropometric characteristic and body composition of athletes from different competitive sports increased over the last decades [9]. But sports participation not only positively influences anthropometric features like body weight, body composition and particularly body symmetry [6, 10]. Tennis is a sport characterized by high physical activity and frequently repeated motions, especially for the dominant upper limb. This creates differences between upper limbs [4] and lead to an asymmetric distribution of muscle mass and unbalanced muscle tonus [1]. Training experiences of tennis players also creates anatomical differences between dominant (DA) and non-dominant (NDA) upper limbs and leads to an asymmetry in the muscle mass distribution [8, 11, 12, 13].

The aim of the study is to estimate the degree of muscle mass asymmetry between the dominant and non-dominant upper limb in young tennis players, using multi-frequency bioelectrical impedance measurements.

Materials and Methods

The study sample included 14 male tennis players (TP) represented a high sport class in tennis clubs in Sofia, Bulgaria. The athletes' mean age was 12 ± 0.38 years, and the length of their training experience: 5 ± 2.51 years.

The control group (NTP) included 11 boys aged 12.51 ± 0.34 years from a middle school from Sofia, Bulgaria. All participants and their parents gave written informed consent (in conformity with the Helsinki Declaration) and voluntarily to fill out a questionnaire for handedness/footedness. Questions in the study regarding functional asymmetry of the boys were summarized in **Table 1**. Eleven athletes played tennis with the right arm and three of them with the left arm. Nine of the non-athlete boys declared being right-handed and two of them were left-handed. Body height was measured using standard procedure to the nearest 0.1cm. Body weight and body composition, along with segmental distribution of muscle and fat mass were determined by bioelectrical impedance analyzer InBody 170 with eight electrodes, which characterized by high precision and accuracy [7]. The bioimpedance analysis (BIA) is widely used method, relatively inexpensive and noninvasive [3, 9]. Segmental body composition analysis allows rapidly and easily determination of asymmetry in the accumulation of lean and fat mass [5, 12].

The data of muscle mass on the right and left arm, right and left leg were analyzed. Asymmetry coefficients of muscle mass accumulation in the upper (AA) and lower (AL) limbs were calculated by Wolanski equation (1957) [15]:

$$WAE = (xd - xnd) / ((xd + xnd) / 2) \cdot 100\% ,$$

where:

Xd – muscle mass on the dominant side [kg];

Xnd – muscle mass on the non-dominant side [kg].

The data from present study were analyzed by statistical software package SPSS 16.0 (SPSS Inc, Chicago, IL, USA). Student's t-test was applied to compare variable means and statistical significance was defined as $P < 0.05$.

Correlation analysis was used for the assessment of relationships between asymmetry coefficients of the upper limbs and mean age and years of training experience in TP (**Table 1**).

Table 1. Questionnaire of handedness and footedness in athlete and non-athlete boys

	Questions
Handedness	Which hand does subject used to draw or write?
	Which hand does subject used to hold a tennis racket?
	Which hand does subject used to cut with scissors?
	Which hand does subject used to hold a dynamometer?
Footedness	Which leg does subject used to kick a ball?
	Which leg does subject used to leap forward to cover a selected distances?
	Which leg does subject used to make the longest jump?

Results and Discussion

The results for height and weight are similar for both groups. The tennis players' mean body height is 162.00 ± 7.06 cm and their mean body weight is 51.46 ± 7.24 kg. In control group of non athlete boys the average body height is 160.00 ± 6.33 cm and body weight - 50.40 ± 8.72 kg.

The segmental analysis of body composition shows that tennis players have larger muscle mass of the dominant upper limb compared with non-dominant. The asymmetry coefficient is high (7.1 %) but statistically significant differences are not observed. Similar results are found for muscle mass distribution of the lower limbs in the same group, but the level of asymmetry is low - 0.57%, only. Significant differences with regard to muscle mass distribution between the dominant and non-dominant upper and lower limbs in controls are not revealed, (asymmetry's level is 3.33% and 1.14%, respectively).

Muscle mass in the upper and lower limbs is also compared between the tennis players and the control group. Significant differences are observed between these groups in terms of the dominant and non-dominant upper limb (**Table 2**).

The relationships between age, training experience and AA in tennis players are assessed by correlation analysis. A positive and moderate relation between age and training experience and a negative and weak one between age and AA of tennis players exist but association between training experience and AA in the same group is not observed (**Table 3**). The established correlations are not significant ($P > 0.05$).

Many researches proved that morphological asymmetry exists in athletes [4, 6, 11, 12]. Some of the studies used a high-technology based methods such as magnetic resonance imaging (MRI), dual-energy X-ray absorptiometry (DEXA), but they are too expensive, time-consuming and cannot be utilized in field experiments [2, 13, 14]. The current study applied BIA to assess the body composition and differences in muscle mass distribution of the upper limbs as a result of sporting activity. In tennis players training experience leads to increased asymmetry in the fat free mass in the arms. The

Table 2. Mean values of measured characteristics and statistical significance between studied groups

Characteristics	Groups		Significance
	TP (n=14)	NTP (n=11)	D: TP-NTP
DA (kg)	2.15±0.48	1.73±0.44	2.1*
NDA (kg)	2.01±0.49	1.68±0.43	1.65
AA (%)	7.10±4.07	3.33±2.1	0.43
T:DA-NDA	p= 0.7	p= 0.25	
DL (kg)	6.56±1.14	6.15±0.98	1.05
NDL(kg)	6.53±1.15	6.09±0.97	1.05
AL (%)	0.57±0.46	1.14±0.57	-0.15
T:DL-NDL	p= 0.07	p= 0.14	

TP - tennis players; NTP - non-tennis players; D: TP-NTP - statistically significant differences at $P < 0.05$ between specified groups; DA - dominant upper limb muscle mass; NDA - non-dominant upper limb muscle mass; AA - asymmetry coefficient of upper limbs; T: DA-NDA - statistically significant differences at $P < 0.05$ between the upper limbs; DL - dominant lower limb muscle mass; NDL - non-dominant lower limb muscle mass; AL - asymmetry coefficient of lower limbs; T:DL- NDL - statistically significant differences at $P < 0.05$ between the lower limbs.

Table 3. Correlation between asymmetry coefficient of the upper limbs, mean age and years of training experience in tennis players

Characteristics		Age	Years of training experience	Asymmetry coefficient
Age	Pearson Correlation	**1**	0.544	– 0.287
	Sig. (2-tailed)		0.163	0.392
Years of training experience	Pearson Correlation		**1**	0.099
	Sig. (2-tailed)			0.816
Asymmetry coefficient	Pearson Correlation			**1**
	Sig. (2-tailed)			

level of asymmetry in athletes corresponds to 7.10%, contrary to non-athletes where the asymmetry's value is only 3.33%. Although our values of asymmetry of upper limbs are lower in comparison with professional tennis players, but they are higher than those observed in Polish tennis players (4.06%) [12]. Sanchis-Moysi et al. determined the volume of the muscle mass of the upper limbs using MRI in 11-year-old boys practicing tennis. The authors concluded that tennis participation five times a week, in time before puberty is associated with muscle hypertrophy of the dominant upper limb, leading to a high level of asymmetry (13%) [13].

Our preliminary study shows that the muscle mass of upper and lower limbs are dominant on the right side in both groups. The value of asymmetry of the lower limb is much lower than this of the upper limb (**Table 2**). These values are similar to Polish tennis players, but they are lower than those of Polish schoolchildren [12]. Although the relationship between age, training experience and asymmetry coefficient values are not significant we consider that sport training revealed an influence on asymmetry in muscle mass distribution in the upper limbs.

Conclusion

Based on our preliminary results we can conclude that the method of bioelectrical impedance can be used to easy determination of muscle mass asymmetry in the limbs. Tennis players have high level of muscle mass asymmetry between the dominant and non-dominant upper limbs. The control group are characterized by lower muscle mass asymmetry coefficient of the upper limbs than the tennis players ($p < 0.05$). The muscle mass of upper and lower limbs are dominant on the right side in assessed groups, but asymmetry value of the lower limb is much lower than this of the upper limb. There are no significant relationships between asymmetry coefficients of the upper limbs, mean age and years of training experience in tennis players' group.

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Degenerative Joint Disease Incidence and Spinal Column Joint Pathologies in the Paleopopulation from Medieval Anchialos, Excavated in Gladston Street, Pomorie. (Preliminary Results)

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Anthropological investigations of the Middle Ages necropolis of Anchialos from 11-12 c. AD register development of degenerative joint disease. In spite of the small sample size, which doesn't allow statistical analysis, obtained results enrich the knowledge about the health status and life quality in the ancient population. Most individuals over 30-40 years of age developed the studied changes, but, except in three cases, where joint changes can be interpreted as secondary developed process to other condition, degenerative joint disease in population didn't lead to heavy disability.

Key words: joint disease, Middle Ages, Anchialos

Introduction

Data about health condition of individuals derived from skeletal series are scarce, but valuable in reconstruction of adaptation of paleopopulations to environmental conditions. This work aims study of the pathological changes on the main joints of limbs and vertebral column in a medieval population of Anchialos excavated in a sector from the modern street Gladston, Pomorie in order to detect age and sex specifics in their dissemination. The degenerative joint disease (DJD), as well as dental caries, is highly dispersed in ancient populations up to modern time, varying through time, geographic zones and populations [13], and is also found in studied population. Their dependence both of genetic predisposition and environmental conditions [6] makes from them a good marker in a study of population health status and social and biological adaptation strategies of past communities.

In the studied material there are also recognized cases of other joint pathologies. The affected individuals possibly exhibited symptoms as the ones suffering from DJD

development and respectively it can be supposed, that they were treated, both, medically and socially, as the affected from the latter condition. This is one of the reasons these cases to be discussed with the problem for spreading of DJD changes in the studied population. On the other hand, it has been noted by researchers that joint disease in paleoanthropological material should be studied with all the complex of pathological changes presented in the available material from the skeleton and a connection is already found between DJD and some other pathological affectations [14].

Materials and Methods

Archaeological excavations on the necropolis of the Middle Age Anchialos, sector from the modern street Gladston, Pomorie, present human bone remains from inhumations [18]. Most of the individuals are recognized in primary burials (remains from 19 skeletons, 73.1%). After singular bone fragments, which duplicate bones or bone locations found in the material of the main skeleton, or fragments, which characterize individuals with distinctly different anthropological identification (mostly of age determination) in some complexes are recognized reburied fragments. These materials identified more seven individuals at least. Only in grave N 12 are registered one skeleton in primary position in a stone sarcophagus and reburied bones and bone fragments from at least 11 more individuals in the sarcophagus and around it (including these secondary buried bones to the statistic the proportion of primary to secondary burials distorts, becoming 19 primary to 18 secondary burials, or 51.35 to 48.65%).

In anthropological investigation age at death is achieved using assessment of cranial sutures obliteration after Olivier-Simpson and Meindl-Lovejoy [2, 19] (60% of cases), pubic simphyseal and iliac auricular surface relief [11, 19] (20% of identified in combination of cranial sutures obliteration and 10% only by latter features). In one case age is ascertained in broad limits after finished skeletal development as grown-up, and in one case – after dental wear. Sex identification is achieved by the summarized methods [1, 5, 20] with priority of results derived from the pelvic girdle bones (50% of buried; only after cranial features are identified 30% of investigated). Measurements of long bones of the limbs – head diameters of humerus, radius and femur and bicondylar breadth of humerus and femur, correlated to tables of Dwight, Krongman, Thieme and Pearson [5, 10] are taken into account in addition (60% of identified).

In age and sex distribution in adults is ascertained a relatively high number of aged males over ca. 50 years at death. Skeletal remains from females point to a younger age than males (**Table 1**). Both sexes are unevenly distributed, males outnumbering the females with 16 skeletons, identified as males (75%) to five females. Among the studied burial complexes special attention attracts grave N 12, the stone sarcophagus. Skeletal remains from this complex allow recognition of at least 12 individuals. They are distributed by age and sex similarly to the remaining material from the necropolis (**Table 1**).

In assessment of pathological changes on skeletal remains are used classifications for paleoanthropological materials [3, 12]. According to the fragmentary state of the material the main joints of limbs are assessed for development of pathology after present fragments, or in most cases after data from a fragment of only one of the bones included in the joint, which lowers to some degree the certainty of the results. This way are studied the shoulder, elbow, wrist, hip, knee and ankle joints. For the wrist joint are used exclusively data for the present distal ends of radius and ulna and results concern mostly radio-carpal joint and in less cases ulno-carpal and radio-ulnar joints. Affectation of the vertebral column was assessed in three main sections: cervical, thoracic and lumbar, to the latter are included data from proximal joint surface of the sacrum. Such

Table 1. Age and sex distribution of individuals with age at death over 18-20 years

	Adultus 20-40 years		Maturus 40-60 years		Senilis Over 60 years		Over 20 years			Identified	Total
Sex	M	F	M	F	M	F	M	F	0		
N	3	2	7	2	4	-	1	1	1	18	21
%	16.67	11.11	38.89	11.11	22.22	-				85.71	
%*	14.29	9.52	33.33	9.52	19.05	-	4.76	4.76	4.76		
Gr. N 12		2	3		1		1	1	1		9

M - males; F - females; N - number of identified; % - percent from identified in main age intervals; %* - percent from total recognized individuals; Gr. N 12 - minimum adult individuals in the grave N 12

approach renders some uncertainty of the results, which is even more raised in relation to the small sample.

For analysis of distribution of changes on the articulations are defined five stages of development as follows: unaffected, pronounced in clear edges of the articulation surfaces and smooth articulation surfaces; initial development – with coarsening of the edges of the articulation surfaces (**Fig. 4 –** *3*); developed – with thickening and exostoses on the edges of articulation surfaces (**Fig. 4 –** *7*); advanced – with big exostoses on the edges of articulation surfaces, abrasion on the articulation surfaces and osteochondrothic changes (**Fig. 4 –** *1, 2, 6*). On this stage is supposed development of disability, which impacts the life of the affected individual. The last stage is defined by high restriction of movements in the joint with advanced disability (**Fig. 4 –** *4, 5*), which supposes surviving of the individual for relatively long period of life exclusively after support by other members of the society. Because of the small sample size the age distribution of joint disease is assessed after calculation of average degree of affectation of joints, which again reduces the possibility for interpretation.

Results and Discussion

All defined stages of development of DJD are registered in the population from medieval Anchialos. With less cases of lack of affectation the elbow appears as most often developing DJD (**Table 2**). In spite of this in studied series more risk appears for development of severe cases of pathological changes on joints of lower limbs – the hip and knee, where are concentrated cases, which suppose developed severe disability. Age distribution of DJD on studied limb joints points to development of these changes from most individuals over 30-40 years, 40-50 years being the peak of frequency of the pathology (**Figs. 1, 2**). The small sample doesn't allow a specific tendency to be derived. It appears a phenomenon of higher affectation of the left side in bones from upper limbs, situation, which could be caused by an insufficient data in the material.

In vertebral column the thoracic section appears as more affected of DJD changes (**Table 1**). The graph, obtained after calculated mean values of affectation for 10-year

Table 2. Distribution of the DJD on the studied joints of limbs

		Shoulder		Elbow		Wrist		Hip		Knee		Ankle	
		dx	Sn	dx	sn	dx	sn	dx	sn	dx	sn	dx	sn
Unaffected	N	4	3	1	1	3	3	3	4	3	4	2	2
	%	30.8	20.0	10.0	8.3	37.5	37.5	27.3	36.4	23.1	30.8	33.3	28.6
Innitial affectation	N	3	4	5	5	3	2	3	1	4	2	1	3
	%	23.1	26.7	50.0	41.7	37.5	25.0	27.3	9.1	30.8	15.4	16.7	42.9
Developed	N	4	4	3	3	2	2	2	4	2	4	2	1
	%	30.8	26.7	30.0	25.0	25.0	25.0	18.2	36.4	15.4	30.8	33.3	14.3
Advanced development	N	2	4	1	2			1	2	2	1	1	1
	%	15.4	26.7	10.0	16.7			9.1	18.2	15.4	7.7	16.7	14.3
Disabled	N				1		1	2		2	2		
	%				8.3		12.5	18.2		15.4	15.4		
Total		13	15	10	12	8	8	11	11	11	13	6	7

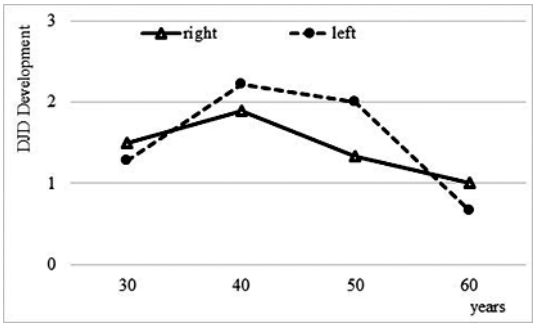

Fig. 1. Age distribution of mean affectation of the DJD on studied joints of upper limbs: 0 – unaffected; 1 – initial affectation; 2 – developed; 3 – advanced development; 4 – disability

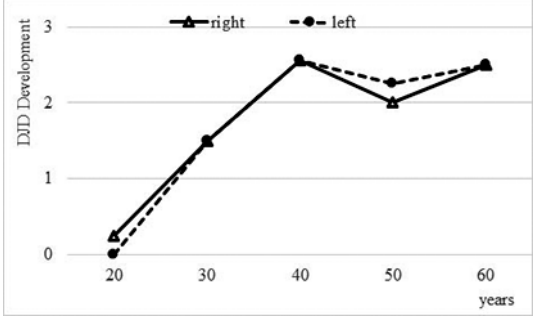

Fig 2. Age distribution of mean affectation of the DJD on studied joints of lower limbs: 0 - unaffected; 1 - initial affectation; 2 - developed; 3 - advanced development; 4 - disability

age intervals (**Fig. 3**) points to highest affectation in the age interval between 30-40 years of death. This situation is explained with worsened health in those, who died at younger age and difficulties to distinguish cases of development of distrophic changes as a secondary process of another main infectious disease, especially in initial stages of development. It can be supposed that most of the people from studied population

Table 3. Distribution of the DJD on the main sections of the vertebral column

	C	%	T	%	L	%
Unaffected	2	28.57			1	12.50
Innitial affectation	1	14.29	1	14.86	2	25.00
Developed	1	14.29	3	42.86	2	25.00
Advanced development	3	42.86	3	42.86	3	37.50
Total	7		7		8	

C - cervical section; T - thoracic section; L - lumbar section (reburied sections from a vertebral column from grave N 12, possible DISH – excluded)

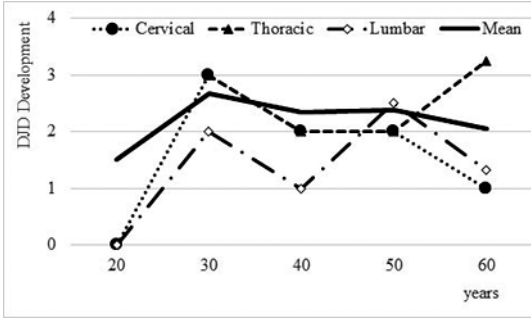

Fig. 3. Age distribution of mean affectation of the DJD on vertebral column:
0 - unaffected; 1 - initial affectation; 2 - developed; 3 - advanced development; 4 - disability

obtained some degree of DJD changes on vertebral bodies at the age of 30-40 years. This pathology rarely reached highly developed stages and respectively rarely led to disability in the population.

There appear two cases of heavy disability after excessive development of DJD changes in limb joints. All they are found in individuals with complicated health status and can be interpreted as connected to other pathological processes. One of these is registered in an individual from the grave N 13, a male, between 40-50 years at death. Here DJD changes from highest degree, after developed scale, are observed in left elbow. The fragments from the left hip joint also show last stage of development of DJD changes with possible heavy disability. Progressive arthritis changes are registered on the found vertebrae. The condition of the hip joint could be explained with possible luxation as inborn predisposition or traumatic consequence. Here the DJD changes can be interpreted as secondary process on the affected joint. In complex with changes on the vertebral bodies the condition of hip joint could be also explained with an infectious disease, which led to rheumatism in childhood and worsened health condition of the individual during his later life. The DJD changes in the left elbow possibly progressed after the fracture on the left radius, which could be a result of the overloading the joint in use of a walking stick in difficult locomotion. Registered fractures on four ribs, recognized as two left and two right, one of which is affected on two places and a defect from a trauma on right tibia could be also a result of incidences in difficult locomotion and week muscle and skeletal composition.

The other case of last stage of development of DJD changes after the used scale is registered again in elbow, on single reburied left humerus in material from grave N 12, which cannot be categorically associated with other fragments from the postcranial

skeleton. From the same complex comes a reburied single bone, right femur, with developed changes on the head, pointing to disability in hip joint possibly after luxation, presenting condition similar to the observed in the individual from grave N 13. Again as in the latter case material from reburied bones from grave N 12 presents advanced vertebral column pathology **(Fig. 4 –** *10*), identified as possible DISH (diffuse idiopatic skeletal hyperostosis). All these bones could have come from one skeleton of a disabled individual. Here the condition of the elbow could be explained again with overloading the arms during walking with a stick. Observed advanced DJD changes in the hip should be regarded as a secondary process on the traumatized joint.

In skeletal remains from studied necropolis are recognized two individuals, with specific changes, who possibly showed similar condition as affected from DJD changes for their contemporaries. It can be supposed that in the period they were treated in similar way in social and medical aspect like those affected from DJD changes. One case is the registered ankylosis of vertebrae from the thoracic section – reburied bone fragments in and around sarcophagus (gr. N 12), discussed above. Five thoracic and first lumbar vertebrae (T-8 – T-12 – L-1) are situated to the north wall of the sarcophagus in anatomical position. Vertebrae from the upper part of the thoracic section, which changes could be evaluated as developing same condition, but couldn't get in correct articulation with the rest of the found vertebra because missing bones are found disarticulated. Outside the sarcophagus, near its east wall are found more two thoracic vertebrae with same pathology. All these bones allow reconstruction of T-1/T-6 and T-8/L-1 sections of the vertebral column of one individual. On the right side of vertebral bodies of all these vertebrae are formed thick bone layers, "wax" appearing, which are most developed on the T-8/T-9, fused together **(Fig. 4 –** *10*). The condition is specific to DISH [12]. The T-2 and T-3 show ankylosis on their neural arches. After developed spondylosis one first cervical vertebra and a lumbar vertebra from the complex could also be associated to the vertebral column of this individual.

In skeleton from grave N 19, female, about 40-50 years at death, is registered other severe abnormality on the spinal column **(Fig. 4 –** *8, 9*). Here C-2 and C-3 vertebrae are fused together in their neural arches and C-4 and C-5 are fused in neural arches and vertebral bodies. On the first cervical vertebra is developed advanced spondylosis on the edges of articulation surface with dens axis of the second cervical vertebra, possibly after abnormal position of the skull according to the vertebral column with bending backward. The find resembles an inborn condition, reminding of Klippel-Feil syndrome [4]. Changes are also found in the pelvic area, where the coccyx fuses with sacrum in bent position to the right side. The sacrum and right iliac bone are also fused together. The individual should have been at least partially disabled. Possible interpretation of the fusion of cervical vertebra in connection of the ossifications on sternal ends of six ribs and sacroiliac joint could be also development of a condition described as ankylosing spondylitis [12], known in Bulgarian paleopopulations as Bechterew disease [17]. The latter interpretation corresponds better to the relatively advanced age of the individual.

Being highly dispersed in the Bulgarian Middle Ages series [7, 8, 9] DJD changes affected most of individuals over 30-40 years of age in different stage of development. This pathology appears as one of the main reasons for disability in the late mature and senile age. With other finds from the Bulgarian Middle Ages series [7, 8, 9] cases of DISH and possible ankylosing spondylitis (Bechterew disease) or Klippel-Feil syndrome point to their dispersal also in the Bulgarian Middle Ages, as not a rare condition [15]. Again, as found from other researchers of spreading of this pathology in the Middle Ages [15, 16], the individual with developed DISH from our investigated series could be apprised as a member of high social strata of the group, which used the graveyard and performed burials in the sarcophagus.

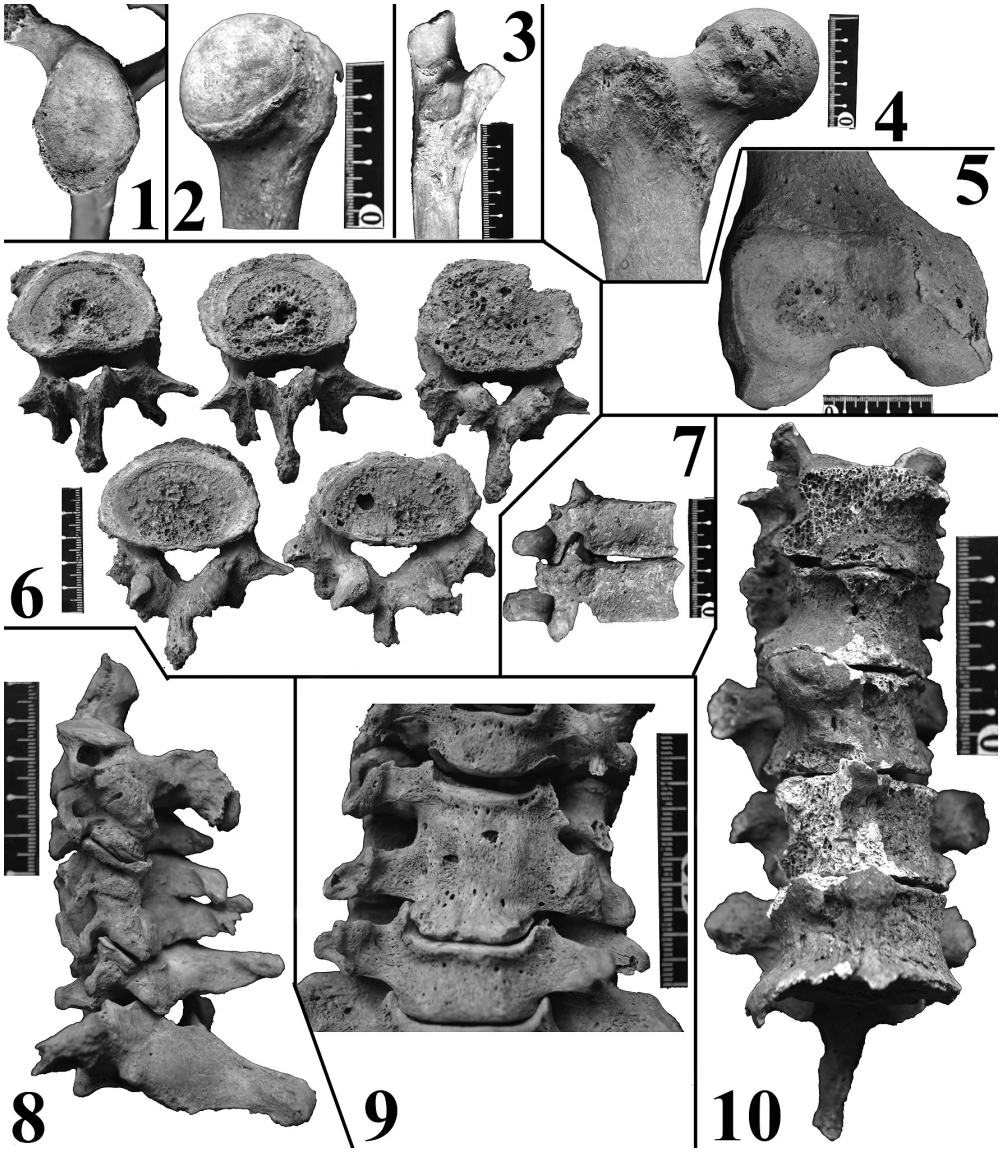

Fig. 4. Degenerative joint disease and spinal column joint pathologies, Anchialos, site in Gladston Str. *1* – Glenoid fossa, left scapula, grave N 18, male, 50-60 years, advanced DJD changes on the articular surface edge and destruction of articulation survace; *2* – Head of right humerus, grave N 24, male, 30-40, up to 45 years, advanced DJD changes; *3* – Right ulna, proximal part, grave N 7, male, 60-65 years, initial development of DJD on the articulation surface edge; *4* – Right femur, proximal part, grave N 13, male, 40-50 years, disability after possible hip joint luxation; *5* – Right femur, distal part, grave N 13, disability after development of DJD as a secondary process to hip joint condition with articulation surface edge exostoses and articulation surface eburnation; *6* – Lumbar vertebrae, grave N 13, advanced development of DJD with osteophitosis of articulation surface edges and osteohondrosis of articulation surfaces; *7* – Two thoracic vertebrae, grave N 24, developed DJD changes, marked by exostoses on the vertebral edges; *8* – C-2 – C-7 vertebrae, grave N 19, female, 40-50 years, ankylosis between C-4 – C-5; *9* – Grave, N 19, ankylosis between C-4 – C-5, detail; *10* – Thoracic vertebrae, reburied in grave N12, development of DISH

Conclusions

Studied population presents relatively favorable situation according to development of DJD changes. Most pronounced cases can be qualified as secondary occurred in connection to other pathological condition, or in life adaptation in disability after progress of another affectation. Cases of advanced disability are in result of development of spinal cord joint diseases, recognized in one case as DISH and in the other as possible ankylosing spondylitis (or Bechterew disease) or Klippel-Feil syndrome. Obtained results will add to the investigation of distribution of this pathology in the Bulgarian Middle Ages.

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Carpal Tunnel Syndrome Treatment with Open Surgical Release: a Study in 292 Patients

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Carpal tunnel syndrome is the most common peripheral neuropathy in the upper extremity due to compression of the median nerve at the wrist. It affects around 3 to 6 percent of adults, predominantly women. Clinically, the carpal tunnel syndrome is presented by numbness of the thumb, index finger, middle finger and the radial side of the ring finger, which becomes painful and aggravates at night. In the present article, we retrospectively review the results of 292 patients treated by simple open surgical decompression of the median nerve due to carpal tunnel syndrome. We also briefly review the literature data concerning this pathology including diagnosis and different treatment modalities. In conclusion, our results confirm that open carpal tunnel release by simple decompression is an effective and safe procedure that could be performed under local anaesthesia with timely return to daily activities and low risk of complications or relapse.

Key words: carpal tunnel syndrome, median nerve, compression, open release

Introduction

Although described for the first time in 1854 by James Paget, the carpal tunnel syndrome (CTS) is still often encountered nowadays. For example, the incidence of CTS in the United States is 1:20 for people between the age of 45 and 60 years [14]. CTS develops as a result of median nerve compression within the carpal tunnel and is the most common neuropathy of the upper extremity [9]. Anatomical variations such as those of the palmaris longus muscle, hypothenar muscles, abductor digiti minimi, persistent median artery, bifid median nerve, etc. are a prerequisite for the occurrence of this syndrome [3-7, 13]. Metabolic disorders (diabetes, thyroid pathology, etc.) can also be a cause of median nerve compression [10]. CTS is characterized by the classical symptoms of numbness and paraesthesia along the distribution of the median nerve and typically aggravate at night. Detection of thenar muscle weakness is a late manifesta-

tion of this pathology [8]. Tinel's (percussion over the median nerve at the level of the carpal crease) and Phalen's (holding the wrist at maximum flexion for 30 to 60 seconds) signs are often positive and could help in establishing the diagnosis. Electromyography (EMG) and ultrasound imaging can be used to confirm the diagnosis. In rare cases with atypical clinical presentation, a magnetic resonance imaging could be used [8, 9].

The aim of the present study was to examine retrospectively the results of patients who suffered from CTS and were treated by simple open surgical decompression of the median nerve, as well as to review the pertinent literature data with regard to diagnosis and the different treatment modalities.

Materials and Methods

The current retrospective study included 292 patients (239 female and 53 male) with CTS treated by open surgical release over a 5-year period. Diagnosis was established on the basis of clinical examination and EMG study. The indications for surgical treatment included: failed conservative treatment (after 3 months of unsuccessful treatment) and signs of muscle damage, confirmed by EMG. The open surgical decompression was performed on 202 right and 110 left hands (20 patients had bilateral CTS). The average follow-up period was 17 months.

Results

In our retrospective study, females (82%) were more commonly affected than males (18%) (**Fig. 1**).

The average age at the point of operation was 57.4 years. Local anaesthesia was used in 98% of cases and regional - in 2% of the patients. The right hand was affected in 182 patients (62%), the left - in 90 patients (31%) and in 20 patients (7%) we performed bilateral decompression (**Figs. 2, 3**).

The hospitalization of all patients was 2 days and post-operative dorsal splints were applied for two weeks. We did not report the occurrence of any infections, intra- or postoperative complications. The outcome of the operation was determined on the basis of recovery of the function of the affected hand compared to the contralateral hand

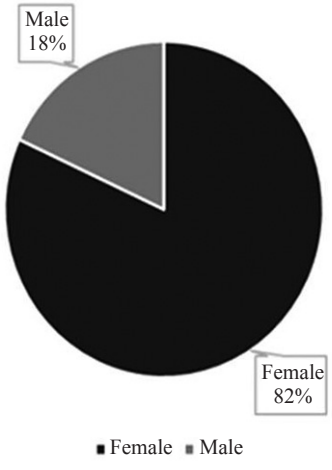

Fig. 1. Chart representing the distribution of carpal tunnel syndrome (CTS) between males and females

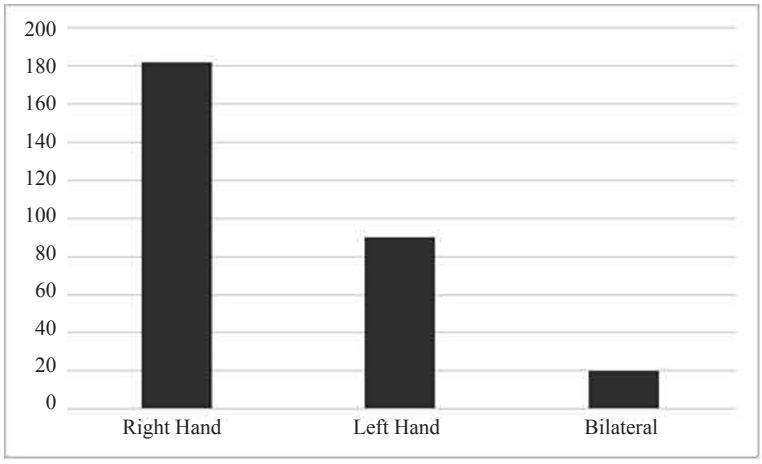

Fig. 2. Chart representing the number of carpal tunnel syndrome (CTS) cases in right hand, left hand and bilateral occurrence

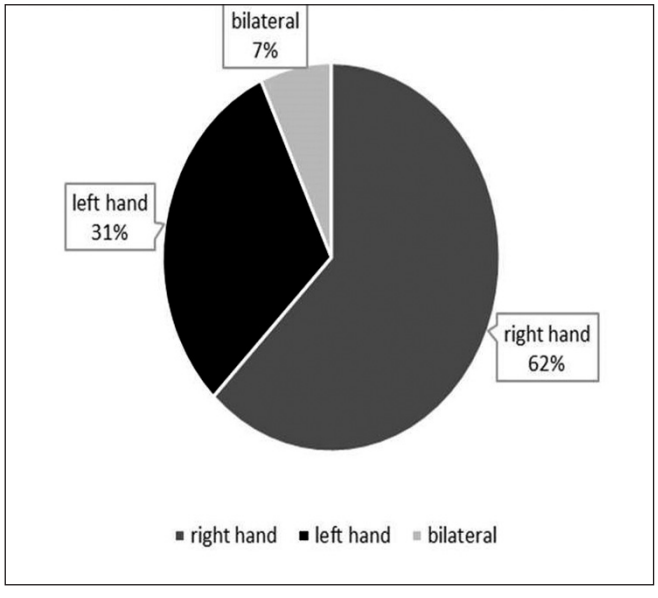

Fig. 3. Chart representing the percentage of carpal tunnel syndrome (CTS) cases in right hand, left hand and bilateral occurrence

and patient satisfaction at six month after surgery. If the patient recovered 100% of the function of the other hand, the results were accepted as "excellent"; 50% recovery or more of the function - as "good"; and no recovery - as "bad". The results of our study included 83% "excellent" outcomes, 13% "good" and 4% "bad" (**Fig. 4**). The "good" and "bad" outcomes were observed in patients suffering from diabetes.

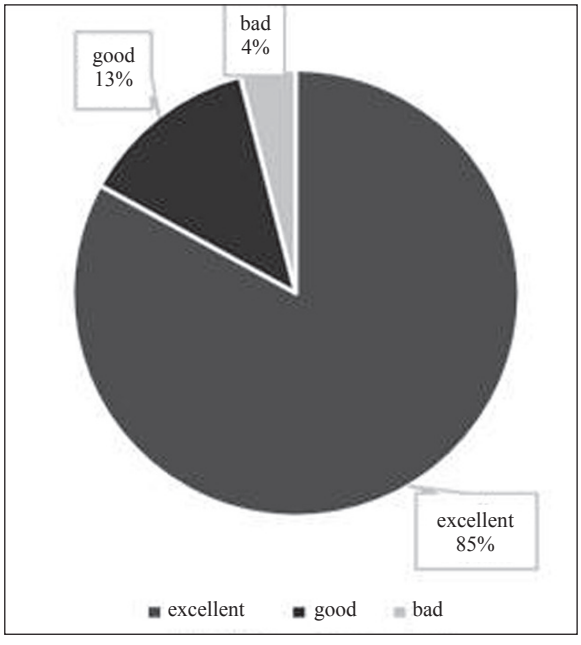

Fig. 4. Chart representing the outcomes of the open surgical decompression of the median nerve in carpal tunnel syndrome (CTS)

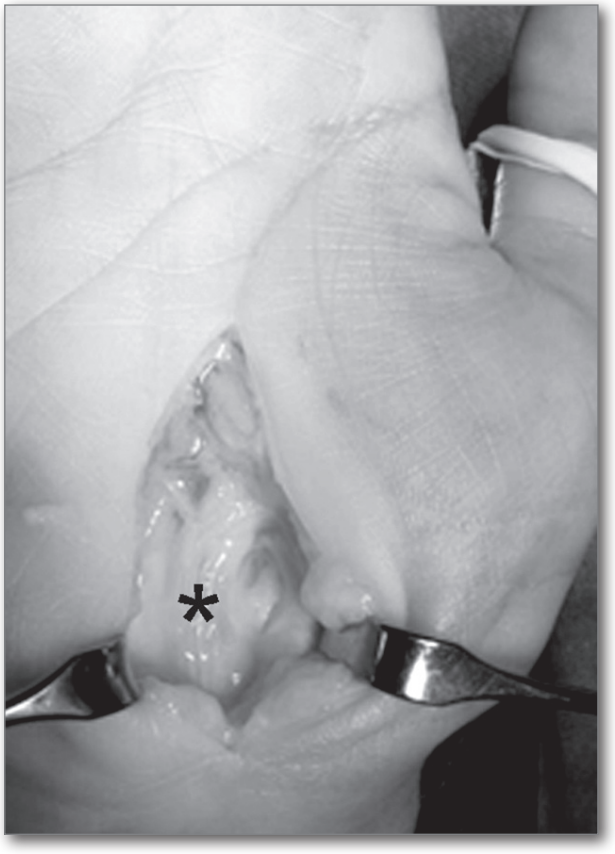

Fig. 5. Intraoperative findings during open nerve decompression due to carpal tunnel syndrome (CTS)

Operative technique

When performing the nerve decompression, the skin incision is on the thenar crease from the distal transversal skin fold of the wrist to the middle of the palm (**Fig. 5**). After incising the fibres of the palmar aponeurosis, it reaches the volar side of the flexor retinaculum, which is explored for perforating branches of the median nerve. Sometimes, the nerve's motor branch can pass through the retinaculum. Under the cover of the Mosquito, the flexor retinaculum is cut from the distal to the proximal end, and it is recommended that this should be done at its ulnar end. After lifting the skin at the proximal end of the wound, the distal fibres of the volar carpal ligament are also cut. The median nerve is inspected. If the epineurium in the compression region is fibrous, it is recommended to cut it along the constriction. Immobilization in the postoperative period is recommended for 2 weeks.

Discussion

In 1933, Learmonth presented the first article regarding the surgical treatment of CTS after releasing the transverse carpal ligament [12]. In spite of the fact that this was the first official publication of surgical median nerve decompression, Galloway may be the first who performed surgical treatment for CTS in 1924, according to a discovered historical letter to his colleague at the start of the new century [12]. Nowadays, median nerve decompression remains also most common operation in the hand. More specifically, the most popular type of surgical treatment remains the open carpal tunnel decompression. Two other approaches have also been described in literature: endoscopic release and mini-incision techniques [11, 15].

The open carpal tunnel decompression should be performed after conservative treatment has failed to produce the desired outcome. Conservative treatment includes wearing splint at night, non-steroidal anti-inflammatory drugs, physiotherapy, local application of cortisone, as well as drugs used to treat neuropathic pain such as pregabalin, gabapentin, amitriptyline, duloxetine, which have a very good effect on night pain and paraesthesia [9].

Different complications after surgical treatment include: infection, haematoma, cutaneous neuralgia, injury of a recurrent motor branch of the median nerve or the superficial palmar arch, excessive scarring and reflex sympathetic dystrophy [16]. Recurrences are rare and due to incomplete division of the flexor retinaculum, inadequate release of the distal part of the antebrachial fascia, lack of normal gliding of the median nerve or iatrogenic trauma. In case of recurrent symptoms, another CTS release is indicated [1, 2]. Tung and Mackinnon [15] proposed surgical techniques such as internal neurolysis, neuroma-in-continuity assessment, neuroma management, nerve grafting and tissue interposition flaps in the second surgery after failed previous operations.

Conclusion

The present retrospective study examined 292 patients after simple open carpal tunnel release. The outcomes confirm that this classic open surgical treatment, which is usually performed under local anaesthesia, is safe, effective and produces excellent results. In our group of patients, we did not report any infections, intra- or postoperative complications. After this procedure, most commonly patients make a fast recovery to their usual life and work. The results of this study are comparable with data available in the literature.

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A Rare Case of Unusual Origin of Extensor Medii Proprius Muscle and its Clinical Significance

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In the current study, we established a variation of the forearm extensor muscles during a routine anatomical dissection of the left upper limb of a cadaver of 64-year-old woman. The variant muscle was represented by the presence of an extensor medii proprius muscle – it originated from the distal part of the extensor indicis muscle and its bundles ran parallel in distal direction. The distal tendon passed through the fourth extensor compartment and inserted into the dorsal aspect of the capsule of the metacarpophalangeal joint of the middle finger. Herein, we describe the unusual origin of this muscle, its relations to the adjacent structures and discuss its possible clinical significance.

Key words: dorsal forearm, variation, extensor medii proprius muscle

Introduction

The extensor indicis muscle is the most medially situated muscle in the deep layer of the dorsal forearm, which originates from the posterior surface of the interosseus membrane and the distal part of the ulna. Its tendon passes through the fourth compartment of the extensor retinaculum and projects into the dorsal aponeurosis of the index finger. In addition, this muscle is widely used for tendon grafting procedures in the field of hand surgery.

In literature, numerous reports describe variations of the extensor tendons, as well as the presence of anomalous muscles in the dorsal forearm. The knowledge of these variant muscles may be useful in the differential diagnosis of pathological lesions such as exostosis, benign adipose tissue tumors, tenosynovitis of the extensor tendons, rheumatoid tenosynovitis, ganglion cysts, etc. Furthermore, the presence of anomalous muscles should be considered in advance in cases of reconstructive surgical procedures in the dorsal forearm and hand. Some of the variants of the extensor indicis muscle include: absence or unusual origin [3, 8]; the presence of two extensor indicis muscles [4]; an accessory slip from the extensor digitorum muscle [13]; the existence of an extensor indicis brevis muscle has also been described [6].

The aim of the current study was to present a rare case of unusual origin of extensor medii proprius muscle in the left dorsal forearm region and discuss its clinical significance.

Case report

During a routine anatomical dissection of the left upper limb of a cadaver, 64-year-old female of Caucasian origin, preserved by an injection of a 10% formalin-based preservative, from the autopsy material available at the Department of Anatomy, Histology and Embryology, Medical University of Sofia, Bulgaria, an additional muscle bundle was found in the posterior forearm region. Dissections were approved by the Medical Legal Office and the Local Ethics Committee. After removing the skin and the subcutaneous adipose connective tissue, the antebrachial fascia and extensor retinaculum were demonstrated and dissected longitudinally in order to observe the deep group muscles. We noted the presence of a variant muscle originating from the distal part of the extensor indicis muscle the bundles of which ran parallel in distal direction. The muscle belly was fusiform, 21 mm in length and 5 mm in width. The distal tendon was about 121 mm long, passed through the fourth extensor compartment along with the extensor indicis and the extensor digitorum muscles and inserted into the dorsal aspect of the capsule of the metacarpophalangeal joint of the middle finger (**Fig. 1**). The dissection revealed that this additional muscle was innervated by branches of the radial nerve.

Fig. 1. Photograph of the extensor medii proprius muscle (arrow)

There was no clinical evidence of trauma or surgical procedures in the dissected region. No other variations in the anatomical structures of the left upper limb were observed.

Discussion

A review of the pertinent literature shows that the described additional muscle is known as extensor medii proprius. It originates from the distal third of the ulna and the interosseous membrane and inserts on the extensor expansion of the middle finger [12]. This anomalous muscle is more frequent in males compared to females and has an incidence between 0.8% and 12% [1, 9, 13]. In addition, meta-analysis reveals that the presence of an extensor medii proprius muscle is significantly lower in Indian and European populations than North American and Japanese populations [15]. In contrast, our study describes an unusual origin of this muscle from the distal part of the muscle belly of the extensor indicis muscle and its bundles have no relations to the ulna and interos-

seous membrane. Li et al. described multiple variations characterized by the presence of bilateral extensor medii proprius with split tendon of extensor indicis proprius [9], while Holmes et al. noted an unusual origin of extensor medii proprius from the lunate bone [5]. The existence of extensor indicis and medii communis muscle has also been discussed [12]. It may be considered as a variation of extensor medii proprius, but its tendon splits into two parts and has attachments to both index and middle fingers [14]. The explanation for the observed variants of the extensor muscles may be associated with the embryonic development. It is known that the dorsal muscle mass of the forearm differentiates into superficial, radial and deep portions. The origin of extensor medii proprius muscle may be associated with the superficial and deep portions, because further they differentiate into muscles, which pass through the metacarpophalangeal joints [11].

Knowledge of anomalous extensor muscles in the dorsal forearm is clinically important. In the field of hand surgery, the tendon of extensor indicis muscle is widely used for tendon grafts [7]. The reason for this is that the extensor digitorum has also an attachment to the index finger. When an additional muscle is present, as the described extensor medii proprius, its tendon may be used for grafting procedures instead of the extensor indicis muscle. Furthermore, the presence of anomalous muscles can be misdiagnosed as ganglion cysts or palpable tumors [5]. Clinical examination may be useful with regard to the differential diagnosis – in a case of variant muscle, it becomes more prominent with active extension of the wrist and fingers, while the wrist flexion demonstrates a ganglion cyst better [2]. Because of the high incidence of variant extensor muscles in the dorsal forearm, any surgical procedures should be planned in advance, because the inappropriate dissection may lead to direct injury to the muscle bundles and postoperative functional reduction can be observed. To help the identification of anomalous muscles, new imaging techniques such as magnetic resonance imaging can be used [10].

Conclusion

The described extensor medii proprius muscle is characterized by an unusual origin. It represents one of the numerous variations of the extensor muscles of the forearm and should be taken into consideration during surgical procedures in the back of the wrist and hand.

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Transradial Catheterization Failure due to High-Bifurcating Hypoplastic Radial Artery: Case Report

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The transradial approach is an excellent alternative to the standard femoral approach for cardiac catheterization with success rates in over 90% of cases and has been widely used. Variations of the radial artery, however, could impede the cardiac catheterization and pose significant challenges to the interventional cardiologist. Herein, we report a failure of transradial heart catheterization due to high-bifurcating hypoplastic radial artery.

Key words: catheterization, hypoplastic radial artery, variations

Introduction

Nowadays, the transradial (TR) cardiac catheterization has been usually performed due to the lower incidence of complications in comparison with the transfemoral (TF) approach [11, 12]. The advantages of the TR approach are due to the fact that the radial artery is located just beneath the skin and is easy to access for hemostasis. In that way, the common complications of haematoma, pseudoaneurysm and arteriovenous fistulas of the femoral approach could be avoided. However, the TR approach could be associated with specific technical challenges and in comparison with the TF approach has relatively high incidence of catheterization failure [10, 11, 12]. TR catherization procedure failures could be due to different anatomical variations in radial artery anatomy and other structures of the upper limb [2-7, 9, 13, 14].

Herein, we describe a case of TR catheterization failure due to high-bifurcating hypoplastic radial artery in a 64-year-old female with clinical and electrocardiography signs of unstable angina pectoris and chest pain.

Case report

A 64-year-old female presented in the Emergency room of our hospital with clinical and electrocardiography signs of unstable angina pectoris and chest pain. A coronary angiogram was planned. Allen's test was performed and TR approach was chosen. The radial artery was successfully accessed with a 6F radial sheath. A 5F Tiger catheter was introduced through a 0.035 guide wire, but the wire could not pass at the level of the proximal brachial region. There was difficulty in catheter advancement, accompanied with pain. The wire was removed and a retrograde contrast injection was given to visualize the obstruction. We found a slender hypoplastic radial artery with high origin from the axillary artery combined with a spasm. We could not pass even with a 4F Tiger catheter. Thereafter, we used an alternative TF access with successful outcome (**Fig. 1**).

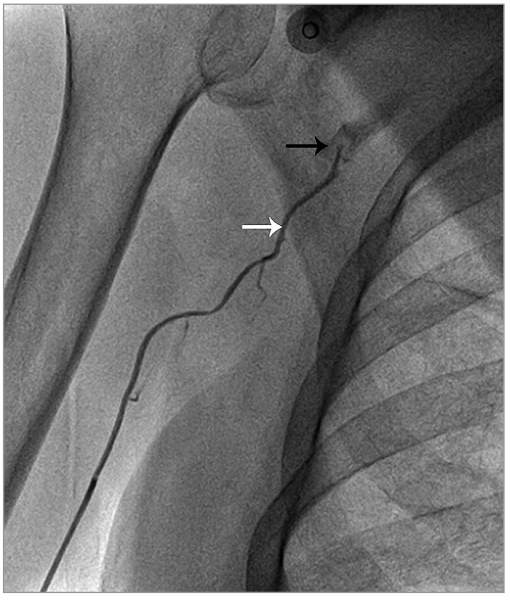

Fig. 1. High-bifurcating hypoplastic radial artery (white arrow) from the axillary artery (black arrow)

Discussion

In 1989, Dr. Lucien Campeau performed the first TR percutaneous diagnostic coronary angiography [1]. After that, in 1993, Dr. Ferdinand Kiemeneij used the TR approach for percutaneous coronary intervention (PCI) [8]. According to literature data, the TR approach has lower vascular complications, lesser hospital stay and healthcare costs in comparison with the TF approach. Moreover, if complications occur, they do not need surgery and usually are treated nonoperatively. The other advantage of the TR approach is the double blood supply to the hand, which prevents hand ischaemia after radial artery thrombosis or spasm [10-12].

Failure of TR approach is between 1-5%. Most commonly, this is due to inability for radial puncture, artery spasm and anatomical abnormalities. Variations of the artery of the arm were between 4-18.5% [10-13]. However, most of them do not impede TR heart catheterization. In rare cases, variations with smaller vascular diameters and especially these of high radial take off associated with a remnant radial artery or a slender radial artery can have such diameter of the radial artery that provokes difficulties in catheter advancement even with a 4F Tiger catheter and can thus lead to failure of TR heart catheterization. Trying to pass in such a remnant artery is painful and commonly associated with spasm and risk of artery perforation.

Conclusion

Interventional cardiologists should be familiar and expect different upper limbs arterial anatomical variations and have a plan to overcome them. Knowledge of such variations will be helpful at their learning curve, and thus in avoiding potential complications. In the case described in the present study, an alternative TF approach is preferable.

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Review Articles

Human Pulmonary Mast Cells: Review

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The aim of the current work was to overview the knowledge regarding the mast cell origin, morphology, mechanisms of mast cell activation and the localization of these cells in normal lung.

In the human respiratory system, the role of mast cells has been examined in two aspects: firstly, as these cells participate into innate and adaptive immunity they are considered to be responsible for lung health, and secondly, mast cell mediators cause and modulate inflammation, and structural and functional remodeling of airways, parenchyma, and vasculature in the respiratory diseases.

The knowledge of mast cell heterogeneity in different lung compartments contributes to clarify the role of these cells in maintaining the homeostasis. Mast cells number in normal lung may be used as referent values in diagnosing lung diseases.

Key words: mast cells, lung, human

Introduction

Mast cells (MCs) play dual role in lung functions. These cells release mediators that contribute to the lung homeostasis and mediators involved in the pathogenesis of lung disorders [7, 25, 32]. Upon activation, MCs release their granule contents, which include proteases, vasoactive amines, proteoglycans, and growth factors [7, 25, 32]. In addition, activated MCs are able to produce a variety of cytokines, chemokines and lipid mediators [7, 25, 32]. The variety of molecules produced by MCs might explain their functions: the recruitment, activation, and differentiation of inflammatory cells [32] and the regulation of vascular permeability [32], smooth-muscle cell contractility [1], and fibroblast growth [32]. Thus, MCs have been found to be involved in the pathogenesis of allergic, chronic inflammatory, and fibrotic diseases. Apart from the mentioned pathological conditions, these cells also play a role in normal physiological processes. The mast cell mediators such as histamine and heparin have been shown to enhance vascularization and endothelial cell proliferation [51]. Furthermore, the bidi-

rectional communication between MCs and sensory nerve cells may provide a homeostatic function such as the regulation of blood flow [32].

Mast cells heterogeneity in human lung has been evaluated concerning their localization, ultrastructure, granule content, receptor expression, mechanisms of their activation and pharmacologic responsiveness [7, 11, 41, 47, 51]. The role of human MCs in the respiratory system has been studied in two aspects: on the one hand, their participation in the maintenance of the healthy lung [4, 7] by contributing to innate and adaptive immunity, and on the other hand, the involvement of mast cells in pathogenesis of lung deseases characterized by allergic, acute and chronic inflammatory, fibrotic and neoplastic processes [2, 7, 41, 51]. The variety of mast cell phenotype is important for development of new effective drugs for respiratory disorders [14].

The aim of this study was to discuss the current knowledge regarding the mast cell origin, morphology and localization in normal lung.

I. Development of mast cells

Mast cell origin

The knowledge regarding the mast cells origin has been undergone considerable evolution over the years. Originally, Combs [12] supposed that mast cells are derived from undifferentiated mesenchymal cells. More precise information about the mast cell progenitors was given by Kirshenbaum et al. [30], who revealed that $CD34^{+}$ $CD117^{+}$ $CD13^{+}$ cells are common precursors for both mast cells and monocytes. Later, Dahlin et al. [15] added that $Fc\varepsilon RI^{+}$ cells development from the Lin^{-} $CD34^{hi}$ $CD117^{int/hi}$ progenitors separates the mast cell lineage from the monocyte lineage and in this regard the Lin^{-} $CD34^{hi}$ $CD117^{int/hi}$ $Fc\varepsilon RI^{+}$ blood cells are considered to be a specific mast cell progenitor. Drew et al. [19] specified that CD34 is lost during mast cell maturation. Therefore, CD34 can be used as a reliable marker to distinguish immature from mature mast cells.

Growth factors involved in mast cells differentiation

Kirshenbaum et al. [29] have found that IL-3 alone and in combination with SCF promoted the growth and survival of human mast cells from bone marrow progenitors. Recently, Toru et al. [46] reported that Interleukin-4 promotes the development of both human tryptase positive mast cells (MCT) and chymase positive mast cells (MCC), whereas stem cell factor (SCF) and IL6 – only MCT.

II. Mast cells heterogeneity

The mast cells heterogeneity in human lung has been evaluated regarding their ultrastructure, granule content, receptor expression, mechanisms of their activation and pharmacologic responsiveness, localization in healthy lungs and in specific lung diseases.

Ultrastructural features

Human MCs possess one nucleus, one or two nucleoli, mitochondria, ribosomes and endoplasmic reticulum, Golgi apparatus, membranous secretory granules with a diameter of 0.5-0.7 μm [11, 47, 50]. Caulfield et al. [11] observed that unstimulated MCs possess about 2 μm long folds of their plasmalemma. Intermediate filaments were described predominantly in perinuclear region, but a few filaments were located in the surface folds and under the plasma membrane. According to the presence of the contents two types of granules were identified - crystalline (0.5 Å in diameter) and amorphous (1mm in diameter) [11].

After immunoglobuline E (IgE)-dependent stimulation, Caulfield et al. [11] observed only amorphous granules. The mentioned authors revealed two ways of granules discharge: firstly, by fusing with the plasma membrane, and secondly, by fusing with other granule membranes to form deep channels or labyrinths consisting of membranes that are involved in the exocytosis. This kind of discharge was described as typical for mast cells and distinguish them from other cell types [51].

Size and shape of mast cells

The size and shape of human lung MCs are described in detail by several authors [3, 11, 47]. According to these authors, human MCs are large cells with a diameter of 10 to 20 μm. Andersson et al. [3] observed mast cells with different size depending on the protease content of their granules. With respect to their size, MCT localized in bronchi and small airways are significantly larger than MCT in pulmonary vessels. Mast cells, containing both tryptases and chymases (MCTC) in pulmonary vessel walls are larger than MCTC in bronchial and small airway walls. Alveolar MCTC are smaller than MCT.

With respect to their shape, MCTC are significantly more circular in all compartments (except the alveolar septa) than MCT in the bronchial wall, small airways and pulmonary vessels [3]. With respect to the FcεRI expression by lung MCs, only the alveolar MCs express no FcεRI [3]. The physiological function of alveolar MCs has been still uncleared [3]. In this respect, the large alveolar mast cells number deserves attention in order to clarify their biological and pathological role.

Mast cells in fetus

Morphological studies on mast cells in human embryo and fetus tissues are scarce. For example, Kitamura et al. [31] described mast cells in human embryo between 15th to 60th days. MCs with metachromatic granules were observed in the tissue of human embryos after the 2nd month. In the 5th and 6th month old human fetus mast cells were defined in the kidney, liver, spleen, skin and muscles, but were of smaller size than in adults [31]. However, we did not find the data regarding mast cells localization in fetal lung.

In normal lung of infants, dos Santos et al. [16] observed larger number of MCT in outer layer than in epithelial layer of airways. However, the authors did not present the data about chymase-positive mast cells. In the airways, the role of these cells has been assumed to be associated with the promotion of the vascular permeability, airway obstruction, and leukocyte recruitment [32].

Mast cells mediators. Histochemical and immunohistochemical features

The heterogeneity of MCs involves differences in morphology, histochemical [36] and immunocytochemical [42] characteristics of mediators in their granules [7, 32]. Mediators secreted by mast cells are usually subdivided into two types: preformed and secretory granule-associated, and newly generated after activation. Preformed mediators include histamine, proteoglycans (heparin, chondroitin sulfate E), serotonin, proteases (such as tryptase, chymase, β-Hexosaminidase, β-Glucuronidase, β-D-galactosidase, cathepsin G and carboxypeptidase), some cytokines such as IL-4 and IL-15, and growth factors (basic fibroblast growth factor, bFGF, tumor necrosis factor alpha (TNF-α), nerve growth factor (NGF), transforming growth factor-b (TGFb), vascular endothelial growth factor (VEGF) [7, 32]. The newly synthesized mediators releaseing after activation are the lipid mediators (prostaglandin D_2 and leukotrienes, generated from arachidonic acid), thromboxanes ($TXAB_2$), 5, 12-hydroxyeicosatetraenoic acid, nitrogen radicals, oxygen radicals, inflammatory cytokines and chemokines [7, 32].

The mast cells were identified as cells which are stained metachromatically with methylene and toluidine blue, and fluorescent binding of berberine to the granules of mast cells due to the presence of glycosaminoglycans [20, 46, 52]. The first classification of MCs into two subtypes: connective tissue mast cells and mucosal mast cells was made in rodents and was based firstly on distinct staining characteristics, fixative properties and anatomical location but later on morphology, granule content and function of the mentioned cells [25, 52].

Based on their protease content human mature MCs were divided in two phenotypes. For example, MCTC concist of tryptases, chymases, and carboxypeptidases, whereas MCT contain tryptases only [25]. A third phenotype of MCs expressing tryptase and carboxipeptidase A3, but not chymase, was described in the airway epithelium in asthmatic subjects (17).

Bradding et al. [6] reported that human MCs are also heterogeneous regarding the cytokine content. IL-4 is expressed predominantly by the MCTC (85%), whereas MCT - 15%. The authors reported that MC phenotypes contain tryptase, heparin, chondroitin-sulfates A and E, histamine and IL4. In contrast, MCT contain also IL-5 and IL-6. This suggests that the different biological functions of mast cell types also depend on their capacity to generate and release different cytokines.

Pulmonary MCs were found to be heterogeneous with respect to both size and granule content [39]. The histamine content in normal human lung ranged from 2.5 ± 0.5 pg/MC for the smallest diameter MCs (up to10 μm) to 10 ±2.5 pg/MC for the largest (16-20 μm). Later, Van Overveld et al. [49] revealed that in lung, histamine content of MCs depends on their formalin sensitiveness.

In lung, the mast cell heterogeneity based on formalin sensitiveness was described by Van Overveld et al. [49]. According to mentioned authors, MCs cells have been defined as formalin sensitive and formalin insensitive. For example, formalin-sensitive MCs release leukotriene C release, whereas formalin-insensitive MCs showed no release of leukotriene C.

Unlike in rodent MCs, histochemical heterogeneity, based on the presence or absence of heparin proteoglycan, is not a useful marker for distinguishing different subtypes of human mast cells. In this respect, Craig et al. [13] revealed that all human MCs contain heparin. Mast cells heterogeneity in humans is based mainly on the protease content and in this relation two subtypes have been identified: tryptase containing mast cells or mucosal mast cells and mast cells containing both tryptase and chymase or connective tissue mast cells [25]. Immunohistochemical studies using antitryptase antibody proved that tryptase is a selective marker for human MCs [25, 32]. The protease content of human MCs is distributed in a tissue-specific manner and it has been suggested that mast cell phenotype is controlled by microenvironmental factors [25].

Mast cell tryptases, a tetrameric neutral serine protease with a molecular weight of 134 kDa, hydrolyzes protein and peptide bonds on the COOH-terminal side of residues [40, 82]. The genes encoding mast cell tryptase are located on the chromosome 16 [40, 42]. There are two main types of mast cell tryptase, α-tryptase (subdivided into α I- and αII-tryptases) and β-tryptase (subdivided into β I-, β II-, and β III-tryptases) [34, 40]. βII-tryptase is stored in the secretory granules of MCs, whereas α-protryptase secreted from MCs as an inactive proenzyme was found in the blood of normal subjects.

Protease activated receptors (PAR)

Mast cell tryptase activates protease activated receptors (PAR) receptors which belong to G-protein-coupled receptors. β-Tryptase activates the PAR-2 receptor [27] icreasing intracellular Ca^{2+} [5]. PARs were observed in airway epithelial and smooth mus-

cle cells, terminal bronchial epithelium, type II pneumocytes, endothelial and vascular smooth muscle cells, and MCs within the respiratory tract [5].

Biologic role of tryptase

Tryptase stimulates the release of high-molecular-mass kininogen (HMMK) – peptide histidine methionine (PHM) and calcitonin gene related peptide (CGRP) which increase bronchial muscle contractility [8; 44]. In epithelial cells, the tryptase has been shown to stimulate a catalytic site dependent release of IL-8, which was also associated with increase of intercellular adhesion molecule-1 (ICAM-1) expression. Thus tryptase may stimulate epithelial repair and in the recruitment of granulocytes following mast cell activation [7].

VIP is known as a mediator of nonadrenergic relaxation of airway smooth muscle [9]. It is known that mast cell tryptase hydrolyzes VIP at two sites: Arg14-Lys15 and Lys2'-Lys21 [9]. On the other hand, there is an evidence that tryptase does not hydrolyze the bronchoconstricting neuropeptide substance P of airway sensory neurons [8]. These studies show the important role of mast cell proteases in modulating neural control of airway tone, which can be achieved by tryptase impact on bronchodilator VIP but not on the bronchoconstrictor substance P.

Mast cell chymases

Chymases are mast cell specific serine proteases with a chymotrypsin-like specificity. Humans express only one mast cell chymase which is encoded by CMA1 gene [51]. The presence of chymases in mast cells was detected originally by histochemical studies. Gomori, 1953 using a-naphthol chloroacyl derivatives as histochemical substrates, defined strong esterase reactivity in MCs. According to Wong et al., [53], the mentioned histochemical technique was used to identify MCs in human skin, whereas for human airway the technique was less useful because only a part of mast cells of human lung contains chymase like esterolytic activity. In this regard, using antibodies against chymase Irani et al. [25] established that only 10% of MCs in the lung contain chymase, but in skin 90% of mast cells are chymase positive. The same authors also found that in the lamina propria of airways, the MCC number is the largest. Therefore, in human lung, the release and actions of chymase depends on the microenvironment.

Biologic role of chymase

Sommerhoff et al., (1989) revealed that more than 70% of the MCs within bronchial submucosal glands contain chymase, suggesting the role for chymase in the physiologic regulation of airway gland secretion [43]. Chymase was found to stimulate cultured serous cell secretion, whereas tryptase had no effect. The findings suggest a potential role for chymase in airway hypersecretion, as in asthma or bronchitis, but the molecular mechanisms for these secretagogue effects are not known [43]. This role of chymase was confirmed by Caughey [10] who also reported other possible roles for chymase including inactivation of sensory neuropeptides, and an increase of histamine dependent vascular permeability [10, 15].

Other role of human chymase is to convert angiotensin I to angiotensin II independent of angiotensin converting enzyme in vivo and in vitro playing a role in blood pressure regulation [23]. Chymase can inactivate bronchoactive peptides such as bradykinin and kaffidin [38] as well as neuropeptides, such as VIP [22]. Thus chymase, unlike tryptase, can inactivate some bronchoconstrictors, resulting in limitation of mast cell degranulation. Mast cell chymase, like tryptase, participate in activation of metalloproteinases MMP-1 and MMP-3 [7, 32].

Histamin leads to smooth muscle contraction

Pietra et al. [37] established that histamine causes increased mucous secretion, pulmonary vasoconstriction and induces bronchial venular permeability resulting in pulmonary edema [37]. Histamine exerts its effects on lung by interacting with two cell membrane-associated receptors: H1 and H2 receptors [18, 48]. H1receptor is responsible for vasoconstriction in lung, bronchial smooth muscle contraction, and systemic vasodepression [48], whereas H2 activation leads to pulmonary vasodepression [18].

III. Activation of mast cells

Mast cells can undergo activation by antigens, superoxides, complement proteins, neuropeptides, and lipoproteins [32]. Since mast cells activation in the airways results in the release of proinflammatory mediators into the surrounding tissue, exposure to environmental stimuli may result in chronic inflammation [32]. The most established involvement of MCs is their activation by surface-bound IgE leading to rapid degranulation, mediator release and development of allergic reaction [32]. Released histamine and serotonin lead to endothelial gap formation in post capillary venules and extravasation of plasma into the airway wall and lumen, causing airway oedema [35]. According to Kennedy et al. [28] alveolar parenchyma has mechanisms for local downregulation of IgE receptor Fc-epsilon-RI which prevent an anaphylactic degranulation in these regions.

Other stimuli may also stimulate degranulation, such as components of the complement, neurotransmitters, osmotic changes and mechanical damage [7, 32]. For example, MCs express Fc-gamma receptors that can be engaged by immune complexes, complement receptors that can be triggered by C3a and C5a [30] and c-kit, the receptor for stem cell factor [7, 32]. Finally, activation of Toll-like receptors (TLR), which are also expressed by MC [7, 32] might be activated by bacterial products which results in production of leukotrienes, cytokines and chemokines.

Activation of MCs can lead to production of effector molecules including prestored mediators (serotonin, histamine, proteases), and actively synthesized mediators released within minutes (prostaglandins, leukotrienes) and a large variety of cytokines and chemokines at several hours after activation. The role these mediators play in tissue remodeling is poorly understood. Mast cells are a source of IL-4 and IL-13 that can influence T cell responses, mucus gland hyperplasia and smooth muscle hypertrophy or hyperplasia [7, 25, 32]. Angiogenesis is another feature of tissue remodeling and MCs can be a major source of angiogenic factors such as VEGF [5].

Apart from their proinflammatory actions, MCs exert their anti-inflammatory effects by releasing the antiinflammatory cytokine IL-10 [26]. Another antiinflammatory action is the ability of mast cell granule proteases to neutralise key cytokines, such as tumor necrosis factor α (TNF-α), IL-4, IL-13 and IL-33 [7, 32]. Thus, MCs are regarded as local immune modulators which regulate the balance between pro- and antiinflammatory responses.

IV. Localization of mast cells in normal lung

In normal lung, many studies described the mast cell localization and density depending on both staining with toluidine blue – toluidine blue positve (MCTB), and proteinase content: tryptase only - MCT, tryptase and chymase - MCTC, and chymase only - MCC positive mast cells. Andersson et al. [4] revealed a lack of a MCC in healthy subjects. According to Marshall and Bienenstock [33] MCT in the alveolar tissue of human lung represent 93%, whereas MCTC - 7% of the total lung MCs. In bronchial wall, the percentage of MCT (77%) is also higher than MCTC (23%). In bronchial wall, the percentage of MCTC is higher than in alveolar tissue.

The number of MCs was evaluated as mean number per square millimeter or mean number per high-power field. In this regard, MCs were found to be localized near blood vessels and within lamina propria of airways in healthy subjects [7, 36]. Hunt et al. [24] estimated mast cell density in the peribronchiolar regions (mean, 13.7 ± 3.5 cells per high-power field). In normal lung, MCs were distributed predominantly within connective tissue around airways and vascular structures. Brightling et al., 2002 revealed that in the submucosal layer of the bronchi of normal lungs, the average number of MCT is 17 cells/mm^2. The number of MCs in smooth muscle was found to be significantly smaller (from 0 to 5).

More precise description of mast cell density in normal lung was presented by Caroll et al. [7]. A few MCs were observed by these authors in the alveolar septa near the small blood vessels. In contrast to the mentioned authors, other researchers revealed that the alveolar parenchyma in the human lung contains significantly more MCs than in the rodents [3, 21, 49]. Important finding is the different mast cells distribution detected in the wall of cartilaginous and membranous airways. In cartilaginous airways, the density of MCs was highest on the smooth muscle (74 MCs/mm^2), intermediate in the inner airway wall (35 MCs/mm^2), in stromal tissue surrounding glands (40 MCs /mm^2) and outer airway wall (7 MCs/mm^2), and lowest in the epithelium (1.5 MCs/mm^2) and lumen (0.2 MCs/mm^2) [7]. In membranous airways the highest density of MCs was on the smooth muscle (201 MCs/mm^2) and in the outer airway wall area (163 cells/mm^2). Mast cell density in the inner wall, in lumen and epithelium was significantly lower (78 MCs/mm^2, 1 MC/mm^2 and 0.8 MCs/mm^2, respectively) [7].

Some authors observed intraepithelial localization of MCs in the bronchial mucosa of normal human lung [7, 36]. However, the number of intraepithelial MCs in healthy lungs is significantly lower than in lungs in pathological state [36].

Conclusion

In human lung, site-specific MCT and MCTC populations are identified in small and large airways, pulmonary vessels and the alveolar parenchyma. Due to their presence and multifunctional capacity, MCs are most probably involved in most allergic and nonallergic diseases in lung. The knowledge of mast cell heterogeneity in different lung compartments contributes to clarify the role of these cells in maintaining the homeostasis. The data regarding the number of mast cells in normal lung can be very useful as referent values in diagnosing lung diseases.

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The Role of Diabetes Mellitus in Male Reproductive Function: Review

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Diabetes mellitus (DM) induced long-term damage, dysfunctions and failures of various organs including abnormalities in male reproductive system. A lot of studies in both human and animals show that both type I and type II diabetes can cause male infertility via action at multiple levels including altered spermatogenesis, sperm count and quality, degenerative and apoptotic change in germ cells, impaired glucose metabolism in Sertoli cells compromised testosterone production and secretion and ejaculatory dysfunction. The aim of current review is to analyze the mechanisms of the two types of DM (I and II) and their impact on spermatogenesis on cellular and molecular level, evaluating hyperglycemia as a risk factor for male infertility.

Key words: diabetes mellitus, hyperglycemia, spermatogenesis, germ cells, male infertility

Introduction

Metabolic syndrome involves various abnormalities like obesity, insulin resistance/diabetes, hypertension, hormonal disorders being serious risk factor for male infertility, often associated with compromised hypothalamic-pituitary-gonadal axis. The prevalence of diabetes mellitus (DM) has risen in recent years and it was estimated that 382 million people suffer from this disease [31]. It is a chronic metabolic disorder characterized by chronic hyperglycemia impairing fat and protein metabolism. DM instigates long-term damage, dysfunctions and failures of various organs including abnormalities in male reproductive system. Type I DM is consider as a chronic autoimmune disease with a strong inflammatory component, characterized by loss of the insulin-producing beta cells of the islets of Langerhans in the pancreas, leading to insulin deficiency [26, 53]. Type II DM is characterized by insulin resistance due to compromised functioning of insulin receptors which in some cases might be combined with relatively reduced insulin secretion [18]. A number of lifestyle factors are known to be important to the development of type 2 diabetes, including obesity, lack of physical activity, poor diet, stress, and urbanization [32, 52]. Glucose metabolism is an important factor for normal spermatogenesis and steroidogenesis and disturbance in these processes can cause male

infertility [14, 36]. A lot of studies in both human and animals show that both type I and type II diabetes can cause male infertility via action at multiple levels including altered spermatogenesis, sperm count and quality, degenerative and apoptotic change in germ cells, impaired glucose metabolism in Sertoli cells compromised testosterone production and secretion and ejaculatory dysfunction [2, 21, 35].

Streptozotocin (STZ) or alloxan are used to chemically induce diabetes in rats in order to study the effect and pathogenesis of DM. STZ and alloxan share structural similarity with glucose and thus can be taken via the cell membrane glucose transporter 2 (GLUT2) causing DNA alkylation and eventual β-cells death [7, 40].

The aim of current review is to analyze the mechanisms of the two types of DM (type I and type II) and their impact on spermatogenesis on cellular and molecular level, evaluating hyperglycemia as a risk factor for male infertility.

Type I DM and spermatogenesis

DM type I is associated with male reproductive dysfunction, reduced fertility and poor sperm quality [44]. Mechanisms including changes in hormonal panels, oxidative stress, DNA damage of sperm and abnormal spermatogenesis have been reported in studies on rodent models [45]. These effects might be mediated through hormonal changes in the hypothalamic-pituitary-gonadal axis or through direct influence of insulin on the testis and spermatogenesis. It is still unclear whether the damage is induced by changes in glucose levels or by impairment in hormonal levels due to compromised hypothalamic-pituitary-gonadal axis [39]. The hypothalamus releases GnRH stimulating the pituitary to secrete LH and FSH. LH and FSH act on Sertoli cells (SCs) and Leydig cells (LCs) of the testis, respectively, stimulating spermatogenesis. DM induced changes in the levels of insulin and leptin alters GnRH release from the hypothalamus producing downstream effects on LH and FSH secretion from the pituitary, testosterone secretion from the LCs, qualitative and quantitative characteristics of spermatogenesis. As FSH and LH considered survival factor for germ cell, the reductions of levels of both of hormones triggers apoptotic cascade in germ cells [19].

The effects of DM on the reproductive axis are mediated by brain signaling. Insulin mediates its effect through binding with insulin receptor resulting in signaling cascade. How exactly insulin action in the brain impacts reproductive axis is still unknown. However, studies using a brain-specific insulin receptor knockout showed the connection between insulin signaling in the brain and fertility. Data from neuron-specific insulin receptor knockout mice (NIRKO) displayed a significant reduction in fertility and impaired spermatogenesis. Histological examinations revealed that many of the seminiferous tubules appeared normal but approximately 20% did not have a lumen and had a little or no mature sperm cells. Another study on STZ induced diabetic rats demonstrated significant decrease in diameter of seminiferous tubules [16].

Insulin/IGF1 signaling is implicated in male reproductive system and both are secreted by Leydig and Sertoli cells, suggesting its regulatory role on spermatogenesis. Consistent with this, receptors for IGF1 and/or insulin have been described at all stages of spermatogenesis in a variety of mammals. Gene knockout studies have further demonstrated the importance of the insulin/IGF1 signaling pathway in growth and reproduction. Deletion of Igf1 causes reductions in body weight, testis weight, testosterone concentration, and sperm counts. In the absence of the insulin family of receptors, male sex determination during embryonic development is inhibited [29].

In type I, deficiency of insulin production is linked with circulating levels of leptin, which is an important molecule secreted by the fat cells, responsible for the signals to the hypothalamus and thus for regulation of the reproductive function. Leptin-treated male animals had significantly elevated serum levels of FSH, increased testicular and

seminal vesicle weight, elevated sperm counts compared to controls, suggesting that leptin stimulates reproduction in males and may serve as a permissive signal to reproductive system of normal animals [13]. Results in humans, showed that leptin levels were decreased in newly-diagnosed type 1 diabetes patients [10]. A recent study showed that leptin-therapy in non-obese diabetic mouse restored normal blood glucose levels suggesting that restoration of leptin levels in type I DM might be able to reverse many of the effects of insulin deficiency on reproductive system [62].

It is well known that both leptin and insulin interact with the hypothalamus regulating the output of GnRH. The relationship between LH and insulin has been shown in transgenic mice that lacked brain insulin receptors [17]. Reduction by 60% in the circulating LH was found which indicated dysfunction of hypothalamus-pituitary-gonadal axis and testosterone deficiency. A significant reduction in number of LCs was reported in this type transgenic animals [12]. Also, insulin is an imperative role on control of cell proliferation and metabolism of LCs [7]. In vitro studies with primary pituitary cultures demonstrate induced release of LH in the presence of insulin-like growth factor-1 (IGF-1) and insulin [64]. In type I DM, the absence of the inductive effect of insulin on GnRH-induced LH and FSH release, inhibits testosterone production by LCs of the testis.

Clinical investigation on diabetic patients reported that LH secretion in response to GnRH signals was lower in this patient population than in healthy controls [11]. Similar results by Lopez-Alvarenga et al. concluded that type I DM affected the hypothalamic-pituitary-gonadal axis resulting in decreased LH secretion and hence decreased testosterone production [39]. Data by Maneesh et al. [42] on diabetic and controlled patients found out that men with DM had reduced testosterone, LH and FSH levels [42].

Another study in sixty-nine men with type I diabetes revealed that only 7% had low total testosterone levels. By contrast 20.3% with type I diabetes had low calculated free testosterone, similar to that observed in type II diabetes. Low testosterone levels were independently associated with insulin resistance in men with type I diabetes as well as type II diabetes. Serial measurements also reveal an inverse relationship between changes in testosterone and insulin resistance [30]. Normalization of the plasma testosterone concentration were observed after 4 days of insulin treatment in newly-diagnosed type I diabetic patients [28]. All these data suggest that testosterone level is impaired by type I diabetes which could lead to inhibition of spermatogenesis.

Taken together the data from animal models and clinical studies about decreased LH secretion, reported disturbance of spermatogenesis, especially later stages, associated by decreased testosterone production by LCs.

One of the key enzymes in androgen biosynthesis is the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) which is a marker for LC steroidogenesis. Data from STZ-induced diabetic rats suggested an increase in smooth endoplasmic reticulum, mitochondrial and lipid content in LCs, together with decreased 3β-HSD activity and serum testosterone levels [7]. Recent studies revealed that the expression of 3 β-HSD was significantly decreased in LCs from the diabetic mice [37]. It is reported that the number of LH binding sites in LCs from diabetic rats was severely lowered after induction of diabetes, leading to suppression of synthesis and secretion of testosterone associated with altered expression of androgen receptor and IGF-1 [12, 34].

It is well known that germ cells are highly differentiated cells. In order to acquire and maintain motility, as well as to complete capacitation and the following acrosome reaction, the male gametes need a lot of energy. The main sources of energy for sperms are the carbohydrates, such as glucose and fructose. Sugars are rich in –OH groups, thus making them polar molecules and impeding their passage through the membrane lipid bilayer. Membrane proteins, such as sodium-dependent glucose transporters (SDGTs)

and glucose transporters (GLUTs) are necessary for sugar uptake. In mature sperm, GLUTs are important for uptake of sugars used as energy source for motility and fertilization ability. The hyperglycemia typical for diabetes cause downregulation of GLUTs which leads to lower intracellular glucose levels in germ cells [49]. Studies in type I DM mice showed that two members of the GLUT family - GLUT8 and GLUT9b were decreased in testes and GLUT9a was undetectable and insulin treatment for 7 days improves sperm motility and fertility [2].

Sperm cells of men with type I DM have reduced motility and decreased *zona pellucida* binding, as well as structural defects and nuclear and mitochondrial DNA fragmentation [2]. A variety of factors can lead to this increased oxidative stress, generation of free radicals and subsequent DNA damage. However, under pathophysiological condition, such as hyperglycemia in diabetic patients the proteins or lipids become glycated as a result of sugar exposure and they form the so called advanced glycation end products (AGEs) [61]. The number of sperms expressing the receptor for AGEs (RAGE) and increased protein amount, were prominently higher in samples from type I diabetic men [41]. The interaction between RAGE and its AGEs-ligands leads to increased reactive oxygen radicals production which in turn can induce DNA fragmentation [33]. The response to cellular oxidative stress required a complex network of sensors and effectors from multiple signaling pathways and in the center of which is the nuclear enzyme poly(ADP-ribose) (PAR) polymerase-1 (PARP-1) [23]. The latter is covalently linked to poly(ADP-ribose) polymers that serve as a signal and a platform for recruitment of proteins associated with the DNA damage response that promote chromatin remodeling, DNA repair, cell cycle arrest, senescence, or cell death. One such protein is tumor suppressor protein p53 that plays a central role in the induction of germ cell apoptosis [55].

Diabetes associated germ cell apoptosis is mediated by two major apoptosis pathways following reproductive dysfunction: the extrinsic death-receptor pathway (Fas-membrane signaling) and the intrinsic mitochondrial pathway. The regulation of these apoptotic pathways occurred through members of Bcl-2 family (pro- and anti-apoptotic proteins) [19]. DM induced germ cells death occurs in the testis of both type I and II DM. A significant increase in germ cell death in the testis of STZ-induced type I diabetic rats and mice has been documented by several studies [60]. In diabetic males, germ cells apoptosis in stage VII and VIII was also reported probably as a result of reduced LH/FSH and low serum testosterone levels. Multinucleated germ cells or giant cells linked with germ cells apoptosis were found in seminiferous tubules in diabetic rats.

Ultrastructural features of germ cell apoptosis were observed such as intense cytoplasm vacuolization of spermatogonia, apoptotic nuclei with dense nuclear chromatin in spermatogonia and spermatocytes. Deposition of intracytoplasmic dark substances (lypofuscin) in germ cells are most frequently observed ultrastructural changes in testis of diabetic rats. Diabetic animals at 1 year of follow-up presented greater amount of spermatids and spermatozoa with defects in the dense fiber complex or in mitochondrial sheet or axoneme [3, 34, 51, 59].

Sertoli cells (SCs) play a key role in testicular development, orchestrating and regulating proliferation and differentiation of germ cells, Leydig cells and peritubular myoid cells. During puberty, the role of SCs are responsible to support germ-cell differentiation. At this terminally differentiated stage the SCs are considered as non-proliferative, becoming the main component of blood-testis barrier (BTB) [54].

In diabetic rats, remarkable changes were observed in the mitochondria of SCs. These organelles had some abnormalities in their shape and quantity which may reflect their abnormal function [34]. Previous studies also indicated that denatured and decomposed SCs cytoplasm were observed in STZ-induced DM in animal models [55].

BTB is responsible for maintaining different levels of substrates and metabolites between germ cells and blood. This is possible due to specific glucose sensitive machinery that is under strict hormonal control mainly by sex hormones and FSH. This hormones receptors are located in SCs and they are very sensitive to extracellular glucose levels [38]. Glucose passive transport across the BTB is executed via glucose transporters which isoforms GLUT 1, GLUT3 and GLUT8 have been found in SCs [20, 27]. Sertoli cells produce lactate at a high rate as it is the main metabolite used by the pachytene spermatocytes and spermatids to meet their energy requirements [5]. The lactate is exported to the intratubular fluid by proton-coupled membrane monocarboxylate transporters (MCTs), mainly MCT4. Studies suggested that insulin deprivation does not affect significantly glucose consumption of SCs due to compensational increase of the expression of GLUT1 but lactate production rate and the levels of MCT4 were severely reduced [46]. Additionally, SCs produce high amounts of acetate, which is essential for the lipid synthesis in the germ cells [4]. Interestingly, some studies suggest a role for alternative substrates in SCs metabolism during hyperglycemic conditions. The main alternative fuels for the SCs that can maintain spermatogenesis are monocarboxylic acids, fatty acids and ketone bodies [22]. By downregulating gene transcript levels of Acetyl-CoA hydrolase, insulin-deprived SCs completely suppress the acetate production thus ensuring Krebs cycle stimulation. This mechanism ensures the survival of SCs while compromising lactate production and germ cells development.

Different cell markers are used in order to better understand the molecular mechanisms behind the DM-induced changes in spermatogenesis. Vascular endothelial growth factor (VEGF) and nerve growth factor beta (NGF-β) are important neurotrophic factors for male reproductive system. Reduction of VEGF and NGF-β levels were observed in diabetic testes and are associated with increase of apoptosis in diabetic rats. Testicular VEGF and NGF-β could be potential novel biomarkers for diabetes induced testicular damage [56]. Proliferating cell nuclear antigen (PCNA) is well known marker for DNA synthesis. PCNA is expressed in spermatogonia and early phase primary spermatocytes at all stages of seminiferous tubules. PCNA positive cells were strongly detected in non-diabetic in contrast with diabetic animals [3]. Decrease of PCNA in the germ cells of diabetic animals indicates reduction of proliferative activity in spermatogenesis.

Our previous studies showed that testicular angiotensin converting enzyme (tACE) is useful marker for developmental stage of germ cell differentiation and fertility [9]. This isoform is expressed in germ cells during spermiogenesis and is localized in elongating spermatids and spermatozoa. Based on the expression profile of tACE in diabetic rats, these results provided the first evidence that prepubertally testis is more affected by hyperglycemia than adult testis [9]. Epidemiological studies are in concert with these findings. Number of live births in a population-based, retrospective cohort of 2819 men with childhood-onset DM I showed that men with diabetes had a smaller number of live births than controls. Later age at onset of DM was associated with a higher rate of having a first child among men [17, 57].

Morphological and morphometric studies on diabetic rats demonstrated decrease in macro parameters as epididymal and testicular weight, diameter of seminiferous tubules, height of seminiferous epithelium. The morphology of the tubules vary from totally or partially disorganized epithelium with impaired organization of spermatogenic stages. Atrophy of tubules with varying degree of spermatogenesis was detected. Vacuolization of epithelium in many areas have been reported. Our previous comparative studies on DM induced neonatally or prepubertally in rats, showed that testicular morphology was more affected in prepubertally induced DM, compared to neonatally induced DM. Spermatogenesis is not completed and different degree of delay in spermatids development was observed [9].

In clinical studies patients with DM I had a lower percentage of spermatozoa with progressive motility and a higher percentage of spermatozoa with abnormal mitochondrial function than controls. Disruption of spermatozoa mitochondrial function due to ultrastructural mitochondrial alterations may be responsible for the decline in spermatozoa motility observed in DM I patients [6, 47]. Semen analysis of patients with both types I and II DM showed alterations in progressive sperm motility and significantly compromised sperm morphology, while the sperm concentration did not show significant differences [24].

In vitro fertilization using sperm from diabetic men produced far fewer pregnancies compared to healthy men [44]. This suggested that sperms of diabetic men are able to fertilize eggs but DNA damage might occur and prevents competent embryo development. Data from animals confirmed that fertilization rates were significantly lower in STZ-injected male mice. Embryo development rates to the blastocyst stage is lower in diabetic animals when compared with controls [58].

Erectile dysfunction is commonly reported condition in diabetic patients and is defined as inability to achieve and/or maintain an erection. Diabetic males showed a threefold higher probability to develop erectile dysfunction in comparison with non-diabetic men [43]. The pathogenesis behind this condition may include vascular insufficiency and neuropathy which further deteriorates into ejaculation dysfunction and decreased libido. Oxidative stress-mediated neurovascular alterations in diabetic patients is responsible for impaired endothelial function and neuropathy in the *corpus cavernosum* in diabetic men [15]. Erectile dysfunction appears at the early stage in diabetic males and its effects increase with the duration of the disease.

Type II DM and spermatogenesis

Type II diabetes mellitus is one of the most prevalent serious metabolic diseases affecting individuals on modern societies. Recently, a prodromal stage called pre-diabetes has been described and considered a high-risk factor for type II DM. These pathophysiological condition affects male reproductive function in particular testicular metabolism, leading to disturbance in testosterone synthesis [50]. Evidence has shown that 40% of men with type II DM present testosterone deficiency [25] leading to abnormal function of SCs [48]. Expression of inhibin B and androgen receptor, which are produced by mature SCs, are dramatically changed in conditions of DM II. The ultrastructural study of testicular tissue in insulin-resistant rats demonstrated remarkable changes not only in SCs but in LCs as well. It has been reported that, in patients with type II DM, the activities of some mitochondrial enzymes decrease and also some abnormalities in the shapes of these organelles have been noted [1]. Low concentration of glucose in *ad luminal* parts of seminiferous tubules exerts effect on metabolism of developing germ cells located in this region of the tubules.

In comparison with alloxan/STZ induced diabetic rats, severe histological changes have been observed in insulin-resistant diabetic animals, resulting in hypospermatogenesis in over 90% of tubules [8]. Histological samples from DM II mice revealed many seminiferous tubules with significant or partial depletion of germ cells, along with several multinucleated giant cells and vacuoles. Diabetes type II mice displayed a significant elevation of germ cell apoptosis, presumably via receptor-mediated caspase-8 activation. Although a significant increase in testicular cell death was observed, there was no concomitant significant change in testicular weight [63].

Conclusion

The great concern of recent society about dropping the average age at diagnosis of DM, required special attention in tandem with clinical and research priorities. Indeed, more than 90% of these patients are diagnosed before age of 30. The epidemic increase in diabetes, one of the human metabolic disorders demonstrate that glucose metabolism is essential for spermatogenesis and either type I or type II diabetes could have detrimental effects on male fertility. More studies on pathophysiological events in reproduction in DM are needed for elucidation of the precise mechanism involved in this metabolic disease that will contribute to development of new strategies for prevention and treatment of reproductive disorders, following DM.

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Clinical Significance of Anatomical Variations in the Carpal Tunnel: Review

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Anatomical variations of the structures related to the carpal tunnel are numerous and involve muscles, tendons, vessels and nerves. The structures passing through the carpal tunnel include the tendons of the flexor digitorum superficialis, flexor digitorum profundus and flexor pollicis longus, as well as the median nerve. Anatomical variations in this region may predispose the median nerve to compression and lead to carpal tunnel syndrome, which is the most often reported compression neuropathy. The most common muscle variations involve the palmaris longus muscle, the flexor digitorum superficialis, the abductor digiti minimi (ADM) and the lumbrical muscles. Vessels anomalies refer to the presence of a persistent median artery and a superficial ulnar artery. The median and ulnar nerves can also be present with variant course, division and anastomoses. The present manuscript reviews literature data on these variations and underlines their clinical implications.

Key words: anatomical variations, carpal tunnel, wrist, median nerve, clinical significance

Introduction

Anatomical variations in the carpal tunnel structures include variations in the nerves, vessels, muscles and tendons of the wrist and hand and represent an often encountered phenomenon [20]. The carpal tunnel is situated directly below the flexor retinaculum (also known as transverse carpal ligament), which in turn extends from the trapezium and scaphoid bones radially to the triquetrum and hamate bones in ulnar direction [20]. The tendons of the flexor digitorum superficialis (FDS) and flexor digitorum profundus (FDP) for the index, middle, ring and little finger and the tendon of the flexor pollicis longus (FPL) pass through the canal, as does the median nerve [20]. This explains why anatomical variations in the region of the carpal canal may predispose the median nerve to compression, a fairly frequent condition known as carpal tunnel syndrome (CTS). CTS is the most common compression neuropathy and its surgical treatment is the most

often performed type of hand surgery [1, 16, 19]. Compression neuropathies in the wrist region can be provoked by different anomalous muscles and vessels, ligamentous attachments, ganglia, neoplastic masses, etc. [26]. Apart from causing these conditions, anatomical variations in the structures of the wrist can in turn suffer iatrogenic injury during diagnostic and surgical procedures in the hand if they are not taken into consideration [20].

The aim of the present article was to review the pertinent literature regarding anatomical variations of the carpal tunnel structures and to discuss their clinical significance with regard to CTS and possible iatrogenic injury to these anomalous structures.

Variations in muscle and tendon anatomy

Muscle variations which are most commonly associated with compression neuropathies in the carpal tunnel include those of palmaris longus (PL), flexor digitorum superficialis (FDS) abductor digiti minimi (ADM) and the lumbrical muscles. PL is one of the most variable muscles in the human body. Its variations include absence, duplication, digastric PL, reversed PL, reversed PL coexisting with an additional ADM, PL with intermediate muscle belly, deep PL, etc. [3-11]. Mitchell et al. [20] indicate that the most significant variations of the PL in the context of CTS include the reversed PL with a possible location of the muscle belly within the carpal tunnel, as well as the passing of the final tendon of the PL through the carpal tunnel and its insertion onto the distal part of the palmar fascia. Some authors associate the hypertrophy of this reversed PL due to overuse in certain professional groups with a higher risk for median nerve compression and development of CTS [8, 22]. Anatomical variations of the FDS include extension of the muscle body inside the carpal tunnel (which is also the most common variation of this muscle), additional muscle belly or an anastomosis between the FDS and PL [3]. Slavchev and Georgiev [26] reported the presence of an additional ADM, which manifested itself as the concomitant presence of a CTS and distal ulnar tunnel syndrome. Anatomical variations of the flexor carpi ulnaris muscle [13] have also been implicated in the possible development of median nerve compression, as well as impairment of the function of the ulnar nerve and thrombosis of the ulnar artery [21]. Such variant muscles are best visualised through the new imaging techniques, such as magnetic resonance imaging [14]. It is also worth mentioning the Linburg-Comstock syndrome which develops on the basis of a tendinous connection between the tendons of the FDP and the FPL. A symptomatic tendonitis of this connection may simulate the symptoms of CTS [20].

Variations in vessel anatomy

Variations in the arterial network of the wrist include a persistent median artery and a superficial ulnar artery [1, 3, 20]. The persistent median artery is a remnant from the embryonic period and can be found in 1-16% of cases [1, 3] or 1.2-23% of cases [20]. This anatomical variation can be found together with a bifid median nerve [3]. Some authors classify the persistent median artery into two types: antebrachial type – a short, thin artery, which does not reach the level of the wrist and palmar type – a longer, large vessel, which reaches the hand [10, 23]. This artery is usually asymptomatic and can play a significant role in the blood supply of the forearm, the hand and the median nerve in particular, which is why an injury to the vessel can compromise blood circulation in that area [10, 20]. The presence of a persistent median artery of the palmar type, however, especially with a diameter of more than 2.0 mm, can cause compression of the median nerve and lead to CTS [2]. As reported by Barfred et al. [2], this artery can be found within the carpal tunnel during surgery for carpal tunnel release and it can also worsen the symptoms of CTS due to induced inflammation [1]. Jelev and Georgiev [15]

also describe an unusual type of median artery, which they refer to as 'superficial brachiomedian artery' due to its high origin from the brachial artery and superficial course throughout the forearm.

The term 'superficial ulnar artery' refers to an ulnar artery situated deep to the antebrachial fascia but superficial to the muscles of the forearm, which is found in 0.7-9.4% of cases [20]. It can be found together with other concomitant anatomical variations such as absence of PL and aberrant superficial palmar arch [25]. This course and location of the artery place it at risk of puncture during injuries, diagnostic and surgical procedures, which is why knowledge of this anomalous course of the ulnar artery is important to physicians and nurses [25]. In particular, extended incision of the carpal tunnel reaching proximally to the wrist crease during various interventions in this area may jeopardize the structural integrity of the superficial ulnar artery [20].

Variations in nerve anatomy

One of the main structures found inside the carpal tunnel is the median nerve. Numerous variations of its usual anatomical course have been described, including bifid median nerve (resulting from a high bifurcation of the median nerve above the level of the carpal tunnel), variations of the motor branch and the palmar cutaneous branch of the nerve, as well as anomalous communications between the median and ulnar nerve [1, 3, 12, 18, 20, 28].

Usually, the median nerve divides into digital branches distal to the carpal tunnel. A bifid median nerve refers to a high point of division of the median nerve, proximal to the flexor retinaculum and has an incidence between 1 and 3.3% [20]. Georgiev et al. [12] studied the prevalence of this variation in the Bulgarian population and found one case of a bifid median nerve among 51 studied formol-carbol fixed cadavers and two cases among 154 upper limbs of patients undergoing open carpal tunnel release. This anomaly is more often associated with compression syndromes, since the cross-sectional area is bigger than in the case of a single nerve [28]. As mentioned above, this variation can coexist with a persistent median artery.

Variations in the take-off of the motor branch of the median nerve were classified by Lanz based on studies by Poisel [18]. The extraligamentous type refers to a motor branch taking off distal to the flexor retinaculum on the radial side of the wrist. This is the most commonly encountered type (46% of cases) and is thus considered a usual anatomical presentation. The second most common type is the subligamentous one (31% of cases), in which the motor branch arises from the median nerve inside the carpal tunnel. The transligamentous type (23% of cases) is characterised by a motor branch piercing the flexor retinaculum before continuing its course towards the muscles of the thenar. Kozin [17] found the presence of more than one motor branch in 4% of cases during a study on 101 fresh-frozen cadavers. Due to the usual presentation and variations of the motor branch of the median nerve, Lanz [18] also underlines the importance of approaching the median nerve from the ulnar side when performing surgical interventions in the carpal tunnel. The palmar cutaneous branch arises from the radial side of the median nerve and passes distally between the superficial and deep layer of the antebrachial fascia, superficial to the flexor retinaculum. Reported variations of this branch include a transligamentous course, as well as a palmar cutaneous branch located ulnar to the median nerve [20]. Knowledge of the anomalous courses of the palmar cutaneous branch is important during surgical interventions in the area of the carpal tunnel, since damage to the nerve fibres may result in a painful neuroma [20].

Anastomoses between the median and ulnar nerves are a frequently encountered phenomenon. A sensory anastomosis between the median and ulnar nerve in the palm is known as Berrettini's anastomosis and is found in as high as 92% of individuals [1].

Riche-Cannieu's motor anastomosis (found in 77-100% of cases) most often refers to a communication between the motor branch of the median nerve and deep branch of the ulnar nerve [1]. The Martin-Grüber's anastomosis refers to a median to ulnar connection in the forearm, which is found in 6 to 31% of individuals and provides alternative patterns of innervation to the intrinsic muscles of the hand [20]. Finally, the Marinacci's anastomosis, referred to also as reverse Martin-Grüber's anastomosis, is a rare, ulnar to median nerve connection, described by Stancic et al. [27] during extended incision of the carpal tunnel. Knowledge of these anastomoses between the median and ulnar nerves is important for the accurate interpretation of electrophysiological studies and reducing the risk of iatrogenic damage during surgical interventions [24].

Conclusion

Anatomical variations of the structures related to the carpal tunnel are numerous and involve muscles, tendons, vessels and nerves. The present manuscript reviews literature data on these variations and underlines their clinical implications. Knowledge of such anomalies is important not only from a descriptive point of view, but also during everyday surgical practise, as they may be associated with pathological conditions, such as carpal tunnel syndrome or can in turn be damaged during diagnostic and surgical interventions, which may impair the normal anatomy and function of the upper limb.

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Sperm Mitochondria-Associated Male Infertility: *Sperm Quality Defects* and Mitochondria (mtDNA) Anomalies: Review

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The main functions of mitochondria in both somatic and germ cells are related with the cellular energy production (by ATP). However, these cellular organelles also have many other functions, depending of the cell life cycle and biological activities, by participation in cellular and molecular events, as cell signalling, proliferation, differentiation and epigenetic control. The injuries in the mitochondrial structure/ultrastructure, mitochondrial genome (mtDNA), transcriptome, proteome, as well as disturbances in mitochondrial membrane potential (MMP) or altered oxygen consumption, have been correlated with loss of sperm functions, which could lead to reproductive problems. Mutations in the mtDNA have been established to be often caused of oxidative stress as a result of free radicals accumulation, but also of other patho-physiological factors, connected with respiration defects in mitochondria and to mutations in the male germ cells.

Key words: mitochondria, mtDNA mutation, spermatozoa, sperm motility, male fertility/infertility

Introduction

The number and distribution of mitochondria on spermatozoa have been characterized as species-specific features [23]. In 1999 World Health Organization (WHO) determined three main criteria for stratification of human sperm quality: spermatozoa morphology, count and motility. Recently, as additional criteria for sperm quality were proposed mtDNA amplification and mtDNA/ß-globin ratio, which could be biomarkers in male infertility [30, 35].

The responsible for sperm motility "movement apparatus" – flagellum, appears in male gametes in the period of their cell differentiation (from spermatids to spermatozoa). In human spermatozoa the mitochondria (usually 10-12/per gamete) are grouped and helically arranged in the region of sperm neck and midpiece – around the outer dense fibers (ODFs) and axoneme [15, 16, 41, 66].

Specifically the internal mitochondrion forms deep cristae from lamellar type [24, 41]. Each sperm mitochondrion carries multiple copies of the paternal mitochondrial genome (mtDNA). In experimental conditions, this paternal mtDNA could be eliminated *in vitro* inside the fertilized ovum by micromanipulation - targeted proteolysis [60].

The anchorage of the mitochondrial sheath - a complex of filaments called sub-mitochondrial reticulum [38], seems to depend on kinesin light chain 3 expression (KLC3). Recent study [67] evaluated that in transgenic male mice, expressing mutant form of KLC3 protein, an abnormal sperm differentiation, low sperm quality and reduced fertility could be registered. On the other hand, the sperm outer mitochondrial membranes (OMMs) [61] are the male gamete structures protecting sperm mitochondria (mtDNA including), and probably contributing to the vitality and improved functions of these organelles. Having in mind that most of the cytoplasm is lost during the spermatogenesis, the quantity of mitochondrial proteins and of mtDNA molecules per cell could be also reduced (paralleled by an increase in the number of mtDNA copies) [5, 14, 26].

1. Morphological and physiological changes in the midpiece of the sperm flagellum related to male infertility

The changes in the mitochondrial integrity/functionality, namely defects in mitochondrial structure/ultrastructure, mitochondrial genome (mtDNA), transcriptome, proteome, as well as disturbances in mitochondrial membrane potential (MMP) or altered oxygen consumption, have been correlated with loss of sperm functions (particularly with decreased spermatozoa motility) [1]. Mitochondria in spermatozoa differ from these in somatic cells in their morphological and biochemical characteristics. The biochemical differences are mainly related to the existence of specific isoforms of enzymes. The morphological and ultrastructural studies of spermatids and spermatozoa

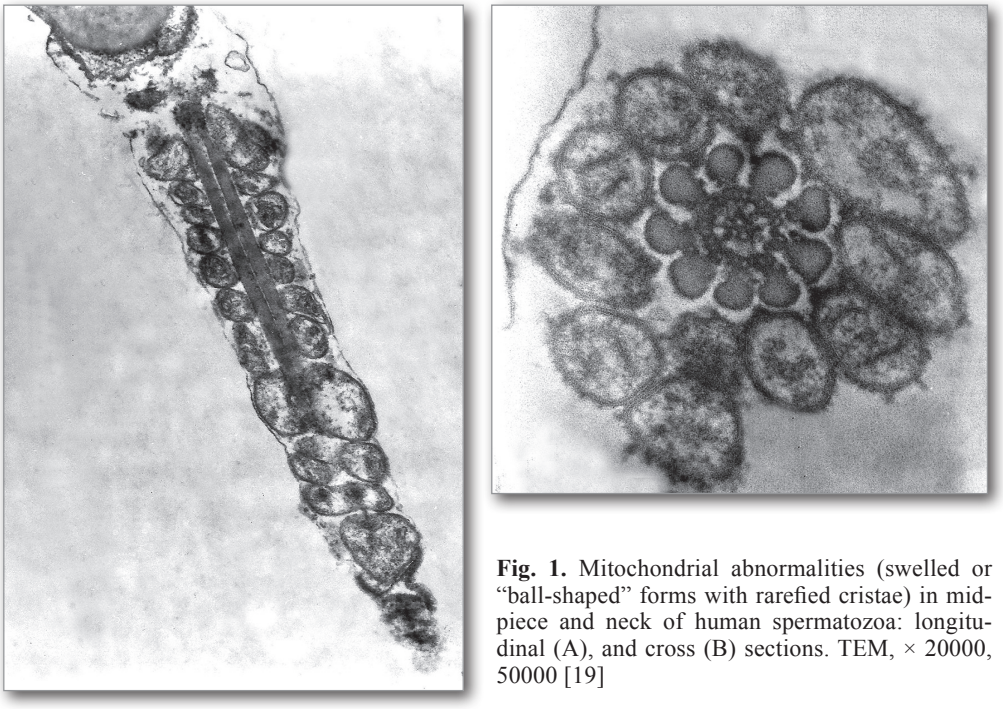

Fig. 1. Mitochondrial abnormalities (swelled or "ball-shaped" forms with rarefied cristae) in midpiece and neck of human spermatozoa: longitudinal (A), and cross (B) sections. TEM, × 20000, 50000 [19]

reveal many injuries, affecting the mitochondrial sheath formation. Disturbances in the ultrastructural components of spermatozoa midpiece were established: changes in the mitochondria number, loss of cristae, lack of mitochondrial membranes, asymmetry in the mitochondria size and distribution have been also observed (**Fig. 1**) [2, 19]. Morphological and biochemical changes lead to the sperm functional disturbances as male gamete hypokinesia and/or asthenozoospermia [8, 25]. The destructive changes in mitochondria could affect the spermatozoa motility, mainly as a result of ATP-synthesis or even because of its lack [25].

The spermatozoa malformations, as "short", "thickened" or "thinner" tails, could be usually related with mitochondria fission and fusion - leading to the increased mitochondrial mass in the spermatozoa midpiece, or with the decreased size - to the full lack of mitochondria [15, 19, 20]. Mundy et al. [34] and Pelliccione et al. [40] described spermatozoa from asthenozoospermic patients as male gametes having short midpieces and few mitochondrial gyres, disordered mitochondria - with swollen inter-membrane spaces, as well as with scattered and disorganised cristae, etc. The spermatozoa defects described in the region of tail [4, 8, 13, 19, 24, 34, 40] could lead to mitochondrial dysfunction as a reason for the low sperm motility and subsequent male infertility.

2. Mitochondria as spermatozoa energy sources and motility forces in male fertility/infertility

The main functional role of mitochondria in the somatic and/or germ cells is related to the cellular energy productions. Recently, mitochondria have been identified as organelles participating in cellular and molecular events, such as cell signalling, cell cycle - proliferation, differentiation, epigenetic control and regulation, etc. [36]. Sperm mitochondria also have specific functional characteristics, closely associated with the spermatozoa motility: spermatozoa resistance to hypotonic conditions and their ability to use lactate as an oxidative substrate [37].

Though sperm receives cellular energy by the glycolytic pathway, spermatozoa are also dependent on oxidative cellular metabolism for its normal physiology [54]. Oxidative metabolism, energy production (by ATP) and free radical generation (ROS) are the main biological reactions, occurring inside the sperm mitochondria. In addition, mitochondria participate in processes of apoptosis and Ca^{2+} homoeostasis. Important phenomenon for reproductive biology is that mitochondria participate in the steroid hormone biosynthesis [44]. As the mitochondrial energy metabolism is a key factor supporting several sperm functions, these organelles host critical metabolic pathways during germ cell development and fertilization [42].

According to data of many authors [3, 11, 47], mitochondria play a pivotal role as bioenergetic sources for spermatozoa vitality, and motility. The ability of mammalian spermatozoa "to swim" is acquired during their "epididymal transit", but it could be observed only upon sperm dilution with seminal plasma fluid at the time of ejaculation [64]. According to the studies of Mortimer [32], the spermatozoa motility force is generated mainly in the tail, in which the binding cytoskeleton components are responsible for both - modulation and performance of this process. The mitochondria provide necessary energy (by production of ATP and the protein dynein) [12, 27]. Glyceraldehyde 3-phosphate dehydrogenase-S (GPDHS) a sperm-specific glycolytic enzyme, appears to be responsible for up to 90% of the ATP synthesis in the spermatozoa, and thus, for their motility maintenance [31]. Confirming the importance of glycolysis in sperm motility, it was shown [28] that porcine and mouse sperm produces an important fraction of ATP anaerobically (mainly via the glycolytic pathway). According other studies [31, 33], the latter energy-generating pathway is more important for sperm motility. This unique feature of the spermatozoa to use different substrates and hence, to activate

different metabolic pathways is closely related to the mitochondrial functional plasticity and is very important for the process of fertilization (insemination) [42]. In some patients with astenozoospermia, the tail length of spermatozoa and their mitochondria volumes correlated with the intensity (frequency) of the vibrations (movements) of the flagellum. The positive correlation between the mitochondria number and spermatozoa motility has been also described [3].

The role of the NADH-tetrazolium-reductase system (NADH-TR) as a biomarker of mitochondrial activity was determined [7, 18, 63, 65]. The data from cytochemical studies on the NADH-TR activity in spermatozoa have been established as well as differences in the enzyme activity on dependence of mitochondrial structure and function [18]. According to the results, received by Edvinsson et al. [7], and Yonkov [65], the activity of NADH-TR system is stronger in spermatozoa from normospermic ejaculates compared with lower enzyme activity in male gametes of patients with astenozoospermia.

Biochemical studies on the activity of NADH-TR system in the sperm mitochondria (from ejaculates of patients with inflammatory diseases - mumps orchitis, prostatitis chr. and epididymitis chr.), indicated two bio-parameters, characterizing the functional status of the male germ cells [63]. First parameter shows the percentage of spermatozoa containing mitochondria with active enzyme systems, and the second one is related to the coefficient of spermatozoa deviation – according to the changes in their enzyme activity. The results demonstrated close relationships between spermatozoa motility and activity of NADH-TR system in their mitochondria.

The investigations, performed by Ruiz-Pesini et al. [47], also evaluated the relationship between the energy production by mitochondria and spermatozoa motility. The susceptibility of the male gametes to the influence of ROS [6], xenobiotics [51], mutations in the mtDNA [22], and other toxic factors influencing physiological status of spermatozoa have been analyzed in medical scientific literature. Spermatozoa, containing defective mitochondria and producing less efficiently ATP, generate reactive oxygen species, which may further cause oxidative stress and damage mitochondrial genome (mtDNA), leading to cellular (male gametes) energy crisis with subsequent decline of spermatozoa motility and male fertility. Additionally, the oxygen consumption (as a result from the effectiveness of the mitochondrial respiratory chains) [9, 55], as well as the influence of many different inhibitors of the electron transfer chains (ETCs) [49, 56], on the motility of spermatozoa, should be further clarified in relationships to male fertility/infertility.

3. Injuries in sperm mitochondrial DNA and male infertility

There is evidence that mitochondrial DNA anomalies in the sperm of mammalian and humans may lead to the male infertility. Point mtDNA mutations, deletions and the presence of mtDNA single nucleotide polymorphisms, as well as of specific mtDNA haplogroups have been associated with low sperm quality [17, 30, 49, 58]. In human sperm, the deletions in mtDNA are associated with a decline in sperm motility and fertility [10, 54]. On the cellular and molecular level, deletions in the mtDNA have been shown to influence the cellular homoeostasis, which could result in reduced sperm functionality and thus - male infertility in mammalian and humans [35, 56].

Folgero et al. [10] first reported data on the reduced sperm motility in individuals with mtDNA defects in spermatozoa: 4977bp deletion in the mitochondrial genome was described to correlate with spermatogenic failure [4].

Mutations at the level of the mitochondrial DNA-polymerase gamma (DNA-Pol-γ) locus were also typified as sperm quality defects associated with male infertility [46]. Concerning specific point mutations/deletions in spermatozoa, it seems consensual that

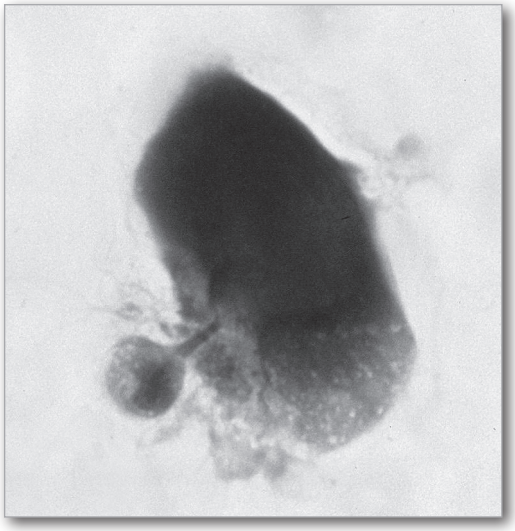

Fig. 2. Fertilization *in vitro:* fertilization cone (protuberance) was formed on the mouse ovum in response to sperm contact and penetration. Methylene blue-fast green (× 450) [*]

the accumulation of multiple mtDNA rearrangements could be associated with loss of normal sperm function. On the other hand, low-quality human sperm has shown an abnormal mtDNA copy number [56, 57]. MtDNA mutations in the ATP generating genes recently demonstrated that mtDNA changes could impair sperm motility [50]. Apart from single nucleotide base substitutions, large deletions in mitochondrial genome have been reported in infertile individuals. Increased levels of mtDNA point mutations and deletions reduce by apoptosis the spermatozoa lifespan [35, 62].

In opposite to the oogenesis, which is associated with a strong amplification of mtDNA copy numbers, the spermatogenesis is related with a drastic reduction in mtDNA content [14]. This mtDNA reduction mainly occurs when the rounded spermatids take on an elongated shape. On the molecular level, the reduction of mtDNA content is due to the down-regulation of the nuclear-encoded mitochondrial transcription Factor A, which is the main cellular factor controlling mtDNA copy number [26]. The reduction in the mtDNA content, together with the action of a specific (ubiquitination-mediated) mechanism of paternal mitochondrial destruction in the early embryo, could explain reduction and/or absence of paternal mtDNA transmission in the zygote and developing embryo [59]. Contradictory messages exist if the sperm midpiece tail is discarded outside the ovum in fertilization (**Fig. 2**) or the paternal mitochondria are degraded inside the zygote, following male gamete penetration [43].

According to May-Panloup et al. [30], the motile sperm from human normal sperm samples were found to contain only 1.4 mtDNA molecules on average (using real-time quantitative PCR). This means that the majority of sperm mitochondria are almost totally devoid of mtDNA, and that many sperm probably do not contain any mtDNA at all [30]. These values are similar and comparable to those established by Shitara et al. [53], who have found an average of 10 mtDNA copies per mouse sperm and 150 copies of mtDNA per mouse spermatid, by using a real-time quantitative PCR technique. Another important finding is the close correlation between the semen quality and the functionality of the respiratory chain in sperm mitochondria [48].

It has been reported that mtRNA transcripts remain highly stable in the mitochondria of sperm, despite the absence of mtDNA replication [45], but the sperm from asthenozoospermic patients have altered levels of specific mtRNAs [30]. The authors indicated that the mtDNA content of motile sperm is up to 28-fold higher in the sperm samples of poor quality, than in normal. Explanation of this epiphenomenon – closely related to abnormal (higher) mtDNA amplification in spermatozoa of low quality, was discussed in the scientific literature [14, 26, 30, 35]. The data show that a low respiratory chain activity of sperm mitochondria leads to the abnormal spermatozoa maturation/differentiation in mammalian and humans. Nakada et al. [35] evaluated that mitochondria respiration defects and genome (mtDNA) mutations in experimental mito-mice induced low sperm number (oligospermia), non-motile sperm (astenozoospermia) and

low sperm quality (sperm morphological abnormalities – preliminary in the midpiece and in the nuclei of male gametes). In addition, testes of the infertile mice showed meiotic arrest (at the zygotene stage) through spermatogenesis and enhanced sperm apoptosis. We described similar morphological changes in spermatozoa of infertile men [20].

Conclusions

Alterations in the mitochondrial genome (mtDNA), transcriptome, proteome or metabolome, as well as any cellular events resulting in compromised sperm mitochondria functionality during the time of sperm travelling and sperm-oocyte interaction (fertilization) (**Fig. 2**), may affect (suppress) sperm motility, functional activity and/or fertility, leading to male sub-fertility/infertility.

Several sperm mitochondrial proteins could be changed in asthenozoospermic patients [29, 39, 52, 68]. The micro-array analysis suggested differential mtRNAs in the sperm from asthenozoospermic patients [21].

ROS activity/oxidative stress and other pathophysiological factors are related to the respiration defects in mitochondria and to the mitochondrial genome (mtDNA) mutations in spermatozoa of patients with male infertility, as well as in other mitochondrial diseases. The data implied clinical applications in cases of male infertility associated to the mitochondrial sperm defects, as the new independent biomarkers of male infertility.

In the scientific literature existed disscussion on the topic: if the sperm midpiece tail is discarded outside the ovum in fertilization or the paternal mitochondria are degraded inside the zygote, following male gamete penetration [43]. The explanation of this interesting biological phenomenon needs of further investigations.

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