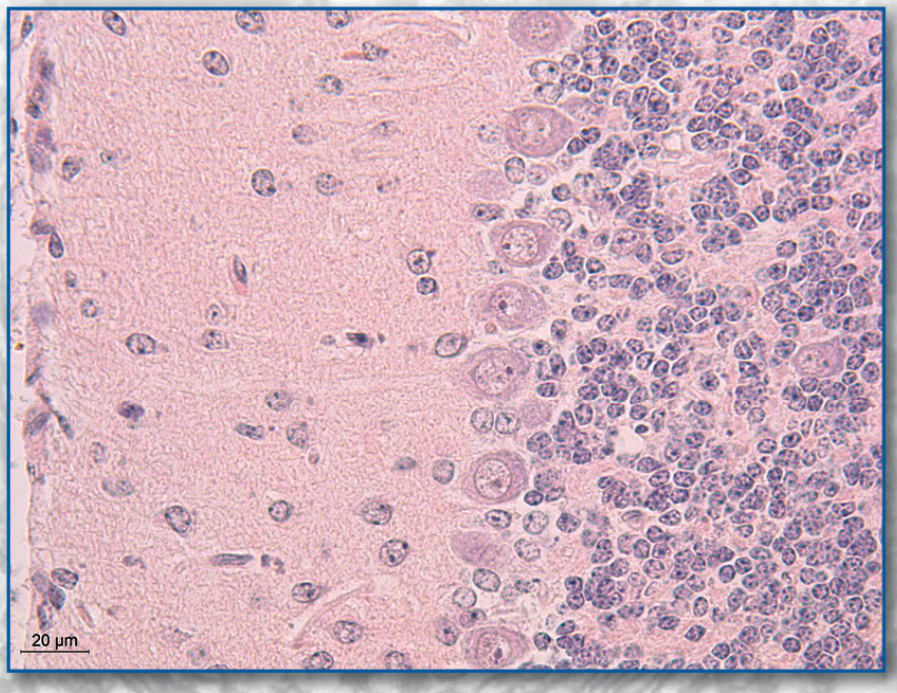
Acta morphologica et anthropologica 27 (3–4)



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Acta morphologica et anthropologica

is the continuation of Acta cytobiologica et morphologica

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Издаването на настоящия том 27, книжки 3 и 4 е осъществено с финансовата подкрепа на Фонд „Научни изследвания“

Prof. Marin Drinov Publishing House of Bulgarian Academy of Sciences
Bulgaria, 1113 Sofia, Acad. Georgi Bonchev Str., Bl. 6

Graphic designer Veronika Tomcheva
Format 70×100/16 Printed sheets 7,50

Printing Office of Prof. Marin Drinov Publishing House of Bulgarian Academy of Sciences
Bulgaria, 1113 Sofia, Acad. Georgi Bonchev Str., Bl. 5

ISSN 1311-8773 (print)
ISSN 2535-0811 (online)

Acta morphologica et anthropologica

27 (3-4)

27 (3-4) ● Sofia ● 2020
Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Contents

MORPHOLOGY 27 (3)

Original Articles

Review Articles

ANTHROPOLOGY AND ANATOMY 27 (4)

Original Articles

Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

MORPHOLOGY 27 (3)

Original Articles

Fast, Easy Staining Method to Visualize Cell Morphology and Apoptosis

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The aim of this work was to combine popular fluorescent dyes into a protocol allowing fast application and easy evaluation of the results regarding the morphology of normal and apoptotic cells. A triple staining method was developed, applying simultaneously the blue fluorescent DNA-binding dye Hoechst 33258, the green fluorescent lipophilic dye DiOC6, and the red fluorescent TRITC-phalloidin binding to filamentous actin in cells with sufficient membrane permeability. It was applied to cultured cells, in some of which apoptosis was induced. Hoechst 33258 visualized the apoptosis-related chromatin condensation, DiOC6 revealed the shape of the cell body, and TRITC-phalloidin penetrated and stained only cells at a late stage of apoptosis, outlining a rounded surface with or without blebs. The described staining protocol is easy to use, requires less than an hour, and provides information about overall cell morphology and convenient identification of apoptotic cells.

Key words: Cytochemistry, apoptosis, DiOC6, Hoechst 33258, phalloidin

Introduction

Although microscopic studies of apoptosis have a long tradition, optimized variations of staining methods are still sought to allow fast, easy and accessible visualization of apoptotic cells. There are assays for specific apoptosis-associated changes, such as TUNEL detecting DNA fragmentation [5] and Annexin V binding to detect translocation of phosphatidylserine from the inner to the outer leaflet of cell membrane [7]. In many

instances, however, it is most appropriate to identify apoptotic cells based on the characteristic appearance of their nuclei. The fluorochrome Hoechst 33258 is often used for this purpose because it produces fast and clear staining of DNA, allowing easy identification of the condensed nuclei of apoptotic cells (e.g. [10]). When used alone, however, it does not provide information about the overall morphology of the cells. In this respect, a dye of interest is 3,3′-dihexyloxacarbocyanine iodide (DiOC6). It is lipophilic, green-fluorescent, and suitable for both living and fixed cells. In low concentrations, it binds to mitochondria and the endoplasmic reticulum [4, 6], while in higher concentrations, it stains all intracellular membranes [1].

Another staining reagent that can reveal the morphology of the target cell, and particularly of its surface, is labeled phalloidin. This fungal toxin binds specifically to filamentous actin and, after conjugation to a fluorochrome, is often used to visualize microfilaments. It does not penetrate live cells, with very few exceptions that possess receptors able to bind and internalize it [8]. Moreover, significant amounts of it do not penetrate even fixed cells, unless they have been permeabilized by detergent [9]. It could be assumed, however, that as cell death follows its course, plasma membrane permeability will increase to a degree allowing labeled phalloidin to diffuse inside the cell. To evaluate the potential of Hoechst 33258 and DiOC6 for fast and easy staining to reveal cell morphology, and to test apoptotic cells for their ability to let in TRITC-phalloidin, we developed a triple staining protocol and applied it to control and apoptotic cultured cells.

Materials and methods

Human gingival keratinocytes were isolated as previously described, using gingival tissue acquired during routine dental extractions of healthy third molars [2]. This was done in accordance with legislature and ethical guidelines concerning participation of human subjects, and after obtaining informed consent. Isolated keratinocytes were cultured in 35 mm diameter dishes at 37°C in an atmosphere of 5% carbon dioxide (CO_2) in EpiLife medium (Cascade Biologics, Portland, OR, USA). After formation of colonies, the cells were transferred to coverslips, placed in 6-well plates (TPP, Trasadingen, Switzerland), and cultured until reaching 80% confluence. Experiments were conducted with cells between 2nd and 4th passage.

The coverslips with the cultured cells were washed in phosphate buffered saline (PBS), pH 7.2. Some of them were left as controls, while others were irradiated with ultraviolet light for 20 min at 37°C to induce apoptosis, followed by incubation in PBS for 40 min at 37°C to allow the events of apoptosis to take place. Then both the induced and the control cells were fixed with 4% paraformaldehyde in PBS for 20 min at 37°C. After washing 3 times in PBS with 0.02% sodium azide, staining solution was applied for 20 min at 37°C in the dark. The staining solution contained 5 μM DiOC6 (Sigma-Aldrich, Germany), 1 μg/ml Hoechst 33258 and 1 μg/ml TRITC-labeled phalloidin (Sigma-Aldrich, Germany) in PBS.

After staining, cells were washed as before and the coverslips were overlaid over microscopic slides with drops of 100% Mowiol (Sigma-Aldrich, Germany). The slides were observed using fluorescent microscope Axioskop 20 (Zeiss, Germany). Absorption by Hoechst 33258, DiOC6 and TRITC was measured at 365, 490 and 560 nm respectively.

Results

Most control cells were in interphase, with irregularly shaped cell bodies revealed by DiOC6 staining. The chromatin, visualized by Hoechst 33258, had a fine structure. It was characterized by diffuse staining of moderate intensity, often with numerous small regions of more intensive staining. The reaction with TRITC-phalloidin was negative (**Fig. 1**). In addition, some mitotic cells could be observed, as well as occasional cells undergoing spontaneous apoptosis (not shown).

In the irradiated slides, most cells had normal appearance but apoptotic cells with condensed, brightly fluorescent chromatin were a common finding. Some of them, with the high degree of chromatin condensation characteristic for advanced apoptosis, were also positive for TRITC-phalloidin, indicating increased membrane permeability. The red fluorescent staining was most intense at the cell surface, presumably corresponding to cortical microfilaments. The labeled cells had a rounded shape, with or without protruding surface blebs (**Fig. 2**).

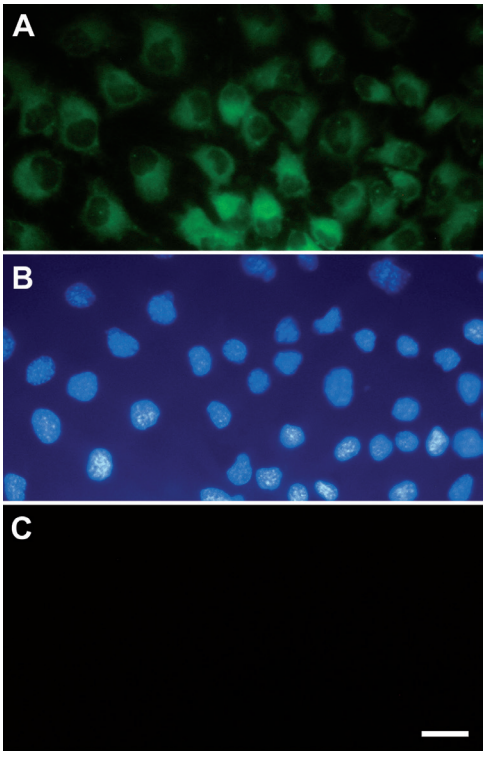

Fig. 1. Control cells stained with DiOC6 (**A**), Hoechst 33258 (**B**), and TRITC-phalloidin (**C**). The cells have irregular shapes, the appearance of chromatin is diffuse, cell membrane is impenetrable to the labeled phalloidin. Bar = 20 μm.

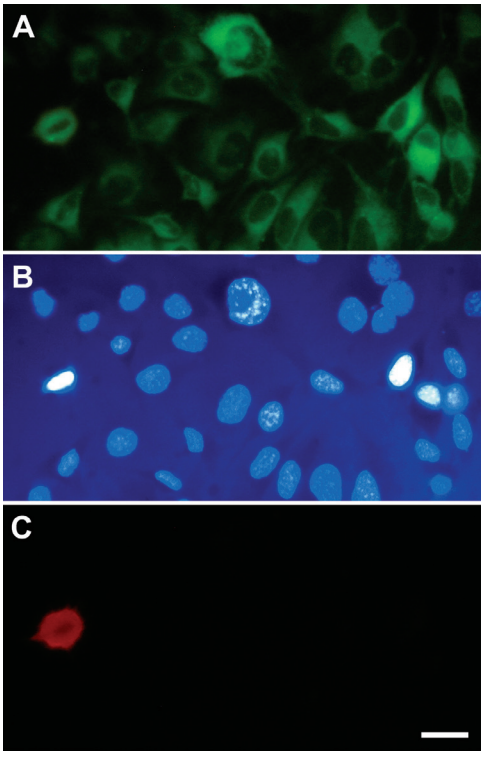

Fig. 2. Cells stained with DiOC6 (**A**), Hoechst 33258 (**B**), and TRITC-phalloidin (**C**) after induction of apoptosis. The Hoechst staining reveals condensed, brightly fluorescent nuclei characteristic of apoptosis both at left and at right. The cell with the most condensed nucleus (left) is also positive for TRITC-phalloidin. Bar = 20 μm.

Discussion

Experiments studying the influence of various factors on cell populations often require comparison of cohorts including vast numbers of cells. In such cases, fast staining methods that allow easy estimation of cell morphology and viability status are very useful. The lipophilic dye DiOC6 already has a specific application in apoptosis research: because its binding to mitochondria is voltage-dependent, it is used on fresh unfixed cells to detect the mitochondrial membrane potential (delta psi) changes associated with apoptosis [3, 10]. In our setting, it was used simply to stain the intracellular membranes in order to reveal the overall morphology of the cell body and so to supplement the information obtained for the nucleus by Hoechst 33258 staining. The simultaneous application of the two dyes allows the morphology and apoptosis status of the observed cell population to be estimated at a glance.

In the present study, we included TRITC-phalloidin because we were interested in the permeability transition that would allow it to penetrate apoptotic cells without detergent treatment. We found that it stained only cells with highly condensed nuclei and rounded cytoplasm indicating a relatively late stage in the course of apoptosis. Staining by TRITC-phalloidin could be omitted from the protocol and, given the high cost of this reagent, in many instances it would be appropriate to skip it. However, its specificity for dying cells and the opportunity to use it simultaneously with the other dyes allows quick evaluation of the effects of apoptosis-inducing factors, even by an inexperienced observer. It should be noted that the membrane disruption associated with necrotic cell death is also expected to let TRITC-phalloidin inside the cell, therefore nuclei of stained cells should always be examined to see whether their morphology (revealed by the Hoechst 33258 staining) corresponds to apoptosis or to necrosis.

Conclusion

The described protocol of simultaneous triple staining of cells with Hoechst 33258 (DNA-binding, blue-fluorescent), DiOC6 (lipophilic, green-fluorescent) and TRITC-phalloidin (actin-binding, red-fluorescent, with low penetrating ability), requiring less than an hour, allows easy estimation of cell morphology and apoptotic status using a fluorescent microscope. Nuclear morphology of phalloidin-stained cells should be examined to distinguish between apoptotic and necrotic cell death.

Acknowledgements: This work was supported by Grants No. D-97/24.06.2020 and D-215/12.12.2018 of the Medical University of Sofia and by the Bulgarian Ministry of Education and Science under the National Program for Research “Young Scientists and Postdoctoral Students”.

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Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

Effect of Anticancer Agents on Viability and Proliferation of 2D and 3D Cultures of Rat Sarcoma Cells, Transformed by Rous Sarcoma Virus Strain Schmidt-Ruppin

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In this study we evaluate the effect of antitumor agents cisplatin, oxaliplatin and epirubicin on viability and proliferation of LSR-SF-SR rat sarcoma cells, transformed by avian Rous sarcoma virus strain Schmidt-Ruppin, that express v-src oncogene. The investigations were performed by short-term experiments (24-72h) with MTT test and neutral red uptake cytotoxicity assay, and long-term experiments (25 days) with 3D colony-forming method. The results reveal that antitumor agents decrease cell viability and proliferation in a time- and concentration-dependent manner. The compounds completely inhibit 3D cell colony-forming ability of the treated cells applied at concentrations ≥ 18.5 μM (for epirubicin), ≥ 33 μM (for cisplatin) and ≥ 75μM (for oxaliplatin). According to the literature available the cytotoxic effect of cisplatin, oxaliplatin and epirubicin in virus-transformed cells and their "interactions" with v-src gene are not clarified yet. The presented cell model and experimental data are an important initial step in this direction.

Key words: Rous sarcoma virus/v-src, rat sarcoma, 2D and 3D cell cultures, antitumor agents, cytotoxicity, model systems

Introduction

Permanent cell line LSR-SF-SR was established from a transplantable rat sarcoma induced by Rous sarcoma virus (strain Schmidt-Ruppin) [2] known to induce tumors in various avian and mammalian species [24]. LSR-SF-SR cells are a suitable model system in the field of experimental oncology, oncovirology, tumor immunology and oncopharmacology because of at least three reasons: i) the cells express oncogene

v-src – the first retroviral oncogene to be discovered. V-src and its cellular counterparts (proto-oncogenes) encode membrane-associated non-receptor tyrosine kinases and are involved in pathogenesis of cancers in avian species, animals and humans [18]; ii) the cell line offers the opportunity to test the putative anticancer activity of new compounds (synthetic compounds, natural products) in retrovirus-transformed tumor cells; iii) Sarcomas are a heterogeneous group (including more than 100 subtypes) of rare malignancies of mesenchymal origin, which represent about 1% of known tumor diseases. With complex treatment (surgical resection of the tumor and chemotherapy – mainly doxorubicin-based regimens), the five-year survival is 60-80%. Metastases are found in 10% of patients at the time of diagnosis, and 25% of patients develop metastases after treatment of the primary tumor. Despite the successes in the treatment of sarcomas, the development of promising new therapeutic strategies in this area is among the main challenges facing modern biomedical science [19, 25, 27].

The possible application of LSR-SF-SR cells in antitumor drug discovery and development requires information about their biological characteristics, including sensitivity to the cytotoxic action of commercially available antineoplastic agents. The aim of our study was to evaluate the effect of cisplatin, oxaliplatin and epirubicin on viability and proliferation of LSR-SF-SR rat sarcoma cells. Platinum-based drugs cisplatin and oxaliplatin are widely used in clinical oncology for the treatment of a wide range of human neoplasms [13]. Cisplatin is one of the most frequently prescribed antitumor agents in veterinary practice [16, 29]. Epirubicin (anthracycline medication smilar to doxorubicin) is included in the treatment of human breast cancer [17]. Moreover, these antitumor agents are included in some combined therapeutic regimens for soft tissue sarcomas (STS). For example treatment with combination of etoposide, ifosfamide and cisplatin has been suggested to be effective in patients with previously treated soft tissue sarcomas [20]. Oxaliplatin-dacarbazine neoadjuvant/adjuvant chemotherapy results in improved prognosis of patients with advanced limb STS in comparison with vincristine, epirubicin, cyclophosphamide combination therapy [31]. According to the literature available the influence of cisplatin, oxaliplatin and epirubicin on viability and proliferation of virus-transformed cells as well as their relationships with v-src gene are not clarified yet. Presenting for the first time data about cytotoxic effect of these antitumor agents in LSR-SF-SR cells is an important initial step in this direction.

Materials and Methods

Materials and Supplies

Antitumor agents cisplatin, oxaliplatin and epirubicin as well as dimethyl sulfoxide (DMSO) and trypsin were purchased from AppliChem (Germany). Purified agar, thiazolyl blue tetrazolium bromide (MTT) and 3-Amino-7-dimethylamino-2-methylphenazine hydrochloride (Neutral red) were obtained from Sigma-Aldrich Chemie GmbH (Germany). Dulbecco's modified Eagle's medium (D-MEM) and fetal bovine serum (FBS) were provided from Gibco-Invitrogen (UK). The antibiotics (penicillin and streptomycin) for cell cultures were from Lonza (Belgium). Ethylendiaminotetraacetic acid (EDTA) and all other chemicals of the highest purity commercially available were purchased from local agents and distributors. All sterile plastic ware was from Orange Scientific (Belgium).

Cell model system andcultivation

The cell line LSR-SF-SR (transplantable sarcoma in rat, induced by Rous sarcoma virus, strain Schmidt-Ruppin) was obtained from the Cell culture Collection of the Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences [2].

The cells were routinely grown as monolayer (2D) cultures in D-MEM medium, supplemented with 5-10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. The cultures were maintained at 37°C in humidified CO_2 incubator (Thermo scientific, Hepa class 100). The passaging was performed using a mixture of 0.05% trypsin and 0.02% EDTA.

Cytotoxicity assays

The cells were seeded in 96-well flat-bottomed microplates for cell culturing at a concentration of 1×10^4 cells/well. After the cells were grown for 24 h to a subconfluent state (~ 60-70%), the culture medium was removed and changed by media modified with different concentrations (0.1-100 μg/ml) of the compounds tested. Each concentration was applied into 4 to 8 wells. Samples of cells grown in non-modified medium served as controls. After 24, 48 and 72 h of incubation, the effect of the compounds on cell viability and proliferation was examined by thiazolyl blue tetrazolium bromide (MTT) test and neutral red uptake cytotoxicity (NR) assay.

The MTT test was carried out as described by Mossman [21]. Briefly, after three hours of incubation with MTT solution (5 mg MTT in 10 mL D-MEM) at 37°C in a humidified CO_2 incubator, the cells were washed by phosphate saline buffer (PBS, pH 7.2; 0.2 mL/well) followed by extraction with a mixture of absolute ethanol and DMSO (1:1, vol/vol) to dissolve the blue formazan.

The NR assay was based on the method of Borenfreund and Puerner [10]. A medium containing NR (50 μg/mL, 0.1 mL) was added to each well. The plate was placed in the CO_2 incubator for 3 h for the uptake of vital dye. Thereafter, the medium with NR was removed and the cells were washed with PBS (0.2 mL/well), followed by the addition of 0.1 mL 1% acetic acid solution containing 50% ethanol to extract the dye from the cells.

Optical density was measured at 540 nm / 620 nm (MTT) and 540 nm (NR) using an automatic microplate reader (TECAN, Sunrise™, Austria).

3D Colony forming method

The method was performed in order to obtain information about cytotoxic activity of the compounds examined for a longer period of time (namely 25 days) in conditions that reproduce better the 3D growth of tumor / tumor cells *in vivo*. The investigations were carried out as it was described earlier [15]. LSR-SF-SR rat sarcoma cells (10^3 cells/well) suspended in 0.45% purified agarose in D-MEM medium containing different concentrations of the compounds examined (ranging from 0.1 to 100μg/mL) were layered in 24 well microplates. The presence/absence of 3D cell colonies was registered using an inverted microscope (Carl Zeiss, Germany) during period of 25 days.

Statistical analysis

Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test (*GraphPadPrizm, GraphPadSoftware Inc., USA,2000*) and Origin 6.1™.

Results

The short-term experiments carried out by MTT test (the gold standard for cytotoxicity assays) and neutral red uptake cytotoxicity technique revealed that applied at a concentration range of 0.1 to 100 μg/ml for 24h, 48h and 72h respectively, anticancer agents cisplatin, oxaliplatin and epirubicin decreased in a time- and concentration-dependent manner viability and proliferation of the treated LSR-SF-SR rat sarcoma cells. Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated for each concentration. Concentration–response curves were prepared (**Fig. 1**) and the compounds effective cytotoxic concentrations – CC_{50} (μM) and CC_{90} (μM) causing respectively 50% and 90% reduction of cell viability as compared to the untreated control were estimated from these curves (Tables 1 and 2). On the basis of their cytotoxic activity (CC_{90}, μM) determined by MTT test after 48 h and 72 h of treatment, the compounds examined were graded as follows (starting with the compound with the highest cytotoxic activity according to Fig. 1, **Tables 1** and **2)**: cisplatin > epirubicin > oxaliplatin (48h) and cisplatin > oxaliplatin > epirubicin (72h).

The long-lasting cytotoxic effect of cisplatin, oxaliplatin and epirubicin on viability and 3D growth of rat sarcoma cells was examined by 3D colony-forming method. The first colonies composed of 12-16 non-treated control cells appeared after 4-6 days of cultivation. The appearance and development of 3D cell colonies were recorded for

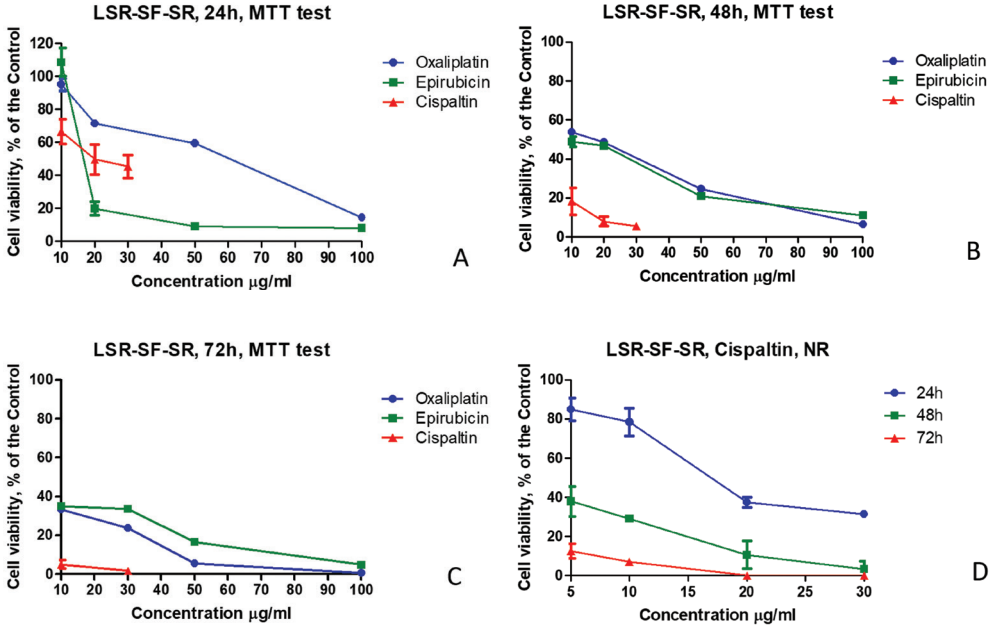

Fig. 1. Effect of anticancer agents on viability and proliferation of LSR-SF-SR rat sarcoma cells. Influence of cisplatin, oxaliplatin and epirubicin on viability and proliferation of LSR-SF-SR cells determined after 24h (A), 48h (B) and 72h (C) of treatment by MTT test. Cytotoic effect of cisplatin in rat sarcoma cells examined by neutral red uptake cytotoxicity assay (NR) after 24h, 48h and 72 h (D). All data points represent an average of three independent assays.

25 days. The compounds examined were found to inhibit completely 3D growth of rat sarcoma cells administered at concentrations ≥ 18.5 μM (for epirubicin), ≥ 33 μM (forcisplatin) and ≥ 75μM (for oxaliplatin). 3D cell colonies were observed although with a smaller number and / or size compared to the untreated control when antitumor drugs were administered at lower concentrations.

Table 1. Effect of cisplatin on viability and proliferation of LSR-SF-SR rat sarcoma cells

Treatment interval (h)	**24**		**48**		**72**	
Method	**MTT**	**NR**	**MTT**	**NR**	**MTT**	**NR**
CC_{50}, μM	66.7	56.8	10.9	8.3	4.8	5.3
CC_{90}, μM	n.d.	n.d.	59.9	66.7	16.7	24.4

MTT = MTT test; NR = Neutral red uptake cytotoxicity assay; n.d. – not determined

Table 2. Effect of oxaliplatin and epirubicin on viability and proliferation of LSR-SF-SR rat sarcoma cells

Anticancer agent	**Oxaliplatin**			**Epirubicin**		
Treatment interval (h)	**24**	**48**	**72**	**24**	**48**	**72**
CC_{50}, μM	151.7	43.4	9.8	30.7	16.8	7.3
CC_{90}, μM	n.d.	228.7	113.4	85.0	184.0	143.6

CC_{50} (μM) and CC_{90} (μM) were determined by MTT test; n.d. – not determined

Discussion

In this study we report for the first time data about sensitivity of retroviral-transformed rat sarcoma (LSR-SF-SR) cells to the cytotoxic activity of three of the most widely clinically applied antitumor agents. Cisplatin, oxaliplatin and epirubicin have been found to decrease viability and 2D/3D growthof LSR-SF-SR cells using methods with different molecular/cellular organelle targets and mechanisms of action (MTT test and NR assay) as well as 3D colony forming method. The MTT test is based on the functional activity of dehydrogenase enzymes in mitochondria, while neutral red dye penetrates the intact lysosomes of healthy cells. Cisplatin shows the highest cytotoxic activity in short-term experiments, and epirubicin most effectively inhibits the 3D growth of sarcoma cells in long-term experiments (25 days). Compared to the traditional monolayer (2d) cell cultures, 3D cell cultures more adequately represent biology and behavior of tumors/ tumor cells, especially their chemosensitivity.

Among the advantages of the 3D colony-forming method we use is that it allows the influence of the compounds examined on the survival and proliferation of the treated cells to be monitored for a long period of time (25 days in the study presented) providing valuable information about stability of their cytotoxic effect. From clinical point of view the presence of even a single 3D cell colony surviving after treatment can lead to relapse and metastasis *in vivo*. That is why our interest was focused on effective concentrations in which the compounds completely suppresses formation of 3D cell colonies [8].

The cell line LSR-SF-SR has a number of valuable biological characteristics that make it a suitable model system for research purposes, such as: i) easy cultivation as 2D and 3D cell cultures;

ii) the cells can be successfully implanted into laboratory animals (for example immunocompetent Wistar rats), leading to the development of tumor formation at the site of inoculation after a typically 7-20 day latency period. This allows *in vivo* studies to be carried out on various aspects of tumorigenesis, including tumor immunology and testing of antitumor activity of various agents and strategies [3-5].

iii) the cells express v-src gene. Src is a member of a superfamily of membrane-associated nonreceptor protein tyrosine kinases that are stimulated by receptors of growth hormones, cytokines and adipokines. Src proto-oncogene plays key roles in cell adhesion, growth, division, migration, and survival signaling pathways and is known to be disregulated in many types of human and animal cancers. Src has been recognized as a promising target for innovative antitumor treatment strategies [26,30].

LSR-SF-SR rat sarcoma cells have been used as model systems to evaluate the cytotoxicity and potential antitumor activity of compounds with various structure and chemical /physicochemical properties including alkaloids [7], photosensitizers [28], disulfiram [14], ammonium vanadate [1], basic salts of zinc(II) and copper(II) [9], metal complexes with different ligands such as ionophore antibiotic monensin [22], non-steroidal anti-inflammatory drugs [11,12], Mannich bases [6], etc.

Comparison of the data on the cytotoxic activity of these substances [1,6,7,9, 11,12,14,22,23,28] with the results obtained in the present study indicate that some of the newly synthesized metal complexes studied (e.g. those of the ionophore antibiotic monensin) are more effective compared to cisplatin – currently the most widely clinically used anticancer agent. Thus, after 72h incubation period, the CC_{50} of monensin and La(III), Nd(III), Mn(II) and Ca(II) complexes with this ionophore antibiotic have been calculated (by MTT test) to be 4.4, <0.23, <0.23, 2.1 and 2.2 μM respectively [22]; [23]; whereas CC_{50} of cisplatin determined at the same conditions (72h, MTT test) is 4.8 μM.

Conclusions

Our study presents original data on the sensitivity of retrovirus-transformed rat sarcoma cells (the cell line LSR-SF-SR) to the cytotoxic effect of three commercially available antitumor agents widely used in human and veterinary clinical practice (cisplatin, oxaliplatin and epirubicin). LSR-SF-SR cells are valuable model system for the needs of experimental oncology and oncopharmacology as they are easily maintained, capable of growing as 2D and 3D cultures *in vitro* as well as in laboratory animals (including immunocompetent rats) *in vivo.* Most importantly, LSR-SF-SR cells express a representative of src gene family (v-src gene) that perform important life-supporting functions and when dysregulated they take part in pathogenesis of a wide range of tumors human and animal cancers. The results obtained are a step forward in better clarification of the biological characteristics of LSR-SF-SR cells and their application in our attempts to identify new antitumor treatment strategies, especially directed against cancer cells expressing the src oncogene, and to elucidate better their mechanism of action.

Acknowledgements: This study was supported by COST Action CA15135 ("MuTaLig"); COST Action CA16119 ("CellFit); Grant ДКОСТ 01/16 from 17.08.2017 and Grant ДКОСТ 01/10 from 22.10.2018, National Science Fund, Bulgarian Ministry of Education and Science.

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Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

Transformation of the Human Prostate Gland in Early Neonatal Period to Later Childhood Period of Development

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Morphometric parameters of the acini of human prostate glands and epithelium, lining gland was carried out from the neonatal period to the second childhood. For the first time, the end pieces of the prostatic glands and their lumens at different age periods according to their lobular structure of the prostate are described. Changes in the sizes of the end pieces of the glands, the gaps of the terminal sections of the glands and their epithelial cells from the early neonatal period to the late childhood age period are described. Histological differences were found in the prostate between different lobules. In the postnatal period, canalization of the ductal system of the prostate glands proceeds through apoptosis and polarization of epithelial cells. In the formed acini of the prostate glands, pronounced changes occur during the children's age periods that correspond to the functions of the organ.

Key words: prostate, epithelium, glands, epithelial cord, apoptosis

Introduction

The genital system of boys, including the prostate, is morphologically formed by the time of birth and its transformations are aimed at further morphofunctional development, which is in the period of 20-45 years, and the age of 41-45 years is considered the beginning of significant involutional changes. Probably this is a reason that all previously conducted studies of the morphogenesis of the prostate glands were performed mainly in the man prostates during mature age and older one. It is widely known that a malignant neoplasm, including the prostate cancer, occurs due to the re-awakening of development processes that occur during organogenesis. The formation of prostatic ducts from epithelial cords and their further transformation into the acini of human prostate glands continues in the postnatal period of prostate development. Clarification of the appearance timing and formation of various structures is important for monitoring the proper development of an organ. There are not many studies of the structural organization of the human prostate glands in its various lobules during the birth period and childhood. Many issues of prostate morphology from birth and throughout childhood are unclear.

The aim of this study was to determine the morphometric parameters of epithelial cords, prostatic ducts, and acini of human prostate glands and their lining epithelium and gland shape in all prostate structural lobules of boys during perinatal and childhood periods of development.

Material and methods

The studies were performed on 36 prostates of boys aged from the first day of life to 13 years (**Table 1**), which were obtained in accordance with the law of the Republic of Belarus no. 55-3 "On the Burial and Funeral Business," as amended by act no. 2/2235 from September 1, 2015. The independent Ethics committee of Vitebsk State Medical University approved the study (Protocol No. 2 of May 7, 2018). Material was collected within 12 hours after death. An organocomplex was removed from the pelvic cavity (bladder, prostate, seminal vesicles, rectum) and its preparation was performed with the release of the prostate. Histological sections were prepared with a Leica RM 2125 RT rotary microtome (Germany), and stained with hematoxylin-eosin and fuxelin according to Hart. Stained preps were examined with a Leica DM 2000 microscope (Germany) equipped with a camera adapter at a total magnification of 100×, 200×, and 400×. Micrographs of the study areas were obtained with a Leica D-LUX 3 digital camera (Germany). The micrographs of the prostate glands were processed with the use of the Image Fiji software using the set of standard tools [4] and plugins that make it possible to change the sharpness and contrast of images and allow smoothing, removal of "noise," and separation of a color image into the color channels. Then, using the Trianable Weka Segmentation plugin, the images were segmented, and their binary masks were obtained. All processed images were calibrated using a standard object micrometer. The morphometric study included the measurements of acini, the acinar lumen areas, and the epithelium height in the inferoposterior, inferolateral, superomedial lobules of the right and left lobes of the prostate. We used the morphological method of verification of apoptotic cells on stained histological preps using the criteria of apoptosis as recommended by Skibo [5]. Statistical hypotheses were tested using Statistica 10.0 and Microsoft Excel 2007 software. The statistical homogeneity of the samples was tested using the nonparametric ANOVA procedures (Kruskal–Wallis test for multiple comparisons). When statistical heterogeneity of several samples was found, subsequent identification of heterogeneous groups was performed using the Mann–Whitney U test and Dunn's post hoc test with Bonferroni adjustment. The critical level of significance was $p < 0.05$.

Table 1. Distribution of the study material according to age groups

Age period	Early neonatal	Later neonatal	Infancy	Early childhood	First late childhood	Second late childhood
	1-7days	8-28 days	1 month- 1 year	1-3 years	4-7 years	8-12 years
Number of cases studied	7	3	9	5	4	8

Results and discussion

In the neonatal and infancy periods, epithelial cords with cleft like lumens, epithelial tubules with lumens of different size and shape, prostatic ducts and acini of human prostate glands were found in all prostatic areas (**Fig. 1**). The epithelial cords are multicellular structures formed by tightly adjoining epithelial cells bounded by the basal membrane. Epithelial cells in the cords contained large nuclei and showed no signs of polarity relative to the basal membrane. In some epithelial cells with changes in the morphology of nuclei and cytoplasm were detected characteristics of apoptosis (**Figs. 2, 3**). In all detected epithelial cords between epithelial cells were present cleft like lumens due to the loss of cell–cell contacts [5]. In infants, epithelial cords were detected up to 3 months of age.

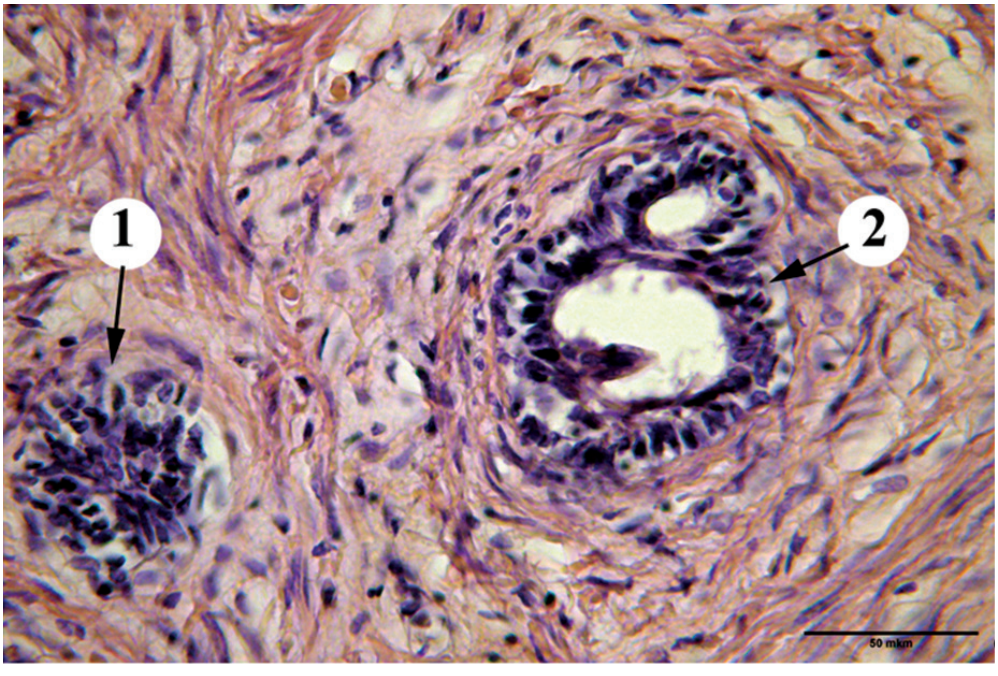

Fig. 1. Prostate preparation of a boy (27 days old). Staining with hematoxylin and eosin. 1) Epithelial cord with epithelial cells tightly adjoining each other. Epithelial cells contain large nuclei without signs of polarization. 2) Epithelial cells of the acini of human prostate glands with basally located nuclei. × 400.

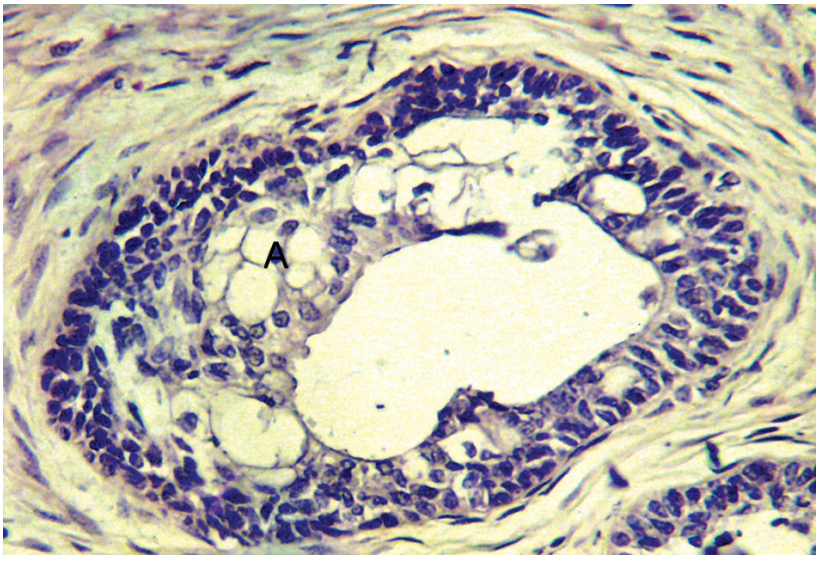

Fig. 2. Prostate preparation of a newborn boy (2 days old). Staining with hematoxylin and eosin. A – conglomerates of apoptotic cells in the prostatic ductal lumens. The epithelial tubules contained epithelial cells with changes in the morphology of nuclei and cytoplasm characteristic of apoptosis: chromatin margination, pyknosis in nuclei, changes in the cytoplasm staining pattern, and gaps between individual epithelial cells due to the loss of cell–cell contacts. × 200.

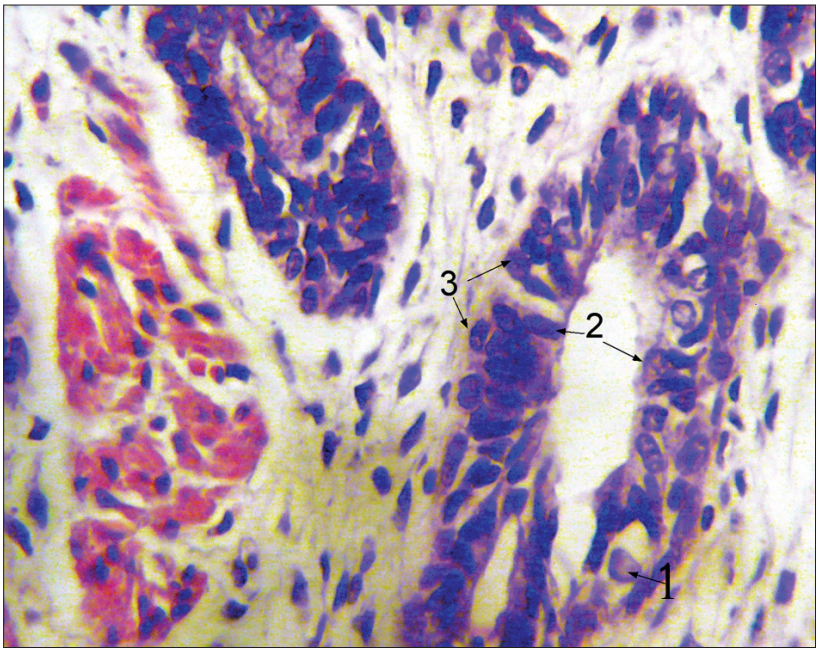

Fig. 3. Prostate preparation of a boy (11 days old). Staining with hematoxylin and eosin. 1. Cell with signs of karyopyknosis of the nucleus and cleared cytoplasm (apoptotic cells). 2. Cubic epithelial cells of prostatic duct. 3. Basal epithelial cells. × 200.

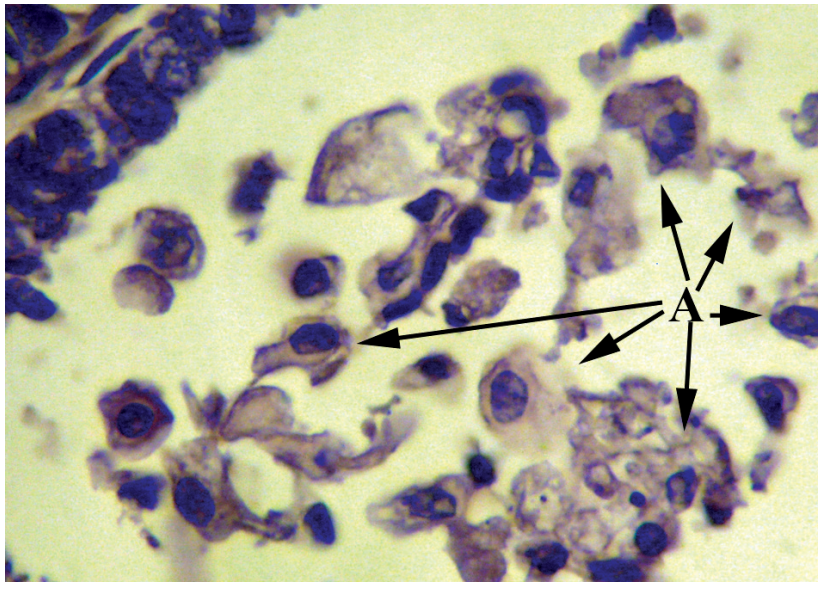

Fig. 4. Prostate preparation of a newborn boy (11 days old). Staining with hematoxylin and eosin. A – conglomerates of apoptotic cells in the prostatic ductal lumens. × 400.

Probably, the canalization of the ductal system of the prostate glands occurring in the neonatal periods [6,7] continues in the postnatal period. Prostatic ducts and acini of human prostate glands, were lined with double-layered epithelium with basally located nuclei. In prostatic ducts located closer to the prostatic part of the urethra, conglomerates of apoptotic cells were observed (**Fig. 4**). This statement is not consistent with the opinion of some authors who claim that remnants of metaplastic cells and / or debris are found inside the prostatic ducts [8]. Starting from infancy, the structure of the glands was studied in place of the definitive inferoposterior, inferolateral, superomedial lobules, which was not possible in the neonatal period. In infancy in competition with the neonatal periods the acinar area and the height of the epithelium lining the acini decreases in all structural prostatic lobules ($p \leq 0.05$) (**Table 2**). The height of the epithelium of the acini significantly increased in early childhood in comparison with the infancy ($p \leq 0.05$) and does not change in other children's age periods ($p > 0.05$). The acinar lumen area of the prostatic glands decreases in all structural lobules of the prostate in early childhood in comparison with the infancy ($p \leq 0.05$) and does not change in other children's age periods ($p > 0.05$) (**Fig. 5**). The results of our studies are consistent with the data of researchers who claim that the prostate develops to a large extent immediately after birth, and then remains relatively inactive until puberty [3, 8]. There is an opinion that in some children's age periods in the prostate there are no histological differences between different lobules [3].

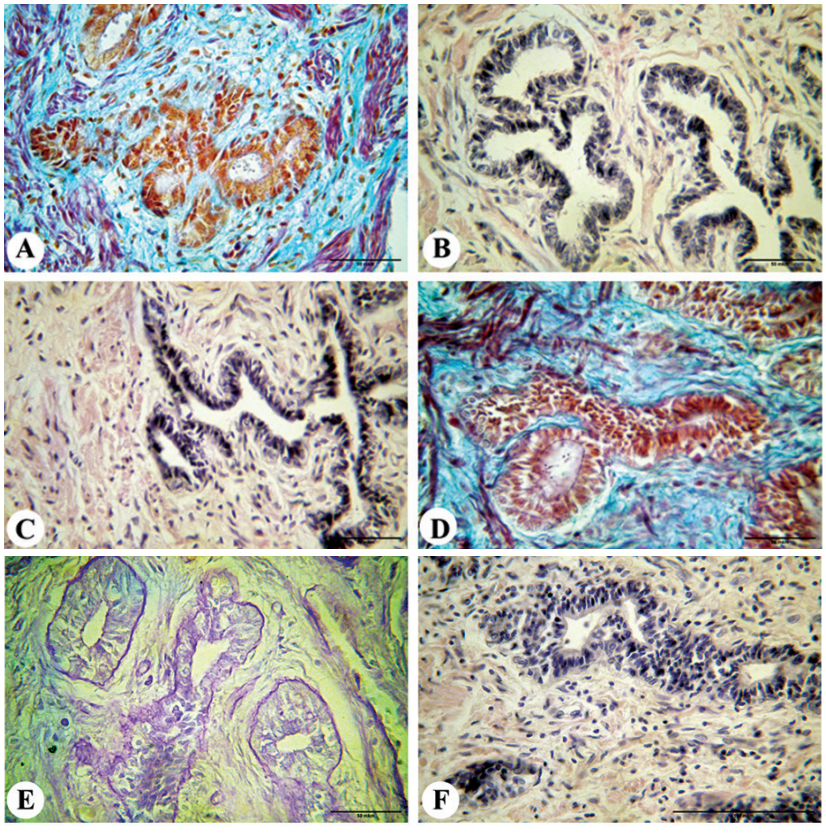

Fig. 5. Prostate preparation of boys at different ages. **A** – Prostate preparation of a boy (1 month old) in the superomedial lobule. Staining according to Heidenhain; **B** – Prostate preparation of a boy (4 months old) in the inferolateral lobule. Staining with hematoxylin and eosin; **C** – Prostate preparation of a boy (2 years old) in the inferolateral lobule. Staining with hematoxylin and eosin; **D** – Prostate preparation of a boy (3 years old) in the posterolateral lobule. Staining according to Heidenhain; **E** – Prostate preparation of a boy (8 years old) in the posterolateral lobule. Staining according to Ritter – Oleson; **F** – Prostate preparation of a boy (11 years old) in the superomedial lobule. Staining with hematoxylin and eosin. Scale bars 50 μm.

We detected that in the superomedial lobule of the prostate of infants, the acinar area, acinar lumen area, height of their epithelium statistically differed from the inferolateral and inferoposterior lobules ($p \leq 0.05$) and did not statiastically differ between the inferolateral and inferoposterior lobules ($p > 0.05$) (**Tables 3,4,5**). The differences among the lobules were "smoothed out" in early childhood and were not detected in the first childhood ($p > 0.05$). In the second childhood, differences in lobules for all studied parameters ($p \leq 0.05$) were revealed among the superomedial and inferolateral lobules and the superomedial and inferoposterior lobules.

Table 2. Prostate gland size in neonatal, infancy, and childhood periods M (1st Qu; 3rd Qu, μm).

		Epithelium height, μm	Acinar area, μm^2	Acinar lumen area, μm^2
Early neonatal		12,5(9,4;16,4)	5002(3390;7484)	24(150;551)
Late neonatal		14,8*(11,9;18,1)	5136*(2751;10072)	852*(465;1425)
Infancy	SM	8,2*(6,58;10,6)	3055*(1694;5554)	823(414;1501)
	IP	10,0 *(8,0;12,9)	4242* (2406;7786)	1029(518;1876)
	IL	10,2 *(8,2;13,1)	3856*(2188;7089)	1061(528;1918)
Early childhood	SM	9,5*(7.1;13.8)	3712*(2049;6858)	508*(204;1099)
	IP	11,4* (8,9;14)	4793(2719;8812)	585*(406;1035)
	IL	10,1 (7,8;13)	4300(2444;7934)	541*(249;1058)
First late childhood	SM	11,1(8,3;12,3)	4529*(2500;8366)	534(214,8;1154)
	IP	10,9 (8,3;16,2)	5752*(3263;10575)	597(305;1164)
	IL	10,6 (8,3;13,7)	5346*(3030;9839)	658(449;1129)
Second late childhood	SM	11,4 (9,0;13,0)	4330(23988;7951)	238(145;417)
	IP	11,6 (9,0;15,0)	5942(33714;10924)	392(213;749)
	IL	16,9*(13,0;25,0)	5453(3091;10026)	389*(223;749)

* – the critical level of significance was $p < 0.05$
SM – superomedial lobule, IP – inferoposterior lobule, IL – inferolateral lobule

Table 3. Comparison of the height of the epithelium among lobules within the age group (adjusted for multiple comparisons).

Options for comparison	Compared groups	p
Infancy	IL-IP	0,32
	IL-SM	<0,001*
	IP-SM	<0,001*
Early childhood	IL-IP	0,6486
	IL-SM	<0,0115*
	IP-SM	<0,0172*
First later childhood	IL-IP	0,2343
	IL-SM	0,1234
	IP-SM	0,1818
Second later childhood	IL-IP	<0,0161*
	IL-SM	<0,001*
	IP-SM	<0,001*

* – the critical level of significance was $p < 0.05$

Table 4. Comparison of the acinar area among lobules within the age group (adjusted for multiple comparisons).

Options for comparison	Compared groups	p
Infancy	IL-IP	0,0703
	IL-SM	<0,0030*
	IP-SM	<0,001*
Early childhood	IL-IP	0,119675
	IL-SM	<0,0259*
	IP-SM	<0,001*
First later childhood	IL-IP	0,1765
	IL-SM	0,6529
	IP-SM	0,3342
Second later childhood	IL-IP	0,19592
	IL-SM	<0,001*
	IP-SM	<0,001*

* – the critical level of significance was $p < 0.05$

Table 5. Comparison of the acinar lumen area among lobules within the age group (adjusted for multiple comparisons).

Options for comparison	Compared groups	p
Infancy	IL-IP	0,6897
	IL-SM	<0,0032*
	IP-SM	<0,006*
Early childhood	IL-IP	0,2534
	IL-SM	0,5392
	IP-SM	0,1686
First later childhood	IL-IP	0,2787
	IL-SM	0,1175
	IP-SM	0,4129
Second later childhood	IL-IP	<0,001*
	IL-SM	<0,001*
	IP-SM	0,9386

* – the critical level of significance was $p < 0.05$

Conclusions

In the postnatal period, the processes of canalization of the ductal system of glands occur in the prostate by apoptosis and polarization of epithelial cells. In the formed acini of the prostate glands, pronounced changes occur during the children's age periods that correspond to the functions of the organ.

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Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

Effects of a Cotinus coggygria Ethyl Acetate Extract on Two Human Normal Cell Lines

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The effect of ethyl acetate extract from *Cotinus coggygria* (sumac) leaves on the viability of two normal human cell lines (BJ and MCF-10A) is tested. Both cell lines are known to express fibroblast activation protein α (FAP) – a serine protease involved in tumorigenesis and tumor progression. The extract is shown to contain one or more components that inhibit FAP. Using the Neutral Red Uptake Test, it is found that in the line of activated human fibroblasts (BJ), the treatment leads to inhibition of cell proliferation. Conversely, the extract has no adverse effect on MCF-10A cells (mammary gland epithelium cells) at low concentrations even increasing the cells proliferation by 13 %. It is concluded that the tested extract contains a potent inhibitor of FAP, which may be useful as an anti-cancer agent, but should be used with caution due to its dual effect on normal epithelial cells.

Key words: Cotinus coggygria, Fibroblast activation protein α, MCF-10A cells, BJ cells

Introduc tion

Secondary metabolism in plants leads to the production of a large number of natural products with historically proven potential for therapeutic use in a number of diseases. *Cotinus coggygria* (sumac) belongs to the family *Anacardiaceae*. Extracts of its roots, stems, leaves and flowers are widely applied in folk and modern medicine because of their antiseptic, anti-inflammatory and hepatoprotective properties [5]. Antioxidant and antitumor activities of those extracts are also documented [1, 3]. Our preliminary studies showed that the ethyl acetate extract of sumac leaves inhibits fibroblast activation protein α (FAP, EC 3.4.21.B28) – a protease involved in the development of a large number of solid tumors. FAP is a trans-membrane serine peptidase, belonging to the S9 family of post-proline specific proteases. It is over-expressed in the stromal fibroblasts of about 90 % of carcinomas and many sarcomas [2]. The enzyme is generally considered as a suitable target for the development of novel anti-cancer therapies [7]. However, there are several malignancies in which FAP acts as a tumor suppressor [8]. For example, the

lack of FAP expression in the membranes of human malignant melanoma cells and non-small cell lung cancer cells is considered to be a part of the tumor phenotype [12, 13]. To elucidate the FAP's dual role in cancer, more studies with the enzyme inhibitors are needed.

The aim of the present study is to assess the effect(s) of ethyl acetate extract of *Cotinus coggygria* leaves as a powerful FAP inhibitor on the cell viability and proliferative activity of two types of human cultured cells, known to express the enzyme – MCF-10A and BJ.

Materials and Methods

Ethyl acetate extract of Cotinus coggygria leaves. Crude ethanol extract of *C. coggygria* leaves was purchased from Vemo 99 Ltd (Sofia, Bulgaria). Five grams of the powdered crude extract were suspended in 20 ml dist. water and 6N hydrochloric acid was added drop wise until pH 3.0. The mixture was extracted twice with ethyl acetate. The organic phase was filtered, washed with brine and dried for several hours over sodium sulfate. Then, the ethyl acetate was partially evaporated *in vacuo* and diisopropyl ether was added in drops. A dark yellow solid fraction was formed, which was filtered and dried.
FAP inhibition by the C. coggygria extract. The inhibitory properties of the sumac leaves extract (1 to 10 μg/ml) were tested on recombinant human FAP (R&D Systems through Biomedica, Bulgaria) in phosphate buffered saline (PBS) with the addition of 1mM EDTA and 80 μM fluorescent FAP substrate Z-glycyl-prolyl-methylcoumaryl amide (Z-Gly-Pro-MCA, Bachem, Switzerland) at 37ºC. Enzyme assays were carried out in 96-well plates on Varioscan Fluorescence spectrofluorimeter at 360 nm excitation and 460 nm emmition every 3 min. The program EnzFilter V2 was used for data processing.
Cell culturing. For the experiments, two human cell lines were used: MCF-10A (immortalized normal epithelial cells of mammary gland) and BJ (activated normal skin fibroblasts). They were cultured in Dulbecco's Modified Eagle's medium – high glucose (DMEM 4,5 g/l glucose), supplied with 10 % fetal bovine serum and antibiotics in usual concentrations in a humidified atmosphere with 5 % CO_2 at 37.5ºC. In the case of MCF-10A cells, epidermal growth factor (EGF), insulin and cholera toxin were added in concentrations according to the cell bank instructions. Cells were plated at a density of 2×10^3 in 100 μl culture medium in each well of 96-well flat-bottomed microplates and allowed to adhere for 24 h before treatment with *C.* coggygria extract. The extract was dissolved in DMSO and diluted in culture medium. A concentration range from 0.75 to 100 μg/ml of the extract was applied for 48 h. The neutral red uptake assay was used for the estimation of the cells viability/proliferative activity, exactly as previously described (9). After treatment with Neutral Red medium for 3 h, washing and application of the ethanol/acetic acid solution (NR Desorb), the absorption was measured on ELISA microplate reader (TECAN, SunriseTM, Grödig/Salzburg, Austria) at a wavelength of 540 nm. GraphPad Prizm5 software was used for the processing of the results. All experiments were performed in triplicate.

Results and Discussion

Our previous experiments showed that extraction of crude plant components with ethyl acetate in acid medium leads to fractions containing polyphenols, chlorogenic acids and flavonoids (glycosylated or not) [10, 11]. Although the composition of the present extract from *C. coggycria* leaves is not determined yet, the mode of extraction may lead to the reasonable assumption, that it contains similar components. Our present experiments prove that one or more of those components are powerful inhibitors of human recombinant FAP with IC_{50}=3.7 μg/ml. As the specific inhibition of FAP would be essential for the development of novel anti-cancer strategies [7], the elucidation of FAP inhibitor(s) structure will be an important objective for our future studies.
According to the data, presented in Human Protein Atlas about the cell distribution of FAP (https://www.proteinatlas.org/ENSG00000078098-FAP/cell), it is expressed in BJ cells as this is the case of the activated fibroblasts all together. Recent studies also show that the enzyme is present in the membranes of MCF-10A cells [4]. While the role of the enzyme in activated fibroblasts is associated with an increase in their proliferative activity, its role in mammary epithelial cells is not known yet. One of the assumptions about the presence of FAP in cells of epithelial origin is that the cells are in the process of preparing for epithelial-to-mesenchymal transition [4].

Our results show that even small amounts of the *C. coggygria* extract induce a decrease of BJ cells viability, most probably due to the inhibition of FAP. The extract IC_{50} on BJ cells was estimated to be 52 μg/ml (**Fig. 1**). This result corresponds to the established importance of the enzyme for the division and migration of activated fibroblasts.

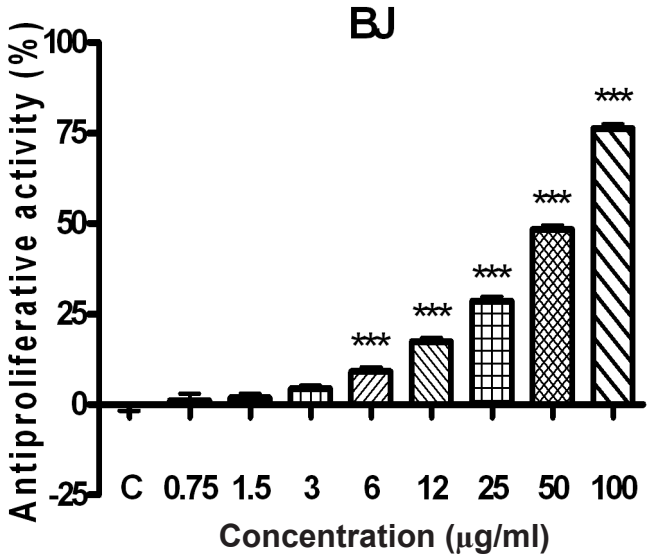

Fig. 1. Effect of *C. coggygria* ethyl acetate extract on the viability/proliferation of BJ cells. Each result represents a mean from three experiments. The anti-proliferative effect of not-treated cells is accepted to be zero.

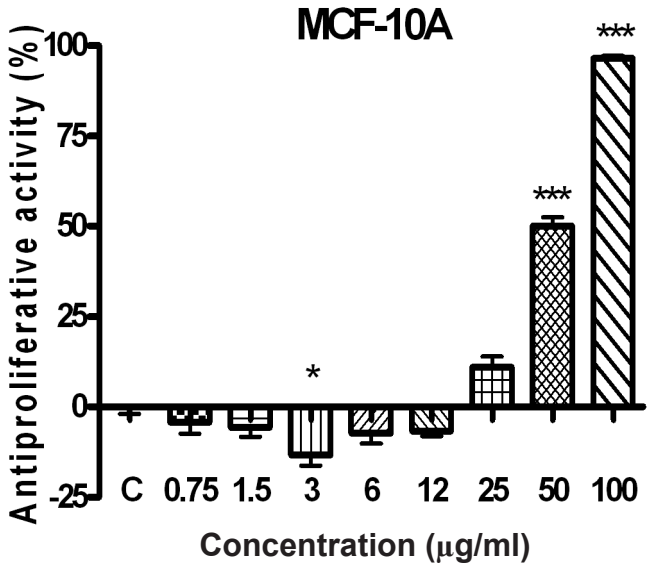

Fig. 2. Effect of *C. coggygria* ethyl acetate extract on the viability/proliferation of MCF-10A cells. Each result represents a mean from three experiments. The anti-proliferative effect of not-treated cells is accepted to be zero.

An interesting result was obtained with MCF-10A cells (**Fig. 2**). Up to concentrations of 12 μg/ml, the *C. coggygria* ethyl acetate extract did not have a negative effect on the cells viability. Conversely, at concentration 3.0 μg/ml a statistically significant increase in cells proliferation activity, estimated to 13 %, was detected. However, concentrations higher than 20 μg/ml decreased the cells proliferation rate with IC_{50}= 52 μg/ml which is equal to that of BJ cells. According to these results, the importance of FAP activity for epithelial mammary gland cells is more complicated. The enzyme may be involved in the mechanisms of cells proliferation control. These results support our hypothesis, expressed in previous studies, that the absence of FAP is a part of the tumor phenotype of human mammary gland epithelial cells [6].

Conclusions

The results, presented here show that ethyl acetate extract of *Cotinus coggygria* leaves possesses one or more FAP inhibitor(s) of yet unidentified structure which may prove to be suited for use both in biomedical research and for the development of novel anti-cancer therapeutic strategies. On the other hand, FAP inhibitors should be considered as convenient tools for suppression of the expansion of tumor fibroblasts, but keeping in mind their dual effect on cells of epithelial origin.

Acknowledgement: This work is financially supported by the National Science Fund of the Bulgarian Ministry of Education and Science, Grant No KP-06-N31/1.

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Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

Influence of Lead on the Secretion of Amyloid Precursor Protein in Mouse Brain

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It is known that heavy metals and especially lead (Pb) are toxic to the organism and particularly to the brain. Lead is assumed to participate in the etiology of neurodegenerative diseases including Alzheimer's disease (AD). Little is known about the relationship between Pb and the metabolism of the amyloid precursor protein (APP) which is considered to have a key role in this type of dementia.

In our study we examined the effect of Pb on the secretion of the amyloid precursor protein and found that it reduces the secretion in the cerebral hemispheres and cerebellum and had no significant effect in the other mice brain regions studied.

Key words: lead, amyloid precursor protein secretion, mice brain regions

Introduction

Changes in the biological levels of essential metal ions, which regulate the function of a number of enzymes, can influence numerous biochemical processes in the body [11]. In the brains of persons who suffered from AD a disruption of metal cation levels has been reported [10], which is associated with subsequent cognitive loss and neurodegeneration. These observations were used by Bush [2] to formulate the "Metals hypothesis of AD", stating that the preservation of metal homeostasis is a critical point for neuronal function.

Wu et al. [14] point out that exposure to heavy metals (such as Pb) during brain development affects the metabolism of APP at later stages, and conceivably the processes of amyloidogenesis.

Lead has long been known as a toxic metal. It can replace divalent cations and affect the concentration of other ions, which are important cofactors of a number of enzymes and are also involved in various metabolic processes.

Lead induces oxidative stress by disruption of the defense mechanisms against reactive oxygen species (ROS). Furthermore Pb can even at picomolar concentrations

replace calcium and affect the sodium ion concentration, thereby affecting vital biological activities in the excitatory tissues like cell to cell interactions, thus disrupting neuronal communication [4].

It has been reported that Pb inhibits APP translation [8] through replacement of iron (Fe)-ions in Fe-regulated pathways. On the other side APP is a protein participating in the maintenance of Fe homeostasis, regulating its efflux [1, 3, 8]. McCarthy et al. [7] proposed that this function is furnished specifically by the secreted APP forms.

The Pb-mediated lowering of APP concentration, and thus the APP-regulated Fe-efflux, results in raised cytosolic Fe levels which can become toxic due to their catalyzing the generation of ROS. Enhanced APP expression counteracts this process [9].

Bringing about elevation of the intracellular Fe concentration, and thus the generation of ROS, Pb has been shown to impair mitochondrial membrane function and to influence the calcium balance in this organelle which results in cell death [13]. This process has also been termed "ferroptosis" [12].

Data on the direct influence of Pb on APP secretion are scarce in the literature which prompted us to address this problem. We found that exposure to Pb decreases the secretion of APP in mice cerebral hemispheres and cerebellum, while in the other studied areas (forebrain, hindbrain) there was no significant effect detectable.

Materials and Methods

The experimental design for inducing subacute Pb intoxication was developed and implemented by Ivanova et al. [5]. The cerebral hemispheres, cerebellum, forebrain and hindbrain of experimental animals were dissected and processed as follows.

The tissues were homogenized and membrane-containing and soluble protein fractions were prepared by centrifugation for 1 h at 100 000 g at 4ºC. The fractions were subjected to dodecylsulfate-polyacrylamide gel electrophoresis on 7% gels. APP was detected through immunoblotting with the monoclonal antibody 22C11 (Boehringer Mannheim) and visualized with the diaminobenzidine-H_2O_2 technique. Quantification of the grey values was performed by densitometric image analysis using the software package TINA 2.0 (Raytest). Results were normalized using actin as internal loading control.

Results and Discussion

Using the experimental protocol for intoxication with Pb, described by Ivanova et al. [5], we studied the effect of Pb on the secretion of APP in cerebral hemispheres, cerebellum, forebrain, and hindbrain of the treated mice.

The monoclonal antibody 22C11 employed in this study binds to an epitope in the N-terminal portion of the APP molecule, i.e. it recognizes both the intact APP molecules as well as the metabolites, obtained as a result of the activity of the proteases acting at the C-terminal. These comprise the secreted forms of APP which are found in the fraction containing the soluble proteins.

To verify the comparison between the studied fractions we used actin for normalizing the results. Under ideal circumstances normalization would not be necessary, but factors as transfer efficiency and sample loading make this step essential. The amount of actin detected allows the correction of deviations in protein content.

Representative immunoblot showing the effect of Pb on the secretion of APP in the cerebral hemispheres is shown on **Fig. 1**, and respectively for the effect of Pb on the secretion of APP in cerebellum – on **Fig. 2**.

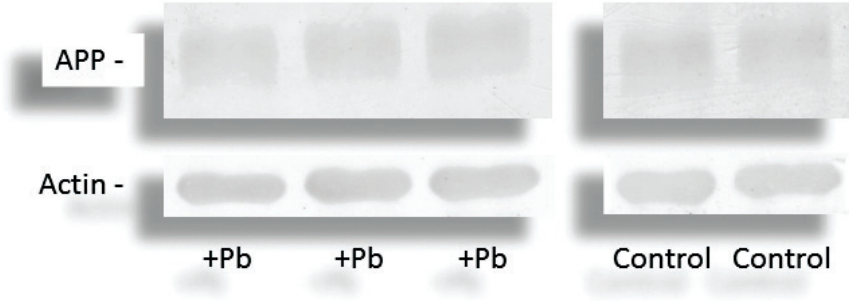

Fig. 1. Representative immunoblot showing the effect of lead (Pb) on the APP secretion in cerebral hemispheres.

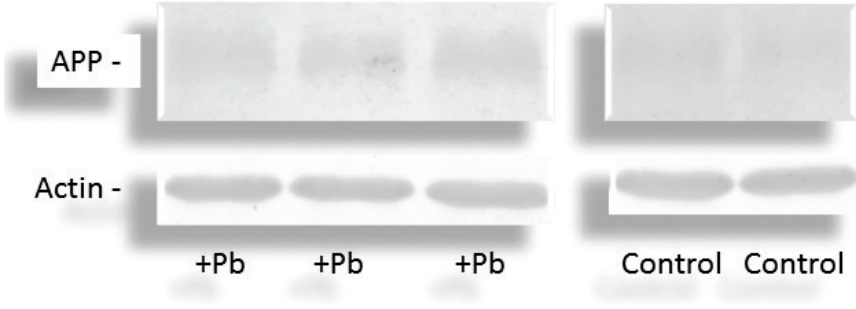

Fig. 2. Representative immunoblot showing the effect of lead (Pb) on the APP secretion in cerebellum.

The numerical quantification of these results is shown on **Fig. 3**. It can be seen that Pb decreases the secretion of APP in the cerebral hemispheres by 10% and in cerebellum by 19%.

We also studied the effect of Pb on the secretion of APP in front brain and hindbrain, where we did not find any significant effects of Pb intoxication (data not shown).

In a previous study we have shown that the content of APP in the cortex containing structures cerebral hemispheres and cerebellum is much higher as compared to that in other brain structures [6] (**Fig. 4**). It could explain the significant influence of Pb in the cerebral hemispheres and cerebellum.

We also performed immunoblotting with the membrane containing fractions of the studied brain regions and found no significant changes in the APP content after ~~the~~ Pb treatment (data not shown).

The results of our study show that exposure to Pb leads to decreased secretion of APP in the cerebral hemispheres and cerebellum. In accordance with the proposal of McCarthy et al. [7] that secreted APP participates in the maintenance of Fe homeostasis, regulating its efflux, we can suggest a mechanism of Pb toxicity – Pb decreases APP

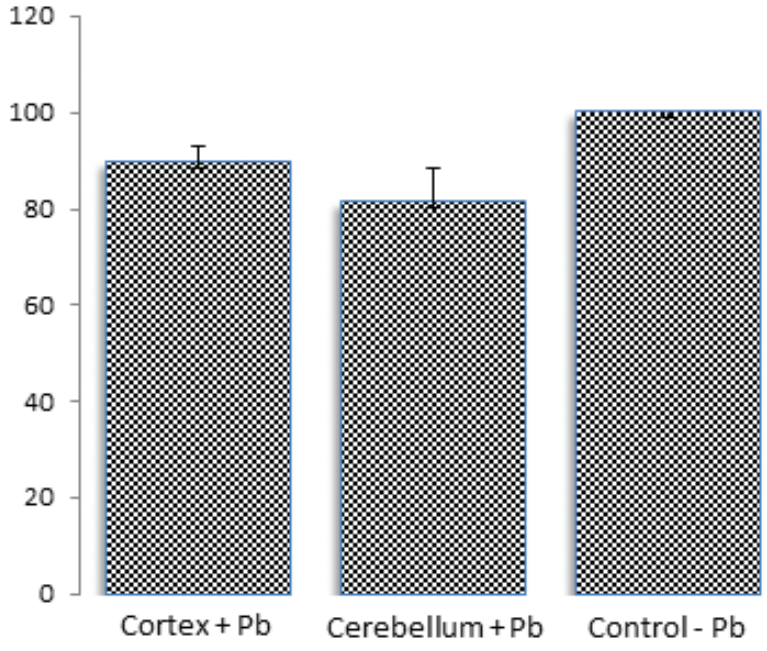

Fig. 3. Numerical quantification of the effect of lead (Pb) on the APP secretion in cerebral hemispheres and cerebellum. The data are calculated as grey values/µg protein and the value of control is taken as 100%. The data are the means of three experiments, each performed in duplicate.

Fig. 4. Expression pattern of APP695 mRNA. Representative autoradiogram from sagittal section through 90-day-old rat brain. The figure is part of the data presented by Kirazov et al. 2001 [6]. For details please refer this publication.

secretion which results in increased Fe concentration and thus causing a number of disturbances in cellular metabolism leading to cell- respectively neurotoxicity.

The mechanism of the effect of Pb on the secretion of APP evidently needs further investigation.

Acknowledgements: This study was financially supported by Sofia University "St. Kliment Ohridski" Science Fund (grant 162/2014).

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Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

Heavy Metals in a Host-Parasite System from a Cooper Mining Region in Bulgaria

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A host-parasite system *Rattus norvegicus/ Hymenolepis spp.* has been used as a bioindicator of heavy metals in the copper mining region of Chelopech, Bulgaria. The region has been polluted from the mining industry for copper and gold. Studies were done for contents of heavy metals zinc (Zn), copper (Cu), cadmium (Cd), lead (Pb), nickel (Ni), aluminium (Al), iron (Fe), manganese (Mn) in tissues (liver and kidney) of rats (infected with tapeworms and non-infected) as well as in strobila of the parasites in comparison aspect using ICP-OES. Bioaccumulation factor was determined as a ratio between the content of metal in cestodes to a content of the same metal in rat tissue (liver or kidney). The high BF-s for Pb and Cu indicated that the amount of metals in the environment may result in significant uptake by the tapeworms and their host.

Key words: Hymenolepis spp., rat, heavy metals

Introduction

The intake and bioaccumulation of trace metals by mammals is known to occur [14]. Rodents and voles are mammals suitable for a bioindication of metals in the environment. These small mammals are widely used for determination of the level of contaminants based on mainly the detoxifying organs (liver and kidneys) due to their high capability for accumulating heavy metals [20]. There is a close interaction between the effects of pollutants and parasites on terrestrial animals [18]. Small mammals most frequently are infected with endohelminths. Both hosts and parasites are being exposed simultaneously to the concentrations of pollutants that are available in the environment. In most cases the intestinal parasites accumulated higher concentrations of heavy metals than their hosts, which could, in turn, even be beneficial for their host [18, 23]. Parasites are accepted as an integral part of the environment, with natural and anthropogenic variables influencing their various life stages [12,13].

Rattus norvegicus is widespread in rural and urban habitats, commonly found living near sources of food and water and is a good bioindicator for heavy metal pollution [9]. A tapeworm *Hymenolepis spp.* is one of the endohelminths with increasing abundance in the

wild rats [10, 19, 21]. A host-parasite system *H. diminuta/R. norvegicus* has been used as a bioindicator of heavy metal pollution in the terrestrial environment [2, 4, 18, 20].

The aim of the study was to determine the main heavy metals content in the host-parasite system /*Rattus norvegicus – Hymenolepis spp.*/ collected in the copper mining region Chelopech, Bulgaria.

Material and Methods

Experimental material was obtained from a total of 96 rats in an area localized in the South West of Chelopech mining, near the village of Chavdar. The lands of the region have been polluted from the mining industry for copper and gold. The analyses of complex soil problems allowed us to point out that the main problem is related to acidity and the lack of nutrient elements. Gold mining has greatly increased copper concentration [3]. Low pH and high copper contamination reflect on the biota [6, 15]. The concentration of heavy metals in the region exceeded the accepted maximal permissible levels in respect to Cd (0.6mg/L), Cu (118,5 mg/L), Zn (98,1 mg/L) and Pb (39,5 mg/L) in randomly spread soil sample [6, 15].

Rattus norvegicus is a very spread rodents in this area. The wild rats were captured during 1 year from May to September 2018. They were captured by snap traps. Only adult rats were used in the study > 2,5 months old, according to body weight (border value 200 g). Their age was determined according to criteria of molar root development and growth [8].

The identification of endohelminths was according [7]. The dominant species of helminths were tapeworms *Hymenolepis* spp. Wild small rodents rarely remain uninfected (**Table 1**). A high prevalence of infection with intestinal helminths may be due to high reproductive potential, moved more often and faster than uninfected rats [15].

Table 1. Number of rats (parasitized and non-parasitized captured around Chelopech (Bulgaria)

Un-infected	Infected
19	52

After trapping dead rats were dissected for removal of the liver, kidney and digestive tract. The digestive tract was investigated according to standard helminthological procedures. Rats infected with trematodes and acanthocephalans and those with mixed infections were excluded from the study. Samples of the target organs were deep-frozen until posterior processing for chemical analysis. Livers, kidneys and tapeworms were taken from each rat, dried and the tissues were digested by dry ashing procedure according [11]. The concentration of samples in the homogenates was determined using ICP-OES. Studies were done for heavy metals Zn, Cu, Cd, Pb, Ni, Al, Fe, Mn in tissues (liver and kidney) of rats (infected and non-infected) as well as in strobila of tapeworms in comparison aspect.

The study was conducted in a compliance with the requirements of the European Convention for the protection of Vertebrate animals used for experimental and other specific purposes and current Bulgarian laws and regulations. All procedures for animals

were reviewed and approved by the Institutional Animal Care and Use Committee of the Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences (Permit number: 96, 22.05.2014). Bioaccumulation factor (BF) was determined as a ratio between the content of metal in cestodes to a content of the same metal in rat tissue (liver or kidney) [19]. The statistical analysis was carried out on the Prism 6 programme. The distribution of data was determined – a Gaussian one (normal for all values). The determination of the distribution was performed using the test of Kolmogorov-Smirnov and D`Agostino-Pearson. In the Grubb test application no extreme value have been found (they are strongly differing from the mean one, usually neglible). Variation analysis was used for determining the mean values, the standard deviation (SD) and the significance criterion (P). The comparison of the mean values of parameters was carried out using the one-way analysis of variance, Dunnett's Multiple Comparison Test. The results from these comparisons were also statistically significant: * ($P \leq 0.05$), ** ($P \leq 0.001$), *** ($P \leq 0.0001$).

Results and Discussion

Data of the study give the possibility to compare the concentration of metals in tissues from non-infected and infected rats as well as to compare concentrations of metals in *Hymenolepis spp.* and host tissues of the infected rats. (**Fig. 1a, b, c**).

The levels of metals in the rat tissues and cestode tissues were in the next descending order:

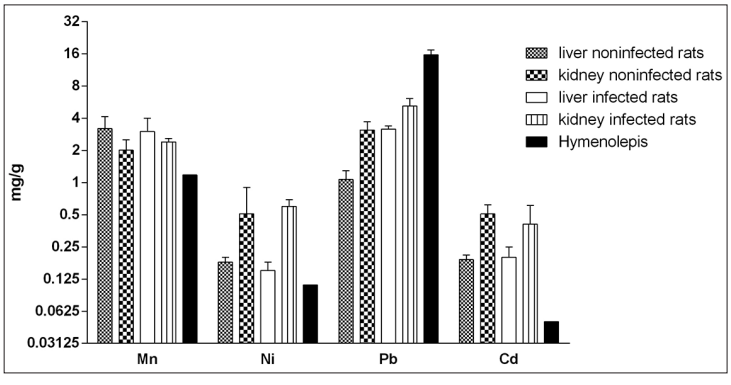

Fig. 1A

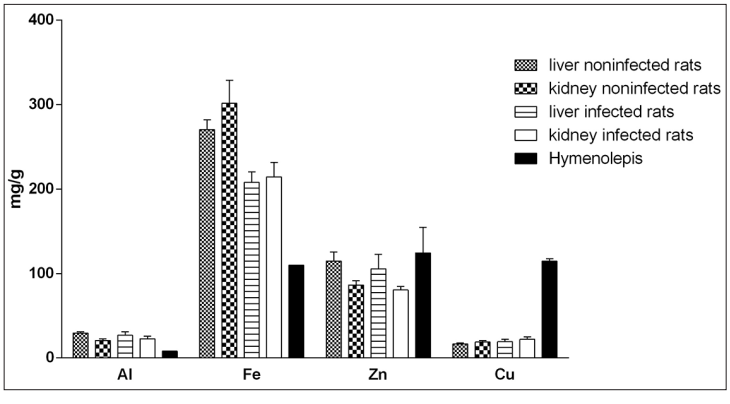

Fig. 1B

Fig. 1C

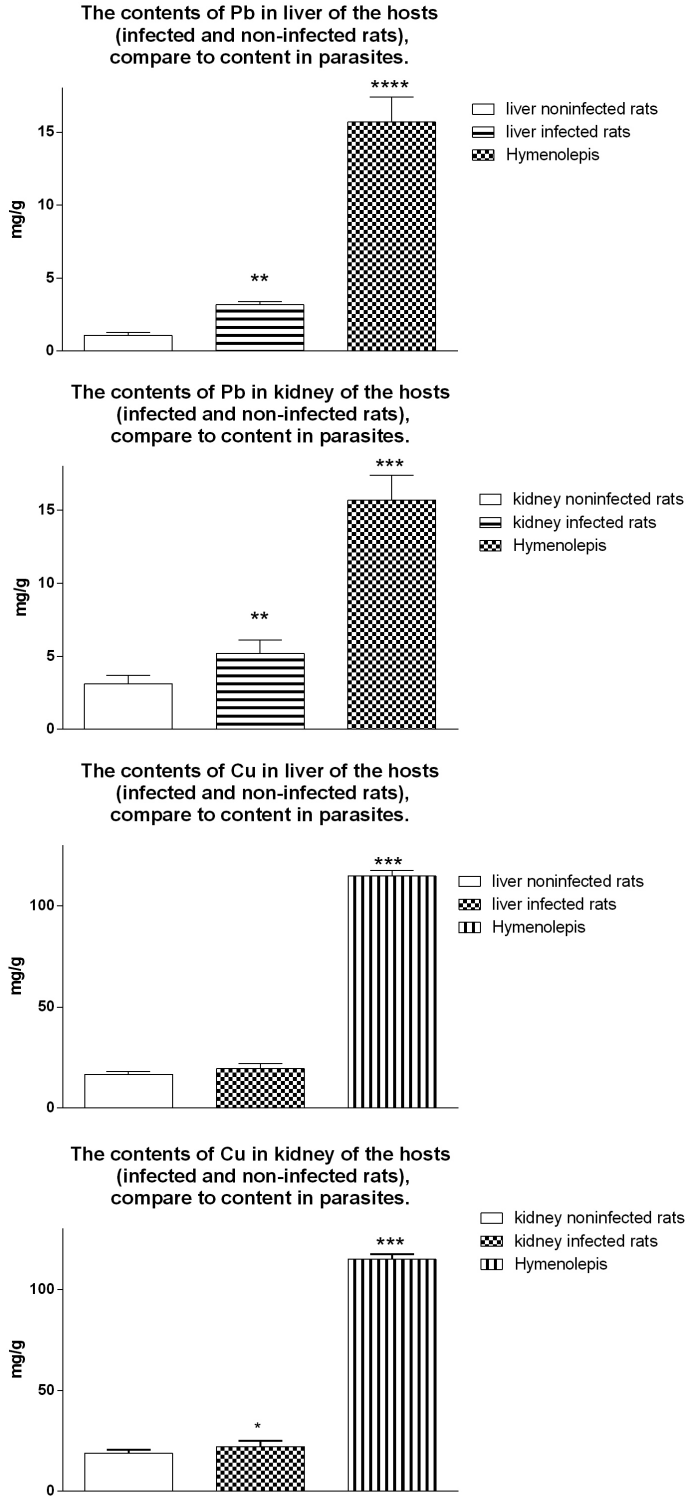

Fig.1. The concentration of metals in tissues from non-infected and infected rats compare to concentrations of metals in *Hymenolepis spp.* and host tissues of the infected rats.

Liver from infected rats: Fe> Zn> Al>Cu> Pb>Mn> Ni> Cd
Liver from un-infected rats: Fe> Zn> Al>Cu> Pb> Mn > Ni> Cd
Kidney from infected rats: Fe> Zn > Cu> Al> Mn> Pb> Ni> Cd
Kidney from un-infected rats: Fe> Zn > Cu> Al> Pb> Mn> Ni> Cd
Hymenolepis spp.: Zn> Fe>Cu > Al> Pb > Mn> Ni> Cd

Concentration of metals in descending order did not differ between these in the livers of infected and non-infected rats. The contents of Fe, Cu, Cd and Pb were higher in the kidneys than the livers of the hosts (infected and non-infected rats). Contents of Al, Cu and Fe were reduced in the livers of infected rats compared with non-infected individual. Concentration of Fe was reduced in infected rats compared with that in non-infected rats. The descending order of metals in the tapeworm was presented in different way to these in the hosts. The levels of Cu in the parasite were about 7 times higher than that in the infected rats. Zn content in the parasite was insignificantly higher compared to Zn level in the host. High ability of cestodes to accumulate heavy metals was present by BF of ratio of metal content in cestodes to that in host tissues. BF for liver and kidney was above 5 for Cu and Pb, and for Zn was above 1. Cu, Zn and Pb were bioaccumulated more in cestodes than in the host tissues. Concentration of Zn is an essential nutrient required for growth and maintenance of many biological systems in a host and a cestode [12, 13]. *Hymenolepis* strobila tissues showed significantly higher Pb and Cu content than the host tissues. It is in a good accordance with studies of [19] which indicated that tapeworms are able to accumulate heavy metals much more rapidly than the host soft tissues.

The system of barriers maintains body homeostasis in animals. One of these is the gastrointestinal barrier, which effectively protects from deleterious effect various factors (biological, chemical etc.) by means of their selective transport. Because the small intestine is simultaneously the predilection habitat of Hymenolepidae and the main site for heavy metal absorption after oral intake, tapeworms may interact with metals in the rat intestine and their absorption into the host body. This interaction may affect the course of heavy metals in the intestine. Helminth paratization may disrupt internal homeostasis, modulates immunological regulation of the host. Cestoda with lacking digestive tract concentrate metals to a higher degree than the host tissues [22].

The studies showed elevated tapeworm BFs in animals living in in polluted areas [5]. Our studies support the hypothesis that cestodes with a relatively large absorbance surface have reached high BFs. It may be due to their lower metabolic activities or due to a fact that the endohelminths lacking digestive tracts (Cestoda and Acanthocephala) and using their teguments for absorbing substances from the host digestive tract [10]. Tapeworms take up certain biogenic elements as Fe, Zn, Cu from the chyme into their tissues, and this uptake could free up transport routes for Pb absorption because these elements use the same pathways. Parasites may also cause an increase in host heavy metals by disrupting their risk elements regulation system. The content of Pb was higher in the kidneys than in the livers in the infected and non-infected hosts. Median values of Pb content differed insignificantly among parasitized and non-parasitized animals. Our results have confirmed those of [5] done only at low Pb exposure. Generally, helminths interfere with the host absorption, metabolism, uptake and regulation of the mineral elements [19]. Helminths may demonstrate higher sensitivity to the toxic effect of Pb.

Endohelminths and their hosts compete for biogenic elements Zn, Fe, Cu. The digestive tracts are absent in the cestodes, therefore they are able to concentrate metals to a higher degree than the host tissues. Worms are not able to synthesize their own cholesterol and fatty acids, so they take bile from a host intestine which in turn reduces their ability to absorb metals [18]. Cestodes use the bile salts for their egg formation and hatching. This could possibly explain the high concentration of metals in the cestodes.

Our data do not fully support the prior hypotheses of [10] which state that intestinal helminths are able to prevent their host from absorbing ingested heavy metals and their accumulation in the tissues of the host. The high Pb and Cu concentration in the tapeworms were not associated with simultaneously significant decrease of these elements in host tissues [5, 19]. Thus tapeworms are not able to actively eliminate pollutants from host tissues. Lead content represents the most important risk, which is probably linked to the nature of the ore exploited, to the low mobility of Pb in the environment and the acidification of the soil in the studied region. The concentration of heavy metals in the region exceeded the accepted maximal permissible levels in respect to Cd (0.6mg/L), Cu (118,5 mg/L), Zn (98,1 mg/L) and Pb (39,5 mg/L) in randomly spread soil sample [17], [16], [6]. Mammals and birds are 100-1000 times more resistant to Cu than other animals. Despite the fact that almost heavy metal values may not indicate a severe risk of toxic effects on wildlife but after a long period of time could exert an impact on individuals, communities and ecosystems.

Conclusions

Concentrations of heavy metals were determined in sub-web a host-parasite system *Rattus norvegicus – Hymenoleps spp*. in copper polluted region. A high ratio C parasite/C host was indicative of acute pollutant exposure or long/chronic exposure of the pollutants correlated with high concentration in both the host and parasite. Parasite infections have been attributed to man-made impact and environmental changes in terrestrial habitat. The position of parasite as a trophic consumer can infer details about the chemical state of the environment as a consequence of food web biomagnifications. The host-helminth system wild rat/ *H. diminuta* reflects the content of heavy metals in the terrestrial environment and could be used as a bioindicator for heavy metal pollution. The high BFs for Pb and Cu indicated that the amount of metals in the environment may result in significant uptake by the tapeworms (**Fig. 2.**). Despite the fact that almost of heavy metal values may not indicate a severe risk of contamination in the environment.

Acknowledgements: This work was supported by the Bulgarian Ministry of Education and Science Grant No DN -14/7, 2017.

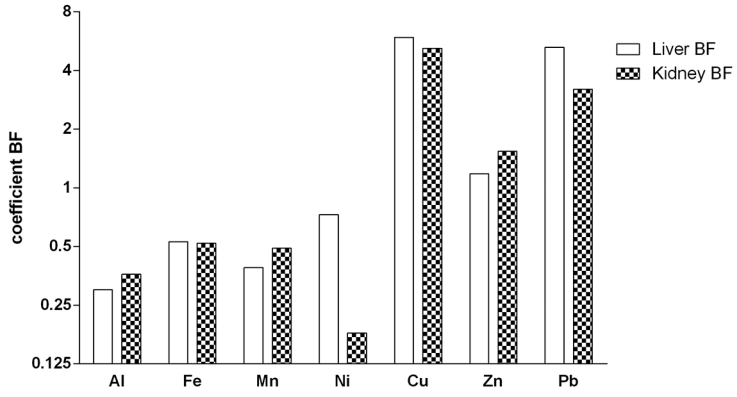

Fig. 2. BF of ratio of metal content in cestodes to that in host tissues.

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Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

Serum Trace Elements and Enzymes in Lambs with Introduced Haemonchosis

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Haemonchosis is an important parasitic infection in sheep and goats. The aim of our study was to evaluate the levels of trace zinc (Zn), manganese (Mn), copper (Cu), iron (Fe), cobalt (Co) and ultra-trace molybdenum (Mo), selenium (Se) elements and the activity of enzymes in serum (alkaline phosphatase, alanine aminotranspherase, aspartate aminotranspherase and lactate dehydrogenase). Levels of Zn, Cu, Fe, Se, Mo and Co were decreased in the serum from infected lambs compared to control. Mn level was non-significantly higher in the serum of infected animals than non-infected ones. Serum ALP, ALT and AST activity was increased in the infected lamb compared to the controls. LDH activity was not significantly changed in the infected lambs compared with healthy lambs. In this study increased pathological marker enzymes and an imbalance in the trace elements profile was observed. *Haemonchus contortus* plays an imperative task as anemia and oxidative stressors on lambs.

Key words: haemonchosis, lamb, trace elements, enzymes, serum

Introduction

Parasitic diseases are a serious problem for livestock economy worldwide. The important nematode species of small ruminants are related to genera *Haemonchus, Ostertagia* and *Trichostrongylus*. Haemonchosis is a prevalent infection in sheep and goats [3], (**Fig.1**) Clinical signs of the infection include anemia, digestion-absorption syndromes and, in many cases the death of young animals [8]. Several factors are involved in the pathogenesis of haemonchosis and the most important of them are parasite virulence and host

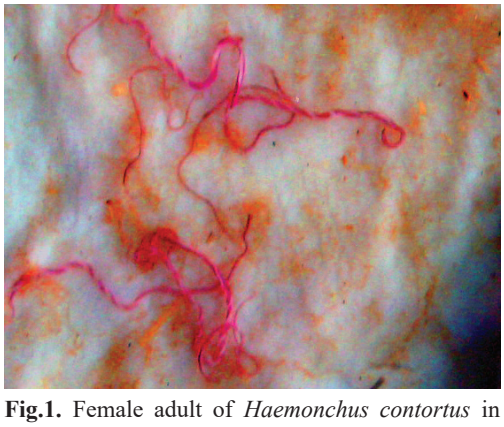

Fig.1. Female adult of *Haemonchus contortus* in abomasum of experimentally infected lamb (16×).

response [11]. Minerals are involved in various fundamental physiological processes in organism. Trace (cobalt, zinc, manganese, iron, copper) and ultra-trace (molybdenum, selenium) elements participate in different responses of infected hosts [9]. Studies have shown that improved mineral status of the animals may reduce parasite burden and improve immunity of the hosts [4,7]. Trace elements e. g. Zn, Se, Mn and Cu are necessary for the immune system and increase the resistance against parasite [8]. Copper has various functions in the organism. It acts as more than 20 metaloenzymes, metallo-proteins in organism. [13] documented that plasma Cu level decreased up to 50% in lambs with haemonchosis compared to non-infected lambs. Zinc is important to build up a successful immune response having capabilities to affect parasites [8]. Investigations indicated a positive impact of dietary Zn in infected hosts to improve immune response against gastrointestinal nematode infections [8]. Iron has presumably no direct effects on gastrointestinal parasites. However, Fe supplementation improves host performance because it restores Fe status in the organism which is lost through blood during the infection [4]. Investigations in animals infected with helminths have shown the positive effect of cobalt salt on their immune system. [7] reported that Co deficiency reduced the resistance of small ruminants against nematode infections. Cobalt is an essential component of vitamin B12. The role of molybdenum in immunity against endoparasites is well established. [10] noted decreased *H. contortus* burden in Mo- supplemented lambs. The authors observed that Mo-supplementation reduced *H. contortus* burden by 78%. Small number of data concerning selenium deficiency and infections in ruminants are noted. [12] reported a reduced burden of *H. contortus* in lambs supplemented with Se. The use of Se may provide better antioxidant defense system in lambs infected with *H. contortus* [9].

Levels of pathological marker enzymes and their interaction with trace elements are not study enough. [16] reported non-significant changes in the serum enzymes ALT, ALP and AST in the infected sheep with *H. contortus*. According [2] the levels of the same enzymes were elevated in the infected lambs and goats.

The aim of the study was to evaluate the level of trace (Zn, Mn, Cu, Fe, Co) and ultra-trace (Mo, Se) elements as well as the activity of enzymes in serum of lambs experimentally infected with *Haemonchus contortus.*

Material and Methods

For this study were used 16 male 6-month-old lambs of the Black head Pleven breed with a middle weight 26-27 kg. The lambs were divided into two groups (eight lambs in a group): Group 2 – lambs infected with *Haemonchus contortus* larvae and Group 1 – controls. Before experimental infection animals were kept in collective pens located in the vivarium of the Institute of Experimental Morphology, Pathology and Anthropology with Museum, BAS, for a month for adaptation to the diet and to the experimental environment. During this period the lambs were treated with antihelmintics – combination of closantel and albendazole. The first group was infected two times every two days with 1 800 *H. contortus* larvae (L3) per a lamb. The Baermann technique was used to extract the L3 larval stages of *H. contortus* intestinal nematodes and counted under a dissecting microscope to determine the larval counts by [17]. The samples

were taken 58 days after infection. Blood samples were collected from the jugular vein in vacutainers. Serum was studied for trace element levels. The study was conducted in compliance with the requirements of the European Convention for the protection of Vertebrate animals used for experimental and other specific purposes and current Bulgarian laws and regulations. All procedures for animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences. (Permit number: 96, 22.05.2014).

The levels of Zn, Cu, Mn, Mo, Co and Fe were determined with an atomic absorption spectrophotometer (PYE UNICAM sp. 2900, PYE UNICAM). Selenium level was determined fluorimetrically.

The statistical analysis was carried out on the Prism 6 program. The distribution of data was determined – a Gaussian one (normal for all values). The determination of the distribution was performed using the test of Kolmogorov-Smirnov and D`Agostino-Pearson. In the Grubb test application no extreme value have been found (they are strongly differing from the mean one, usually neglible). Variation analysis was used for determining the mean values, the standard deviation (SD) and the significance criterion (P). The comparison of the mean values of parameters was carried out using the one-way analysis of variance, Dunnett's Multiple Comparison Test. The results from these comparisons were also statistically significant: * ($p \leq 0.05$), ** ($p \leq 0.001$), *** ($p \leq 0.0001$).

Serum levels of alkaline phosphatase (ALP), alanine aminotranspherase (ALT), aspartate aminotranspherase (AST) and lactate dehydrogenase (LDH) were determined using a semi-auto chemistry analyzer BA-88 A (Mindray, China).

Results and Discussion

Serum enzyme activities: Serum ALP activity was increased in the infected lamb compared to the healthy ones by 40 %. Activity of ALT and AST was higher in the infected host than controls by 21-22 %. LDH activity was not significantly changed in the infected lambs compared with healthy ones (**Table 1**).

Table 1. Enzymes activity in serum of lambs IU/L

	ALP	ALT	AST	LDH
Control	155,40 ±10,16	28,70 ±2,18	120,49 ±3,50	271,40 ±19,50
Infected	216,79 ±18,44	34,80 ±9,62	146,93 ±28,11	265,33 ±29,40

Trace element levels: Zinc serum level was significantly reduced in the infected animals compared with controls. Copper level was lower in the infected lambs compared with non-infected ones too. Higher Manganese level was observed in the infected lambs than the controls but non-significantly. Serum cobalt level was reduced in the infected animals compared to the normal range. Iron serum level was significantly decreased in the infected animals than in the controls (**Table 2**).

Ultra trace elements: Mo and Se were significantly reduced in the infected lambs compared to controls (**Table 2**).

Table 2. Serum trace elements level mg/L

	Zn	Cu	Mn	Co	Mo	Se	Fe
Control lambs	0,97 ±0,04	0,83 ±0,05	0,22 ±0,06	0,09 ±0,0	0,011 ± 0,00	0,22 ±0,01	1,94 ±0,09
Infected lambs	0,46 ±0,01	0,70 ±0,06	0,28 ±0,01	0,02 ±0,0	0,002 ±0,00	0,13 ±0,07	0,97 ± 0,11

In this study increased pathological marker enzymes and an imbalance in the trace elements profile was observed. The elevation of serum ALP, ALT and AST level indicated some disruptive activities in organs of the origin or of altered membrane permeability. Their level could also rise due to lack of excretion or decrease due to impaired synthesis [16]. Ahmat and Ansari [2] reported a significant rise in serum ALP in sheep and goats with haemonchosis. The rise in enzyme activity might be attributed to the damage to abomasal mucosa by the parasites similar to that described by Alam et al. [3]. Specific hepatic function is affected by a wide variety of the pathological conditions of extrahepatic origin especially gastrointestinal origin. Similar findings were reported by Ahmat and Ansari [2] that the increase in enzymatic activities reflect the cellular abnormalities directly related to damage occurred in the hepatocytes and pathological lesions of intestines. As ALP is widely distributed in the body and found in high concentrations in intestinal mucosa, liver, increase of the ALP value is suggestive to damage to mucosal cells of intestine due to parasite pathogenesis in gastrointestinal disorders. Our results for serum enzymes in the infected with *H. contortus* lambs are in good agreements with those of Ahmat and Ansari [2]. Similar data for elevated levels of the enzymes ALP, AST and ALT are observed by Sharma et al. [16] in goats. Analysis of serum is an effective method to analyze trace elements profile of animals.

Trace elements are important for optimal immunity and disease resistance in infected animals [8]. Metabolism of trace elements was seriously affected by the haemonchosis [3]. Their role as cofactors of certain enzymes has been recognized by Kojouri et al. [8]. Most of them act as antioxidants [8] as like Zn, Fe, Cu, Se. Zinc is a part of several enzymes. The active sites of most anhydrases contain Zn. This biogenic element also is required for the regulation of genes that are required for signal transduction, response to stress, growth and energy utilization [9]. The enzyme superoxide dismutase contains zinc, which is necessary to protect cells from superoxide radicals and participates in the antioxidant defense system [3]. Zn serum level was reduced in the infected animals compared with controls. The present Zn level is similar to that established by Kojouri et al. [8]. Zn concentration falls in a variety of disease associated with the anorexia [9]. Reduced Zn content could be associated with increased adult worms using the host Zn ions for their own metabolism.

Copper is an essential element for the synthesis of various enzymes, including cytochrome-c-oxidase, superoxide dismutase, ceruloplasmin. Clinical signs related to Cu-deficiency are anemia, severe diarrhea, fragile bones and depressed growth. Cu level was lower in the infected lambs compared than the non-infected ones. Reduced hydrolysis in the abomasum and proximal regions of the intestine when pH is elevated

by nematodes may contribute to reduced Cu availability and absorption [9]. Due to the biologically significant homeostatic relationship between Zn and Cu, both elements have been used as an indicator of severity of disease [16].

Manganese participates in the metabolism of lipids and carbohydrates. Manganese is a cofactor of the superoxide dismutase (MnSOD). Higher Mn level in the infected lambs observed by us is parallel to the observations by Ahmad et al. [1]. It is in a good agreement with our data for increased Mn-content in the lamb.

Cobalt is an essential component of vitamin B12, which is a cofactor for the enzymes methionine synthase and methylmalonyl Coenzyme A mutase [1]. Similar reduced serum Co content in our study was noted by Kojouri et al. [8]. Cobalt deficiency leads to developed of anemia [12].

Molybdenum is an essential component of the enzyme xanthine oxidase catalyzing the oxidation of hypoxanthine to xanthine and of sulfite oxidase which is required for the metabolism of sulfur-containing amino acids. Mo content in serum from infected animals was reduced compared to the control content. Molybdenum is an important co-factor in the integral complex that is important in weight gain [1].

Iron is an important element of hemoglobin and myoglobin, and is an essential component of cytochrome, catalase and peroxidase [4]. Deficiency of iron in ruminants leads to anemia, reduced food intake and chronic blood loss during parasitic infections [1]. The reduction of serum Fe level could be attributed to the expended erythropoiesis to compensate for blood loss leading to depression of Fe stores [4]. Serum deficiency of Fe observed by us confirms the data by Murad et al. [12]. Decreases in blood Fe were correlated to elevations in ALT observed by us now and by [4, 15].

Selenium is a component of glutathione peroxidase which plays an important role in the metabolism of lipid hydroperoxides. Selenium is a component of thioredoxin reductase which regulates transcription, recycles vitamin C and E, and absorbs calcium [8]. Serum Se level was reduced in the infected lambs compared to non-infected hosts. It is in a good agreement with the lowed activity of glutathione peroxidase [13]. Se deficiency leads to compromised immune function, weight loss and diarrhea.

H. contortus has serious effect on serum biochemistry and enzymatic assays like ALT, ALP and AST as well as on biogenic elements in serum. Interactions between reduced enzyme activity of ALT and a decrease in Zn serum level have been associated with a variety of liver diseases [16]. Zinc level is a significant predictor of ALT level [2]. Animals with high serum ALT were more likely to have lower Zn compared with normal level.

Data in the present study, on trace elements levels and enzymes activity changes, caused by haemonchosis in lambs carry importance as they may indicate the extent of damage to the abomasal mucosa and thereby help in better understanding of the pathogenesis of anemia especially in the absence of other possible factors which may influence those changes. *H. contortus* manipulates the lamb gastrointestinal microbiome, modifying the balance between host and gastric microbiota [5]. Our data evidence the role of trace- and ultra- trace minerals in these mechanisms. It can be concluded that *Haemonchus contortus* plays an imperative task as an anemia and oxidative stressors on lambs.

Conclusion

In generally accepted that trace elements have beneficial effect for controlling and prevention of gastrointestinal parasites in animals. There is a need to identify important trace elements needed for mucosal immunity function and whose imbalance and deficiency act as a predisposing factor for susceptibility to gastrointestinal parasitism in small ruminants.

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Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

First Report of Feline Thelaziosis in Bulgaria and Morphometric Data of Thelazia callipaeda Railliet et Henry, 1910 in Present Materials

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Thelazioses are ocular parasitic diseases in a number of mammals, including humans. The genus *Thelazia* includes 14 species, among which is *Thelazia callipaeda* Railliet et Henry, 1910, which is known to parasitize in domestic dogs, raccoon dogs, domestic cats, red foxes, European rabbits, monkeys, and humans. In 2019 one domestic cat from Sofia region, Bulgaria, was referred to veterinary practitioner with symptoms of conjunctivitis. Thirty-five nematodes of *T. callipaeda* species were collected from both eyes of the cat. They were examined by light microscopy, and the most important morphometric features of the species were described. The obtained results were compared with existing ones in the literature. This is the first reported case of thelaziosis in a domestic cat from Bulgaria.

Key words: eyeworm; domestic cat; *Thelazia callipaeda*; morphometric data; Bulgaria

Introduction

Thelazioses are infections caused by parasitic worms of the family Thelaziidae (Nematoda; Spirurida). The adults of these parasites inhabit the eyes and associated tissues (such as eyelids, lacrimal ducts, etc.) of definitive hosts – various mammals and birds, including humans. Intermediate hosts of *Thelazia* are non-biting diptera which feed on animal lachrymal secretions and become infected [16].

A number of species belong to the genus *Thelazia*: *T. rhodesi,* parasitizing in cattle, buffaloes, zebu, bison, horses, sheep and goats; *T. brevispiculata,* parasitizing in cattle; *T. californiensis* – in domestic dogs, domestic cats, coyotes, sheep, Mule deer, American black bears, and humans; *T. callipaeda* – in domestic dogs, raccoon dogs, domestic cats, red foxes, European rabbits, monkeys, and humans; *T. erschowi* – in pigs; *T. ferulata* – in cattle; *T. gulosa* – in cattle and yaks; *T. hsüi* – in cattle; *T. iheringi* – in agouties; *T. kansuensis* – in cattle; *T. lacrymalis* – in horses and donkeys; *T. leesei* – in camels; *T. petrowi* – in cattle; *T. skrjabini* – in cattle and yaks [19].

The helminths of the genus *Thelazia* are known for the Bulgarian fauna. The species *T. gulosa* and *T. rhodesii* have been reported as parasites on cattle and buffaloes

60 years ago [21], and *T. callipaeda* has been found in domestic dogs recently [4,10]. In the present work, we provide brief data on a case of parasitic conjunctivitis in a domestic cat caused by *T. callipaeda* and a morphometric description of the species in the collected materials.

Materials and Methods

In the summer of 2019, a male cat referred to a veterinary practitioner with conjunctivitis was found on clinical examination to have adult nematodes in the conjunctival sac. The cat was of a mixed breed and lived freely in the yard of its owners in a village near Sofia (42° 46′ 7.79″ N, 23° 24′ 23.21″ E). After removal of the worms by flushing with saline solution, the cat was treated topically with Advocate® for cats as per label recommendations.

The collected parasites were stored in 70% ethanol. Aiming to differentiate their species 10 male and 10 female specimens were studied after enlightening in lactophenol. The imaging and measurement of the parasitic structures was performed using a "Motic Images Plus 3.0" camera connected to an "Amplival" microscope and the accompanying software. The identification of the helminths was carried out on the basis of their host species, localization and morphological characteristics [19]. The obtained specimens were deposited in the collection of the Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Sofia, Bulgaria.

Results and Discussion

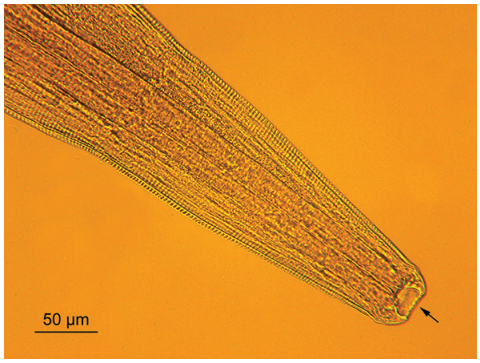
Thirty-five parasitic nematodes were collected from both eyes of the cat – 19 female and 16 male. They were identified as *Thelazia callipaeda*.

At the physical examination the cat was in good general condition, but with conjunctival redness and a serous secretion from both eyes, as well as chemosis of the 3rd eyelids.

According to Otranto and Dutto [17] infestation by *Thelazia* in animal and human hosts may be asymptomatic, though it frequently causes watery eyes, conjunctivitis, corneal opacity, or ulcerative keratitis. According to the literature, conjunctivitis is the most common symptom of *T. callipaeda* infestation, with more reports of unilateral conjunctivitis [3, 6, 11, 14] than those describing involvement of both eyes [5, 8] as in the present case. Complications have been also observed, which are not always related to the number of parasites affecting the eyes. For example, severe ocular changes (conjunctivitis, edema, keratitis, and mucoid secretions) have been observed in a domestic dog infested with 77 specimens of *T. callipaeda* [5] as well as in European rabbits infested with only three specimens each [8]. In the latter gross lesions compatible with haemorrhagic viral disease have been described.

Morphometric data of *T. callipaeda* in present materials

Threadlike, whitish body, with slightly narrowed ends. Well-defined transverse striations of the cuticle (**Fig. 1**). Presence of a vase-shaped buccal capsule with a wide middle part and a narrower bottom. The esophagus starts directly from the buccal capsule and expands slightly in its distal part (**Fig. 2**).



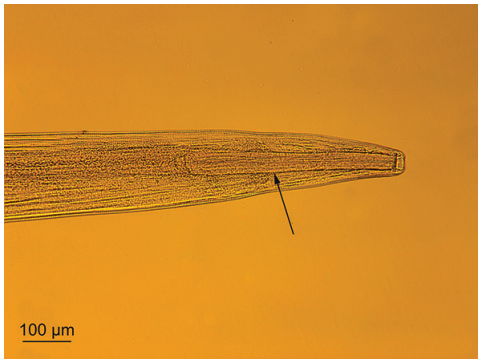


Fig. 1. **Fig. 2.**

Thelazia callipaeda found in a domestic cat from Bulgaria: **Fig. 1.** buccal capsule (arrow); **Fig. 2.** oesophagus (arrow).

Male: The posterior end is strongly curved (**Fig. 3**). The spicules are different in shape and size. One is long, thin, with wavy curves, and the other is much shorter, thick with the shape of a gutter. Perianal and postanal genital papillae are observed (**Fig. 4**). Female: The vulva opens at the anterior end of the body, at the level of the last third of the oesophagus (**Fig. 5**). The eggs in the uterus are embryonated. The posterior end is rounded, with 2 small lateral papillae (**Fig. 6**). The metric data on the specimens of the present materials are given in Table. 1. It contains measurements for the species by other authors too.

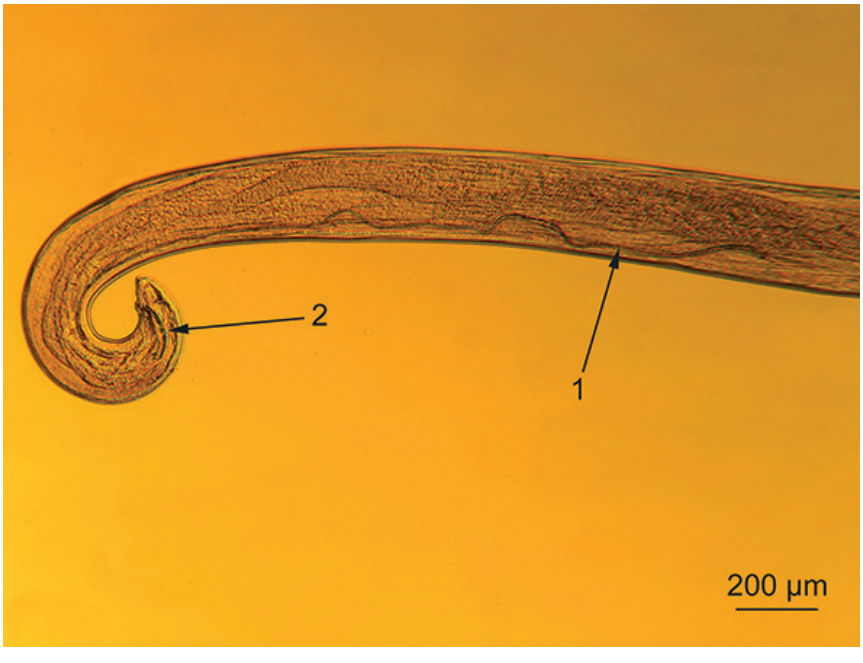
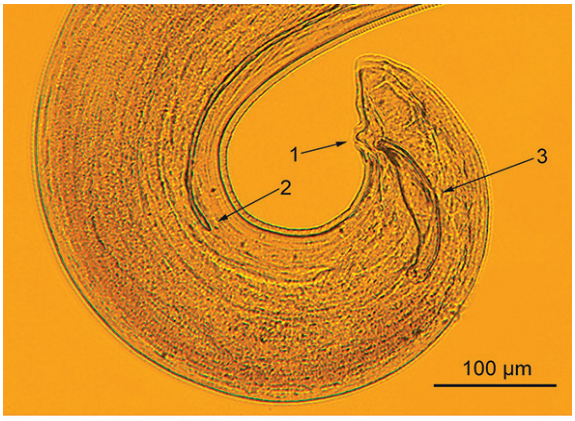


Fig. 3. Posterior end of male *Thelazia callipaeda* found in a domestic cat from Bulgaria: arrow 1 – large spicule, arrow 2 – small spicule.



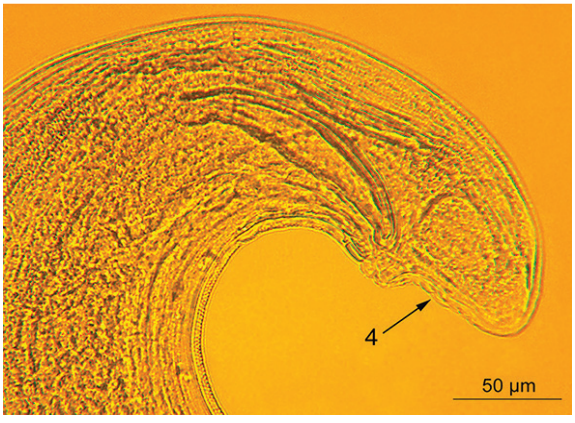


Fig. 4. Posterior end of male *Thelazia callipaeda* found in a domestic cat from Bulgaria: arrow 1 – perianal genital papillae; arrow 2 – distal end of the large spicule; arrow 3 – small spicule; arrow 4 – postanal genital papillae

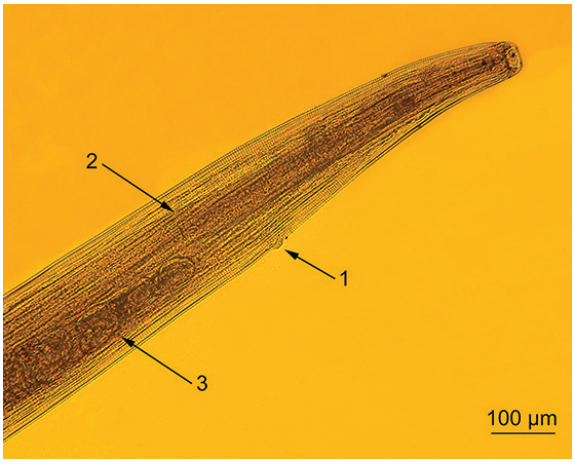


Fig. 5. Anterior end of female *Thelazia callipaeda* found in a domestic cat from Bulgaria: arrow 1 – vulva; arrow 2 – oesophagus end; arrow 3 – uterus with embryonated eggs

Fig. 6. Posterior end of female *Thelazia callipaeda* found in a domestic cat from Bulgaria: lateral papillae (phasmidae) – arrows

When comparing the metric data established for the species in the current materials with those indicated by other sources (**Table 1**), it makes an impression that: 1). The average body length of our specimens falls within the range indicated by most authors [2, 7, 9, 19] but exceeds that indicated by Kanchev et al. [10] and Čabanová et al. [1]. 2). Body width was measured in different sections by different authors. In most studies, data were missing for many structures such as the buccal capsule, oesophagus, the distance between the cloaca and the anus, and the posterior body ends. 3). The length of the large spicule of our specimens exceeds that indicated by other authors. 4). The distance between the vulva and the anterior body end varies, and in most cases it is similar except as indicated by Čabanová et al. [1], but this is logical since the body length of the female specimens in this case is also much smaller.

In general, it can be seen that the size limits of the morphological structures in the species are wide. This may be due to the population pecularities of the thelaziae obtained from different regions and hosts, as well as to the different methods for fixation and processing the parasites. For a more accurate concept, a larger number of future in-depth morphometric studies on materials from various sources are needed.

Thelazia callipaeda has been established in animals and humans in a number of countries in the southern and western parts of the European continent, and autochthonous cases have recently been identified in Central Europe as well [1, 2]. According to Motta et al. [14] in cats thelaziosis is diagnosed sporadically. According to the literature, cases of infestation of domestic cats with *T. callipaeda* have been reported in Italy [15], France [6], Switzerland [12; 14], Portugal [13, 20], Serbia [7], Bosnia and Herzegovina [9] and Greece [18]. This is the first case in which it is found in a domestic cat from Bulgaria.

Acknowledgments: This work was supported by the Bulgarian Ministry of Education and Science under the National Program for Research „Young Scientists and Postdoctoral Students“ (Decision of the Council of Ministers № 577/17.08.2018).

Table 1. Metric data of *Thelazia callipaeda* Railliet et Henry, 1910 in different materials

	Authors; Source of the matherials						
Structure	Our data; Domestic cat, Bulgaria	Skrjabin et al., 1967	Kanchev et al. 2013; Dom. dogs, Bulgaria	Hodžić et al., 2014; Red foxes, D.dogs, D.cats, Bosnia and Herzegovina, Croatia	Gajič et al. 2014; Dogs, cats, Sarbia	Čabanová et al., 2017 D.dogs, Slovakia	Čabanová et al., 2018; Red foxes, Slovakia
Body length ♂ (mm)	10-12.3 (11.14)	9-13	8,2-10,5	7.5-13	10.46-13.91	8.99 mean	9.3-15.4
Body width ♂ (µm)	119-179 (147.9) *	210-270 *	-	340-430 max	385-438 max	318-455 max	290-657 ^
Buccal capsule length ♂ (µm)	19-22 (20.4)	27-30	-	-	-	-	16.8-23.2
Buccal capsule width ♂ (µm)	30-39 (33.8)	32-36	-	-	-	-	32.3-45.8
Oesophagus length ♂ (µm)	440-592 (526.9)	580-608	-	-	-	-	512.5-634.9
Max. oesophagus width ♂ (µm)	49-63 (51.7)	50-68	-	-	-	-	-
Distance cloaca – tail tip (µm)	70-80 (75,4)	70-90	-	-	74-85	-	-
Big spicule length (mm)	1.497-2.1 (1.823)	1.3-1.7	-	-	1.421-1.800	-	1.782
Small spicule length (µm)	120-145 (131.7)	128-160	-	-	140-159	-	125
Body length ♀ (mm)	11-18 (16.75)	10.45-15	11,5-15,4	12-18.5	14.48-17.95	11.20 mean	15.7-25.4
Body width ♀ (µm)	122-183 (142.4) *	220-260 *	-	370-510 max	420-453 max	336-391 max	265-688 ^
Buccal capsule length ♀ (µm)	22-26 (23.6)	30	-	-	-	-	14.2-33.0
Buccal capsule width ♀ (µm)	35-42 (38,6)	36	-	-	-	-	38.0-48.1
Oesophagus length ♀ (µm)	540-637 (597.4)	560-720	-	-	-	-	317-717
Max. oesophagus width ♀ (µm)	51-57 (54)	90-100	-	-	-	-	-
Distance vulva-anterior body end (µm)	447-556 (501.6)	350-570	-	-	573-640	261 mean	485.0-703.6
Distance anus – posterior body end (µm)	70-81 (74.86)	70-100	-	-	-	-	-

* At the end of the oesophagus; ^ at the middle portion

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Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

Supraorbital Leiomyoma – a Case Report

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We present a case of a 49-year-old woman who is receiving treatment with a formation above her left eyebrow. 10 years ago, the patient suffered trauma in this area without compromising skin integrity, and for about two months noticed a small bump in the area. Under local anesthesia, a skin incision was made and a 1.5-1.8 cm formation was extirpated. The material was sent to a histological examination laboratory, on the basis of which the patient was diagnosed with Leiomyoma.

Key words: facial leiomyoma, maxillofacial surgery, leiomyocytes, formation extirpation, rare benign neoplasm

Introduction

Leiomyomas are rare benign soft tissue neoplasms that arise from smooth muscle [1, 7]. These tumors are common in the skin, gastrointestinal tract, and female genital tract, especially the uterus, and represent well-described neoplasms [3, 5]. Oral leiomyomas are extremely rare, with a frequency of less than 1% of all benign soft tissue tumors, as smooth muscle cells are relatively rare in the oral cavity compared to the gastrointestinal tract [8]. In practice, leiomyomas in the face are extremely rare.

According to literature, leiomyomas have been described as neoplasms that develop as a result of a mutation that results in the loss of mechanisms to regulate the growth of smooth muscle cells [8, 9]. The development of a benign tumor depends on a complex interaction between growth factors and cytokines, hormones (estrogen, progesterone), as well as genetic predisposition [6, 4]. Risk factors include: middle-aged (between 40 and 60 years of age), women with a history of chronic disease, including recurrent gynecological infections, dark skin and high body mass index [2]. Leiomyomas are clinically manifested as slow-growing, asymptomatic or painful lesions, which are most commonly described as being in purple [1]. Histologically, three types of leiomyoma can be distinguished [3]:

1) leiomyoma (hard leiomyoma)
2) epithelioid leiomyoma (leioblastoma)
3) angioleomyoma (vascular leiomyoma)
piloleimyoma and genital leiomyoma.

The diagnosis is based on histopathological examination and surgical excision is the treatment of choice, with recurrence being extremely rare [6].

Case Report

We present a case of a 49-year-old fimale patient who is receiving treatment with a formation above her left eyebrow. Anamnesically, the patient reported that she had suffered trauma in this area 10 years ago without disturbing the skin integrity, and for about two months had noticed a small bump in the area that was beginning to grow. The patient consulted a dermatologist who directed her to consult a specialist in maxillofacial surgery. Clinical examination revealed a formation above the left eyebrow that is not painful and the skin above it is unchanged. Under local anesthesia, a skin incision was made and a 1.5-1.8 cm formation was extirpated. The material was sent to a histological examination laboratory.

Histological analysis of the tissues was perfomed and stained haematoxylin-eosin (HE).

Immunohistochemical procedure for SMA detection

Sections were cut at 4 microns, de-paraffinized in xylene, hydrated in graded ethanol concentrations to distilled water, and digested with pepsin (5 mg/mL 0.01 N HC1 for 45 minutes at 37°C). The slides then were washed in distilled water, treated with 0.5% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase, and washed further in distilled water and phosphate-buffered saline (PBS) (pR 7.3). Normal mouse antiserum (DAKO) was applied at a 1:20 dilution for 10 minutes, followed by two washes in PBS. All further incubations were at room temperature and in a humidified chamber. The primary antiserum, anti SMA antibody, was applied at a 1:200 dilution for 30 minutes, followed by swine anti-rabbit immunoglobulin antiserum (DAKO) at a 1:20 dilution. The sections were washed in PBS and incubated with a rabbit peroxidase antiperoxidase complex (DAKO) at a 1:30 dilution for 30 minutes. The sections were then treated with fresh 3'-diaminobenzidine in 0.05 M Tris HC1, pH 7.6 (activated by hydrogen peroxide) for 1-5 minutes, to visualize the brown color indicative of peroxidase activity. Hematoxylin was used as a counterstain, after which the sections were dehydrated in graded ethanol concentrations, cleared in xylene.

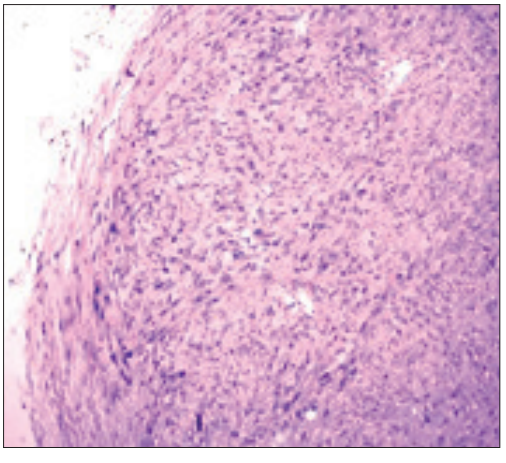

Fig. 1. Hematoxylin and eosin staining at low magnification reveals the fibrous capsule at left margin. × 100

We obtained the following results at magnification × 200 – the presence of a lesion that is made up of elongated

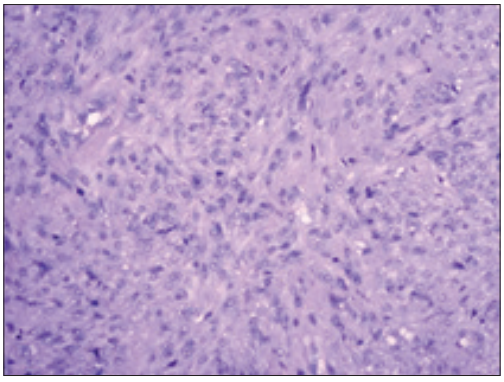

Figure 2. Haematoxylin and eosin staining revealed bundles of uniform, spindle-shaped cells. × 400

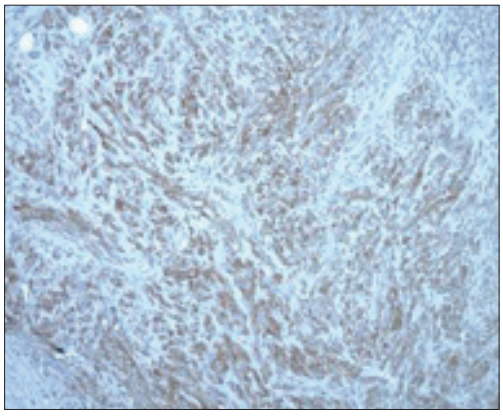

Figure 3. Immunohistochemical staining showed the diffuse staining with SMA (smooth muscle actin). × 100

eosinophilic cells with fusiform nuclei, fibrillar cytoplasm and distinct cell membranes, with less than 3 mitotic figures per 10 high power fields in most mitotically active area, and with no atypia (**Fig. 1, 2**). It was circumferentially delimited by a fibrous capsule (**Fig.1**). Strong diffuse immunohistochemical staining for SMA (Smooth muscle actin) was revealed (**Fig. 3**).

Discussion

The extraocular muscles develop from three preotic somites. These three somites correspond with the distribution of cranial nerves III, IV, and VI. These are the somites founds anterior to the developing ear of the embryo and are responsible for the development of the extraocular muscles [4].

Baden et al. described leiomyomas as a benign neoplasm of smooth muscle origin [2]. Leiomyomas can develop wherever smooth muscle is present. Skin is the second commonest site for leiomyoma comprise approximately 5% of all leiomyomas, right after uterus which accounts for 95% of cases [2]. Nevertheless, in extremely rare cases, they may become supraorbital and in cervix (only 1-2% of cases). The origin of leiomyomas in eyebrows has been a matter of speculation, it is most probably developed from arrector pili muscles or pericyst in vascular wall. Cutaneous leiomyomas are more common in adults, and without predilection for either sex.

The pathogenesis of leiomyoma can be caused by trauma, venous stagnation, hormonal changes and genetic alterations [7].

Previous studies [3, 5] have reported oral leiomyomas mainly in the lips, followed by the palate, mandible, tongue or cheeks [8, 9]. With this in mind, the case of leiomyoma reported here on the skin of a 49-year-old woman's face may be considered relatively rare. It is clinically difficult to distinguish facial leiomyomas from other lesions or tumors, such as nevi, fibroids, lipomas, hemangiomas [3, 5].

Beneficial characteristics of leiomyomas include lack of mitoses and necrosis, as well as cellular atypia and pleomorphism in histopathological evaluation.

Leiomyomas are only rare, although prognosis depends on the completeness of surgical excision of the tumor.

Conclusion

Supraorbital leiomyoma of the skin is a very rare benign neoplasm. With this report, we have presented a case of leiomyoma located on the skin above the left eyebrow of a patient at risk age for the appearance of predisposing factors. Tumors of this type are usually asymptomatic and can develop for months or even years. Despite variable clinical manifestations, majority of the lesions have a favorable prognosis. The diagnosis of this type of lesion requires histopathological evaluation.

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Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

Review Articles

Toxic Effects of Heavy Metals (Lead and Cadmium) on Sperm Quality and Male Fertility

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Male infertility with idiopathic etiology may be attributed to various environmental or occupational exposures to toxic substances, such as heavy metals. The accumulation of lead, cadmium, arsenic, bismuth, and other elements, even in low concentrations, induces strong toxic effects on the reproductive tract. It has been suggested that the risk is usually connected with both increased concentrations and exposition duration. The current review focuses on the toxic metals lead (Pb) and cadmium (Cd) as the main environmental pollutants, and their effects on the function of the male gonads (testes and spermatogenesis), as well as the related reproductive consequences as poor sperm quality and male infertility. Characteristic of all heavy metals is that they generate reactive oxygen species (ROS) and induce oxidative stress (OS) in a variety of systems, including the reproductive system. Massive degeneration of germ cells and alterations in the levels of LH, FSH and testosterone are also reported.

Key words: heavy metals, testis, male reproductive system, male fertility

Introduction

There are still debates concerning what exactly means the term „heavy metal" and which chemical/natural elements are classified as such. In general, this term is widely used about metals and/or metalloids with potential for toxicity (i. e., which form toxic soluble compounds in the organism) in humans, animals or the environment. Lead (Pb), cadmium (Cd), mercury (Hg) and arsenic (As) are prime examples of ‚heavy metals', not just because their chemical characteristics show a relatively high atomic number and atomic weight or density (>5g/cm^3), but also because they are major polluters of the environment [68]. Occupational exposure to metals can occur in different sectors of industry and can lead to significant morbidity and mortality in people working there. A large part of these elements can be eliminated from the body. Approximately 12% of the absorbed by the organism metal ions enter the bloodstream. From the blood, they

accumulate and are proven to cause severe damage to a variety of organs such as bones, kidney, liver, lungs, and brain [31]. The heavy metals' ions' toxic effects are expressed mainly by blocking and/or changing of the normal action of different enzymes [55, 57]. People exposed to heavy metals for a long time get chronic poisoning. However, other metals as zinc (Zn), cobalt (Co), and iron (Fe), besides toxic, are essential also about many biochemical processes in the human organism [19]. For instance, zinc is an important co-factor in many enzyme reactions in the human body, vitamin B_{12} contains cobalt ion (Co^{2+}), and the hemoglobin – iron ion (Fe^{2+}), respectively, in the active centers of its molecules. Analogically, copper, manganese, selenium, chromium, and molybdenum are trace elements that are essential for a living organism. Another subgroup of metals and metalloids, such as aluminum, bismuth, gold, gallium, lithium, silver, mercury, arsenic, etc., are included in the arsenal of pharmacological resources and have therapeutic application in medicine and cosmetics. Furthermore, radioactive metals have both chemical and radiation toxicity. Each one of these elements could be harmful to the organism, if it is applied in high dose or when the normal mechanisms of its elimination and/or metabolism are disrupted [68].

In their accumulation in the organism, the different metals could cause serious damage to various organs – more often in the lungs (as chronic bronchitis, pneumofibrosis, emphysema), liver (cirrhosis), kidneys (different types of nephropathies), the heart (cardiomyopathy and hypertonia), testes, brain (paraesthesia, polyneuritis disorders and different types of encephalopathies in the central nervous system – CNS), blood system (different types of anemia) and bones (osteoporosis, osteomalacia) [56]. They could also participate in the replacement of different substances in tissue structures. In this way, the tissues, constructing the arteries, joints, bones, and muscles show reduced function and/or morphological instability due to the replacement process. Potential risks are both acute and chronic intoxications with ions of heavy metals because of the impossibility about the regeneration of the normal functions of the injured tissues in the organism.

The known ways of metals to enter in the organism are: by inhaling metal vapor and smoke (in the industry), by ingestion (with food and water), as well as by absorption through the skin and mucous membranes during work, play, etc. The main moment in the study of the toxic effects of different substances on the body is the determination of the exposure time and dose-response relationship in the experimental model. In this regard, the reference values of the elements in the blood and urine are of important diagnostic matter. For example, the reference values of lead (Pb) in urine and blood in humans, are: 6.3-13.0 mkg/l, 8.0-269 mkg/l, respectively; for the cadmium (Cd) – 0.5-4.7 mkg/l in urine and 1.0 mkg/l in blood, and their increased levels indicate toxicity and necessity application of measures for prevention and/or treatment. Doses of 0,2-0,3 mg/kg body weight per day lead compounds (as Pb-acetate) are sufficient about the appearance of symptoms of intoxication. Usually, the risk is directly related to both the increase in lead concentration and the duration of exposure [4, 53]. Epidemiological studies in men (working in a Pb environment) with blood Pb levels ranging from 10 μg/dl to <40 μg/dl have shown an increased risk of infertility [53]. In another study, 4000 workers with blood Pb levels higher than 25 μg/dl, have significantly decreased insemination potential (childless men or with a small number of children) has been shown, compared with the control group of 5000 individuals [40].

From the most of the heavy metals, Cd has been the most widely studied and is considered a reproductive toxicant in experimental animals when administered

as a single high dose [61]. Cd concentration in blood is a marker of both recent and cumulative exposure, whereas urinary concentration mainly mirrors cumulative exposure [6, 19]. After smoking, the Cd content of smokers is 4-5 times higher than that of non-smokers [59]. On average, the daily Cd intake of humans is 1.06 μg/kg body weight [65]. The absorption of Cd and Cd compounds, such as Cd-chloride ($CdCl_2$), Cd-acetate and Cd-carbonate in the people, varies from 3,0% to 8,0% of the respectively applied dose [31]. Several other studies, however, have also reported adverse effects at lower environmental doses [68]. Despite the lower intake of Cd, the elimination half-life of Cd is longer (~ 20-40 years in humans) and can accumulate in the body [65]. Besides, the testis is the tissue in which Cd can accumulate in large amounts [61]. Cd exerts direct cytotoxicity within the testis, mainly targeting two specific cell populations, the Sertoli cells (SCs) and the Leydig cells (LCs), with consequent impairment of spermatogenesis and endocrine function [50].

The current review focuses on the toxic metals Pb, and Cd as the main pollutants of the environment, and their effects on the function of the male gonads (testes and spermatogenesis), as well as the related reproductive consequences as poor sperm quality and male infertility.

Testicular and hormonal effects of heavy metals

Chronic heavy metal intoxication (and/or their compounds) also affects the endocrine glands, including the testes and spermatogenesis. Studies with exposure of experimental animals to lead (Pb) have demonstrated macroscopic changes in the testes and accessory glands, such as testicular atrophy, decreased weight of the seminal vesicles, epididymis, ventral prostate, and alterations in semen quality [51, 58, 60]. Histological preparations have shown morphological damages in the testes with decreased population of germ cells [1], as well as disruption of the hypothalamic-pituitary-testicular (HPT) axis [58, 62] caused namely by the increased levels of Pb in rats. Other studies performed on mice (exposed to Pb) have shown the seminiferous tubules degeneration [25] and abnormal cytology of all types of germ cells [22]. According to one experiment of monkeys, exposed to Pb the electron-microscopic assay revealed that from even an early age, prolonged exposure caused testicular changes that persisted in their later life, even when blood Pb concentrations were significantly reduced [23]. Accumulation of lead preferentially in the epididymis and other accessory glands has also been noted [42]. Furthermore, the exposure of Pb leads to suppression of testosterone (TE) synthesis, which suggests that the probable target cells of Pb in the testis are Leydig cells [62], while Sertoli cell function does not appear to be affected.

According to literature data, cadmium (Cd) has been determined as a reprotoxic element, which affects directly the selected cellular populations in the testis, including impairment (cytotoxic and functional) of Sertoli and Leydig cells and induced oxidative stress in both somatic and germ cells. Data showed that Cd affects the male reproductive system from embryonic stages to adulthood, and has adverse effects on gonadal development [50, 61]. At doses that did not affect most organs, Cd caused damage to the testes within 24-48h. Men with varicocele usually show an increased accumulation of Cd in the testicular circulatory system, correlating with the increased percentage of apoptotic germ cells in the seminiferous tubules [9]. Indeed, it has been proven that Cd attacks and causes specific morphologic alterations and dysfunction in

blood vessels [5], to the internal spermatic artery, its testicular and epididymis branches, and the pampiniform plexus (in species with a scrotal testis including human), making them strongly permeable.

Exposure of rats to Cd of 1 mg/kg daily by gavage for 28 days can cause severe ultrastructure changes in adult SCs [27], and at a single dose of Cd (3μmol/kg) show vacuolation in the Sertoli cell cytoplasm and irregular chromatin condensation in late spermatids [21]. In adult mice exposed to Cd by inhalation for 28 days can cause severe mitochondrial changes in SCs [11]. Various studies have shown that Cd has a much stronger effect on the gonadal structure than Pb, by damaging vascular endothelium and blood-testis barrier (BTB) integrity [57], inducing in this way apoptosis and inflammation processes in testicular tissue [34]. BTB is a complex structure whose main component is SCs (by forming tight junctions – TJ between them) and which spatially divides mitotic from meiotic and post-meiotic germ cells in the seminiferous tubule structure. Cd attacks BTB by inducing defragmentation of actin filaments of SCs in rodents [69] and humans [70]. Molecular biology findings indicate that Cd perturbs the cytoskeleton of actin by disrupting F-actin organization in human SCs at 0.5-20μM after altering the expression of actin regulatory proteins Arp3 and Eps8 *in vitro* [70]. BTB damage is associated with germ cell loss and reduced total spermatozoa count, which determine subfertility or infertility conditions. *In vitro,* the addition of Cd to cell cultures of Sertoli [57] or SC and spermatocytes [17] disrupts the tight (occludin) connections between cells, thus facilitating the entry of toxic substances into cells. These studies also show that Cd has a dose-dependent effect on BTB integrity, by inhibiting the establishment or inducing the disruption of the TJ assembly between rat SCs, through downregulation of occludin (a TJ integral membrane protein) and urokinase plasminogen activator, without causing any apparent cytotoxicity and TE can protect it [17, 57]. Cd can down-regulate the focal adhesion kinase (FAK – non-receptor protein tyrosine kinase to regulate BTB) expression [65], by regulates TJ proteins (e.g., ZO-1 and occludin) in the rat testis [57]. In this connection, studies have indicated the negative influence of Cd on E-cadherin in the epithelial cells of seminiferous tubules [17, 36], which makes the testes particularly susceptible to the toxicity of this element. This is probably due to interaction of Cd with the putative Ca^{2+}-binding motif in the molecule of E-cadherin, suppressing in this way the cadherin-based cellular adhesion. Furthermore, E-cadherin is a separate part of a common structure with tight junction-proteins (e.g., occludin, claudins, JAM-A) at the BTB, which allows maximally fast access of Cd to E-cadherin in the epithelial cells. An important role in these processes probably plays the testosterone, which counteracts the disruptive effects of Cd by inducing the expression of TJ integral membrane proteins such as occludin, regulating in this way Sertoli cells TJ-permeability barrier [17]. These observations confirm the view that TE promotes BTB integrity and cell adhesion function in the testis [66], as well as the likelihood that this androgen (or a manipulation of the androgen receptor in SCs) will be a potential target factor to manage Cd-induced testicular pathology. Another investigation demonstrates that Cd disturbs BTB in the rat testis *in vivo* by up-regulating transforming growth factor β3 (TGF-β3), which in turn activates p38 MAPK signaling [41]. Interestingly, Cd also activates the JNK pathway at the same time to up-regulate α2-macroglobulin to counteract its adverse effects because JNK specific inhibitor can aggravate Cd-induced damage on BTB [69], indicating the JNK signaling is the protective mechanism in SCs after Cd treatment [74]. Cadmium also significantly decreased hCG-stimulated TE production by Leydig cells *in vitro* or

*in viv*o at doses that did not affect viability, which indicates that Leydig cells probably also suffer adverse effects. Independently of that, SCs are much more sensitive to Cd and Cd compounds than LCs (in contrast to the effects of Pb), showing major structural and functional changes after exposure, even at doses of Cd that do not lead to visible testicular damage [43]. The reproductive toxicity of Cd (compared to other heavy metals) has been studied in more detail in experimental animal models when administered as a single high dose [61]. An *in vivo* experimental rat model showed that Cd exposure induced testes inflammation. The Cd-induced inflammatory process in the testis develops with enhanced expression of the characteristic inflammation markers (including inducible nitric oxide synthase/iNOS, tumor necrosis factor-α/TNF-α, nuclear factor-kB/NF-kB, cyclooxygenase-2/COX-2, and heme oxygenase-1), leading to vacuolization of the seminiferous epithelium cells, together with hemorrhage and interstitial tissue edema and subsequent widely spread necrosis [24]. These pathological changes were associated with impairment of spermatogenesis [19]. Experimentally exposed to Cd rats (at different doses and duration), showed disorganization of the seminiferous epithelium, germ cell depletion, and release of immature gametes into the lumen [18, 49], but multinucleated giant cells increased [32] as well as rat's sperm count, motility, and viability declined, respectively [47].

The effects of Cd related to human fertility have been reviewed in several papers [19]. The data from epidemiological studies support the positive correlation between Cd and male subfertility/infertility. Sharma et al. analyzed the association between cases of hypospadias and serum Cd concentrations and established that the boys with hypospadias have significantly higher serum Cd levels compared to healthy control boys [54]. Similar studies give reason to suppose that most cases of unexplained subfertility in infertile couples or hypospadias, and/or other idiopathic diseases, a probable factor may be Cd reproductive toxicity [13].

Spermatogenesis-related hormonal disruptions.

Androgen hormones play a complex and important role in the regulation of spermatogenesis and maturation of male germ cells. The results of experimental studies in rats show many locations where the effects of heavy metals are involved in the dynamics of male sex hormones, mainly in the hypothalamic-pituitary-testicular axis [51]. For example, dependently of the levels and longevity of exposure of the rats under the influence of Pb, damage to the signaling systems in the hypothalamus and pituitary gland, including their hyperfunction, leading to over-production of gonadotropin hormone (gonadotropin-releasing hormone/GnRH) and luteinizing hormone (LH) [58] have been established. In support of animal experiments, studies of workers (industry workers with elevated Pb levels) have reported a positive relationship between serum LH levels and the duration of occupational exposure to Pb, finding confirmed, and in mean blood Pb levels of 35μg/dl [48]. In response to the stimulation with LH, the Leydig cells in the testes produce the main androgen hormone testosterone. In this aspect, studies on the serum TE in men professionally-exposed to Pb interpret in different ways the concentrations of this hormone. Semen Pb concentrations at a mean of 2 μg/dl have been reported to be inversely related to serum TE among occupationally-exposed men [4]. Smoking was correlated significantly positively with the levels of both TE and estradiol, suggesting it may have confounded those relationships. In

increased blood Pb levels have been observed significantly decreased levels of markers for prostate secretory function (seminal zinc, acid phosphatase activity, and citric acid). In experiments with mice exposed to Pb for 30 days [52], increased steroid-binding globulin levels and suppression of testicular TE levels were shown to be associated with increased duration of Pb exposure. Decreased testosterone levels and increased androgen binding protein levels have also been established in the cells of the epididymis [51]. According to other literature reports, increased serum testosterone concentrations have been assessed in men, exposed in both low (around 5 μg/dl) [60] to comparatively high (more than 40 μg/dl) Pb serum levels. In all cases, however, the continuous exposition in the presence of Pb influences the serum testosterone concentration. These findings suggest that except disrupting TE secretion (under the action of Pb) relative to the reproductive hormonal axis, it is possible to include other hormonal and/or feedback hormonal pathways, such as a lack of reflex in response to plasma testosterone, direct inhibitory androgen biosynthesis in LCs, or defects in LH regulation at the pituitary level [58]. This hypothesis could be confirmed by the molecular mechanisms (based on histopathological studies), revealing degenerative changes in LCs in experimental rats [68], which gives reason to consider these cells as a target of Pb intoxication. On the other hand, due to the imbalances in the hormonal axis caused by Pb exposure, pituitary cells release inappropriate LH levels and alter the steroid negative feedback loop [51], usually at the hypothalamic level [26]. Elevated concentrations of other sex hormones, such as follicle-stimulating hormone (FSH), secreted by the pituitary gland, have been observed after exposure to Pb in men [48] and rats treated with this metal. Furthermore, inappropriate inhibin B overproduction in excessively lead exposed subjects may be induced by Sertoli cells dysfunction, which suggests spermatogenesis impairment [44]. Investigations with male monkeys, however, have suggested as eventual reason about the changed functions of the Sertoli cells the decreased levels of inhibin/FSH [23], rather than the direct influence on the cells. Similar results have proposed the role of the Sertoli cells as indirect targets of Pb toxicity, and the disruption FSH levels by the lead's effects are the most probable reason about reproductive dysfunction, instead of direct influence. These studies have also demonstrated the protective role of BTB on the testicular cells against direct exposure to high levels of serum Pb. For these reasons and considering the wide spectrum of Pb toxicity on the hormones, it probably influences the male reproduction mainly by altering the gonadal hormonal axis, as well as on the hormonal control of the spermatogenesis, rather than by a direct effect on the cells of seminiferous tubules in the testes [63].

Cadmium (Cd) has also been shown to accumulate in the hypothalamus and pituitary glands, decreasing the blood prolactin level [38]. Once Cd enters cells through any damage, its influence has been attributed primarily to its interference with zinc-mediated metabolic processes, probably by molecular mimicry of Zn [12]. As a whole, data suggest both direct (via testicular and hypothalamus-pituitary toxicity) and indirect (via altered hormone secretion) effects may be involved in cadmium's reproductive influence and consistent with this is a hypothesized role of Cd as a metallohormone [14]. According to studies, performed by Jurasovic et al. [33], in men attending infertility clinic with median blood Cd levels of 0.85 mg/L, significant negative associations between serum Cd level and testis size have been observed, but not with the sperm parameters in models adjusted for potential confounders. Besides, serum Cd was positively associated with FSH, TE, and estradiol levels, as well as with seminal fluid acid phosphatase, an

indicator of prostate function [2, 33]. According to a study of 98 industrial workers (median serum Cd level 3.40 mg/L) and 51 subjects not occupationally exposed (median Cd level 1.83 mg/L) found significant positive correlations between pathologic sperm, LH, TE levels with blood Cd levels, but also a negative correlation with prolactin level in the total study population [60]. In another study conducted in Nigeria, in 60 male (with unusually high Cd levels) partners of couples attending an infertility clinic (excluding men who smoked, consumed alcohol, used steroids or fertility drugs, or had medical conditions that might impair spermatogenesis) showed that serum levels of LH and FSH, TE, and prolactin were significantly higher in the men with low or no sperm [2]. Other data, however, showed that Cd (compared to Pb) was positively associated with serum inhibin B levels in a model adjusted for age, BMI, and current smoking, and this association persisted even when the effects of the other metals were controlled, but no effects were seen on FSH or TE [45]. Inhibin B is considered the best available endocrine marker of spermatogenesis in subfertile men, but another study found it did not correlate with sperm parameters [37]. Various investigations conducted on a large scale prove that there is a negative association between serum Cd levels with total (or free) TE and sex hormone-binding globulin (SHBG) [15].

By taking in consideration the ethical limitations, many of the described studies on the reproductive organs have been performed on experimental animals (mainly rodents), where large doses of the heavy metal ions (administered by injection) have been applied to reveal their influence on cellular and tissue levels. However, the used experimental animal models substantially differ from human occupational and environmental exposure conditions (including that through smoking).

Influence of heavy metals on sperm quality and male fertility

The general effects of metal toxicity on the testicular tissue and endocrine function inevitably lead to impaired spermatogenesis and sperm quality. In most professionally men exposed on contact with Pb, a lot of changes in the parameters affecting the morphology and physiology of sperm are observed with increasing Pb concentration in seminal plasma or blood serum (usually at levels > 40 μg/dl, but often even at <10 μg/dl). These Pb levels are associated with reduced ejaculate volume (hypospermia) [26], decreased number/concentration (oligospermia) or sperm density [4, 46], and at higher levels (53 μg/dl) correlation has been shown with other pathological conditions, such as asthenospermia (immobile sperm) and teratozoospermia (gametes with various morphological defects) [39]. Other studies have found higher percentages of immature and abnormal sperm (with round or microcephalic heads, and/or short tails) in the ejaculate of workers exposed to both high and lower serum Pb levels [60]. Lead has been shown to incur detectable negative effects on blood, semen and/or spermatozoa quality in workers, such as inducing prolonged liquefaction time, decreasing sperm motility [46] and viability, even in serum levels ≤ 10μg/dl [28]. The harmful effect of this metal is also associated with reduced functional maturity of the germ cells (sperm chromatin condensation), especially in men with a mean serum Pb level of 45μg/dl [67]. On the other hand, concomitantly, significant improvements in the number of motile sperm has been reported after mean serum Pb decreased from 42 μg/dl to 20 μg/dl among the Pb factories workers [64]. According to studies in the field of the assisted reproductive techniques, a relationship between induced by Pb abnormal functions of the male gametes, premature capacitation and acrosome reaction has been observed [7], leading to decreased

ability of the sperm to penetrate through the *corona radiata* and *zona pellucida* and thus, fertilizing of the ovum [5]. The same authors have established an increased frequency of post-implantation embryonic loss in 0-2µg/ml Pb-acetate in the samples and at 40µg/dl serum Pb levels (or at 25-50mg/kg in food), respectively [5]. Benoff et al. (2003) have found a strong negative correlation between Pb levels in seminal plasma and rates of successful *in vitro* insemination in humans [8]. According to these results, Pb significantly increases the frequency of sperm function disorders in exposure cases both before and after ejaculation.

In several larger studies, associated with low levels of Pb exposure in different male groups/populations, adverse reproductive effects or sub-fertility were also observed. Results of regression models adjusted for confounders (age, smoking, alcohol, blood cadmium, and serum copper, zinc, and selenium), the serum Pb level (median serum Pb level: 49.2 mg/L or 57 mg/L and upper) correlate negatively with sperm count, the number of motile sperm (significantly lower percentage of progressively motile sperm), normal sperm and serum prolactin and positively associated with abnormal sperm morphology and, the percentage of slow sperm, serum TE and estradiol, respectively [60]. Also, a decrease in d-aminolevulinic acid dehydratase (ALAD), an indicator of long term lead exposure, was associated with decreased seminal plasma zinc levels indicating the adverse effects of lead on prostate function [60].

Studies focusing on the direct relationship between cadmium exposure to the environment and sperm quality in humans are often contradictory, despite the conclusion that Cd accumulation in germinal cells and Cd effects on sperm count and sperm motility are dose- and time-dependent [3]. Zhang et al. collect 11 research articles (1093 infertile subjects and 614 controls) and perform a meta-analysis and find that a high level of Cd in semen causes male infertility [73]. Some studies have suggested a significant negative correlation between Cd serum concentrations and sperm parameters, but other investigations have not shown any clear relationship between these two characteristics [19]. For instance, Asian [16, 71] and Nigerian men [2] with fertility problems and working in conditions with increased levels of Cd, who have had medium, even low levels of Cd in the serum, have shown decreased semen quality. At serum Cd levels between 0.78 mg/L and 1.31 mg/L, a decrease ejaculate volume and sperm density were observed [71], as well as an increase in the number of gametes with midpiece defects and immature forms (in mean Cd serum level – 1.35 mg/L) [16]. The mean level of serum Cd is higher only in men without sperm in the ejaculate (serum Cd levels – 460 mg/L) than in men with low or normal sperm count (serum Cd levels – 230 mg/L; vs. 210 mg/L) [2]. Besides sperm concentration, sperm motility is also severely affected by Cd. Men with low sperm motility had significantly higher serum Cd levels than did men with normal sperm motility [71]. In the investigation of fifty healthy men, de Franciscis et al. have found that serum Cd concentrations were positively associated with a reduction of sperm motility and the appearance of teratozoospermia [20]. Contradictory results have been reported by other smaller studies (Finnish and German) that did not detect significant effects of cadmium exposure on sperm parameters [30]. In comparative studies on the concentrations of Cd in the seminal fluid in industrial or refinery workers (n=27) and consecutive sperm donor candidates (n=45), the levels in the sperm of the industrial group have been significantly higher (mean Cd levels – 0.04 mg/kg) than in the sperm donors (0,005 mg/kg). However, the higher Cd levels in the first group did not show significant changes in sperm morphology and motility compared to the second group [30]. On the other hand, no correlation between the

semen Cd level and the sperm parameters has been observed. Several other studies also have not found significant correlations between serum and seminal plasma (or sperm) Cd levels [35], making it especially difficult to interpret the data. Analogous difficulties have also been reported when comparing low levels of Pb in the serum to semen parameters [4]. Contradictory opinions are probably based on the fact that different studies apply different methodologies in which specific variables, such as smoking and alcohol use by the men involved, are not sufficiently controlled, and thus may disguise differences related to male fertility. All the same, many experiments with animal models confirm the harmful influence of Cd on the sperm parameters, even in low concentrations. In a single low dose of Cd (0.05 or 1.0 mg/kg body weight), administered to adult rats, has resulted in failure of spermiation, the final phase of sperm differentiation [29], and also to reduced sperm concentration and motility [72]. Sperm motility is recognized to be more sensitive to this trace element, as the reduced sperm motility has been observed at a dose far below the dose affecting sperm production [10]. The data show that while animal experiments support an adverse effect of low Cd exposure on semen parameters, more research is needed to clarify this relationship in human males.

Conclusion

The presented data characterize the heavy metals as strongly toxic to the male reproductive system. Each of them shows a selective influence (directly or indirectly) on germ cells populations in the testes and functionally damages Sertoli cells and Leydig cells. These heavy metals also affect the endocrine system (hypothalamic-pituitary-gonadal axis), alter the normal expression of FSH, LH, TE, and other biologically-active proteins, which could disrupt spermatogenesis and lead to male infertility. Most of the literature points to the fact that probably the wide range of adverse effects is due to increased production of ROS and cause OS with pathological tissue damage as a result of prolonged exposure to high levels of heavy metals in humans and animals. Although the presented data are impressive, additional studies are necessary on the mechanisms of influence of the levels of the heavy metals on the male reproductive system and the quality of human sperm before making any conclusions in this direction.

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Bulgarian Anatomical Society

Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

ANTHROPOLOGY AND ANATOMY 27 (4)

Original Articles

Sexual Dimorphism in Odontometric Dimensions of Maxillary Teeth in Bulgarians

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The aim of the present study was to determine the sex differences in odontometric dimensions of maxillary teeth in Bulgarians. The study included 169 subjects of Bulgarian origin in the age group of 20-40 years. Buccolingual, mesiodistal and cervicoincisal dimensions of maxillary teeth were measured by Dentistry Sliding Vernier Caliper and analyzed with SPSS 23.0. Cervicoincisal dimensions in maxillary incisors, canines, premolars and molars were significantly higher in males compared to females. Similar significant differences were found in mesiodistal dimensions in maxillary canines and molars. Buccolingual dimensions in upper molars were significantly higher in males compared to females. The results of the present measurements exhibited significant sex differences in maxillary teeth in Bulgarians. Our results showed that maxillary canines and molars exhibited the greatest sexual dimorphism. In accordance with similar odontometric studies, teeth may differentiate both genders and thus determine the need for further investigations in this field.

Key words: sex differences, odontometric dimensions, maxillary teeth.

Introduction

Sex determination in forensic anthropology is an essential step for medicolegal purposes and crucial for identification as the number of possible matches is reduced to 50% [2]. The identification of sex is of significance not only in cases of mass fatality incidents where bodies are damaged beyond recognition but also in situations where only fragments

of jaw bones with teeth (or teeth alone) are found. Teeth, being the central component of the masticatory apparatus of skull, are excellent material in living and nonliving populations for anthropological, genetic, odontologic and forensic investigations, because they are the hardest and chemically the most stable tissue in the body [3]. Odontometrics help us determining sex in young individuals in whom secondary sexual characters have not developed. It is cheaper than the DNA analyses and does not require specific techniques. Sex determination using dental features is based on the comparison of tooth dimensions in males and females, or upon the comparison of frequencies of non-metric dental traits, like Carabelli's trait of upper molars, deflecting wrinkle of lower first molars, distal accessory ridge of the upper and lower canines or shoveling of the upper central incisors. Therefore, odontometrics provide information on sex. There are numerous studies in which differences in male and female odontometric features in specific populations have been identified. Since there are such differences within the same population, it is necessary to determine specific population values in order to make identification possible on the basis of dental measurements. Standards for one population are not useful for other populations. The aim of this study is to evaluate the degree of sexual dimorphism in the maxillary teeth for the South region of Bulgarian population and thus to present odontometrics as an easy-to-use additional technique to determine sex.

Materials and methods

The present study included 86 males and 83 females of Bulgarian origin living in Pazardzhik and Plovdiv in the age group 20-40years. Before starting the study, subjects were informed about the nature of the study and written informed consents were obtained. Patients were included based on the following criteria:

- presence of complete set of fully erupted and periodontally healthy maxillary teeth
- presence of non-carious and non-worn maxillary teeth
- no dental history of any crown restorations or bridges
- normal occlusion

Exclusion criteria:

- History or clinical evidence of cleft palate
- Orthognathic surgery or trauma
- History or clinical features suggestive of endocranial disorders, metabolic disorders, developmental disorders and history of prolonged illness

Buccolingual, mesiodistal and cervicoincisal (coronal height) dimensions of maxillary teeth were measured by Dentistry Sliding Vernier Caliper, Ridge Mapping Caliper Type A and Type B. We used the technique of Martin-Saller, 1957, modified by Prof. Yordanov [18]. According to him the mesiodistal dimension is the greatest mesiodistal distance between the contact points of maxillary teeth, usually it is in the upper or middle third of coronal height. It is also termed the dental width. The buccolingual (vestibulolingual) dimension, also termed as the dental thickness is the greatest dimension between buccal and lingual surfaces of crown, taken at right angle

to the plane in which mesiodistal diameter is taken. Cervicoincisal (cervicoocclusal) dimension, also termed as the coronal height is the greatest dimension by vertical axis from the tip of the highest tubercle to the cervical line on the buccal side. For the coronal height of the maxillary molars we used the technique of Zubov, 1968, modified by Prof. Yordanov, 2012. According to it it is better to measure the height between the occlusal surface (the lowest point between the two vestibular tubercles) and the cervical line, without considering the height of the tubercles.

The measurements were analyzed with SPSS 23.0 using Student's t-test. The level of statistical significance was set at $P< 0.05$. The degree of significance was considered weak ($P<0,05$), moderate ($0,01>P>0,001$) or high ($P<0,001$).

Results

1. We found statistically significant differences between the two sexes in the coronal height of the maxillary central and lateral incisors. In the right and left central incisors and in the right lateral incisor there was moderate degree of significance ($0,01>P>0,001$), while in the left lateral incisor the degree of significance was weak ($P<0,05$). The mean values in males were higher than in females (**Table 1**).

Table 1. Comparison between coronal height of maxillary incisors in Bulgarian males and females.

	Males				Females				Sexual differences
Tooth	N	Mean	SD	SE	N	Mean	SD	SE	P
I11H	86	9.30	0.94	0.14	83	8.77	0.90	0.14	0.008
I12H	86	7.88	1.05	0.16	83	7.32	0.78	0.12	0.006
I21H	86	9.30	0.83	0.13	83	8.74	0.90	0.14	0.004
I22H	86	7.79	1.06	0.16	83	7.26	0.88	0.13	0.013

2. Similar differences were found in the coronal height of the maxillary canines, but they were with high degree of significance ($P<0,001$). Males show higher values than females (**Table 2**).

Table 2. Comparison between coronal height of maxillary canines in Bulgarian males and females.

	Males				Females				Sexual differences
Tooth	N	Mean	SD	SE	N	Mean	SD	SE	P
C13H	86	9.40	0.69	0.11	83	8.67	0.78	0.12	0.000
C23H	86	9.42	0.70	0.11	83	8.65	0.81	0.12	0.000

3. Coronal height of the maxillary premolars showed statistically significant higher values in males compared to females. The degree of significance in the first premolars and the second left premolar was weak ($P<0,05$), while in the second right premolar we found moderate degree of significance ($0,01>P>0,001$), (**Table 3**).

4. We found statistically significant differences in the coronal height of the upper molars between the two sexes in favor of males again with the exception of the left

second molar where no statistically significant differences were found. In the right molars the degree of significance was moderate (0,01>P>0,001), while the first left molar showed weak degree of significance (P<0,05), (**Table 4**).

Table 3. Comparison between coronal height of maxillary premolars in Bulgarian males and females.

	Males				Females				Sexual differences
Tooth	N	Mean	SD	SE	N	Mean	SD	SE	P
P14H	86	6.95	0.87	0.13	83	6.58	0.63	0.10	0.026
P15H	86	6.65	0.72	0.11	83	6.21	0.60	0.09	0.003
P24H	86	6.95	0.87	0.13	83	6.53	0.59	0.09	0.011
P25H	86	6.58	0.70	0.11	83	6.23	0.57	0.09	0.013

Table 4. Comparison between coronal height of maxillary molars in Bulgarian males and females

	Males				Females				Sexual differences
Tooth	N	Mean	SD	SE	N	Mean	SD	SE	P
M16H	86	6.19	0.55	0.08	83	5.81	0.55	0.08	0.002
M17H	86	6.12	0.50	0.08	83	5.74	0.49	0.08	0.001
M26H	86	6.16	0.75	0.11	83	5.81	0.59	0.09	0.019
M27H	86	5.95	0.62	0.09	83	5.72	0.55	0.08	0.068

5. Mesiodistal dimensions of the maxillary canines showed statistically significant higher values in males compared to females with high degree of significance (P<0,001), (**Table 5**).

6. Similar significant differences between the two sexes were found in the mesiodistal dimensions of upper first right and left molars again in benefit of males with high degree of significance (P<0,001), (**Table 6**).

Table 5. Comparison between mesiodistal dimensions of maxillary canines in Bulgarian males and females.

	Males				Females				Sexual differences
Tooth	N	Mean	SD	SE	N	Mean	SD	SE	P
C13MD	86	8.72	0.63	0.10	83	7.95	0.65	0.10	0.000
C23MD	86	8.72	0.59	0.09	83	7.95	0.62	0.09	0.000

Table 6. Comparison between mesiodistal dimensions of maxillary molars in Bulgarian males and females.

	Males				Females				Sexual differences
Tooth	N	Mean	SD	SE	N	Mean	SD	SE	P
M16MD	86	10.70	0.67	0.10	83	9.95	0.62	0.09	0.000
M17MD	86	10.00	0.53	0.08	83	9.56	1.48	0.23	0.070
M26MD	86	10.58	0.82	0.13	83	10.00	0.65	0.10	0.000
M27MD	86	9.98	0.51	0.08	83	9.77	0.65	0.10	0.100

7. We found statistically significant differences between males and females in the vestibulolinqual dimensions of the maxillary first right and left molars with moderate degree of significance (0,01>P>0,001). Males showed higher mean values than females (**Table 7**).

Table 7. Comparison between vestibulolingual dimensions of maxillary molars in Bulgarian males and females.

	Males				Females				Sexual differences
Tooth	N	Mean	SD	SE	N	Mean	SD	SE	P
M16VL	86	10.84	0.53	0,08	83	10.53	0.59	0.09	0.015
M17VL	86	10.47	0.55	0.08	83	10.23	0.57	0.09	0.058
M26VL	86	10.84	0.53	0.08	83	10.56	0.59	0.09	0.024
M27VL	86	10.35	0.53	0.08	83	10.26	0.58	0.09	0.440

Discussion

Our results showed sexual dimorphism in some of the dimensions of the maxillary teeth. The mean values were statistically higher in males than females. They are in accordance with similar results found by Ditch and Rose [3] who were the first proved that teeth can be used successfully for defining the sex. Other authors confirmed these results - Iscan and Kedici [5], Pettenati- Soubayroux [14]. We found statistically significant differences in the coronal height of all maxillary teeth in favor of males which is probably related with the fact that males have larger cranial sizes and more specifically lower third of the face [6]. Avinash Tejasvi measured the circumference of the cranium in Indians and also proved larger cranial sizes in males compared to women [18]. Differences in the coronal height of maxillary teeth proving sexual dimorphism in favor of males were described by Bhuvan Nagpal [9], Lakhanpal [7], Garn[4].

According to the present study there were statistically significant differences in the mesiodistal dimensions of the maxillary canines and molars and in the vestibulolingual dimensions of the maxillary molars. The mean values in males were again higher compared to females. The reasons for the bigger odontometric dimensions in the male maxillary teeth are probably the differences in the differentiation of the dentition in males and females. According to Schwartz and Dean [17] the concentration of the sexual hormones during the development of the tooth germ is related with that. The differences in the odontometric dimensions between the sexes is due to the thickness of the tooth dentin which is more in males because the mitotic cellular activity in the dental epithelium and papilla are influenced by the Y- chromosome. This chromosome induces genesis of dentin which defines the size of the enamel-dentinal junction. These findings are in accordance with the results of Smith [16] and Saunnders [15] who claimed that there was larger dentinal zone in males which leads to greater odontometric dimensions in the male maxillary teeth.

Garn proposed formula for calculating the percentage of the sexual dimorphism: ((Xm/Xf)-1) *100 where Xm is the mean value of the dental size in males and Xf – in females [4].

Our results showed that the mesiodistal dimensions of the maxillary canines have the highest degree of sexual dimorphism (9,68 %) with statistically significance of high level (P<0,001). These results are similar to the ones from Acharya [1]. Krishnamurthy studied the teeth in South Indians [6], while Lund and Mornstad measured the dental size of the Sweden population [8] and both claimed that the mesiodistal dimensions of the maxillary canines showed the highest degree of sexual dimorphism.

Our results showed high degree of sexual dimorphism in the mesiodistal dimensions of the maxillary molars (7,54%) also in favor of males. These findings are in accordance with the ones from Narang SR [10] who studied North Indians and Iscan and Kedici who described similar significant differences in the maxillary molars of Turkish population [5].

In contrast to our study where the mesiodistal dimensions of the maxillary canines and molars showed the highest degree of sexual dimorphism other authors claimed that the vestibulolingual dimensions of the maxillary canines and molars showed higher degree of sexual dimorphism. Such results were published by Nikola who studied the odontometric dimensions in Austrian population [11].

We did not find statistically significant differences between the two sexes in the mesiodistal and vestibulolingual dimensions of the maxillary central and lateral incisors. In contrast to that Peckmann TR found significant sexual dimorphism in the maxillary central incisors of Chilian population [12]. Similar results were published by Pereira C who found sexual dimorphism in the maxillary lateral incisors of Portuguese population [13].

The fact that in different populations different maxillary teeth show sexual dimorphism proves that the odontometric dimensions are population specific which defines the need of data for each population.

In some studies mesiodistal dimensions show higher degree of sexual dimorphism, in others vestibulolingual dimensions show higher degree of sexual dimorphism. Due to these reasons it is recommended measuring of all the odontometric dimensions.

Conclusion

Sexual dimorphism in tooth size and the accuracy of odontometric sex prediction is found to vary in different population and therefore it is necessary to determine specific population values in order to make identification possible.The present study revealed the existence of sex differences in the coronal height of maxillaryincisors, canines, premolars and molars, mesiodistal dimensions of maxillary canines and first molars and vestibulolingual dimensions in maxillary molars in Bulgarians. Our results showed that maxillary canines and molars exhibited the greatest sexual dimorphism. In accordance with similar odontometric studies, teeth may successfully differentiate both sexes and thus determine the need for further investigations in this field.

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Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

Palmar and Finger Ridge Count in Two Isolated Slavic Muslim Populations (Zhupa and Gora) from Kosovo in Comparison with Kosovo Albanians

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In order to compare two geographically and culturally isolated ethnic groups of Slavic Muslims from Zhupa and Gora regions in Kosovo, we analyzed their quantitative dermatoglyphic traits, compared them with each other and with the majority, Albanian population. The dermatoglyphs were collected from a total of 263 Zhuplyani, 145 Gorani, and 213 Albanians of both sexes. The ANOVA analysis showed more differences between the Albanians and both minority populations, than between Zhupa and Gora regions populations themselves. We also detected selective inertia in Slavic Muslim women. The canonical discriminant analysis grouped the Gora and the Zhupa women together, and at the same time closer to the Gora men than to the Kosovo Plain women. The Gora and Zhupa men were much closer to each other than to the men from Kosovo Plain. To conclude, the Gora and Zhupa populations differ less from each other, than any of them differs from Kosovo Albanians.

Key words: quantitative dermatoglyphic traits, Slavic Muslim populations, Zhupa region, Gora region, Kosovo

Introduction

Genetic structure of the population reflects not only the balance between gene flow and genetic drift within and among interbreeding populations; it also documents the genetic variability due to migrations, founder effects, the size and composition of these populations [5]. Consequently, from the aspect of population history, it is questionable whether the degree of similarity between populations is the result of exchanging mates or a common ancestry [16]. Hence, when interpreting the geographical pattern of genetic

variation, one should join the population structure and population history data, and combine structural and historical factors [45], as confirmed in several studies [21, 39, 40]. Biological variations between populations are directly related to geographical, migratory, linguistic and socio-cultural characteristics [18]. Dermatoglyphics have been used extensively to characterize human populations and to assess biological affinities between them [1, 9, 18, 19, 27, 43], and quantitative dermatoglyphic traits, when used as phenotypic markers, were shown to be conserved with respect to plastic environmental influences and stochastic processes of evolution [13, 25, 26, 29, 30, 32, 38].

Kosovo, one of the youngest European countries, has a population of more than 1.73 million [8], and most of the inhabitants are ethnically Albanians (>92%). Albanians are an ancient population that lives in the Southwest Balkan Peninsula: they speak Albanian, the only surviving Indo-European language, which is an extreme case of a relict language that has survived through thousands of years of continuous linguistic turnover in neighboring regions [14]. Since this area was inhabited by Dardans, an Illyrian tribe, from about 400 years BC [12], it is probable that Albanians are their descendants [6, 35]. In spite of the country`s turbulent history due to numerous invaders, Albanians managed to avoid the assimilation and to keep their identity and language intact. Beside the Albanians, the most numerous population in Kosovo, it is also inhabited by less numerous populations such as Serbs, Bosniaks, Romani, Ashkali, Gorani, etc. [8].

The inhabitants of Gora, the geographical region in Southern Kosovo surrounded by the tall Albanian Accursed Mountains in the west and by the high Šar Mountain (2,748 m) from two sides, have the only route in and out of the Gora region through hilly area with relatively slight slope via Prizren direction. Gorani, which means "people from the mountain", are a South Slavic ethnic group and despite still vivid debates about their collective identity and their ethnic origin, Gorani have since the 1990s been recognized as ethnic minority group in Kosovo [8]. It is believed that their ancestors came in this area in 6th and 7th century across the mountains, Christianized in Middle Ages, but later been converted from Christianity to Islam. This population is mentioned for the first time in 1348 in the edicts of Serbian Emperor Stefan Dushan. They speak the Gora dialect, known as "Našinski/Nashentski", meaning roughly "ours" - it is an Old Slavic dialect, part of a wider Torlakian dialect. They are adherents to Islam and have a rich and varied folk culture [46].

Zhupa (Župa) is the eastern periphery of the ethnographic region of Gora, but it seems that this area has rarely been counted as a part of the Gora [26]. Administratively Zhupa belongs to the district of Prizren and geographically is a part of Šar Mountain: some of Župa villages are in the basin of river Prizrenska Bistrica. This is a sparsely populated region whose residents call themselves Zhuplyani and Gorani, one group being contained within the other [26]. Most of them are also Muslim Slavs who speak "Nashentski", but elucidating the identity of Zhupa region residents is even more complex because this region is even more heterogenous than the Gora region since Zhuplyani were, over the time, subject to many national ideologies [3].

These two minority populations, residents of Zhupa (who will be further called Zhuplyani) and Gorani, could be regarded as two different groups according to their dialect, customs and tradition [36], plus marriages between them are rare [42]. In order to test this hypothesis, we compared the quantitative dermatoglyphic traits between the populations from Zhupa and Gora region, and since both regions are surrounded by the

Albanian villages, we also compared their digito-palmar patterns with the autochthonous Albanian population from the Kosovo plain.

Materials and Methods

The analyses of dermatoglyphic patterns of digito-palmar complex were carried out in two minority populations and in the majority Albanian population, all from Kosovo. Their finger and palm prints on both hands were taken by the standard ink method and scored according to Cummins & Midlo [11] and Holt [17]. The prints which were not complete were excluded. The final sample consisted of 263 residents of Zhupa region (125 men and 138 women), 145 Gorani from Gora region (80 men and 65 women) and 213 Albanians from Kosovo Plain (103 men and 110 women) (**Fig. 1**). All of the participants participated in this study voluntarily and signed informed consent.

We analyzed the following quantitative digital dermatoglyphic traits: ridge count on each finger on right (FRCR 1-5) and left hand (FRCL 1-5) Of the palmar traits, we analyzed number of ridges between digital triradius a and b (a-b ridge count – a-b rc), b

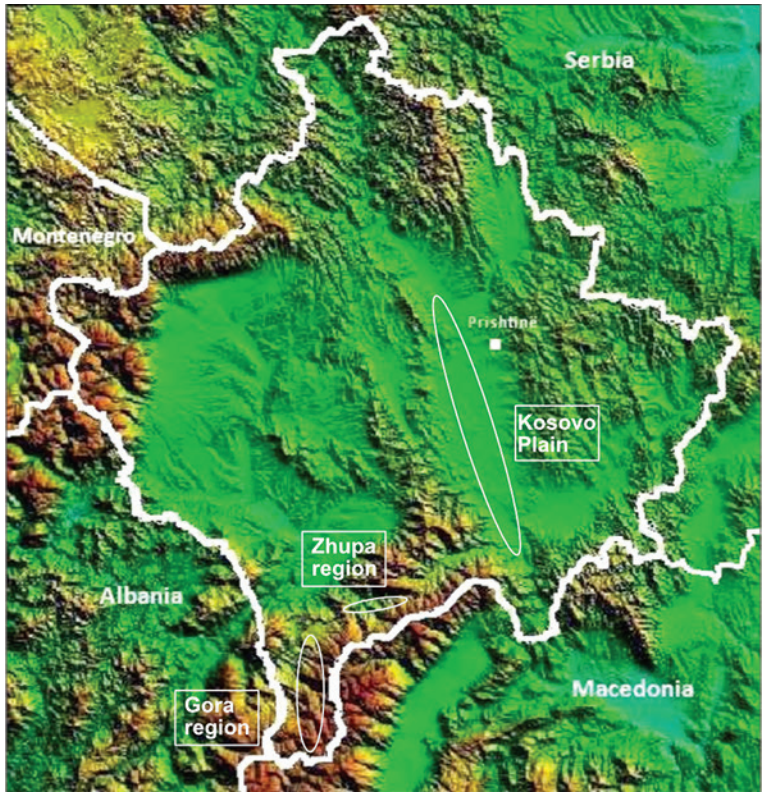

Fig. 1. The geographical map of Kosovo selected explicitly to display the natural barriers between Gora and Zhupa regions and the location of Kosovo Plain (taken and adapted from http://www.kosovo-mining.org/kosovoweb/en/kosovo/geography.html).

and c (b-c rc) and c and d (c-d rc), and the atd angle of both hands measured in degrees. The dermatoglyphic prints were analyzed by the single observer (G. Temaj).

The analyses of these quantitative dermatoglyphic traits included descriptive statistics, "One-way" ANalysis Of VAriance (ANOVA) and post-hoc Tukey HSD method, and Canonical Discriminant Analysis for the six (3 male and 3 female) groups of examinees. These statistical analyses were conducted using SPSS Statistical package 7.5.

Results

The descriptive statistics results of the comparison of quantitative digito-palmar dermatoglyphic traits from two minority and one majority Kosovar populations are presented in **Table 1** for men and **Table 2** for women. The men from Gora (Gorani) differed from men from Zhupa (Zhuplyani) only in one trait, the fourth finger ridge count right (FRCR4, $p<0.05$) (**Table 3**). More differences, and only on palms, were found in comparison of Zhuplyani and Gorani with Albanians from Kosovo plain. The Gorani differed from the Albanian men on a-b rcR ($p<0.05$) and rcL ($p<0.001$), and on the b-c rcL ($p<0.001$) and atd angle right ($p<0.001$). Differences between Zhuplyani and the Albanians were found in three variables on the left palm; a-b rcL ($p<0.001$), b-c rcL ($p<0.001$) and c-d rcL ($p<0.001$); and only atd angle on the right palm ($p<0.001$).

A comparison of women from Gora with those from Zhupa revealed no differences in quantitative digito-palmar traits (**Table 4**). However, both these populations differed from the Albanian women population. The Gorani women significantly differed from the Albanian women on digital FRCL1 ($p<0.05$), on palmar variables b-c rcL ($p<0.01$), c-d rcR ($p<0.05$) and c-d rcL ($p<0.001$), and the differences were found also in atd angle right ($p<0.05$). The women from Zhupa differed from the Albanian women from Kosovo plain on the FRCL4 ($p<0.001$), and on palms in b-c rcL ($p<0.001$), c-d rcR ($p<0.001$) and c-d rcL ($p<0.001$). Differences were also found in atd angle right ($p<0.001$).

The results of discriminant analysis for the three examined groups and original variables revealed that out of five canonical discriminant functions, the first three were significant at $p<0.05$ level, and that cumulative percentage of variance explained by those three functions was high (94.9%). Structure matrix showing correlations among original variables and canonical discriminant functions, eigenvalues and chi-square tests for 5 canonical discriminant functions discriminating 6 groups is presented in **Table 5.** Three discriminant functions were statistically significant: function 1 (eigenvalue = 0.601, χ^2 test = 491.968, $p<0.001$), function 2 (eigenvalue = 0.244, χ^2 test = 205.802, $p<0.001$) and function 3 (eigenvalue = 0.074, χ^2 test = 72.993, $p<0.012$). **Table 6** shows coordinates of group centroids in discriminant space for statistically significant functions. Discriminant function 1 clearly separated men from Kosovo Plain from all the other groups, discriminant function 2 separated the Kosovo Plain women from all the men and other female groups, whereas the discriminant function 3 separated men from Zhupa from the other examinees. When graphically presented in 3D (**Fig. 2**), the Gora and the Zhupa women were closer to each other and at the same time closer to the Gora men than any of them to the women from Kosovo plain. Besides finding a difference between the Gora and the Zhupa men only in one trait, discriminant functions grouped them closer than any of them to the men from Kosovo Plain, from whom both groups differed in several traits.

Table 1. Dermatoglyphic variables (mean ± standard deviations) of the digito-palmar complex in men from Gora and Zhupa regions, and in the Albanians from Kosovo Plain.

	Men from Gora region (N = 80)		Men from Zhupa region (N = 125)		Albanian men from Kosovo Plain (N = 103)	
Variables *	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
RIGHT HAND						
FRCR1	14.79	5.88	15.56	5.21	14.48	5.17
FRCR2	8.73	6.05	8.64	6.05	7.81	5.48
FRCR3	9.79	5.41	9.75	5.11	8.95	5.23
FRCR4	10.99	4.84	12.77	4.99	11.52	5.49
FRCR5	9.21	4.66	10.51	4.51	10.32	4.42
a-b rcR	32.90	5.92	33.79	6.23	35.27	5.89
b-c rcR	21.83	6.16	23.58	5.21	22.24	5.36
c-d rcR	28.71	5.86	29.42	6.84	29.63	6.03
atd R	43.86	7.78	44.02	6.52	36.65	8.58
LEFT HAND						
FRCL1	12.91	5.08	13.35	4.84	13.17	5.03
FRCL2	7.99	5.46	7.51	5.90	6.49	5.46
FRCL3	9.16	5.21	9.90	5.49	8.80	5.48
FRCL4	10.98	3.94	11.91	4.77	11.39	5.28
FRCL5	9.00	4.14	10.09	4.12	10.16	4.75
a-b rcL	32.71	6.43	34.46	6.67	40.01	7.37
b-c rcL	21.54	5.95	21.98	5.10	27.75	8.00
c-d rcL	27.51	6.29	29.03	6.09	25.77	6.28
atd L	45.09	8.52	46.66	5.74	43.84	5.27

*Abbreviations: FRCR – finger ridge count right; FRCL – finger ridge count left; a-b rcR, b-c rcR, and c-d rcR – palmar ridge count right; a-b rcL, b-c rcL, and c-d rcL – palmar ridge count left; atd R – right angle; and atd L – left angle.

Table 2. Dermatoglyphic variables (mean ± standard deviations) of the digito-palmar complex in women from Gora and Zhupa regions, and in the Albanian women from Kosovo Plain.

	Women from Gora region (N = 65)		Women from Zhupa region (N = 138)		Albanian women from Kosovo plain (N = 110)	
Variables *	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**
RIGHT HAND						
FRCR1	15.68	4.35	15.01	4.72	14.08	5.90
FRCR2	8.82	6.28	9.43	6.26	10.51	5.99
FRCR3	9.98	4.57	10.06	5.11	9.55	4.30
FRCR4	12.18	4.46	11.38	5.07	13.75	5.27
FRCR5	9.38	3.72	9.69	4.20	10.41	4.18
a-b rcR	35.62	4.81	34.76	5.02	34.53	5.89

b-c rcR	23.17	4.19	22.83	4.42	23.56	5.75
c-d rcR	28.95	5.64	27.46	4.70	31.37	7.41
atd R	46.82	8.01	47.44	8.53	43.16	7.95
LEFT HAND						
FRCL1	13.86	4.63	13.16	5.00	11.83	5.17
FRCL2	8.48	5.94	7.96	5.84	9.56	6.12
FRCL3	8.66	5.05	8.90	5.84	10.44	5.24
FRCL4	11.22	4.48	10.97	5.00	12.34	5.32
FRCL5	9.74	4.48	9.73	4.62	10.25	4.22
a-b rcL	33.86	5.58	32.95	5.03	32.06	7.52
b-c rcL	21.94	4.86	21.26	4.38	24.66	7.08
c-d rcL	29.57	5.89	28.49	4.95	33.65	9.46
atd L	46.40	7.05	46.72	8.41	45.62	6.99

*Abbreviations: FRCR – finger ridge count right; FRCL – finger ridge count left; a-b rcR, b-c rcR, and c-d rcR – palmar ridge count right; a-b rcL, b-c rcL, and c-d rcL – palmar ridge count left; atd R – right angle; and atd L – left angle.

Table 3. Results of comparison between the three groups of men from different geographic regions of Kosovo (Zhupa, Gora and Kosovo Plain) using One-way ANOVA test.

	Gora men/ Zhupa men		**Gora men/ Albanian men from Kosovo Plain**		**Zhupa men/ Albanian men from Kosovo Plain**	
Variables *	**F**	**p**	**F**	**p**	**F**	**p**
RIGHT HAND						
FRCR1	0.77	0.575	0.30	0.925	1.07	0.290
FRCR2	0.09	0.994	0.92	0.544	0.83	0.534
FRCR3	0.03	0.999	0.84	0.531	0.80	0.483
FRCR4	**1.78**	**0.040**	0.54	0.762	1.24	0.162
FRCR5	1.30	0.110	1.11	0.227	0.19	0.946
a-b rcR	0.89	0.557	**2.37**	**0.023**	1.48	0.156
b-c rcR	1.76	0.067	0.42	0.867	1.34	0.161
c-d rcR	0.70	0.718	0.92	0.594	0.22	0.965
atd R	0.15	0.989	**7.21**	**0.001**	**7.37**	**0.001**
LEFT HAND						
FRCL1	0.44	0.810	0.26	0.933	0.18	0.961
FRCL2	0.48	0.826	1.50	0.174	1.03	0.359
FRCL3	0.74	0.605	0.37	0.893	1.11	0.274
FRCL4	0.94	0.353	0.41	0.829	0.52	0.686
FRCL5	1.09	0.187	1.16	0.175	0.07	0.993
a-b rcL	1.74	0.177	**7.30**	**0.001**	**5.55**	**0.001**
b-c rcL	0.44	0.882	**6.21**	**0.001**	**5.77**	**0.001**
c-d rcL	1.52	0.201	1.75	0.142	**3.27**	**0.001**
atd L	1.42	0.270	1.25	0.392	0.17	0.978

*Abbreviations: FRCR – finger ridge count right; FRCL – finger ridge count left; a-b rcR, b-c rcR, and c-d rcR – palmar ridge count right; a-b rcL, b-c rcL, and c-d rcL – palmar ridge count left; atd R – right angle; and atd L – left angle.

Table 4. Results of comparison between the three groups of women from different geographic regions of Kosovo (Zhupa, Gora and Kosovo Plain) using One-way ANOVA test.

	Zhupa women/ Zhupa women		Gora women/ Albanian women from Kosovo Plain		Zhupa women/ Albanian women from Kosovo Plain	
Variables *	**F**	**p**	**F**	**p**	**F**	**p**
RIGHT HAND						
FRCR1	0.67	0.657	1.60	0.112	0.93	0.330
FRCR2	0.61	0.787	1.69	0.185	1.08	0.356
FRCR3	0.07	0.994	0.44	0.823	0.51	0.673
FRCR4	0.80	0.539	1.157	0.403	**2.37**	**0.001**
FRCR5	0.30	0.875	1.03	0.240	0.73	0.344
a-b rcR	0.85	0.532	1.09	0.388	0.23	0.937
b-c rcR	0.34	0.887	0.39	0.864	0.74	0.465
c-d rcR	1.49	0.221	**2.42**	**0.026**	**3.91**	**0.001**
atd R	0.63	0.868	**3.65**	**0.013**	**4.28**	**0.001**
LEFT HAND						
FRCL1	0.70	0.617	**2.03**	**0.025**	1.32	0.095
FRCL2	0.52	0.831	1.09	0.685	1.61	0.088
FRCL3	0.28	0.955	1.77	0.096	1.54	0.072
FRCL4	0.24	0.944	1.12	0.326	1.37	0.084
FRCL5	0.01	0.999	0.51	0.747	0.51	0.639
a-b rcL	0.91	0.583	1.80	0.145	0.89	0.494
b-c rcL	0.68	0.697	**2.73**	**0.005**	**3.40**	**0.001**
c-d rcL	1.08	0.566	**4.09**	**0.001**	**5.16**	**0.001**
atd L	0.32	0.959	0.78	0.791	1.10	0.500

*Abbreviations: FRCR – finger ridge count right; FRCL – finger ridge count left; a-b rcR, b-c rcR, and c-d rcR – palmar ridge count right; a-b rcL, b-c rcL, and c-d rcL – palmar ridge count left; atd R – right angle; and atd L – left angle.

Table 5. Discriminant analysis: structure matrix showing correlations among original variables and canonical discriminant functions (upper part of the table), and eigenvalues and chi-square tests for 5 canonical discriminant functions discriminating 6 examined groups (lower part of the table). First three discriminant functions were significant, explaining 94.9% of variance. Discriminant function 1 strongly correlated with atd angle of the right hand and c-d ridge count of the left hand, discriminant function 2 with c-d ridge counts of both hands, b-c ridge count of the left hand and FRCR4, whereas discriminant function 3 had the highest correlation with atd L.

	Discriminant function				
Variable *	**1**	**2**	**3**	**4**	**5**
atd R	0.553	-0.241	0.110	0.239	-0.100
c-d rcL	0.278	0.596	-0.049	0.286	0.134
atd L	0.151	-0.022	0.460	0.097	-0.057
FRCL2	0.150	0.215	0.114	-0.046	0.189
FRCR2	0.126	0.187	0.117	0.009	-0.244
FRCR3	0.089	-0.054	0.002	0.008	-0.007

	Discriminant function				
Variable *	**1**	**2**	**3**	**4**	**5**
b-c rcR	0.071	0.115	-0.203	0.410	-0.040
FRCR4	0.052	0.316	-0.260	0.354	0.119
FRCR1	0.044	-0.149	-0.181	0.186	0.242
FRCL3	0.039	0.194	-0.228	-0.050	-0.146
FRCL4	-0.003	0.182	-0.189	0.126	-0.003
FRCL1	-0.014	-0.214	-0.069	0.208	0.283
FRCL5	-0.036	0.099	-0.073	0.344	-0.155
a-b rcR	-0.047	0.009	0.291	0.627	0.130
FRCR5	-0.056	0.126	-0.224	0.252	-0.306
c-d rdR	-0.067	0.378	-0.172	0.016	0.397
b-c rcL	-0.437	0.354	0.264	0.293	0.013
a-b rcL	-0.502	-0.180	-0.023	0.521	0.096
Eigenvalues	0.601	0.244	0.074	0.034	0.016
Cumulative % of variance	62.1	87.3	94.9	98.3	100
chi-square tests when preceding roots were removed	491.968	205.802	72.993	29.755	9.686
p (chi-square test)	**<0.001**	**<0.001**	**0.012**	0.478	0.785

* Variables are ordered by absolute size of correlation within first function (largest absolute correlation between each variable and any discriminant function). Abbreviations: FRCR – finger ridge count right; FRCL – finger ridge count left; a-b rcR, b-c rcR, and c-d rcR – palmar ridge count right; a-b rcL, b-c rcL, and c-d rcL – palmar ridge count left; atd R – right angle; and atd L – left angle.

Table 6. Discriminant functions at group centroids but only for the statistically significant functions: discriminant function 1 separates Kosovo Plain men, discriminant function 2 separates Kosovo Plain women, whereas discriminant function 3 separates Zhupa men.

	Discriminant function		
Group	**1**	**2**	**3**
Gora women	0.522	-0.273	0.179
Zhupa women	0.590	-0.393	0.226
Kosovo Plain women	0.315	1.030	0.076
Gora men	0.137	-0.254	0.018
Zhupa men	0.096	-0.120	-0.522
Kosovo Plain men	-1.679	-0.057	0.122

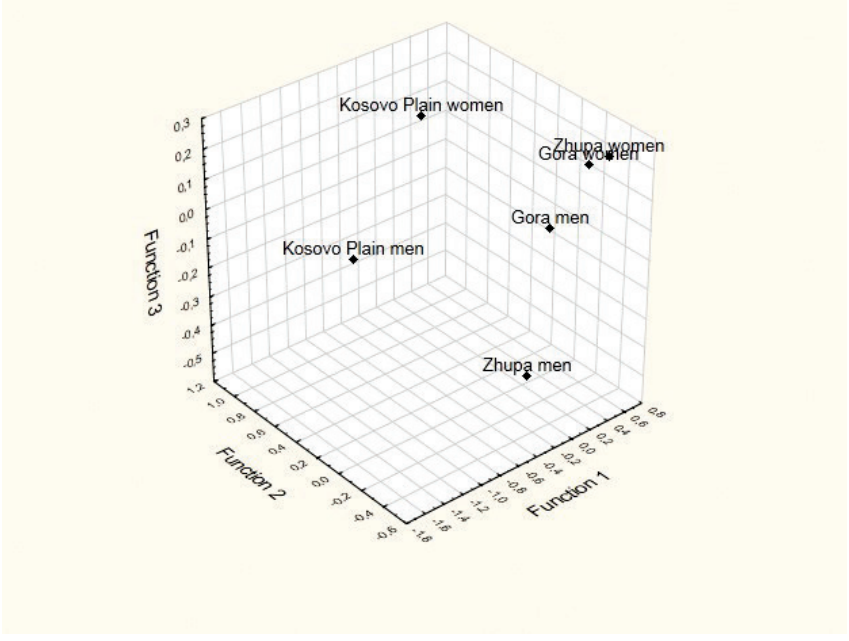

Fig. 2. Graphical presentation of group centroids in discriminant functions 1, 2 and 3. Women from Gora and Zhupa are closer to each other than any of them are to women from Kosovo plain. Additionally, the Gora and Zhupa women are closer to Gora men than to Kosovo plain women. Gora and Zhupa men are closer than any of these two groups to men from Kosovo Plain.

Discussion

The variability in dermatoglyphic patterns reflects the existence of differences dating from the fetal period [11]. The development of palmar dermatoglyphics has a relatively longer growth period when compared with fingers [10], and the palmar patterns are found to correspond better to the ethno-historical background of the populations than characteristics of fingers [24]. Also, genetic and linguistic evolutions are found to correspond closely [7, 34], although not in every study [41].

Quantitative dermatoglyphic traits change slower than qualitative ones, but are at the same time very sensitive to the events which took place during microevolution of the contemporary populations [20, 40]. Geographic isolation and small relative size of the populations, the optimal conditions for the operation of the genetic drift, have almost certainly been present in the Zhupa and Gora regions. Still, our findings indicate the relative lack of genetic differences between these two investigated populations, although at the same time they support the expectation that in environmentally stressful areas, where all local populations are subjected to the same pressures, male and female measures of differentiation should be smaller than in other areas [37]. As we already mentioned, several studies demonstrated that quantitative dermatoglyphic traits are conservative with respect to plastic environmental influences and stochastic processes of evolution, showing selective inertia on changes mostly in females, as is the case in populations living in Zhupa and Gora regions [13, 21, 31, 32, 33].

The men of Gora and Zhupa differed from the Albanians in a-c and b-c ridge counts, and the men from Zhupa additionally differed from the Albanians in c-d ridge counts. In women, both the Gora and Zhupa groups differed from the Albanian group in one digital trait and in three palmar traits (b-c rcL, c-d rcR and c-d rcL). Arrieta et al. [2] investigated influence of genetic and environmental factors on a-b, b-c and c-d interdigital areas, and found that the genetic influence in palmar variables in men was stronger for b-c interdigital area, while a-b ridge count seemed to be more influenced by environmental factors. In women they found stronger genetic than environmental influence for all three counts in the interdigital area, but lowest was for b-c ridge count.

So, in the context of Arrieta`s finding, the differences between the Zhupa and Gora male groups and the Albanian male group might be the result of the influence of both genetic and environmental factors, while the differences between the Zhupa and Gora females on one side, and Albanian women on the other side, might be caused by their genetic differences. The prenatal sex differences in environmental sensitivity should also be taken into account when discussing dermatoglyphic sexual dimorphism [23, 28]. The 3D graphical presentation of the results of the canonical discriminant analysis showed that the Zhupa and Gora women differed from women from the Kosovo plain, and at the same time they were closer to the Gora men than to the Kosovo plain women. The Gora and Zhupa men were grouped closer than any of them to the men from Kosovo Plain.

Conclusion

In conclusion, the Zhupa and Gora populations from Kosovo showed less difference in quantitative dermatoglyphic variables than any of these population groups alone when compared to the Albanians. This indicates that the admixture between these two minorities and Albanian population living in Kosovo has been very small, and that Zhuplyani and Gorani have retained their genetic identity for several centuries. Most probable factors responsible for the detected dermatoglyphic variations might be geographic isolation, stressful environment, and turbulent history of this area, linguistic specificity and socio-cultural differences between the investigated populations. The forces of random genetic drift and local gene flow adequately describe the observed microgeographic variations [4].

Acknowledgements: We are grateful to all volunteers for their kind participation in this study. This study was supported by the Ministry of Science, Education and Sports, of the Republic of Croatia (projects 196-1962766-2736 and 196-1962766-2747) and the Ministry of Sciences of Kosovo.

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Institute of Experimental Morphology, Pathology and Anthropology with Museum
Bulgarian Anatomical Society

Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

Anthropological Characterisation of Balkanjis from Razgrad District

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Anthropological data from 126 adult men of the Bulgarian ethnographic group Balkandjis, collected in 1975 in anthropological expedition in the district of Razgrad are processed statistically and analyzed. The results show a population with mean height 168,5 cm, cephalic index 84,4%, morphological face index 86,7%, nasal index 64,3%, mixed and variable pigmentation (average eye colour 6,5 after Martin scale, mean hair color 49,8 after Michalski scale or T after Fischer-Saller). The individual analysis presents the prevalence of Subnordic, Dinaric and Alpine anthropological types, the elementary analysis – a combination of Nordic, Lapponoid and Armenoid elements. These anthropological characteristics place the population of Balkandjis among the Centraleuropean populations. The Balkandjis of Razgrad district are relatively close to the Bulgarians from Middle North Bulgaria, former Botevgrad county or Eastern Sofia district.

Key words: Balkandjis, Michalski's methods, Razgrad district, ethnic anthropology, Centraleuropean populations

Introduction

National wide ethnoanthropological surveys have been conducted in Bulgaria – by Acad. Stefan Vatev around 1899, by Acad. Methody Popov in 1938-43, by Aris Poulianos in 1963 and the National Anthropological Program in 1989-1993 [11, 12, 17, 18]. Their results show that the anthropological structure of the present Bulgarian population is very heterogeneous in territorial aspect. Unfortunately, the results from these studies are published only at national and regional level. Only few data are published on local level [11, 17]. The survey of Krum Dronchilov [2] perhaps the best exact and best known outside Bulgaria, presents anthropological data on local level, but does not cover the whole territory of Bulgaria. The materials of the extensive local anthropological studies of Peter Boev, Luchia Kavgazova and their collaborators, collected during the 1970s and 1980s are only partly published [4, 5, 6]. Resent review and analysis of some incomplete data of Methody Popov study also support the idea that more attention to the investigation of the anthropology of local Bulgarian populations should be paid [13].

That's why the author of this paper in the last years processed statistically and analyzed data of few local populations from the rich collection of unpublished archive materials [14, 15].

One of the ethnographic groups of Bulgarians are the Balkanjis (Mountaineers). They originally inhabited the Northern branches of Middle Stara Planina from about Etropole to Elena and spoke Balkan Eastern Bulgarian dialects. Characteristic for them are the Balkan house, black men's traditional costume, sukman women's costume. Because of shortage of arable land they early turned to crafts, manufacturing and trade. In 19-th century and especially after the establishment of new Bulgarian state (1878) they en masse migrated to the plains **(Fig.1).** They actively participated in the formation of Bulgarian nation and state and the Bulgarian language standard is based on one of their dialects. The social position of the women among them was higher than in other Bulgarian ethnographic groups. Because of this and because of the lack of arable land they something early accepted the limiting of the family size. [7, 16, 17]. Anthropologically they are higher than average Bulgarians, more brachycephalic, with mixed pigmentation [11, 17].

The present paper is dedicated to the anthropological characteristic of Bulgarians from the ethnographic group Balkandjis from Razgrad district, where their ancestor migrated from the Midle Stara Planina region.

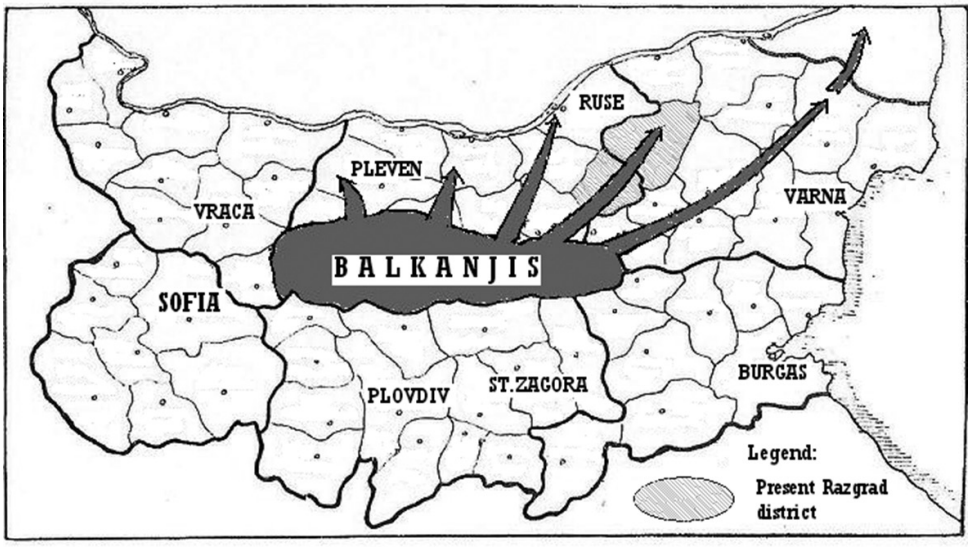

Fig. 1. Migrations of Balkandjis to the plains – 19th century and early 20th century

Materials and methods

Anthropological data from the archives of the former Department of Ethnic Anthropology of the former Institute of Morphology of BAS are processed statistically and analyzed. They include individual data of 126 adult men of the Bulgarian ethnographic group Balkandjis, collected in 1975 in anthropological expedition in the district of Razgrad (under the leadership of P. Boev).

The anthropological cards contain data about not only the ethnographic group but about the birth place of the parents of the investigated persons. Thus some misincluded in the group of Balkandjis persons (n = 9 of 135 in the folder) were excluded before processing and analizing.

Only the major anthropological traits are processed and analyzed in this paper. The analysis of the anthropological structure has been made according to the methodic of Michalski [3, 8, 9, 10]. In the methodic are made some minor modifications, which are described in previous article of the author [14].

The primary examination of the crude data has shown that morphological face height and nasal height are measured from *ophrydion* and not from *sellion*, thus being too high. Since the methods of Michalski-Henzel for determining the anthropological type is based on measurements from sellion [3, 8, 9, 10] the usual correction of 5 mm has been made [1]. Also the hair colour has been corrected by one number in the Fischer-Saller scale (From Y to X, from X to W and so on) – otherwise the proportion of black hairs (X-Y or 70 after Michalski's scale) shall be too high for each Europoid population.

Results and Discussion

According to their anthropological traits and basically on the pigmentation the Balkandjis from Razgrad district belong to the populations with mixed Southeuropoid and Northeuropoid traits **(Table 1).** They are relatively tall, brachycephalic, mesoprosopic, leptorhinic population of mixed pigmentation, with relatively prominent noses (straight and convex). Eastern Eurasian traits are rare as in most Bulgarian populations.

Table 1. Major anthropological traits and indices in the population under study

Anthropological trait	**n**	**M**	**SD**	**SE**
Height, cm	125	168,5	6,4	0,9
Head length, mm	126	186,0	6,1	0,5
Head breadth, mm	126	157,0	6,6	0,6
Cephalic index, %	126	84,5	3,5	0,3
Bizygomathic face breadth, mm	126	139,8	7,4	0,7
Morphological face height, mm [1]	126	126,0	7,1	0,6
Morhological facial index, % [2]	126	86,7	6,5	0,5
Nose height, mm [1]	126	57,8	4,2	0,4
Nose breadth, mm	126	33,7	3,5	0,3
Nasal index, % [2]	126	64,3	8,3	0,7
Eye color after Martin, 1-16	124	6,5	4,0	0,4
Hair colour after Michalski, 10-70 (after Fischer-Saller) [3]	120	49,8 (T)	10,4	0,9
Nasal form after Michalski, 10-100 [4]	126	68,3	18,8	1,7

[1] – uncorrected, after correction by 5 mm shorter

[2] – calculated from corrected values of morphological face height and nose height

[3] – 10 – light blond – 70 black without brown or red nuances, after correction

[4] – 10 – strongly concave – 100 strongly convex

A comparison of the major anthropological characteristics of the population under study has been made with data from the studies of M. Popov in the beginning of 1940s [11] and from the National Anthropological Program in the beginning of 1990s [18] **(Table 2).** When making such comparisons one should not forget that in each of the regions included (Middle North Bulgaria, Ruse, Varna) not only Balkandjis are living, there are also other Bulgarian ethnographic groups (Polyantsi, Hartsoi, Kapantsi, Chengentsi, Vayatsi, etc.). There may also be some differences in the result of secular changes – acceleration, brachycephalization, debrachycephalization) and because of use of different scales for scopic traits. However, the similarity between Razgrad Balkandjis and the population of Middle North Bulgaria is evident.

Table 2. Anthropological traits in Razgrad district Balkandjis and data from some other anthropological studies

	After [11], around 1940			**After [18], around 1990**			**Balkandjis, 1975**
Region (district)	**Pleven**	**Ruse**	**Varna**	**Lovech**	**Ruse**	**Varna**	**Razgrad**
Height, cm	171,0	170,7	171,0	171,5	169,8	170,7	168,5
Head length, mm	188,0	188,1	188,7	190,0	189,8	189,9	186,0
Head breadth, mm	156,6	156,3	154,6	158,9	157,7	157,7	157,0
Cephalic index, %	83,3	83,2	82,0	83,5	83,2	83,1	84,4
Bizygomathic face breadth, mm	141,2	141,2	140,2	139,8	145,1	144,4	144,0
Morphological face height, mm [1]	127,6	127,5	128,1	128,5	126,3	126,0	126,0
Morphological facial index, % [2]	86,8	86,7	87,8	85,1	84,0	84,0	86,7
Nose height, mm [1]	59,0	58,9	58,5	57,8	56,4	56,0	57,7
Nose breadth, mm	34,1	34,1	34,0	35,0	35,0	35,1	33,7
Nasal index, % [2]	*63,2*	*63,3*	*63,6*	*66,3*	*68,1*	*68,8*	64,3
Eye colour after Martin, 1-16	*7,0*	*6,4*	*6,6*	*6,1*	*6,1*	*6,0*	6,5
Hair colour after Michalski, 10-70 (after Fischer-Saller)	*45,8 (T)*	*46,9 (T)*	*45,3 (T)*	*54,7 (U)*	*54,6 (U)*	*56,6 (V)*	49,8 (T)

[1] – uncorrected, after correction shall be by 5 mm shorter

[2] – calculated from corrected values of morphological face height and nose height

The numbers in italic are just a rough estimate. Pleven administrative region in 1940-s and Lovech region in 1990s are the same geographic region – Middle North Bulgaria. Razgrad district before 1940s was divided between Ruse and Varna administrative regions **(Fig.1)** , but around 1990 was part of Ruse region.

According to the typological analysis the population of Razgrad district Balkandjis is heterogeneous. Among the individual anthropological types prevail Subnordic (AL) – 24%, Dinaric (AH) – 20% and Alpine (HL) – 15% **(Table 3)** – a typical Centraleuropean combination [1, 14,15]. Eastern Eurasian admixture (AM, AZ, HM) can be traced only in 5,6% (7 persons) of the sample.

Table 3. Individual typological structure of Balkandjis from Razgrad district (classification of Michalski-Henzel)

Anthropological type	**Symbol**	**N**	**%**
Mediterranean	EE	2	1,6
Armenoid	HH	5	4,0
Laponoid	LL	1	0,8
Atlanto-Pontic (Northwestern)	AE	6	4,8
Amoritic	AK	3	2,4
Dinaric	AH	**25**	**19,8**
Subnordic	**AL**	**30**	**23,8**
Euromongolic	AM	2	1,6
Eurasian	AZ	1	0,8
Finnish	AQ	1	0,8
Atlantic	YE	1	0,8
Pseudoalpean	YH	3	2,4
Levantinian	BH	1	0,8
Suboriental	EK	1	0,8
Litoral	EH	**8**	**6,3**
Sublaponoid	EL	5	4,0
Subarmenoid	KH	2	1,6
Western asian	KL	1	0,8
Souteastern	EQ	2	1,8
Alpine	**HL**	**19**	**15,1**
Turanian	HM	4	3,2
Pseudolitoral	HQ	3	2,4
Total		126	100

In bold – anthropological types with significant (over 5%) presence)

The analysis of the elementary anthropological structure **(Table 4)** presents a pattern close to the Bulgarians of Middle North Bulgaria – combination of Nordic, Armenoid (Balkano-Caucasian – h) and Laponoid (l) elements [15]. Thus they both belong to the populations of Central Europe [1]. Other close group are the Gagauzes from Kavarna, which also present Centraleuropean characteristics [14]. Also to this group belong the populations of the Eastern part of Sofia district (materials of Krum Dronchilov [2], in processing by the author). In fact the mountain part of Middle North Bulgaria (including the Eastern parts of former Botevgrad (Orhanie) county near Etropole) is the region from which the ancestors of our local group have migrated in Razgrad district. The populations of Southern Bulgaria mostly present other anthropological characteristics and belong to other, the Atlanto-Pontian group of populations [13, 17] with prevalence of Mediterranean and Nordic elements and mesocephalic heads.

Table 4. Elementary anthropological structure after Michalski

Sample	n	Anthropological elements, %										Eastern complex, %	South complex, %	For-mula
		a Nordic	**y** Croma-gnoid	**b** Berberic*	**e** Mediter-ranean *	**k** Oriental*	**h** Armenoid*	**l** Lapo-noid	**m** Mongolic*	**z** Pacific	**q** Uraloid			
Balkandjis	126	29,8	3,0	0,6	11,3	3,2	27,4	22,0	1,2	0,2	1,4	24,8	56,5	ah(le)
North Bulgaria [15]	226	31,2	0,6	0,9	6,1	6,7	27,3	14,2	8,1	3,3	2,0	27,2	56,4	ah(l)
Gagauzes [14]	109	31,9	2,5	2,3	10,3	6,9	18,8	21,6	0,7	0,9	4,1	27,3	52,7	al(he)

Synonims: Berberic = Mediterranoid, Mediterranean = Ibero-insular, Oriental = Eastern Oriental, Armenoidal = Balcano-Caucasian, Mongolic ^ typical Mongoloid

Remarks: Eastern complex = l+m+z+q , Soutern complex = (b+e+k+h)/(a+y+ b+e+k+h), formula – elements over 20% and in brackets element from 10 to 20%.

Conclusion

The anthropological characteristics of the Balkandjis from Razgrad district place them among the Centraleuropean populations. They are relatively close to the Bulgarians from Middle North Bulgaria, Botevgrad county or Eastern Sofia district and also to the Gagauzes from Kavarna.

The results from the study are an interesting testimony of centuries of demographic and ethnographic processes in Bulgaria.

Acknowledgements: The author thanks Lukasz Macuga from Torun for consultations when working on these materials.

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Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

Using of Diaphonization for Study of Domestic Pig's Auditory Tube

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By diaphonization the cartilage and bone in the pig's auditory tube are visualized "*in situ*", with aim to estimate its suitability for that animal species. The study was carried on 16 head halves from 8 six months old (4 males and 4 females), 90-100 kg/b.w. pigs, slaughtered for meat consumption in licensed abattoir. For staining of cartilage part in blue – Alcian blue 8G was used, and in violet-red for the bone section – Alizarin Red S, respectively. Trypsin was used for achieving of transparence. Excellent results were obtained, as both cartilage and bone parts were very clearly delineated in three-dimensional images. The transparency enhanced precise and attraction of visualization the received specimens. In conclusion, the data obtained give enough detailed information for investigation structure of pig's auditory tube. The prepared durable specimens could be used many times from specialists in their work.

Key words: auditory tube, diaphonization, pig

Introduction

As an organ connecting the pharynx (*nasopharynx*) with the middle ear, the auditory tube (*tuba auditiva*) plays an important role not only in the drainage and ventilation of this part of the ear but is also a natural way for the pharyngitis to pass into the auris media cavity, causing otitis media. From here, it is possible for the inflammation to spread to the meninges [1, 2, 3, 5]. The tube consists of bone (*pars ossea tubae auditivae*) and cartilaginous (*pars cartilaginea tubae auditivae*) parts [4].

Diaphonization is a method that is widely used to visualize in a definite way the bone and cartilage tissues in different parts or in the whole vertebrates. The structures stained by a special method with different colors allow detailed research with different purposes, in which very accurate data and useful information about them are obtained [7].

This method allows to study the bone and cartilage elements of the skeleton in fish, amphibians, reptiles, as well as parts of the skeleton of mammals of different sizes [6]. The latter authors also used diaphonization to study in detail the structure of the tooth root canal.

The lack of more specific data on both parts of the auditory tube in domestic pigs, and in particular on its cartilaginous part, as well as the growing importance of this animal species as a model in biomedical research relevant to humans [8], motivates us to undertake the present study.

In this study, we used diaphonization for more precise differentiation and subsequent measurement of the bone and cartilage part of a transparent native preparation from auditory tube in a domestic pig. We believe that this is a suitable alternative method that allows conducting a precise morphometric study without the risk of mechanical damage to the studied structures**.**

Materials and Methods

Animals

The study was conducted on 16 halves of heads from 8 pigs at six months of age (4 males and 4 females), 90 – 100 kg/b.w., of the breed Bulgarian white × Landrace, slaughtered for local consumption in a licensed slaughterhouse, in accordance with the Bulgarian legislation.

Experimental design

Fixation: Immediately after the machine halving of the heads, they were placed in containers and transferred in a cooler bag to the Department of Veterinary Anatomy, Histology and Embryology of Stara Zagora. After rinsing on tap water for 30 minutes, the preparations were placed in distilled water for 10 minutes. Immediately after that they were immersed in buffered 10% formaldehyde at pH 8.0 for 97 hours.

Water rinsing: After fixation, the samples were placed for rinsing under tap water for 4 hours and then placed in distilled water for 10 minutes.

Bleaching: The tissues were further depigmented in a solution of 0.25% hydrogen peroxide and 0.5% potassium hydroxide (KOH) for 2 hours, followed by rinsing with distilled water for 30 minutes.

Dehydration: Placing the samples in 90° ethanol until they sink to the bottom of the container.

Alcian blue 8 GS staining: The halves were then placed for 24 hours at room temperature in a solution of Alcian blue 8G, dissolved in 95° ethanol, mixed with glacial acetic acid with pH 1.6 (90 mg Alcian blue 8G, 480 ml 95° ethanol and 120 ml glacial acetic acid) to stain the cartilage structures.

Rinsing in distilled water and processing in sodium borate solution: Removed from this solution, the halves were placed in a solution of 700 ml of distilled water and 300 ml of saturated sodium borate solution for 12 hours to neutralize the acid reaction.

Treatment with 1,5% Trypsin solution: To achieve transparency, the preparations were placed in a pre-prepared 1.5% solution of trypsin with saturated sodium borate solution – 3 parts and 7 parts distilled water for 72 hours.

Alizarin Red S staining: The Alizarin Red S working solution was prepared by adding 600 ml of 0.5% potassium hydroxide to 600 mg of Alizarin Red S. The samples are kept in this solution for 30 hours at 25°C.

Additional processing in 0.5% potassium hydroxide and glycerol: The preparations were then placed sequentially in a mixture of 0.5% potassium hydroxide and glycerol in the ratio as follows – 1:1 for 48 hours at room temperature, 1:2 for 72 hours at room temperature.

Pure glycerol: Finally, the preparations were placed in pure glycerol for 7 days at 28°C.

The samples were then removed from the solution, carefully drained, and placed on glass, illuminated with additional light from below for better visualization of the cartilage from the bone structures. The length of the auditory tube in the bone canal was carefully measured with a digital caliper with an accuracy of 0.01 mm and photographed with a digital camera. The measurements of the width and height of the cartilage part of the auditory tube were done by a steroscope (MBS-10).

Results

The position and views of treated auditory tubes with clear distinct outlines of both cartilaginous and osseous parts are show on **Fig. 1** and **Fig. 2**.

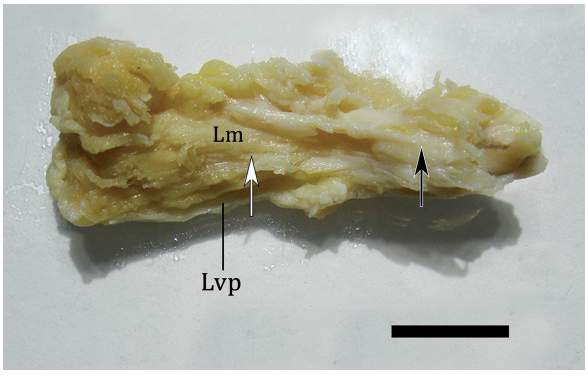
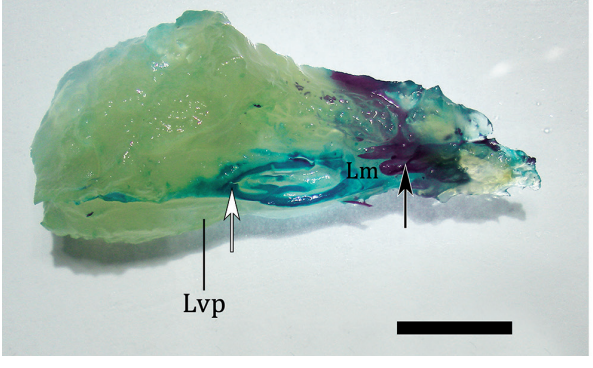


Fig.1. Medial view of a right auditory tube of a female pig – native preparation; **white arrow** – *pars cartilaginea tubae auditive*, **black arrow** – *pars ossea tubae auditive,* **Lm** – *lamina medialis*, **Lvp** – *m. tensor veli palatini.* Bar = 1 cm.



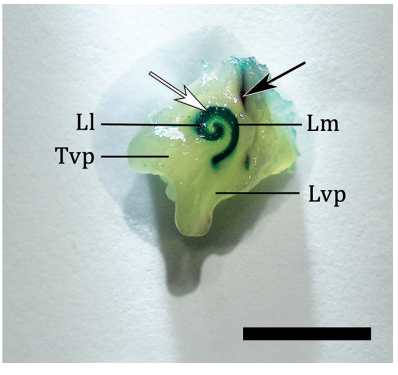


Fig.2. Left: Medial view of a right auditory tube of a female pig – diaphonized preparation (left)); **white arrow** – *pars cartilaginea tubae auditive*, **black arrow** – *pars ossea tubae auditive,* **Lm** – *lamina medialis*, **Lvp** – *m. tensor veli palatini.* Bar = 1 cm. **Right:** Transversal cut of a right auditory tube trough its bone part (right); **white arrow** – *pars cartilaginea tubae auditive*, **black arrow** – *pars ossea tubae auditive,* **Ll**– *lamina lateralis,* **Lm** – *lamina medialis*, **Tvp** – *m. tensor veli palatini,* **Lvp** – *m. tensor veli palatini.* Bar = 1 cm.

The measurements with a steroscope showed that the values of the width and height of cartilage part of left auditory tube in males the width varies from 2.10 to 3.05 mm (ẋ-2.61 mm), height – from 3.65 to 3.90 mm (ẋ-3.76 mm), and in females – the width varies from 2.80 to 3.40 mm (ẋ-3.14 mm), height – from 3.60 to 4.40 mm (ẋ-4.0 mm), in both sexes are higher than those of the right tube in males the width varies from 2.25 to 2.70 mm (ẋ-2.50 mm), height – from 2.80 to 3.10 mm (ẋ-2.96 mm), and in females – the width was 2.45-3.15 mm (ẋ-2.79 mm), and height – 3.25-3.80 mm (ẋ-3.61 mm). In male pigs, the measured parameters showed lower values compared to those in females.

The length of the auditory tube in the bone canal was measured with a digital caliper. Measurements of the length of the left and right tubes in both sexes showed similar values. In male pigs the length of right tube was 10.73-11.80 mm (ẋ-11.17 mm), and the left tube 9.60-11.84 mm (ẋ-10.97 mm),in female pigs – 10.52-12.40 mm (ẋ-11.5 mm), and 10.15-11.93 mm (ẋ-11.27 mm), respectively.

Discussion

Diaphonization is a method that is widely used to study the bone and cartilage elements of the skeleton in fish, amphibians, reptiles, as well as parts of the skeleton of mammals of different sizes [6]. The latter authors also used diaphonization to study in detail the structure of the tooth root canal. However, there is no information about the application of this method in studying the morphological features of the auditory tube in animals and humans. In the current study, the method of diaphonization was used for the first time in order to visualize the cartilage and bone parts of porcine auditory tube without the risk of mechanical damage of the studied structures. This method allowed precise measurements of the width and height of the cartilage part of auditory tube and the length of the bony part of the tube. We showed that the diaphonization can be very useful in studying the anatomy details of bony and cartilaginous structures of the animal and human body.

Conclusion

The data obtained give enough detailed information for investigation the peculiarities in position, form and structure of pig's auditory tube. The prepared specimens can be stored for unlimited period of time and they could be used many times from both medical and biological specialists in their work.

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Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

Rare Variation of Submental Artery Extending Into Inferior Labial Artery

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During routine anatomical dissection of a 68-year-old Caucasian male cadaver we found a large sized left submental artery which after supplying the structures in submandibular triangle crossed the mandibular body and further extended into the inferior labial artery. After reviewing the literature, we discuss the variations in the inferior lip blood supply and their possible clinical significance.

Key words: submental artery; inferior labial artery; arterial variations; clinical significance; human

Introduction

The submental artery is the largest branch of the facial artery in the neck, which can be identified within the submandibular triangle, parallel to the body of the mandible. At this location, it supplies the overlying skin and adjacent muscles and also the chin and contributes to inferior lip blood supply by anastomosing with the inferior labial and mental arteries [7, 8]. These anastomoses are named vertical labiomental arteries [2, 11]. Sometimes such arterial anastomose may enlarge and become the major blood supply to the lower lip together with the inferior labial artery [5].

Case report

We discovered a rare variation of the lower lip blood supply during a routine anatomical dissection of a 68-year-old Caucasian male cadaver from the authopsy material of the Department of Anatomy, Histology and Embryology at the Medical University of Sofia. By a layered neck dissection, the skin was removed, followed by platysma layer and the investing layer of the cervical fascia. While dissecting the content of the left submandibular triangle, we found an unusually large left submental artery (external diameter 2.8 mm). This vessel started from a large sized facial artery (external diameter 3.6 mm) within the submandibular triangle and followed a direction parallel to the body of the mandible just underneath the investing layer of the cervical fascia. In this

location, the variant submental artery had nearly the same size as the ascending in the face part of the facial artery. At a point nearly 2 cm aside from the anterior midline, the submental artery curved superiorly over the mandibular body, giving a small but discernible bony indentation, and ascended to the mental region (**Fig. 1a, b**). Further dissection revealed that the variant submental artery continued to supply the lower lip. After precise dissection in the buccal region we established that instead of usual inferior labial artery, the facial artery was only providing a small anastomosing branch (external diameter of less than 1 mm) to the lower lip. In the reported case obviously the submental artery was extending into the left inferior labial artery. On the right side, no variations in the origin and distribution of the submental, inferior and superior labial arteries were observed.

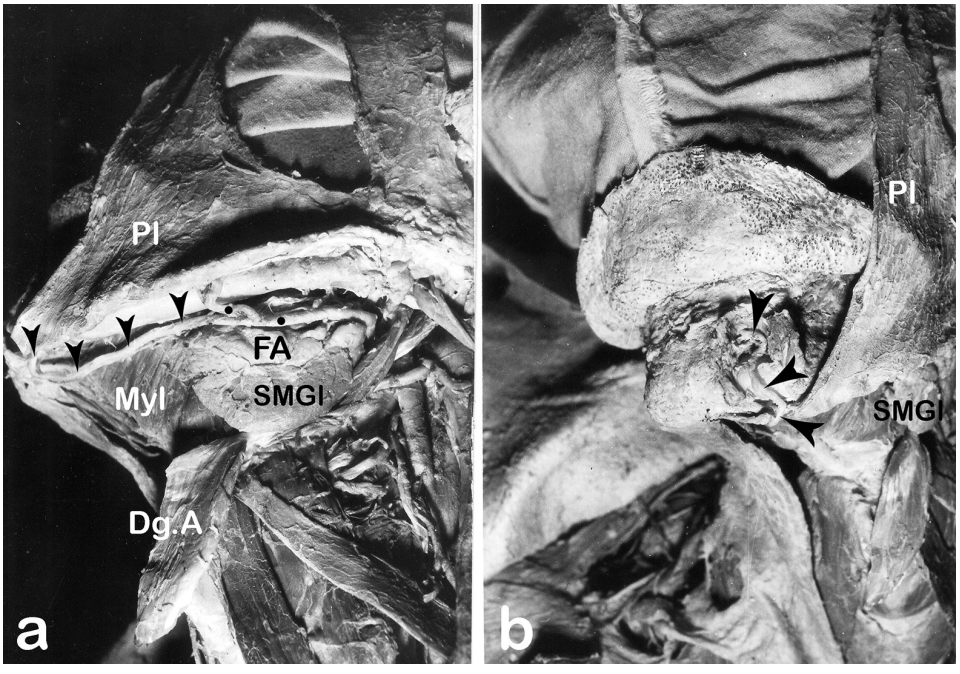

Fig. 1. Photographs of the neck dissection demonstrating the large sized submental artery (black arrowheads) arising from the facial artery (a) and extending into the inferior labial artery (b). Muscles: **Pl** – platysma; **Dg.A** – digastric anterior belly; **Myl** – mylohyoid. **SMGl** – submandibular gland. **FA** – facial artery.

Discussion

The blood supply of the lower lip and chin – mentolabial region, is complex and is provided by inferior labial and submental arteries as well as anastomosing branches between them called horizontal and vertical labiomental arteries [2,11]. The mental artery is only supplemental to this anastomosing network [11]. The patterns of blood supply may be different, with different enlargement of individual anastomotic branches, but the inferior labial artery is always present according to Pinar et al. [4,5], Al-Hoqail and Meguid [1] and Schulte et al. 2001[6]. Very rare, the inferior labial artery might

be absent and then it is replaced by its contralateral fellow [9]. In 4 to 20%, according to two different studies [4, 5], vertical labiomental artery – a direct continuation of submental artery, is the major blood supply to inferior lip, together with a small inferior labial artery – a finding consistent with our report.

Because of its large size and unusual point of passing over the mandible, the reported here submental/inferior labial artery variation might be important in surgical procedures of the submandibular and mentolabial regions. In plastic and reconstructive surgery, rising platysma myocutaneous flap [6, 10] based on submental artery extending into inferior labial artery might impair inferior lip blood supply [3, 11].

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Acta morphologica et anthropologica, 27 (3-4)
Sofia ● 2020

Possibilities of Silicone Rebasing Materials for Increasing the Hermetization of Maxillary Obturators. Case Report.

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The main problem in treatment with maxillary obturators is defect's hermetization and creation of stable border between oral and nasal cavity. This requires the application of specific methods and appropriate materials for prosthetic treatment. A methodology of prosthetic treatment with silicone relining materials is presented in patient with unilateral defect of the maxilla after oncological operation. A buccal flange obturator was fabricated, due to defect's size and localisation. The obturator was finished by heat cured acrylic resin with low quantity of residual monomer and the substitution part was designed as a hollow part for better retention and stability. Direct relining with silicone material was performed for improvement of defect's hermetization. The conducted treatment provided the necessary retention and stability of the obturator. Patient's chewing, feeding and speaking have been successfully restored. The silicone relining material facilitated the insertion of the denture and protected the tissues from damages.

Key words: maxillary resection, maxillary defect, obturator, silicone relining materials.

Introduction

Prosthetic treatment methods take main role in rehabilitation of patients with maxillary resection [18]. Silicone materials are used in fabrication of the obturator or some of its parts [11, 20, 24]. Clinical studies reveal that their application provides optimal retention and stability of the denture [8, 12]. Silicone materials make the insertion in the defect easier and can be corrected or replaced quickly if it is needed [29]. The investigations show that they are non-toxic, bio compatible materials, which do not cause trauma or irritation of soft tissue [2, 15]. This makes them very useful in the treatment of patients with retentive tubers and alveolar bone atrophy [19]. Electromyographic researches report, that their application increases masseters and temporal muscles activity, such as chewing efficiency [25, 28].

The main problem, which limits the application of silicone materials, is the stability of the connection with the acrylic resin, which needs preliminary preparation of the surface [33]. For this purpose, some authors [10, 13] use phosphoric acid for etching denture's borders and others create micro retentions with lasers [9]. Studies

show that application of monomer on denture's surface stabilizes the adhesion to the silicone material, but sandblasting weakens it [16]. Adhesion increase is observed when heat cured silicone materials are used, especially if their polymerization is performed at the same time as the acrylic resin [3, 17].

Essential disadvantage of silicone relining materials is their porous surface, which creates a prerequisite for growth of Candida [4, 14]. The studies by Wieckiewicz et al. [35] prove the presence of Candida in 90% of the prosthetic treatments with silicone obturators. According to other data, polysiloxane base of the silicone materials allows preserving of the soft smooth surface, which prevent plaque accumulation and facilitates denture's cleaning [1, 22]. The application of disinfectants is recommended, despite the risk of denture's coloring and breaking the connection with the acrylic resin [6]. Studies show, that the right choice of silicone and cleaning material prolongs dentures' life [23, 27]. The application of silicone relining materials is very appropriate in two-stage prosthetic treatment in patients with maxillary resection, when the obturator's base is fabricated at first, and then its substitution part [30, 34]. In this case, the base is fabricated by heat cured acrylic resin and substitution part- from silicone material, which provides retention and stability of the denture [12, 20, 24]. This type of prosthetic treatment is used for fabrication of two-part obturators in patients with trismus [32, 34]. Very often the substitution silicone part is connected to the base through cobalt-samarium magnets [12, 21, 24]. Acrylic locks and system type "interlock" are used as well [7, 20].

The indications and contraindications for application of silicone materials in patients with maxillary resection are controversial. Most authors [5, 31] claim, that their application is possible only in small palate defects. In cases with large defects it is recommended stabilization of the silicone obturation part with methyl methacrylate pin [26].

Materials and methods

A case of 58-years-old female patient with defect of the upper jaw after cancer operation is presented as an example for evaluation of silicones' relining properties. The intraoral examination revealed a defect in the right side of the jaw, which reach the midline and involves soft and hard palate. The alveolar bone in the left side was very much resorbed, without any teeth left and the lower jaw had all the teeth. Due to the recently conducted radiotherapy, the patient had very severe trismus and pain in the masticatory muscles. That fact and the defect's size lead to treatment plan with buccal flange obturator. For this purpose, impression was taken with standard metal tray and additive silicone material (**Fig. 1**). The defect was tamped with gauze in advance. The occlusion height and centric relations were fixed by occlusal wax rims in the next clinical stage. The trial denture did not show any mistakes or deviations and the obturator was fabricated from heat cured acrylic resin with low quantity of residual monomer. The substitution part was formed as open hollow part for better retention and stability (**Fig. 2**). The adjustment showed unsatisfactory hermetization, which required additional rebasing. A silicone material Reviler **(**Kulzer GmbH) for direct rebasing was shaped functionally in the mouth (**Fig. 3**).

Fig. 1. An impression from the defect

Fig. 2. Buccal flange obturator

Fig. 3. The obturator, rebased with silicone

Results

The initial results revealed unsatisfactory retention and stability of the obturator. The hollow bulb substitution part did not provide optimum defect hermetization, especially in the area of soft palate. This didn't provide successful restoration of feeding, speaking and swallowing. Additional rebasing procedure was needed and silicone material was used

for this purpose. The silicone material was functionally designed on the defect's borders and denture bearing area after preliminary preparation of the acrylic surface, according to manufacturer's instructions. This provided the necessary retention and stability of the denture. The successful hermetization provided restoration of the damaged functions. The creation of stable border between the oral and nasal cavity helped for normalization of speaking and facilitated liquids reception. The occlusal closure was restored, which improved patient's chewing and feeding. The usage of silicone material provided non traumatic transmission of masticatory pressure and facilitated denture's insertion. The occurred positive changes improved significantly patient's life quality and restored her self-esteem and social activity.

Discussion

The prosthetic treatment of patients after maxillary resection is accompanied by many difficulties and problems. The main problems were connected with taking functional impressions, as most of the cases. The impression registration was very tough, due to trismuss, occurred after radiotherapy, and defect's localization. Very limited mouth opening required treatment plan for fabrication of open obturator, which could be easily inserted by the patient. The fabrication of hollow bulb substitution part has been creating a precondition for better retention of the obturator, especially when all the teeth were missing. Despite the described advantages, a satisfactory hermetization was not achieved. The main reason for this was defect's localization, which involved part of the soft palate and did not allow obturator's stability during feeding and swallowing. To resolve this problem we rebased directly the obturator with silicone material, which allowed maximum use of the defect's retentive areas and complete hermetization in the area of A-line. The achieved results confirmed the state, that silicone materials improve dentures' retention and stability [8, 12]. Their application allowed easy and non traumatic insertion of the denture in the defect that is their main advantage, according to many authors [2, 15]. The successful treatment suggested that silicone materials are really appropriate in the prosthetic treatment of patients with retentive tubers and alveolar bone atrophy [19]. The advantages of the two-stage technique, which provides optimum obturator's retention and stability, was confirmed, as well [12, 20, 24]. It was suggested that the described method could be used as an alternative technique of two-part obturators, which are the only devices for trismuss treatment, as some authors reported [32, 34]. The clinical results did not confirm, the thesis, that silicone relining materials can be used only in small defects [5, 31]. The described clinical report is not very common in daily dental practice. Despite the achieved positive results obtained, more research is needed to investigate the durability between the acrylic resin and silicone material, such as microbiological studies of obturator's surface.

Conclusions

The application of silicone rebasing materials improves stability and increases hermetization of the maxillary obturators and it is very appropriate in patients with large defects and complete edentulism.

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IN MEMORIAM

Prof. Dr. Georgi G. Markov

On 12.9.2020 in New York passed away Prof. Dr. Georgi G. Markov, born on 13.4.1924 in Pleven.

Prof. Dr. G. Markov is known as a cytologist and molecular biologist, one of the founders of the Institute of Molecular Biology of Bulgarian Academy of Sciences. However, he has an outstanding contribution to the Bulgarian anthropology.

After the death of his uncle Acad. Methody Popov in 1954, Georgi Markov took the task to arrange and systematize the anthropological materials collected by Methody Popov during 1937-1943 that survived the bombing of Sofia. Professor Markov got responsibility to process them and on the base of the primary notes of M. Popov to write a coherent text and to publish it. This was not an easy task because these materials were very vast (data for 5759 men and 2160 women in a period when modern computer technology did not exist) and because in this period Acad. M. Popov was an object of attacks because of political reasons for not accepting Michurin-Lysenko's biology.

The result of this work was "Anthropology of Bulgarian people" (published in 1959 by authors M. Popov and G. Markov) – a fundamental work, giving anthropological characterization of Bulgarians born around 1920s before the great migrations on national, regional and even on county level. The data included in this book are still a base for comparison with other anthropological studies and an object of analysis with modern statistical methods. There is no other study in the field of ethnic anthropology in Bulgaria like that by M. Popov and G. Markov.

In the following years G. Markov worked in the field of cytology and of molecular biology. But he never forgot anthropology and he supported publication of materials of the National Anthropologic Program in the beginning of 2000s.

Rest in peace!

From: The Department of Anthropology and Anatomy, Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences.

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ISSN 1311-8773 (print)
ISSN 2535-0811 (online)