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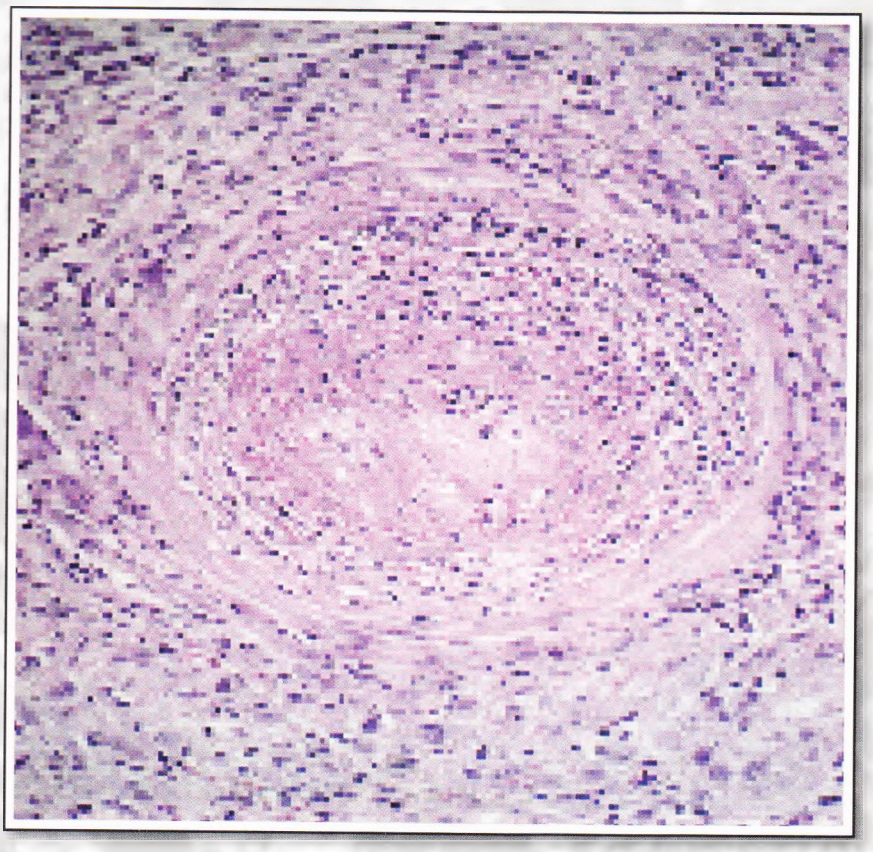

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# Acta morphologica et anthropologica (15)

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Institute of Experimental Morphology and Anthropology with Museum
Bulgarian Anatomical Society

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*Editorial*

# Fifty-Fifth Anniversary of the Institute of Experimental Morphology and Anthropology with Museum

Dear colleagues, friends and guests,

The foundation of the Institute of morphology at the Bulgarian Academy of Sciences, whose 55th anniversary we notice today is a result and beginning, as well. A result concerning the achievements of the Bulgarian scientists (histologists, embryologists, anatomists, anthropologists) in the field of cell, tissue and system morphology, and start of a new period connected with the development of the structure basis of the biological and medico-biological sciences.

The Institute of Experimental Medicine was founded in 1947 as a part of the Biological branch of the Bulgarian Academy of Sciences. On the base of the Department of histology, embryology and experimental medicine a new independent unit was established in 1953 – the Institute of Morphology at the Department of biological and medical sciences in BAS. This provided the elaboration of institutionalized morphological and anthropological studies.

Founders of the Institute of Morphology at BAS are the outstanding scientists Acad. Asen Ivanov Hadjiolov and Corr. Member Dimitar Dimitrov Kadanov – renowned university lecturers with long practice, founders of the Bulgarian morphological school and supervisors to a large number of young scientists, postgraduate and graduate students.

The Institute of Experimental Morphology and Anthropology at BAS (IEMA) was established in 1995 being successor of the Institute of morphology, and since 2006 named Institute of experimental morphology and anthropology with National anthropological museum – IEMAM, BAS.

IEMA is a leading national institute in the field of anthropology, experimental morphology and cell biology.

Nowadays IEMAM has 89 employees and it consists of 4 Departments (Department of Neuro-morphology, Department of Cell Differentiation, Department of Experimental Cytology and Department of Anthropology); 2 general Laboratory (Laboratory of Electron microscopy and Radio-isotope Laboratory), as well as an administrative unit (accountant's office, library and animal house).

The scientists from IEMAM participate in the educational and research activities of various Universities by teaching students, working on projects funded by the Ministry of Education and Science or international collaborative projects. Throughout 2000-2007 alone international collaboration of 20 projects have been put into effect with the following countries and institutes: Russia – RAS, Institute of Physiology in Sankt-Peterburg; Hungary – HAS, National Institute of Psychiatry and Neurology in Budapest; Germany – Institute of Brain Investigations "Paul Flexig", Leipzig, Institute of Cell Biology and Bio-Systematic Techniques in the University of Rostock, Institute of Anatomy in Hamburg, University of Duseldorf; Great Britain – Center of Human Reproductive Science in Edinburgh; France – University No7 in Paris, Office of Medical Investigations, Commissariat of Nuclear Energy in the Defense Ministry; Austria – Institute of Medical Chemistry and Biochemistry in Innsbruck, and USA – VICAM Company in Boston, University of Alabama, Birmingham.

The achievements of the IEMAM that are confirmed in Bulgaria and abroad could be presented shortly as follows:

Peculiar completion of the purposeful national study concerning physical development of the Bulgarian population at the end of the past century is the detailed anthropological characterization presented in the monograph "Anthropology of the Bulgarian Population at the End of the 20$^{th}$ Century", Sofia, Prof. Marin Drinov Academic Publishing House, 2006. The data could serve also as a starting point for coming anthropological studies. The anthropological investigation of children from birth till 17 years of age characterize the specific processes in children's growth and development, the acceleration and deceleration, and the morphological and functional status of the young population in our country.

The paleoanthropological studies of material from archaeological excavations characterize the populations who have lived in our lands during different epochs, as well as the paleo-demography and paleo-pathology. By means of the method concerning the plastic anthropological reconstruction of head on the skull, images of people who have inhabited the Bulgarian lands from the Neolithic period till the Renaissance are visualized.

In the realm of Neuromorphology research is aimed at the establishment of: the participation of neurons in myelinogenesis and in the pathogenesis of multiple sclerosis (MS); the participation of the amyloid beta-peptides in the etiology of Alzheimer's disease; the changes of the lipid constituents of brain subcellular fractions after experimentally evoked cerebral ischemia and hypoxia and under various feeding regimes; the topography and reactivity of the cells in the central nervous system through immunohistochemical studies of degenerating brains and of the brain mononuclear phagocytic system.

Control mechanisms of cell proliferation and differentiation in reproductive and blood tissues are studied by application of experimental animal models and clinical investigations. In the field of reproductive biology and medicine, the regulatory events of spermatogenesis, oogenesis, steroidogenesis and programmed cell death in the testis and ovary are elucidated. Specific changes in different cell types of gonads and reproductive tract are established at various functional and pathological conditions that can be used as biomarkers for infertility. In the field of hemopoiesis, an in vitro model for culturing of hematopoietic progenitor cells was developed as a useful tool for morphological and biochemical studies. New data were generated about influence of hematopoietic factors on differentiation of erythroid and myeloid cells and their biochemical properties with potential use for transplantology and regenerative medicine.

The positive activity of the growth factors in the colostrums on the development of the newborn gut and in the whey to ameliorate the pathological effects of some medi-

cines was proved, several immunomodulators of lymphocyte proliferation of plant origin were found and exploited, the optimal cryoprotective media and regimen for cryoprotection of corneal stem cells were investigated, fluorescent substrates for localization of peptidases in tumor and normal cells were applied and diagnostic kit prepared; studied and characterized were the morphological substrates of skin vasculutis.

In 1997 has been founded a permanent anthropological exposition "Man in the Past", which was conferred statute of a museum collection of IEMA at BAS by the Managing Board of BAS and the Ministry of Culture and has served as basis of the National Anthropological Museum foundation. The visiting version of the exposition, showed in various Bulgarian towns, was visited by thousands of people in the country. On March 21st 2008 was the inauguration of the National Anthropological Museum – the fourth one in the Bulgarian Academy of Sciences, registered also by the Ministry of Culture.

The results obtained from the researches carried out in the Institute are presented in scores of monographs (books) and hundreds of articles reported on many national and world congresses and being multiple cited in Bulgaria and abroad. The IEMAM publish two journals – the "Acta morpgologica et anthropologica" and the "Journal of Anthropology".

Nowadays, the Institute of Experimental Morphology and Anthropology with Museum supported and promoted by the Governing Body in the Bulgarian Academy of Sciences evolve the morphological and anthropological sciences in conformity with the contemporary realms of the European and world science and with the requirements of our days, as well. This fact could guarantee the successes of the Institute in the future and could help the confirmation of the traditions in the Bulgarian morphological school.

Happy Fest!

***Corr. Memb. Dr Yordan Alexiev Yordanov***,
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Institute of Experimental Morphology and Anthropology with Museum
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# Celebration of the 80th Anniversary of Prof. Vassil Vassilev

Dear Prof. Vassilev,
Dear guests,

I have the honour and pleasure of opening our meeting relevant to the 80th anniversary of Prof. Dr Vassil Assenov Vassilev – Honorary Chairman of the Bulgarian Anatomical Society and Honorary member of the Bulgarian Anthropological Society.

The name and activities of Prof. Dr Vassilev are widely and well known in our medical community being lecturer of long in human anatomy teaching many generations medical and dental doctors and pharmacists, Head of the Department of Anatomy, Chancellor of the Medical University in Sofia, socially active person, awarded many honorary titles, he is a figure in the Bulgarian Scientists Union and the Union of the medical Scientific Societies, as well as a good Bulgarian.

The wide administrative experience of Prof. V. Vassilev is of good benefit for his participation in the High Testimonial Committee – a support concerning his objectivity and impartiality as reviewer, speaker and consultant for the Committee.

His scientific production is noteworthy not being only numerous, but being a very good model for high quality, exactness and honesty in the scientific work. An example for the connection between generations and guarantee about traditions' confirmation is the fact that Prof. V. Vassilev is disciple of the great anatomists Dimitar Kadanov, Milko Balan, George Galabov and Teodor Schibler. Prof. Vassilev himself writes: "For a young man everyone could be a teacher by whom he could learn anything." Let us remember this!

Everything begins from the point to which it stands. Prof. V. Vassilev has continued and confirmed his predecessors' work and gave new directions to the scientific investigations. He is one of the few very active popularizers of the morphological science.

The development of Prof. V. Vassilev as teacher and scientist followed its normal progress – from a long service as demonstrator up to professor degree; PhD, DSc, Head of the Department of anatomy and so on. This more than forty years activity is a result of many years of enormous labor, constancy and perseverance. It looks like easy to get it, but only at first sight. Only a wish could not be enough, skillfulness is needed too and Prof. Vassilev's makings prove it.

What are the few things that we know or not about the man – Prof. V. Vassilev?

Vassil Assenov Vassilev is a holistic worker – not only concerning his profession but also in life. Being very active, he is also extremely exact person in everything he does.

Vassil Assenov Vassilev is married to Dr Margaret Vassileva, the only wife for him till now. The pride of the parents is both their children – Dr Ekaterina Vassileva and Dr Ivan Vassilev. I shall omit the grandchildren meaning not that they are persons of no importance, but because of the fact that the Family Vassilev is proud of five great-grandchildren also. Let them be happy, healthy and smart!

As a man coming from Boyana district, Prof. Vassilev is enthusiastic skier. Nowadays, he is playing basketball ambitiously and passionately. A good example to be followed!

The constant endeavors of Prof. Vassilev are extraordinary for the consolidation and development of the Bulgarian Anatomical Society that he has done during the past years and has carried on till now. The tradition enjoins him and undertakes him to do it being connected with the names of Asen Hadjiolov, Dimitar Kadanov, Milko Balan, George Galabov. For Prof. Vassilev the professional, alive consolidation of the Bulgarian morphologists is a precondition about contemporary, high-grade development of the morphological science in Bulgaria, it could support and stabilize the international contacts and could confirm our science.

The high professionalism of Prof. Vassilev as anatomist is well known and universally accepted – excellent dissector, brilliant lecturer, objective examiner, organizer of the teaching process.

In support of my words I shall give some data.

Vassil Assenov Vassilev was born on August 15, 1928 in Sofia. He graduated from the Medical Faculty in Sofia in 1952. Since 1953 he occupied the position of an assistant professor in the Chair of Anatomy in Sofia and passing over all scientific stages he attained the academic rank Professor in 1975.

In 1959 he gets the clinical specialty in orthopedics and traumatology. Prof. Vassilev worked and made specializations in several Universities in Bulgaria and abroad – Wurzburg, Berlin, Lubek, Leipzig, Munich. He is author and co-author of 12 textbooks and high school appliances, as well as of over 200 scientific works published in authoritative foreign journals and books; he participated with papers in more than 180 Congresses – International and National with international participation.

His scientific work and publications cover a wide frame of reference:

Macroscopic anatomical investigations;

Neuromuscular investigations of sense innervations of different organs;

Spinal column studies

Investigations of the joints' structure;

Investigations of connective tissue in the skeletal muscles;

Ultrastructure and histo-chemistry of the serous membranes;

Ultrastructure of the meninges.

Summarizing the scientific contributions of Prof. Vassilev one might say he is the best authority on the connective tissue. He contrived to build up a good scientific group that successfully applies the new morphological methods (transmission and scanning electronic microscopy, histo- and cytochemistry, immunohistochemistry and experiments with markers – peroxidase, Ruthenium red). The problems are markedly related to practice being result of his clinical specialty in orthopedics and traumatology. That's why Prof. Vassilev could be thought a pioneer of the clinical anatomy at microscopic and submicroscopic level. Considerably are the international scientific contacts of Prof. Vassilev thanks to the possibility of him to work in authoritative German Institutes, as well as to his participations in many international congresses. He has contacted with our clinical doctors taking part in lot of the orthopedic and surgeon conferences.

The short presentation of the entire and abundant activity of Prof. Vassilev could outline clearly his image of a thorough and knowledgeable scientist, a perfect organizer of science and education, an active public figure.

Please, let me congratulate and wish him on yours behalf and myself longevity and creative activity, healthy and happy days.

Happy anniversary, honoured Prof. Vassilev!

Many happy returns!

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*Morphology*

# Projections from the Central Amygdaloid Nucleus to the Mesencephalic Trigeminal Nucleus in the Rat

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The efferent connections of the central amygdaloid nucleus (AmCe) to the mesencephalic trigeminal nucleus (Me5) were investigated in rats. High molecular weight (10 000 mw) biotinylated dextran amine (BDA 10k), an established anterograde axonal tracer, was stereotaxically injected in the AmCe. The AmCe axons, labelled with BDA 10k ascend from the injection focus and follow two routes. The main axonal stream runs in the stria terminalis, and a smaller fiber component builds the ventral amygdalofugal pathway. From the latter deviate the axons descending in the brainstem. After the innervation of the lateral portion of substantia nigra, the labelled axons take a dorsomedial course in the mesencephalic tegmentum and reach the periaqueductal gray, innervating also the rostral (mesencephalic) portion of Me5. The axons followed to the pons descend ventrally to the motor trigeminal nucleus and bend dorsally towards the dorsolateral pons. This bundle terminates with dense axonal arborizations in the parabrachial nuclear complex and in the caudal (pontine) portion of Me5. It appears that both the pseudounipolar and multipolar neurons receive axons from AmCe. The present data indicate that the amygdala, a key structure of the limbic system, is also strongly involved in proprioception.

*Key words*: anterograde axonal tracing, biotinylated dextran amine, limbic system, orofacial proprioception.

## Introduction

The mesencephalic trigeminal nucleus (Me5) is a unique structure in the CNS, mainly composed of pseudounipolar primary sensory neurons. In the rostral pons Me5 neurons are located in the triangle between the locus coeruleus and the medial parabrachial nucleus, and in the mesencephalon they border laterally the periaqueductal gray [5, 13]. Mesencephalic trigeminal neurons innervate the masticatory muscles, the periodontal

ligament and a subset of the extraocular muscles [1]. Unlike the "ordinary" pseudounipolar neurons in the sensory ganglia, the mesencephalic trigeminal neurons display axosomatic synaptic contacts [5, 7, 8]. The Me5 receives afferent connections almost exclusively from the brainstem structures [reviewed in 5, 6].

We presently report an afferent projection to the Me5 from a key structure of the limbic system – the amygdaloid nuclear complex, studied by anterograde axonal tracing.

## Material and Methods

Ten adult Wistar rats weighing 220-260 g were used. The animals were anesthetized with Thiopental and then mounted in David Kopf stereotaxic apparatus in the flat skull position. Stereotaxic coordinates of the central amygdaloid nucleus (AmCe) were obtained from the atlas of P a x i n o s and W a t s o n [10]. Under aseptic conditions small craniotomies were performed. In the AmCe 0.25-0.5 $\mu$l biotynilated dextran amine (BDA;10%, 10.000 mw; Molecular Probes Europe BV, Leiden, The Netherlands) dissolved in phosphate buffer (PB, 0.1M, pH 7.2) was injected with a Hamilton microsyringe (Hamilton Co, Reno Nevada, USA) using a dorsal approach. At the end of the injection, the injection canula was held in place for 15 min to insure that the injected tracer had been absorbed into the tissue. After survival time of 8-21 days, the rats were deeply re-anaesthetized and perfused transcardially with phosphate buffer saline (PBS), followed by 500 ml of 4% paraformaldehyde in PB. The removed brains were postfixed overnight in the same fixative, blocked in the coronal plane and soaked in 0.5% paraformaldehyde in PB containing 20% sucrose at 4°C. Serial sections were cut at a thickness of 40 $\mu$m on Reichert Jung freezing microtome, collected in a free-floating state in PB and then processed for tracer histochemistry. A commercial avidin-biotin-HRP complex (ABC) kit was used to visualize BDA (Vectastain ABC Kit, Vector Laboratories Inc., Burlingame, USA). Briefly, the sections were preincubated in PB containing 0.1% bovine albumin (fraction V; Sigma Chemical Co, St. Louis, USA) for 20 min, and rinsed in PB for 30 min. Subsequently they were incubated in the avidin-coupled biotynilated HRP solution for 45-60 min, and rinsed again in PB for 30 min. The reaction product was developed with 0.06% 3,3'-diaminobenzidine (Sigma Chemical Co, St. Louis, USA) and 0.02% $H_2O_2$ in Tris buffer (0.05 M, pH 7.6) for 10-15 min in the dark. The sections were then rinsed in distilled water, mounted on chrome alum gelatin coated slides and air dried overnight. Finally, the sections were examined in Zeiss Axioplan 2 microscope and selected areas were taken with AxioCam MRc digital camera. The results from the present experiments on the amygdaloid projections to the forebrain were presented in our previous study [15].

## Results

In all examined cases the injection site involved the AmCe (Fig. 1**A**). By the five cases, in which a minute injection of 0.25 $\mu$l BDA was performed, the injection foci were completely selective, e.g. there was no spillage of the tracer upon surrounding structures: intercalated cell masses of the amygdala (ventrally), basolateral and lateral nuclei of the amygdala (laterally), medial nucleus of the amygdala and optic tract (medially), and neostriatum (dorsally). By the five animals, in which a larger quantity of BDA was delivered (0.5 $\mu$l), in three cases selective injection foci were present (Fig. 1**A**), and in the remaining two cases there was a minimal spillage of the tracer along the most ventral part of the cannula track, upon the most ventral portion of the amygdalostriatal transition area and globus pallidus. Despite the minimal contamination, these two cases were excluded from systematic examination.

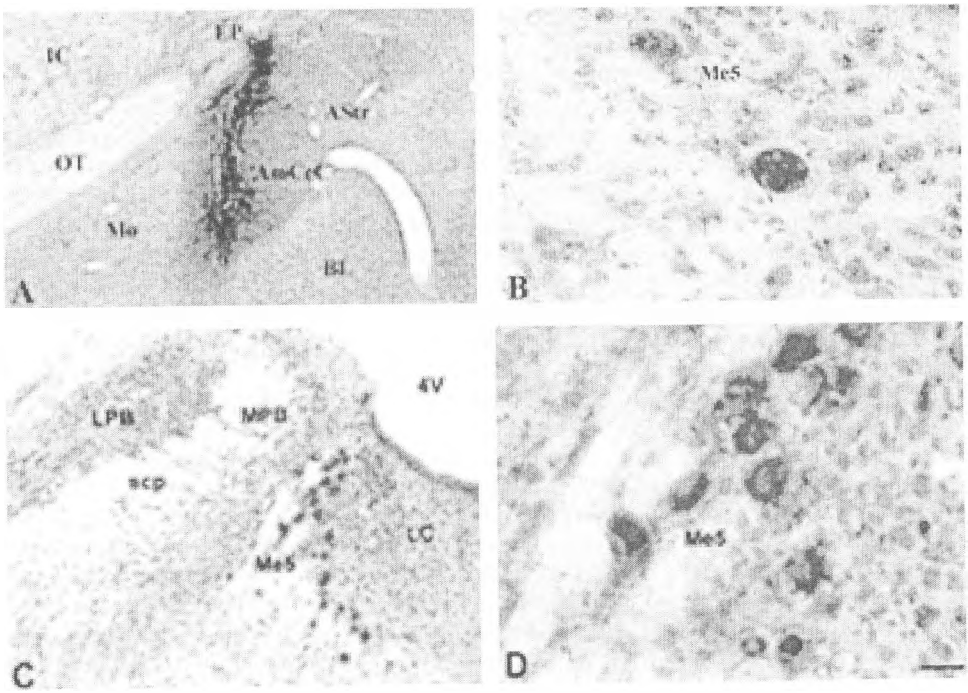


Fig. 1. **A** – A selective injection focus in the central amygdaloid nucleus (AmCe); **B** – BDA-labeled fibers and terminals in the rostral portion of Me5; **C** – Low-power view of the dorsolateral pons at the level of Me5; **D** – shows the dense field of BDA-labelled terminals in Me5. Scale bars = 100 $\mu$m (**A, C**); 50 $\mu$m (**B, D**)

The labelled efferent axons of the AmCe ascend from the injection site. Most fibers run in the major efferent bundle of the amygdala, the stria terminalis. A smaller number of labelled axons bend medially above the optic tract, a component of the ventral amygdalofugal pathway. The most caudally located labelled axons in the ventral amygdalofugal pathway descend to brainstem structures, and the first target is the lateral portion of substantia nigra. Afterwards, the labelled axons run dorsolaterally in the mesencephalic tegmentum towards the periaqueductal gray. Entering this area, the labelled axons run among the pseudounipolar neurons of the rostral portion of Me5 (Fig. 1**B**) that are located at the lateral border of the periaqueductal gray and innervate also the small multipolar neurons in the Me5. Further caudally the labelled axons proceed in the pontine tegmentum. They descend along the ventral border of the motor trigeminal nucleus and bend in dorsal direction towards the dorsolateral pons. The fibers run through the motor trigeminal nucleus, medially to it in the peritrigeminal nucleus, and laterally to it in the intertrigeminal nucleus. In the motor trigeminal nucleus most, if not all, labelled axons represent passing fibers, whereas in the peritrigeminal and intertrigeminal nucleus also discrete terminal bursts are present. Such are seen also in the supratrigeminal nucleus. In the dorsolateral pons dense terminal fields are present in the medial and lateral parabrachial nuclei and in the Me5, whilst no labelled axons enter the locus ceruleus (Fig. 1**C**). In the Me5 dense pericellular baskets surround the large pseudounipolar neurons (Fig. 1**D**), and the small multipolar neurons in this region are also contacted by amygdalofugal axons.

## Discussion

The amygdala is relatively voluminous gray substance, located in the depth of the ventromedial temporal lobe, ventral to the caudolateral striatum and to the pallidum. It is a very complicated structure and consists of several nuclei, divided on the basis of cytoarchitectonic, hodological, histochemical and immunohistochemical studies [reviewed in 3]. The amygdala is involved in the modulation of neuroendocrine functions, visceral effector mechanisms, and in complex patterns of behavior: learning and memory, aggression and defense, pain modulation, reproduction, food intake, etc. [reviewed in 16]. The classical hodological studies, carried out by silver impregnation of degenerating axons described projections to certain basal telencephalic and hypothalamic structures but not to the brainstem [2, 9]. The introduction of modern, more sensitive techniques for tracing axonal connections led to the description of a much more extensive subcortical distribution of amygdaloid fibers [reviewed in 11]. H o p k i n s and H o l s t e g e [4] followed amygdaloid tracts to the caudal brainstem but did not describe a projection to the trigeminal nuclear complex. A projection to the caudal (pontine) portion of Me5 was noticed in the autoradiographic experiments of P o s t and M a i [12] and of P r i c e and A m a r a l [13]. The use of the most effective modern anterograde tracer enabled us to describe an unexpectedly strong projection from AmCe to the entire rostrocaudal extent of the Me5. The projection is so massive that the density of BDA labelled terminals in the Me5 rivals the density in the generally appreciated strong projection of the amygdala to the parabrachial nuclear complex (see Fig. 1**C**). On the other hand, we were unable to confirm the finding of P r i c e and A m a r a l [13] on a connection to the ventral part of locus ceruleus. Species differences (monkey versus rat) might explain this discrepancy. Our findings suggest that both neuronal types in Me5 receive an amygdaloid input. The projection to the pseudounipolar neurons is especially evident in the pontine part of the Me5, where the densely arranged mesencephalic trigeminal perikarya are surrounded by numerous labelled endings. The amygdaloid axons are also in contact with the small multipolar neurons located in close vicinity. The affiliation of latter neurons to Me5 is a matter of debate, but L a z a r o v [5, 6] provided firm evidence that the small multipolar neurons belong to the Me5, and represent GABAergic interneurons.

There is growing evidence that the amygdala is unexpectedly important subcortical nociceptive centre [16, 17]. The input of pain sensation is conducted by the spino (trigemino) – parabrachial – amygdaloid pathway, and we recently demonstrated that the amygdala receives a monosynaptic input from the dorsal horn of the spinal cord and from the spinal trigeminal nucleus [16, 17]. The present data suggest a further sensory involvement of the amygdala, e.g. a very strong monosynaptic influence over both the primary proprioceptive neurons of the Me5 and their interneurons.

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# Serum Ganglioside GM3 Changes in Patients with Early Multilpe Sclerosis

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Gangliosides GM3 are the major gangliosides in the endothelial cells forming blood-brain barrier (BBB) and in normal human blood serum. The relative distribution of GM3 was determined in the serum of patients with relapsing – remitting multiple sclerosis (RRMS) during the first attacks of the disease, when there was a significant BBB damage and of healthy subjects. A statistically significant decrease of serum GM3 in early RRMS was observed. These findings revealed for the first time a correlation between the decrease of serum GM3 during the first clinical signs of multiple sclerosis and the destruction of BBB. Therefore, serum gangliosides GM3 could be used as biomarkers of blood-brain barrier destruction.

*Key words*: ganglioside GM3, multiple sclerosis, serum, blood-brain barrier.

## Introduction

Gangliosides (sialic acid – containing glycosphingolipids) are particularly abundant in the nervous system. Up to 95 % of cell gangliosides are present in the plasma membrane [10]. The major gangliosides in the endothelial cells forming blood- brain barrier (BBB) are GM3 (62 % of total gangliosides) [3, 6]. Gangliosides occur also in non-cell associated forms in blood plasma and other body fluids. The ganglioside spectra of normal blood plasma are remarkably stable, but show pronounced changes in pathological conditions [1, 2, 4, 8 ]. In multiple sclerosis (MS) significant changes of gangliosides GM1, GD1a and GT1b were revealed in the serum of patients during the first attacks of relapsing-remitting form of MS (RRMS) [12-15]. There are no data available on the serum GM3 level in MS patients. It was convincingly demonstrated that the damage of BBB occurred very early in the pathogenesis of MS [9].

The purpose of this study was to evaluate the serum level of GM3 in the patients with early MS when there was a significant BBB destruction. The relative distribution of GM3 was determined in the serum of patients with RRMS during their first attacks.

## Materials and Methods

Sera were obtained from 7 patients with first attacks of MS of what later was definitely diagnosed as RRMS according to Poser's criteria [7] and from 30 healthy subjects.

Isolation of serum gangliosides was performed by the method of Ilinov et al. [5]. It includes the following stages: a) dehydration of the sample by azeotropic distillation of the mixture of serum water/n-propanol = 1:10 (v/v); b) total lipid triple extraction with cyclohexane (I), chloroform : methanol = 1:1 (v/v) (II), and chloroform : methanol = 1:2 (v/v) (III); c) non-polar lipids removal by preparative TLC with a mobile phase: chloroform : methanol: 0.3 % $CaCl_2$= 30:18:4 (v/v/v); d) elimination of the blood sugar by Sep Pak technique according to Williams and McCluer [11]; e) HPTLC of the ganglioside fractions with a mobile phase: chloroform: methanol : 0,1 M sodium lactate = 55:40: 10 (v/v/v). The spots were visualized by spraying with orcinol reagent followed by local heating at 110°C and the gangliosides were quantified densitometrically. Bovine brain gangliosides (GM1, GD1a, GD1b and GT1b) (Calbiochem) and GM3 ganglioside (Sigma) were used as a test for identification. The Student's test was used to determine statistical differences between the MS patients (I group) and healthy subjects (II group) using $P<0.05$ as the level of confidence.

## Results

The relative percentage of GM3 gangliosides in patients with early MS and in healthy subjects was recalculated on the basis of the densitograms. The relative proportion of GM3 decreases from 69.10 % in the healthy subjects to 48.60 % during the first attacks of RRMS (Fig. 1). The relative portion of GM3 content during the first attacks of the disease and in healthy subjects was statistically significant ($P<0.05$) (Table 1).

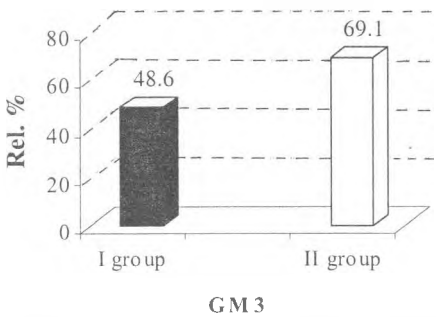


Fig. 1. Diagram of serum ganglioside GM3 of RRMS patients with first attacks of the disease (I group) and in healthy subjects (II group)

Table 1. Relative Percentage of GM3 Serum Gangliosides in RRMS Patients with First Attacks of the Disease and in Healthy Subjects

| Ganglioside | I group (n=7) M ± SEM | II group (n=30) M ± SEM |
|---|---|---|
| GM3 | 48.60 ± 3.88 | 69.10 ± 0.45 |

M – mean value; SEM – standard error of mean; I group – RRMS patients with first attacks of the disease; II group – healthy subjects

## Discussion

In this study the relative distribution of ganglioside GM3 was determined in the serum of patients with RRMS during their first attacks of the disease and in healthy subjects. The results demonstrated in comparison to healthy individuals a statistically considerable decrease of serum ganglioside GM3 in MS patients. The decrease of serum GM3 observed by us correlated with the significant destruction of BBB during the first attacks of MS, revealed by S h a r i e f et al. [9]. The endothelium of brain capillaries represents the structural basis for the BBB in vertebrates. D u v a r et al. [3] found that the major gangliosides in a new human cerebromicrovascular endothelial cell line are GM3. K a n d a et al. [6] established a method to yield sufficient quantities of highly purified human brain microvascular endothelial cells and compared their glycosphingolipid composition to that of human unbilical cord vein endothelial cells, as the representative of endothelial cells not forming BBB. They also detected that GM3 are major gangliosides of the BBB endothelial cells.

In conclusion, the findings of this study permits us to find, for the first time, that a considerable decrease of serum GM3 in early MS correlate with a severe destruction of blood-brain barrier. Therefore, we could suggest that serum ganglioside GM3 may be monitored as biomarkers of early damage of blood-brain barrier, which provides impetus to initiate early therapy.

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# Investigation of the Early Postnatal Neurogenesis in Rats

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After birth in mammals, neurogenesis continues in restricted regions throughout life. We used the thymidine analog, 5-bromo-2'-deoxyuridine (BrdU), that labels DNA, and cell-type-specific markers for neuronal cell lineage (nestin, Dcx, MAP(2) and NeuN). We statistically investigated two parameters of neurogenesis (cell proliferation and neuronal differentiation) in 10 different regions of the developing rat brain at various time points after birth from P1 to P51 (P, postnatal day). Both of these parameters showed regional distribution and age dependency during the postnatal period. Peak neurogenesis was found in the dentate gyrus during the first week of life with a progressive decline after P9. Since adult mammalian neurogenesis consists generally of the same processes as early postnatal neurogenesis, our investigaton might provide usefull information for studying normal and pathological neurogenesis.

*Key words:* Neurogenesis, BrdU, rat, dentate gyrus.

## Introduction

In the mammalian central nervous system (CNS), neurogenesis occurs intensively during the first weeks after birth picking at P7-P9. Thereafter, it remains longlife in the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus[1] (DG). Adult neurogenesis repeats the stages of generation and functional integration of new neurons similarly to the developing brain. Therefore, data on early postnatal neurogenensis might give a better understanding to adult neurogenesis. The goal of the present study was to provide additional data on basic neurogenic mechanisms of the developing and adult brain.

## Material and Methods

All animal experiments were done in accordance to the institutional guidelines. Wistar rats (BgVV, Berlin) at different age between P0 and P30 were injected i.p. with BrdU (Sigma, St. Louis, MO) using two paradigms: 1) Type 1-100 mg/kg BrdU on P0, P3, P7, P10, P14, P21, P30; perfusion 24 h or 21 days later for each time point (14 groups, $n$=3

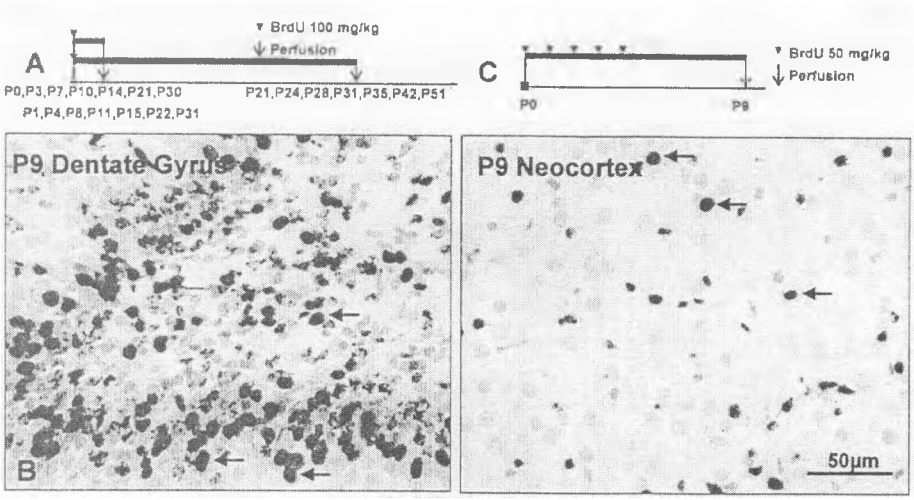


Fig. 1. **A** – Type 1 BrdU paradigm (1 × 100 mg); **B** – Light micrographs showing BrdU(+) cells (arrows) in dentate gyrus/neocortex on P9, VNR substrate, hematoxylin counterstaining; **C** – Type 2 BrdU paradigm (50 mg daily for 5 days) on P9 corresponding to the images in **B**

per group) (Fig. 1A); 2) Type 2-50 mg/kg BrdU daily for 5 days starting on P0, P3, P6 and P14; perfusion 9 days later. A similar group as P6 was designed with a survival time of 28 days (n=5) (Fig. 2A). Animals were anesthetized and transcardially perfused with 0.01M PBS, followed by 4% paraformaldehyde in 0.1M PB. The brains were removed and postfixed in the same fixative.

Paraffin sections were processed for single or double immunostaining for peroxidase or fluorescence labelling. First antibodies to detect BrdU, Nestin, Doublecortin (Dcx), NeuN and MAP(2) were used. Biotinylated second antibodies, ABC reagent and DAB/VNR substrate were then applied. By fluorescence labeling, Alexa Fluor 594 or 488 and Vectashield mounting medium with DAPI were used. Morphometric countings evaluating BrdU(+) nuclei on P9, P12, P15, P23 and P34 or Dcx(+) cells, BrdU(+) nuclei and Dcx(+)/BrdU(+) colocalization using confocal images were done in a blinded fashion. Values are presented as mean ± S.E.M.

## Results

We first compared same age groups with different BrdU paradigms or survival timings between P1-P51 (Fig. 1A). We found that in both groups, 24 h and 21 days, older animals expressed progressively less BrdU and longer survival timings reduced singnificantly the number of labeled cells. At the end of the second postnatal week BrdU/neuronal marker expression was restricted mainly to two regions – SVZ of the lateral ventricle and SGZ of DG. BrdU staining in DG and cortex on P9 is shown in Fig. 1B; corresponding BrdU paradigm is indicated in Fig. 1C. Summarized total proliferative scores of BrdU(+) cells on P9, P12, P15, P23 and P34 (BrdU paradigm in Fig. 2A) showed statistical significance (Fig. 2B). With few exceptions, cell countings in ten brain regions have shown significant decrease in proliferative rate to the previous age group (Fig. 2C). Compared to P23, values for P34 were higher because of younger animals by P34. We quantified numbers of immuno(+) cells for Dcx(+) (77.12%), BrdU(+) (50.60%) and cells coexpressing both of these markers (39.48%) in the DG on P15 (Fig. 3).

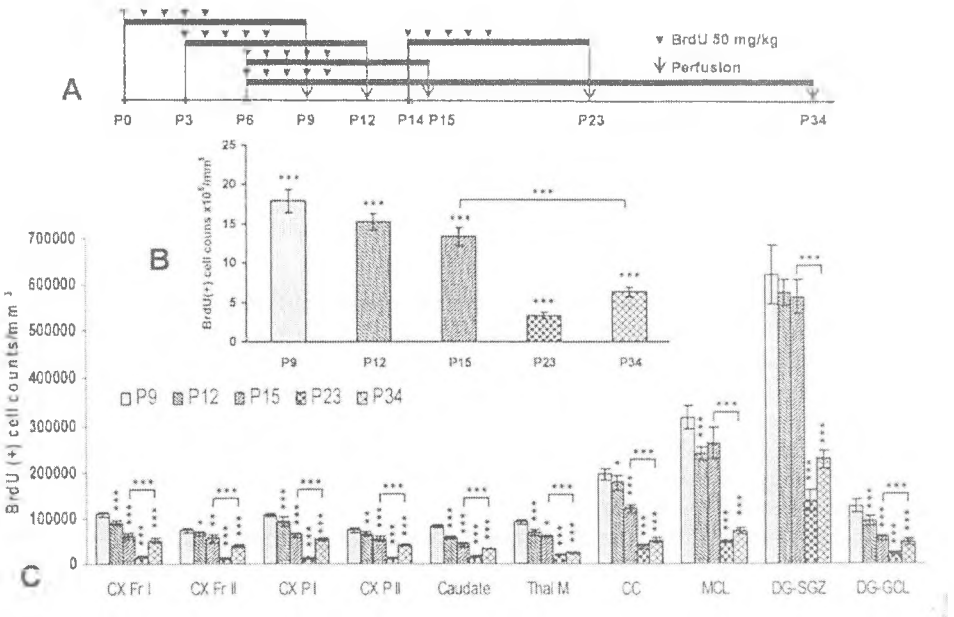


Fig. 2. **A** – Type 2 BrdU paradigm (50 mg daily for 5 days); **B** – Summarized total proliferative scores of BrdU(+) cell counts on P9 ($n$=9), P12 ($n$=9), P15 ($n$=8), P23 ($n$=8) and P34 ($n$=5); **C** – BrdU(+) cell counts in ten individual brain regions (frontal cortex layer I = CX Fr I, frontal cortex layer II = CX Fr II, parietal cortex layer I = CX P I, parietal cortex layer II = CX P II, caudate nucleus = Caudate, mediodorsal thalamus = Thal M, corpus callosum = CC, molecular layer of cerebellum = MCL, subgranular zone of the dentate gyrus = DG-SGZ, granular cell layer of the dentate gyrus = DG-SGL on P9, P12, P15, P23 and P34. Student't $t$-test; ***$P$<0.001, **$P$<0.01 or *P<0.05, when compared with total or individual score of the previous time point or between P15 and P34 as indicated

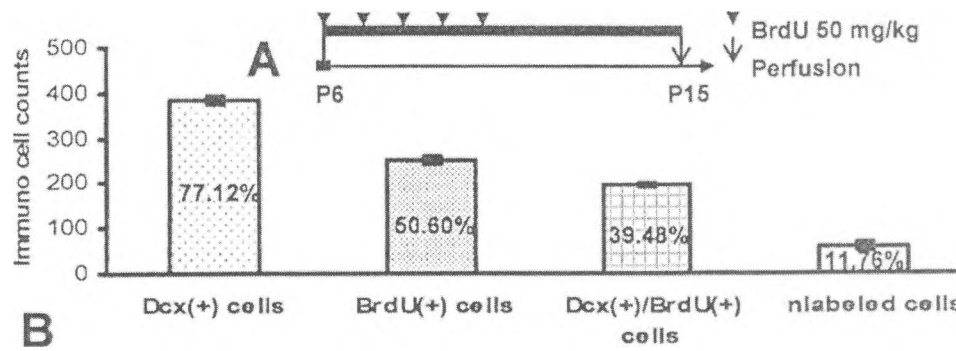


Fig. 3. **A** – Type 2 BrdU paradigm (50 mg daily for 5 days) on P15; **B** – Percentage of Dcx(+) cells, BrdU(+) cells, Dcx(+)/BrdU(+) cells and unlabeled cells in the dentate gyrus on P15 ($n$=5)

During the first two postnatal weeks, Nestin(+) radial glia and Dcx(+) cells underwent a considerable reduction. MAP(2) and NeuN immunoexpression increased after P9 and remained constant by older animals. Frequent colocalization for BrdU(+)/Nestin(+) or BrdU(+)/Dcx(+) was found in specific regions such as the SVZ, cerebellum, corpus callosum and DG-SGZ. BrdU(+)/MAP(+) or BrdU(+)/NeuN(+) colocalization was seen rarely by later timings.

## Discussion

Cell proliferation and neuronal differentiation in the developing rat brain display regional distribution and age dependency. BrdU labeling shows dose, age and survival time dependency. Expressions of Nestin and Dcx decrease, especially for nestin(+) radial glia in neocortex. Radial glia are known not only to guide migrating neurons to outer cortical layers but also to generate neurons [3]. We registered also an increasing expression for MAP(2) and NeuN showing that neuronal migration and differentiation are still going on during the early postnatal period. This is one of the vulnerable periods to pathological factors leading to disruption [2].

We found that after the first three weeks, postnatal neurogenesis in the rat is considerably reduced and restricted to two brain regions: the SVZ of the lateral ventricle and SGZ of the DG. In both zones multipotent neural precursors are preserved lifelong [1] and may contribute to repair following CNS diseases [4]. Our data on early postnatal neurogenesis might contribute to a better understanding of basic neurogenic events in the mammalian brain.

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# Innervation of the Muscle Coat of the Recto-Anal Region in the Rat

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The distribution of nerve cell bodies and fibres which contain adenosine triphosphate (ATP), nicotine amide adenine dinucleotide phosphate-diaphorase (NADPH-d), choline acetyltransferase (ChAT), tyrosine hydroxylase (TH) and substance P (SP) have been studied in rat recto-anal region using quinacrine fluorescent, NADPH-d histochemical and ABC immunohistochemical methods respectively. Single ATP fluorescent nerve perikarya were observed in the myenteric ganglia of the rectum and anal canal. Numerous NADPH-d nerve cell bodies in myenteric ganglia of both investigated regions were found. Single nerve cell bodies immunoreactive for ChAT and SP were found in the myenteric ganglia of the rectum and anal canal. High number of immunoreactive nerve fibres in the myenteric ganglia and in the internodal strands were observed. Large number nerve fibres, demonstrating immunoreactivity for all three peptides in the muscle coat between the smooth muscle cells were observed. In the internal anal sphincter ChAT-, TH-, SP-immunoreactive and NADPH-d-positive varicose and folded nerve fibres were found.

*Key words*: adenosine triphosphate, nicotinamide adenine dinucleotide phosphate-diaphorase, choline acetyltransferase, tyrosine hydroxylase, substance P.

## Introduction

The maintenance of continence is a complex process of interrelating factors which includes the sphincter muscles, rectal and anal sensation and stool composition. The regulation of activity of muscles in recto-anal region depends on the intrinsic myogenic properties and external innervation. Projections from nerves within the enteric nervous system serve to control the internal anal sphincter [2]. The localization and function of nerve types innervating the distal part of the intestinal canal has been intensively examined in view of their involvement in process of maintenance of the bowel continence [1, 4, 6, 10]. Generally, these studies investigate only the single neurotransmitter in norm [6] and in some pathological conditions, such as chronic constipation, anal fissure, Hirschprung's disease [5, 7].

The aim of this study was to investigate the presence, localization and distribution of the ATP, NADPH-d-, SP-, ChAT- and TH-positive nerve elements in the myenteric ganglia and in the muscle coat of rat recto-anal region.

## Materials and Methods

All experimental procedures were carried out in agreement with the Bioethic Commission of the Institute of Neurobiology of the Bulgarian Academy of Sciences. To investigate ATP in myenteric ganglia, the quinacrine fluorescent technique of O l s s o n et al. [10] was used. The tissue sections were incubated in $10^{-6}$ quinacrine solution for 30 min at 37°C. Appropriate filter combination was applied. Under deep ether anesthesia 6 young male Wistar rats were perfused transcardially with 0.05 M phosphate buffered saline (PBS), pH 7.3, followed by a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. The rectum with the anal canal and the internal anal sphincter were cut out and postfixed in the same fixative over night at 4°C. Then, the specimens were cryoprotected in 20% sucrose in PB. Thirty μm thick tissue sections were cut on "Reichert Jung" freezing microtome. The sections from muscle coat were collected in PBS and washed in the same solution over night. The histochemical staining procedure was performed according to the NADPH-diaphorase technique [11].

The immunohistochemical reaction was realized by using the avidin-biotin-peroxidase complex (ABC) technique of H s u et al. [3]. Mouse anti-TH monoclonal, rabbit anti-SP polyclonal and goat anti-ChAT polyclonal antibodies and respective (anti-mouse, anti-rabbit or anti-goat) IgG and ABC complex were used. Visualization of the reaction was made by 3,3'-DAB/$H_2O_2$ and nickel ammonium sulphate in some experiments was applied for magnification intensity of the reaction. Following the development of the reactions the sections were mounted on chrome-gelatin-coated slides, air dried, dehydrated in alcohol, cleared in xylene and embedded in Entellan. Control sections were processed by omission of the substrate β-NADPH and or respective primary antibody and the results were negative.

## Results

Single quinacrine-fluorescent, numerous NADPH-d- (Fig. 1a), single SP- (Fig. 2a) and ChAT-positive (Fig. 3a) nerve cell bodies in the myenteric ganglia of the distal rectum and anal canal were observed. TH-immunoreactive neuronal perikarya in the myenteric ganglia of both regions were not found. Quinacrine-fluorescence nerve fibres were not presented. The immunoreactive nerve fibres were observed in the myenteric ganglia. The nerve fibres were of different sizes and had a characteristically varicose appearance. The number of SP-positive nerve fibres around the neurons was greater than the number of the other positive varicosities. The immunoreactive nerve fibers formed a network in the neuropil of the ganglia (Figs. 2a, 3a) and they could be traced for long distance in the internodal strands. NADPH-d- and peptide-positive fibres as single fibres or in nerve trunks run parallel to the muscle cells of the muscle layers. In the circular muscle layer they occurred more often than in the longitudinal one. In the internal anal sphincter NADPH-d- (Fig. 1b), SP- (Fig. 2b), ChAT- (Fig. 3b) and TH-immunoreactive nerve fibres were observed such as varicose, folded and run parallel to the smooth muscle cells. The NADPH-d-positive nerve fibres were more numerous than the peptide immunoreactive ones.

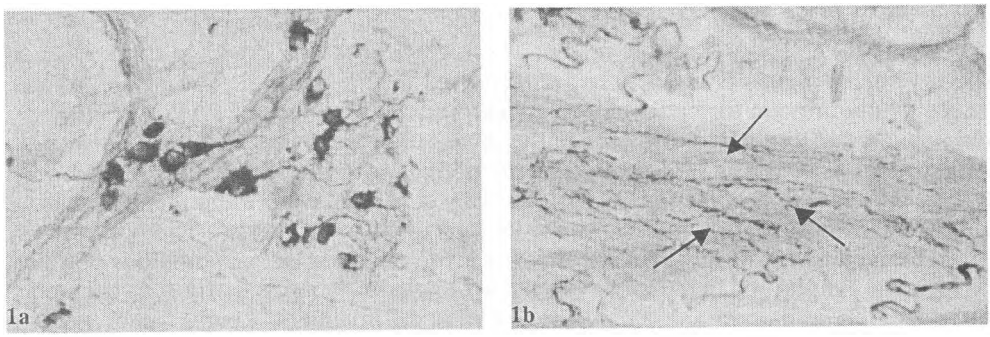


Fig. 1. NADPH-d-positive perikarya in myenteric ganglion of the anal canal (**a**) and positive, varicose and folded nerve fibres (arrows) in internal anal sphincter (**b**)

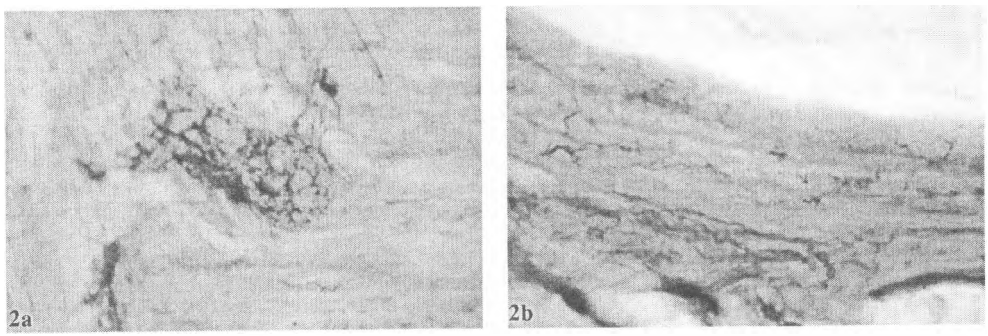


Fig. 2 **a** – SP-immunoreactivity in myenteric ganglion of the anal canal. Single SP-positive perikarya (head arrows) and varicose axons branching to form basket-like structures apparently around unlabeled myenteric nerve cell bodies (arrows); varicose and folded SP-positive nerve fibres (arrows) in internal anal sphincter (**b**)

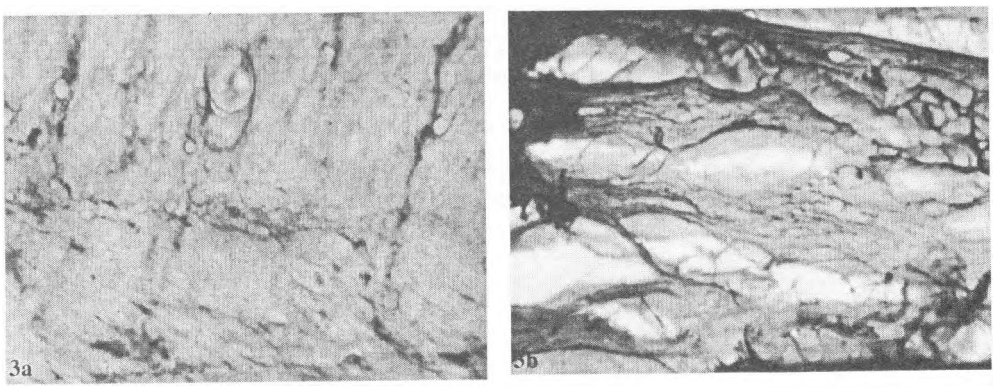


Fig. 3. ChAT-positive nerve perikarya (arrows) and varicose nerve fibres in myenteric ganglia of the distal rectum (**a**); positive nerve fibres (arrows) in internal anal sphincter (**b**); scale bar – 50 μm

## Discussion

The myenteric plexus is a major target of nerves in the gut wall. The results of the present study provide further evidence concerning the existence of ATP-, NADPH-d- and peptide-positive nerve elements in the recto-anal region in rat model. Our results for distribution of NADPH-d-positive nerve perikarya in the recto-anal region are in agreement with the observations of O ` K e l l e y et al. [6]. We present the distribution of NADPH-d-positive nerve fibres in the internal anal sphincter also. The presence of single SP- and ChAT-positive perikarya in the myenteric ganglia and numerous positive axons in the ganglia and in muscle layers did not exclude of extrinsic origin. In our study the lack of TH-immunoreactive nerve perikarya in the myenteric ganglia but numerous positive axons suggests that these axons could be of extrinsic origin, probably of postglionic sympathetic neurons. This finding corresponds to the data of O l s s o n et al. [9], received by using combined tract tracing and immunohistochemical studies.

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# *De novo* Expression of Neuropeptides in the Mesencephalic Trigeminal Nucleus of the Rat after Masseteric Nerve Injury

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In normal circumstances, galanin (GAL) and neuropeptide Y (NPY), two putative neuromodulators, are not expressed in the neurons of the mesencephalic trigeminal nucleus (MTN), a unique structure of (pseudo)unipolar sensory neurons in the CNS. Here we demonstrate GAL and NPY immunoreactivity in MTN neurons after an axotomy of *n. massetericus* in rats. Following survival periods of 7 and 14 days, the MTN neurons on the ipsilateral (axotomized) side display immunostaining, while on the contralateral side they are negative. It can be inferred that axonal injury initiates a cascade of intracellular events, leading to a *de novo* synthesis of neuroactive substances, such as GAL and NPY, which otherwise are not specific for the MTN neurons under normal conditions. The newly synthesized peptides possibly play a trophic role in the revival process or actively take part in re-establishing the MTN sensory modalities in the orofacial region.

*Key words:* axotomy, neuropeptides, mesencephalic trigeminal neurons, neurochemical plasticity, rat.

## Introduction

Primary afferents in the trigeminal system have their cell bodies both in the trigeminal ganglion and the mesencephalic trigeminal nucleus (MTN) [6, 9]. The MTN is a distinctive structure because it is the only nucleus in the CNS predominantly consisting of (pseudo)unipolar neurons of a nerve crest origin, much like these in the peripheral sensory ganglia. The MTN perikarya are located in the midbrain and the rostral portion of the pons and send out their peripheral processes to muscle spindles of the jaw-closing muscles and extrinsic ocular muscles, and to mechanoreceptors in the periodontal ligament [8]. In normal circumstances the MTN utilizes in synaptic transmission a variety of neuroactive substances, although certain ones are expressed only under pathological conditions such as injury and pain. To date there exist no data on neuropeptide synthesis or expression in the MTN perikarya in health, notwithstanding the presence of fibre networks of varying density containing different neuropeptide substances amidst the neuronal bodies [6–8]. However, when subjected to injury of their peripheral processes,

the production of neuroactive substances and neuromodulators in MTN neurons, which normally are not a constituent of their milieu, is initiated [1, 4].

Thus, we set it as a goal of this study to test the MTN neurons in the rat for the expression of two neuropeptides, galanin (GAL) and neuropeptide Y (NPY) as a result of an acute trauma inflicted to the masseteric nerve unilaterally and to register chronologically the emergence and intensity of immunoreactivity, and its possible decrease.

## Materials and Methods

Four adult rats underwent a unilateral transection of the *n. massetericus*. The untreated side served for a control. The animals were left to survive for 7 or 14 days. Then they were re-anesthetised and perfused with 4% paraformaldehyde. The brains were removed and the brainstem cut at the level of the MTN. After cryoprotection, the samples were cut on a freezing microtome at 20-30 μm. For immunostaining the sections were processed in accordance with the avidin-biotin-peroxidase complex (ABC) method [5]. Briefly, they were treated with 1.2% $H_2O_2$ in absolute methanol and preincubated in 3% normal goat serum in 0.01 M PBS containing 0.3% TritonX-100. Then they were incubated in primary polyclonal antibodies against GAL and NPY respectively, diluted 1:1000, in the preincubation medium for 48 h. The sections were consequently treated with biotinylated goat anti-rabbit IgG and the ABC complex. The peroxidase activity was visualized with 3,3′-diaminobenzidine. The sections were mounted on gelatin-coated glass slides and subsequently observed with a Zeiss AxioPlan 2 microscope.

## Results

In the MTN of the animals subjected to unilateral transection of *n. massetericus*, prominent immunoreactivity (IR) was registered on the ipsilateral side of the axotomy 7 days after the intervention, while no IR was seen contralaterally (Figs. 1, 2). The IR was best expressed in the MTN neurons as well as in the surrounding nuclei, i.e. the locus ceruleus and the medial parabrachial nucleus. In the case of GAL, the IR was notably

Fig. 1. GAL-IR on the ipsilateral side of axotomy seven days after the intervention

Fig. 2. NPY-IR seven days after axotomy. Although less pronounced, it is still well seen on the ipsilateral side of the intervention, while the contralateral remains negative

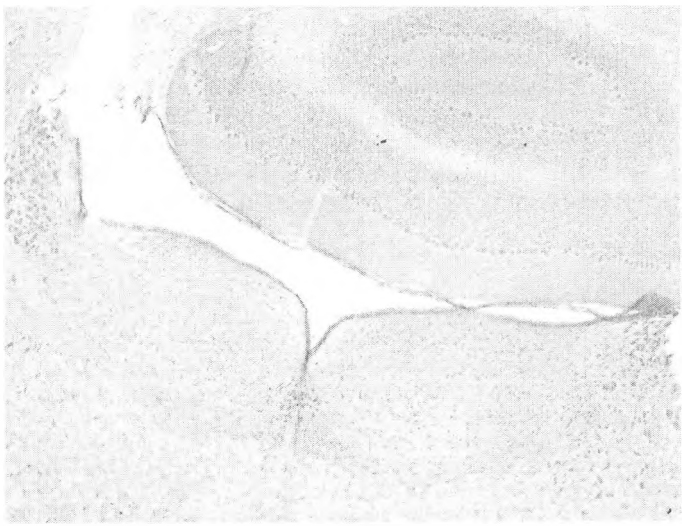

Fig. 3. Fourteen days after axotomy GAL-IR is still present, albeit in a weaker manner

expressed in the population of MTN neurons throughout the whole length of the nucleus, where they were visualized as dark-stained round-to-ovoid cell bodies against a negative background (Fig. 1). The GAL-IR was evenly dispersed throughout the perikarya. In the case of NPY the IR was clearly visible in the MTN neuronal population along the entire extent of the nucleus, although its intensity was somewhat weaker than that of the GAL-IR (Fig. 2).

We noted that 14 days following axotomy the overall IR, both of GAL and NPY, although much preserving the patterns as 7 days after the procedure, is in general notably weaker, which is a feature of a down-regulation in the expression of the two neuropeptides already commenced (Fig. 3).

## Discussion

This study reveals the *de novo* synthesis and expression of two putative peptide neurotransmitters, GAL and NPY, in the MTN of the rat after peripheral transection of the *n. massetericus*. The obtained results are in agreement with the already published data by other authors, as well as earlier studies of ours [1, 2], confirming the induction of synthesis and upregulation of neuropeptides in the MTN neurons of the rat [1] and cat [6] following injury of their peripheral axons. For the first time we note that while there is a pronounced neuropeptide expression 7 days after axotomy, the intensity of the reaction diminishes two weeks following the intervention, which may be regarded as a sign of an already commenced down-regulation. The fact that MTN neurons react to peripheral axotomy by a *de novo* synthesis of neuropeptides such as GAL and NPY leads to the notion that these substances may play some central role in the trophic responses of neurons to the altered environmental cues. In this respect the study is in conformity with other sources reporting on the significant changes in the neurochemical content of primary sensory neurons following peripheral axotomy [1, 4, 9]. We support the view of these authors that obviously nerve damage causes the surviving neurons to shift their functional activities from normal maintenance and neurotransmission away to sustaining survival and regeneration. In our opinion, the newly synthesized neuropeptides, GAL and NPY, possibly play a supportive role as neurotrophic factors in the course of the adaptive processes that initiate and develop in response to injury. Thus they may protect the peripherally axotomized MTN neurons in their pathway back to re-establishing the usual functional modalities. Very recent data *in vitro* confirm that the MTN, similar to certain hypothalamic nuclei, possibly has a determinative role in setting the circadian rhythms and serves in regulating daily feeding behavior [3]. It may be speculated that the *de novo* expression of neuropeptides is one way of adaptation to a newly geared biological clock in accordance with the changed environmental conditions and that it assists in re-establishing the MTN sensory modalities in the orofacial region.

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# Distribution of the Tomato Lectin-Reactive Objects in Healthy and Degenerative Hamster Brain

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The distribution, topography and morphology of the TL-reactive objects in hamster cerebral hemispheres were studied by histochemical procedure with tomato lectin –TL (*Lycopersicon esculentum*) immunohistochemistry. The analysis reveals TL-positive objects in the healthy and degenerative cortex, hippocampus and thalamus of hamster central nervous system (CNS). A little number of them are sparse lectin-reactive cells identified as ramified microglia exhibiting elongated forms and branched processes. The bigger number of TL(+) objects are components of brain microvasculature. Details of morphology and topographical distribution of the TL(+) objects in hamster CNS are described.

*Key words*: TL (tomato lectin) immunohistochemistry, ramified microglia, brain microvasculature.

## Introduction

Tomato lectin (TL) from *Lycopersicon esculentum* as neurobiological marker was proposed earlier [1] but its specificity remains till now non-well determined. It was known that the lectin of *Lycopersicon esculentum* has affinity for poly-N-acetyl lactosamine sugar residues [23]. Tomato lectin binding in CNS is related to some of the glial cells [6]. Microglial cells are basic potential target for TL binding in CNS [1, 3, 6, 22, 23]. Now microglial reactivity usually is estimated by a number of other markers as for example monoclonal antibodies 5D4 against keratan-sulfate [9] or wide-used macrophage specific markers (MAC-1). At the same time many investigators propose TL for this aim [5, 8, 12, 15, 21]. In several cases they use to confirm the results with a second specific microglial marker like MAC-1, RCA-1 or Isolectin B4 [7, 17, 20]. Equally TL is proposed for electronmicroscopical immunohistochemisry [19]. A high specificity of TL for microglial cells in the normal adult brain is presumed [4, 11]. Here, we demonstrate that TL immunohistochemistry applied in healthy and degenerative brain tissue is marker simultaneously for several objects of CNS microanatomy.

## Material and Methods

Adult five-week-old female outbred golden Syrian hamsters (45 animals) were source of healthy and degenerative brain tissue. A procedure for obtaining degenerative brain tissue was described previously [14]. Commercially available biotinilated – *Lycopersicon esculentum* (tomato) lectin (VECTOR Labs, Cat.No. B-1175) diluted in working solution of 10 micrograms/ml buffer PBS (phosphate buffer saline), enriched with $CaCl_2$ (1mM), $MgCl_2$ (0.1 mM) and stabilized with Natrium azide (0,08%) was used for procedure. Incubation of the brain sections with TL was performed at 20°C for two hours followed by application of ABC reagent (VECTOR Labs, Cat.No.PK-7100). DAB substrate kit for peroxidase (VECTOR Labs, Cat.No. SK-4100) was used as diaminobenzidine chromogen source for 2-10 min.

Light microscopy and interferential contrast microscopy (Nomarski optics) was performed.

## Results

In healthy or degenerative hamster cortex, thalamus and hippocampus of hamster CNS TL immunohistochemistry reveals different kinds of positive objects – ones with determined cellular shape bodies and others obviously non-cellular elements of the brain microanatomy. TL(+) cells are very irregularly shaped with ramified and amoeboid morphologies (Fig. 1). Elongated cells with several processes and rounded cells are labelled generally. These microglia-like cells are seen abundantly in the degenerative hamsters brains. A microgliosis could be registered during the terminal stage (after 80$^{th}$ day of the agent inoculation) of the experimental transmissive spongiform encephalopathy, provoked by the strain scrapie 263K (Fig. 2). Detailed study of the immunostained CNS fields in cortex, thalamus and hippocampus reveals many other objects positive to TL

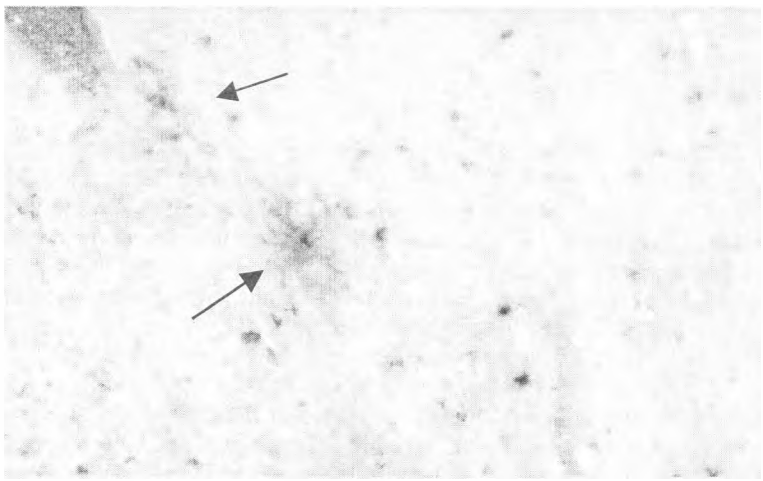

Fig. 1. TL(+) cellular objects are very irregularly shaped with ramified and amoeboid morphologies. Cortex, × 200

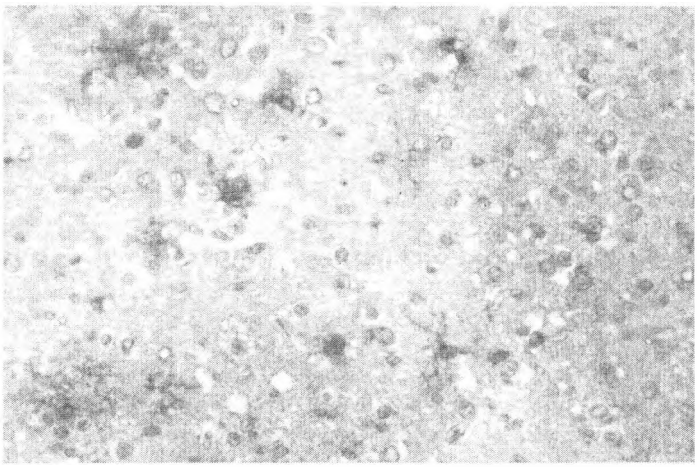

Fig. 2. Microgliosis could be registered by TL immunohistochemistry during the terminal stage (after 80$^{th}$ day of agent inoculation) of the experimental transmissive spongiform encephalopathy, provoked by the strain scrapie 263K. Thalamus, ×200

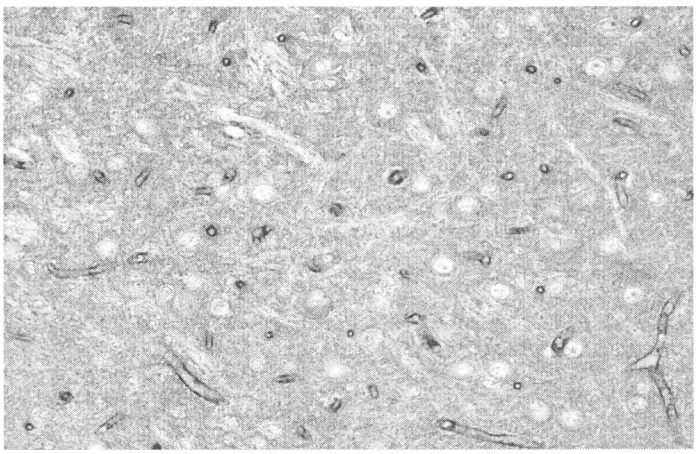

Fig. 3. Other CNS objects positive to TL are components of the brain microvasculature. Thalamus, ×100

(Fig. 3). The light microscopy reveals rounded and prolonged objects situated everywhere in the brain tissue both in healthy and degenerative CNS. The interferential contrast microscopy shows that lectin positive objects in studied regions are components of the brain microvasculature.

## Discussion

TL immunohistochemistry reveals a lower density of labelled microglia in comparison with other procedures for microglial visualization (5D4 monoclonal antibodies) [14]. Second, a great number of the TL(+) objects in hamster brain are components of the thinnest network of the brain microvasculature.

During degenerative changes in hamster CNS a marked response of the microglia occurred in the terminal stages of the experimental scrapie 263K in the cortex, hippocampus and thalamus. The universal phenomenon of microgliosis could be registered with TL immunohistochemistry as it is proposed earlier [13]. The widespread extracellular TL labelling found by us in the hamster CNS corresponds to fine microvasculature components. A sure explication of this fact is that tomato lectin is specific for blood brain capillary endothelium [10, 16, 18].

The staining of the vascular network by TL immunohistochemistry in healthy and diseased CNS is higher efficient than this with commonly used vascular markers as EMS laminin or PECAM-1 [16].

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# Experimental Model of Parkinson's Disease: Antioxidant Defense System in Rat Brain

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Parkinson's disease (PD) is characterized by progressive degeneration of dopaminergic neurons, arising in substantia nigra pars compacta and terminating in the striatum. Oxidative stress has been implicated in the pathogenesis of PD based on its role in the cascade of biochemical changes that lead to dopaminergic neuronal death. The aim of the present study was to measure *in vivo* the levels of antioxidant enzymes and their lateralization in different brain regions (cortex, striatum, hippocampus) in the experimental model of Parkinson's disease in rat brain. Our results suggest that lipid peroxidation is elevated while the activities of antioxidant enzymes (glutathione reductase, glucose-6-P-dehydrogenase, superoxide dismutase and catalase) are altered in Parkinson's disease model, underlying a hemispheric asymmetry.

*Key words:* Parkinson's disease, antioxidant enzymes, striatum, cortex, hippocampus.

## Introduction

Parkinson's disease (PD) is a common age-related neurodegenerative disease that is pathologically characterized by the selective loss of dopaminergic neurons in the substantia nigra. Oxidative stress has been implicated in the pathogenesis of PD based on its role in the cascade of biochemical changes that lead to dopaminergic neuronal death. In PD there is a progressive death of substantia nigral cells leading to less availability of dopamine to the striatum, which participates in movement control. Neurons of substantia nigra may be particularly vulnerable to oxidative stress, because the oxidative metabolism of dopamine (oxidized by either monoamine oxidase or undergo autooxidation) has the potent to generate cytotoxic free radicals. The body possesses a complex protective antioxidant system against these potentially toxic products such as vitamin E, vitamin C, vitamin A, glutathione and antioxidant enzymes. These enzymes include glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase [4]. The aim of this study was to measure *in vivo* the levels of antioxidant enzymes and their lateralization in different brain regions (cortex, striatum, hippocampus) in Parkinson's disease model.

## Material and Methods

The experiments have been performed according to the "Principles of laboratory animal care" (NIH publication No. 85-23), and the rules of the Ethics Committee of the Institute of Neurobiology, Bulgarian Academy of Sciences (registration FWA 00003059 by the US Department of Health and Human Services).

### A) Surgical procedures

A total of 24 male Wistar rats, weighing 150-200 g at the time of surgery, were randomly divided in groups and housed in cages with free access to rat chow and water. The rats were anesthetized with chloral hydrate (400 mg/kg, i.p.), had their heads shaved, and placed in a stereotaxic apparatus. The scalp was cleaned with a iodine solution, incised on the midline and a burr hole was drilled through the skull at the appropriate location. The target coordinates were: AP = +0.2; LR = –3.0; H = –5.6 according to the stereotaxic atlas [8]. The experimental group received an injection of 20 μg/2 μl of 6-OHDA (Sigma-Aldrich, St. Louis, MO, USA; calculated as free base, dissolved in ice-cold saline with 0.02 % ascorbic acid) while the control group received an injection of 2 μl saline. All injections were made into the right striatum area by a Hamilton microsyringe at a rate of 1 μl/min. The needle was left in place an additional 2 min before being slowly withdrawn. The wound was closed with stainless steel clips and the rat was allowed to recover before being returned to its cage.

### B) Biochemical procedures

Protein content was measured by the method of L o w r y et al. [7]. Lipid peroxidation in the absence and in the presence of an inducer ($5.10^{-5}$ M $Fe^{2+}$) was determined by the amount of the thiobarbituric acid-reactive substances, formed in fresh preparations for 60 min at 37°C [5]. The absorbance was read at 532 nm against appropriate blanks; the absorbance at 600 nm was considered to be a non-specific baseline and was, therefore, subtracted from A532. Total glutathione level was measured according to T i e t z e [11]. Cu, Zn-superoxide dismutase activity (SOD) was determined according to B e a u c h a m p and F r i d o v i c h [1]; one unit of SOD activity was the amount of the en-

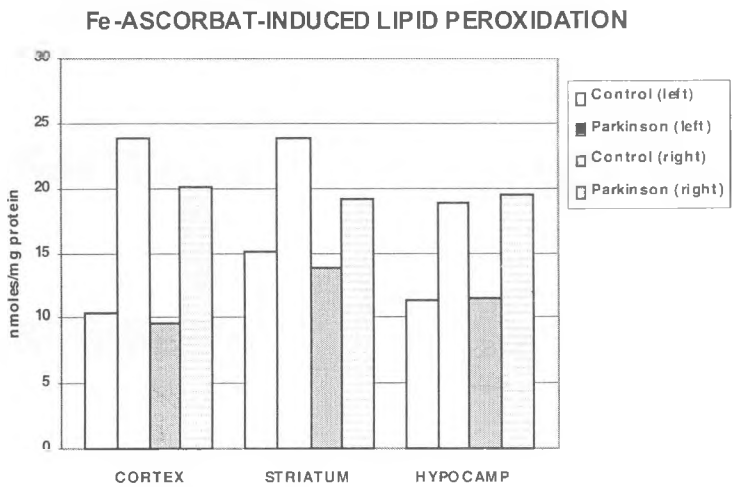


Fig. 1. Levels of lipid peroxidation in cortex, striatum and hippocampus in control and Parkinsonian rats

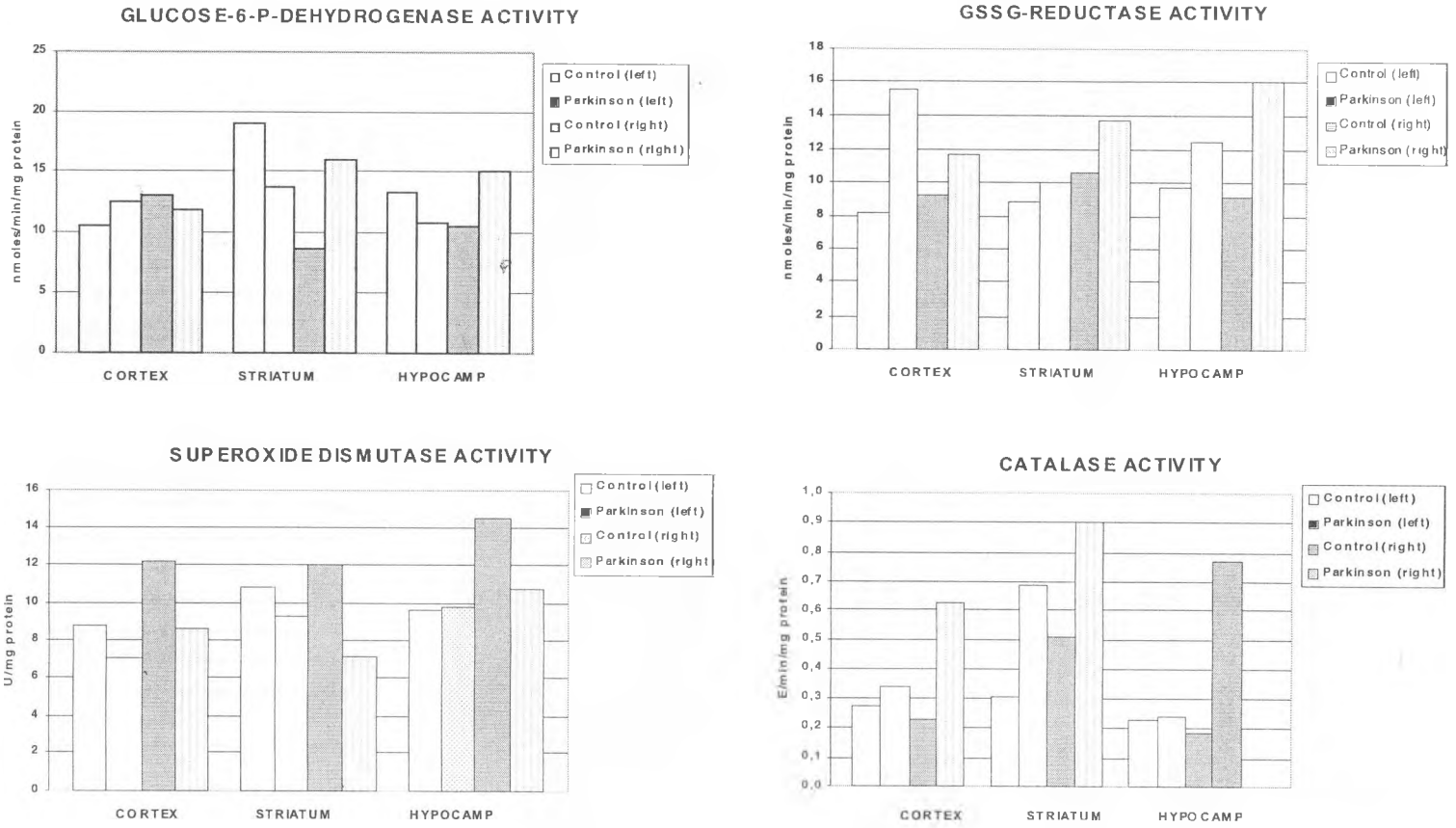


Fig. 2. Levels of antioxidant enzymes and their lateralization in cortex, striatum and hippocampus in control and Parkinsonian rats

zyme, producing 50% inhibition of nitro-blue tetrazolium-reduction. Glutathione peroxidase activity was measured by the method of Gunzler et al. [3]. Glutathione reductase activity was measured by the method of Pinto and Bartley [9]. Glucose-6-phosphate dehydrogenase activity was determined according to Cartier et al. [2].

## Results and Discussion

Levels of lipid peroxidation (Fig. 1) and antioxidant enzymes (Fig. 2) in the left and right striatum, hippocampus and cortex of control (saline 2 μl into the right striatum) and Parkinsonian rats (6-OHDA 20 μg/2 μl into the right striatum) were evaluated at the 21st day after surgery. The experimental model of Parkinson's disease was proved by the rotational behavior of rats induced by apomorphine (0.5 mg/kg, s.c.) two weeks after surgery [10, 6].

The presented results of our investigation suggest that lipid peroxidation is elevated while the activities of antioxidant enzymes (glutathione reductase, glucose-6-P-dehydrogenase, superoxide dismutase and catalase) are altered in Parkinson's disease model, underlying possible hemispheric asymmetry.

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# Functional Morphology of the Rat Choroid Plexus in Experimental Models

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In the present study were carried out ultrastructural and morphometrical investigations of the rat choroid during development and after hypokinesia and low doses of ionizing irradiation. Investigations of the rat choroid plexus during development provide evidence that light and dark epithelial cells finish their differentiation on 30 days postnatum. Changes of the epithelial cells during development suggest that dark and light cells are modulations of the same basic cells with possible functional differentiation starting from 17 days postconception and continue to 22 months. The observed ultrastructural and morphometrical data during hypokinesia may be related to the increased functional activity of the dark epithelial cells and earlier adult changes, for which epithelial ultrastructure gives evidence in the investigated period. Investigations after total-body irradiation of rats with low doses have shown that changes of the epithelial and endothelial cells of the plexus choroideus are more marked after irradiation with oxygen ions and neutrons in comparison with gamma rays.

*Key words:* rat choroid plexus epithelial cells and blood vessels, ultrastructure and morphometry, development, hypokinesia and low doses irradiation.

## Introduction

The choroid plexuses are specialized highly vascular anatomycal structure which protrude into the lateral ventricle, as well as in the third ventricle and fourth ventricle. The surface of the choroid plexus consist of numerous villi each covered with single layer of epithelial cells surrounded by vascular connective tissue cells [8]. As a secretory source of vitamins, peptides and hormones for neurons, the choroid plexus provides substances for brain homeostasis [4]. Most blood vessels in the plexus choroideus are wide-calibre (approximately 15 μm) fenestrated capillaries [7]. Investigation of the influence of hypokinetic conditions indicates that immobilization of pregnant rats for 5 days considerably influences the morphology of the corpus luteum and luteal cells [6]. X-rays, neutrons, alpha- and beta-particles come from environment or are produced by human activities. The real long-term effects of this background radiation are nevertheless a mystery, which is why they are currently being investigated by the scientific projects.

The *aim* of the present study is to investigate the ultrastructural and morphometrical changes of the rat choroid plexus during development and after hypokinesia and exposure to low doses of ionizing irradiation.

## Materials and Methods

*Development.* Wistar rats ($n$=60) aged 17 and 20 days postconception, 5, 15, 30, 45 and 60 days postnatum and 4, 7, 10, 13 and 22 months were used. The animals were fixed by immersion [14] and by intracardial perfusion [5].

*Hypokinesia.* One-month aged Wistar rats were divided into two groups: I group ($n$=20) rats subjected to 3, 6, 9 and 12 months of hypokinesia in specially constructed individual cages for physiological immobilization [15] and II group – control rats ($n$=20) under normal vivarium conditions.

*Irradiation.* Three-months aged female Wistar rats were divided into four group: I group –irradiated with single dose of $10^4$ particles /cm$^2$ of oxygen ions ($n$=3), II group – irradiated with fast neutrons ($n$=3) to 1.5 MeV at the dose of 1.0 Gy, III group – irradiated with gamma rays $Co^{60}$ ($n$=3) at the dose of 1.0 Gy and IV group – control six months aged rats ($n$=4). Three months after irradiation the animals were intracardially perfused [7]. The choroid plexuses were embedded in Durcupan and examined with JEOL JEM 1200EX transmission electron microscope.

We obtained morphometric data from the light microscope at 1000× magnification using a square grid system [13] calibrated for linear measurement in μm and area measurement in μm$^2$. All values were expressed as mean ± SEM, and statistically analyzed by Student t-test.

## Results and Discussions

In our investigations of the rat choroid plexus from aged 17 days postconception to 22 months we established the three periods of the ***development***. The **first period** may be divided into three phases of the differentiation. During the *first phase* (17-20 days postconception) the epithelial cells are pseudostratified and they have electron-light cytoplasm with many glycogen granules and scanty cell organelles, concentrated at the apical part (Fig. 1 A, B). During the *second phase* (20 days postconception – 15 days postnatum) the epithelial cells come to low columnar, in the apical part of the epithelial cells there are many cytoplasmic protrusions with glycogen, short and fine microvilli (Fig. 2 A). The most marked ultrastructural changes of the epithelial cells on the 15 days postnatum are many cytoplasmic protrusions, filled with glycogen, many vacuoles, granular endoplasmic reticulum and mitochondria with unformed cristae (Fig. 2 B). The

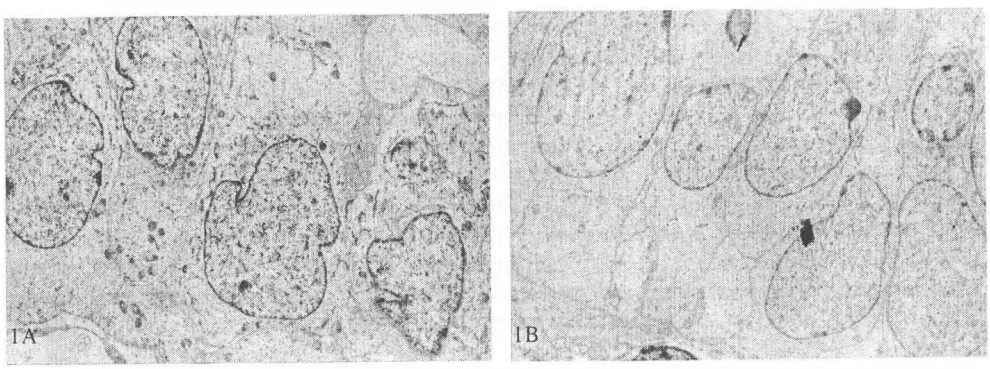
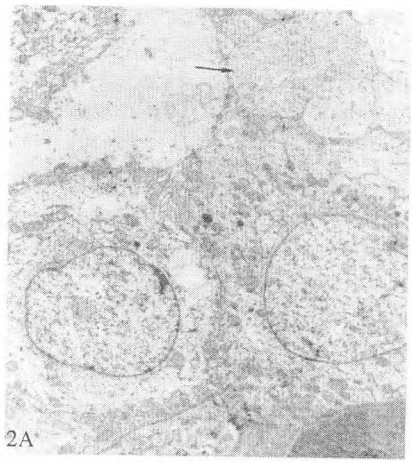



Fig. 1A – Pseudostratified epithelial cells of the rat choroid plexus aged 17 days postconception. × 3 000; 1B – High columnar epithelial cells of the rat choroid plexus aged 20 days postconception. × 3 000



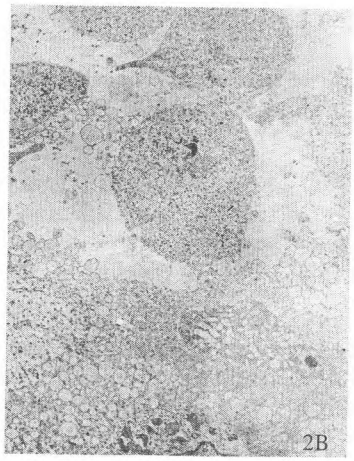




Fig. 2A – Rat choroid plexus aged 5 days postnatum. ×2 500; 2B – Rat choroid plexus aged 15 days postnatum. ×3 000

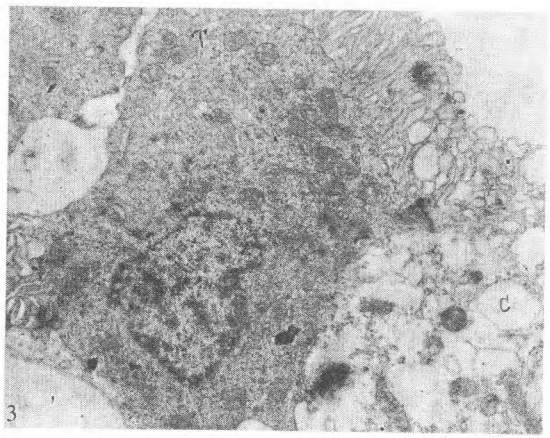



Fig. 3. Light and dark epithelial cells of the rat choroid plexus aged 30 days postnatum. ×6 000

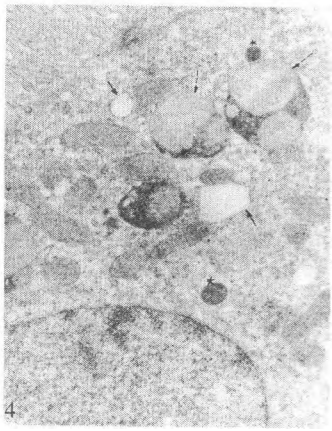



Fig. 4. Rat choroid plexus aged 22 months. × 12 000

concentration of glycogen in the rat choroid plexus epithelial cells increased to 5 days postnatum and decreased at the 15 days postnatum. S t u r r o c k made similar observations in the mouse choroid plexus [12]. During the *third phase* of the rat choroid plexus development (15-30 days postnatum) the epithelial cells come to cuboidal, the electron density of the epithelial cytoplasm is increased, the microvilli are well shaped and tight packed, and the connective tissue and the blood vessels are well differentiated (Fig. 3). From morphometrical analysis of the rat choroid plexus during development it was established that the nuclear, cytoplasmic and cell area of the dark epithelial cells is smaller than the same parameters of the light epithelial cells during the whole investigated period. The nuclear, cytoplasmic and cell area of the light and dark epithelial cells increased from 17 days postconception to 5 days postnatum and decreased from 5 days postnatum to 15 days postnatum. Changes of the nuclear, cytoplasmic and cell area of the light and dark epithelial cells from 17 days postconception to 15 days postnatum are proceeded simultaneously with the ultrastructural data for glycogen accumulation. On

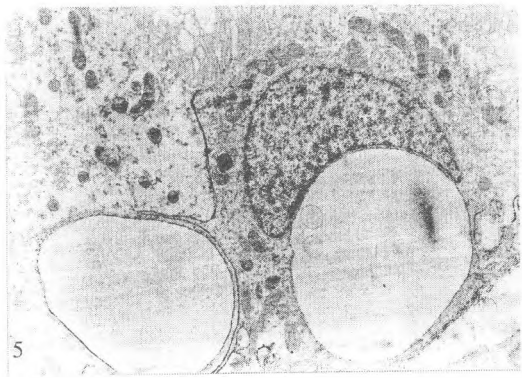


Fig. 5. Rat choroid plexus after 3 months hypokinesia. × 5 000

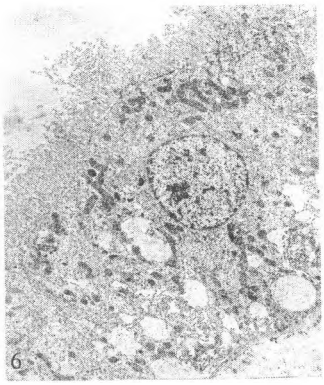


Fig. 6. Rat choroid plexus after 6 months hypokinesia. × 3 000

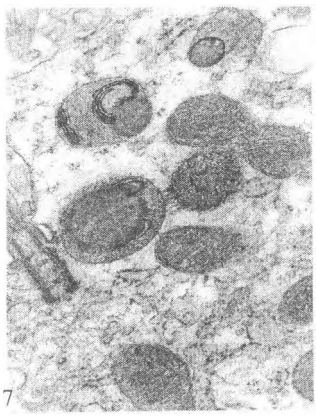


Fig. 7. Apical part of the epithelial cell of the rat choroid plexus after 9 months hypokinesia. × 30 000

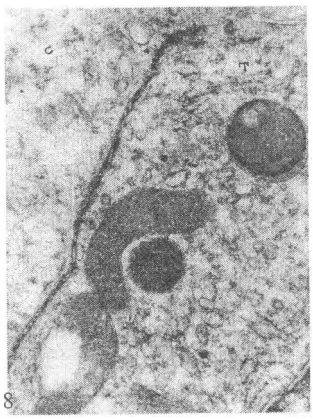


Fig. 8. Rat choroid plexus after 12 months hypokinesia. × 25 000

the basis of the ultrastructural and morphometrical investigations of the rat choroid plexus during the **second period** of development (45 and 60 days postnatum, 4, 7, 10 and 13 months) it was established that the epithelial cells are cuboidal, as the average area of the light cells is 204.5 $\mu m^2$, and of the dark cells – 137.2 $\mu m^2$. The nuclei of the epithelial cells are rounded, located basally and have homogeneous chromatin. The large numbers of mitohondria are present, concentrated at the apical ends of the cells. The most marked ultrastructural changes of the epithelial cells during the **third period** of development (13-22 months) are the presence of many lipid droplets, second lysosomes, imbibing mitochondria and dense bodies (Fig. 4). The main morphological changes noted with age suggest a decrease in efficiency of choroid plexus cells in old age [4].

The prolonged ***hypokinesia*** provoked significant ultrastructural and morphometric changes of the rat choroid plexus. The most marked ultrastructural changes of the rat choroid plexus during 3 and 6 months of hypokinesia are elongated mitochondria, giant electron-light vacuoles, dense bodies, multivesicular bodies and coated vesicles in the epithelial cytoplasm (Figs. 5, 6). These changes pointed out the increased absorptive ac-

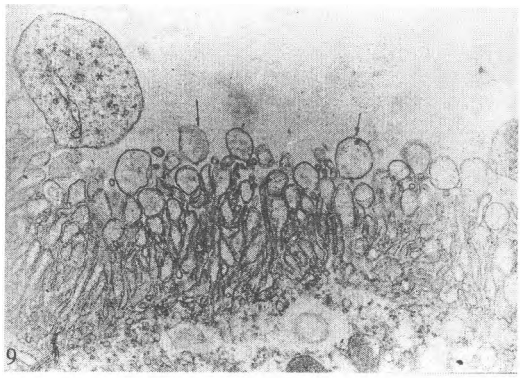


Fig. 9. Rat choroid plexus 3 months after irradiation with high energy oxygen ions. × 10 000

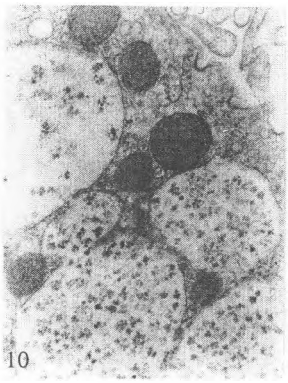


Fig. 10. Rat choroid plexus 3 months after irradiation with high energy oxygen ions. × 25 000

tivity of the choroid plexus epithelium and probably depend on the increased vasopressin [11]. The epithelial cytoplasm of the rat choroid plexus cells during 9 months of hypokinesia, in contrast to control, contains a great number of dense bodies, multivesicular bodies, coated vesicles and electron-dense lysosome-like bodies with lamellar structures (Fig. 7). These changes may be associated with increased transcellular transport by fenestrated capillaries and earlier adult changes. Destructive changes of the choroid plexus epithelium (numerous swollen mitochondria, lipid droplets, second lysosomes and electron-dense bodies with lamellar structures) observed during 12 months of hypokinesia are an evidence for earlier adult changes (Fig. 8). The obtained morphometrical data point out that the increased relative part of the dark cells, the large value of the nuclear, cytoplasmic and cell area of the dark cells, and decreased nuclear, cytoplasmic and cell area of the light cells more significantly during 12 months of hypokinesia, in comparison with dark cells, may be related to the increased functional activity of the dark epithelial cells and earlier adult changes [9].

Choroid plexus of the brain is an ideal model for studying the development of ***radiation*** damage due to a close contact between vascular and epithelial cells, which normally have very slow turnover [1]. On the apical surface of the epithelial cells of the rat choroid plexus 3 months after irradiation with *high energy oxygen ions* were seen cytoplasmic protrusions and elongated and dilated microvilli (Fig. 9). In the epithelial cytoplasm there are seen dense bodies, vesicles, multivesicular bodies and vacuoles, containing glycogen granules (Fig. 10). In the epithelial cytoplasm of the rat choroid plexus 3 months after irradiation with *fast neutrons* were seen well defined Golgi apparatus, coated vesicles, pinocitotic vesicles and multivesicular bodies (Fig. 11). Most characteristic ultrastructural changes of the rat choroid plexus 3 months after irradiation with *gamma rays* were elongated mitochondria, many pinocytotic vesicles on the basolateral intercellular junctions as well as in the endothelial cells of the capillaries. Many epithelial cells possessed two nuclei after exposure with oxygen ions and gamma rays. The obtained morphometrical data after irradiation of the rat choroid plexus have shown that the nuclear and cytoplasmic area of the light and dark epithelial cells is changed statistically significant after irradiation with oxygen ions and gamma rays. The relative part of the capillaries, i.e. vessel <16$\mu$m in diameter were 70.66% in control rats, and 34.86% in fast neutrons, 56.34% in oxygen ions and 70.52% in gamma rays irradiated rats. The initial loss of capillaries and the increase in number of large vessels in plexus choroideus 3 months after irradiation were consistent with the effects attributed to re-

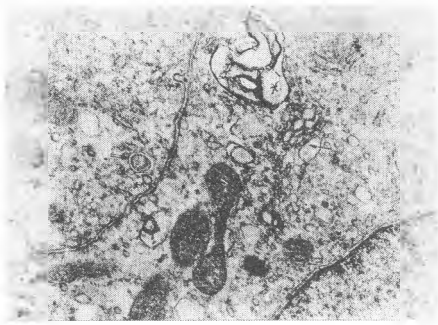


Fig. 11. Rat choroid plexus 3 months after irradiation with fast neutrons. × 15 000

generation in the plexus choroideus [10]. These changes may be indicative of compensatory reactions in the organism following radiation exposure. Experimental study have shown a significant reduction in the number of blood vessels > 16 $\mu$m in diameter and atrophy of the choroid plexus epithelial cells only after 25 Gy of X-rays [2, 3]. These results clearly demonstrate that the effect on blood vessels after irradiation can be induced in the choroid plexus by single dose of 1.0 Gy fast neutron, oxygen ions and gamma rays. We suggest a hypothesis that the vascular damage is predominant factor leading to development of late effects in irradiated normal tissues.

## Conclusion

Plexus choroideus performs a multiplicity of functions for the central nervous system. From fetal period of development through adult, and extending into terminal physiological stages, plexus choroideus actively engages with building, maintaining and repairing of the brain homeostatic mechanisms which are vital to neuronal system.

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# Lipid Profile of Rat Brain Nuclear and Microsomal Subcellular Fractions during Ischemia

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In this study, we report lipid changes in brain nuclei and microsomes in a rat model of cerebral ischemia. We found a decrease of total phospholipids and an increase of total cholesterol and glycolipids in both fractions. Total free fatty acids (FFAs) tended to decrease in microsomes and to increase in nuclei. These changes indicate a disturbance of lipid metabolism and may be interpreted as a physiological adaptive response to ischemia.

*Key words*: lipids, cerebral ischemia, nuclei, microsomes, rat brain.

## Introduction

Among the biochemical events occurring during ischemia, membrane alterations have important consequences on the brain metabolism and function. Despite the intense research on lipid metabolism during cerebral ischemia, little is known about lipid changes in the brain subcellular fractions. The aim of the present investigation is to evaluate the level of phospholipids, cholesterol, glycolipids and free fatty acids in nuclei and microsomes in rat model of cerebral ischemia.

## Materials and Methods

Three-month-old male Wistar rats were used in the experiment. Animals were subjected to cerebral ischemia according to the model of S m i t h et al. [8] with minor modifications. Nuclear and microsomal subcellular fractions were isolated according to the method described by V e n k o v [10]. Lipids were extracted according to the technique described by K a t e s [11]. The content of cholesterol and FFAs was determined by gas chromatography as we previously described [5, 6]. The content of total glycolipids was determined according to H a m i l t o n et al. [3]. Total phospholipids were determined by the method of B a r t l e t t [1]. Glycolipid and phospholipid classes were separated by thin-layer chromatography.

The data were analyzed with Student's t-test.

Table 1. Changes of the phospholipid content in nuclei (Nuc) and microsomes (Ms) after cerebral ischemia

| Brain fraction | | Phosphatidic acid | Lysophospho-lipids | Phosphatidyl-inositol | Sphingo-myelin | Phosphatidyl-serine | Phosphatidyl-choline | Phosphatidyl-ethanolamine | Total phospholipids |
|---|---|---|---|---|---|---|---|---|---|
| Nuc | Control | 2.07±0.05 | – | 0.055±0.01 | 0.304±0.03 | 5.249±0.05 | 13.515±0.06 | 24.803±0.07 | 45.995±0.07 |
| | Ischemia | 0.786±0.02 $p<0.001$ | – | 0.668±0.04 $p<0.001$ | 1.131±0.01 $p<0.001$ | 6.562±0.04 $p<0.001$ | 11.048±0.04 $p<0.001$ | 12.411±0.02 $p<0.001$ | 32.606±0.08 $p<0.001$ |
| Ms | Control | 2.996±0.07 | 0.261±0.03 | – | 0.871±0.02 | 9.763±0.05 | 20.288±0.07 | 29.421±0.09 | 63.599±0.1 |
| | Ischemia | 0.735±0.02 $p<0.001$ | 0.188±0.02 $0.01<p<0.001$ | 0.563±0.04 | 2.036±0.04 $p<0.001$ | 4.459±0.04 $p<0.001$ | 17.325±0.08 $p<0.001$ | 12.195±0.08 $p<0.001$ | 37.501±0.06 $p<0.001$ |

Values are expressed in mg/g dry lipid residue/ml. A dash indicates trace amounts.

## Results and Discussion

Our results showed a decrease in total phospholipid content by 29% in nuclei and by 41% in microsomes which is due to hydrolysis by different phospholipases (Table 1). The total cholesterol increased 2.4-fold and 7.2-fold in nuclei and microsomes, respectively (Table 2). Ischemia caused nearly 2-fold increase in total FFAs in nuclei and 3.7-fold decrease in total FFAs in microsomes (Table 3). The content of total glycolipids rose 6-fold in nuclei and 20-fold in microsomes (Table 4).

The various changes of the phospholipid classes may be influenced by differences in their turnover. Another possible reason is the difference in the substrate specificity of each phospholipid or in the accessibility of phospholipase $A_2$ to phospholipids due to a different distribution of each phospholipid in the membrane [2]. In nuclei, 12-fold increase in phosphatidylinositol (PI), and 2.6-fold decrease in phosphatidic acid (PA) were the most pronounced changes. Probably the low content of PA is due to its mobilization in the synthesis of PI or to the action of nuclear PA-phosphohydrolase. In microsomes, all phospholipid classes tended to decrease except for sphingomyelin whose concentration was increased by 134% (Table 1).

The concentration of the free cholesterol was increased 3-fold in nuclei and nearly 2-fold in microsomes. In the latter, we found 22-fold increase in esterified cholesterol (Table 2). The high concentration of sterol esters can apparently be explained with a role of the ester to serve as a carrier and storage site for some toxic free fatty acids [7]. It is reported that the accumulation of cholesterol and cholesterol esters represents a durable adaptive response to different forms of cell injury and there is a striking correlation between the severity of tissue injury and the extent of cholesterol accumulation [9].

The gangliosides increased 6-fold and 12-fold in nuclei and microsomes, respectively, while cerebrosides rose 6-fold and 41-fold in nuclei and microsomes, respectively (Table 4). Probably the high content of cerebrosides makes the membrane steadier because cerebrosides contribute to a dense network of H-bonding between three hydroxy groups of cholesterol, the hydroxy group of the sphingosine, the hydroxy groups of the acyl chains and the amide bond of the sphingolipids. Considering gangliosides as neuroprotectors [4], these changes may be interpreted as a defensive and compensatory mechanism against the ischemic shock.

In nuclei, the concentration of stearic acid ($C_{18:0}$) increased 7-fold (Table 3). In microsomes the content of arachidonic acid ($C_{20:4}$) decreased 2.6-fold and it can be due

Table 2. Changes of the cholesterol content in nuclei and microsomes after cerebral ischemia

| Brain fraction | | Free cholesterol | Esterified cholesterol | Lano-cholesterol | Total cholesterol |
|---|---|---|---|---|---|
| Nuc | Control | 0.293±0.06 | 0.131±0.05 | 0.005±0.001 | 0.431±0.05 |
| | Ischemia | 0.921±0.06 $p<0.001$ | 0.102±0.05 | – | 1.025±0.17 $p<0.001$ |
| Ms | Control | 0.392±0.06 | 0.138±0.02 | – | 0.531±0.09 |
| | Ischemia | 0.708±0.05 $p<0.001$ | 3.11±0.05 $p<0.001$ | – | 3.824±0.33 $p<0.001$ |

Values are expressed in mg/g dry lipid residue/ml. A dash indicates trace amounts.

Table 3. Changes of the FFAs content in nuclei and microsomes after cerebral ischemia

| Brain fraction | | $C_{14:1}$ | $C_{16:0}$ | $C_{16:1}$ | $C_{18:0}$ | $C_{18:1}$ | $C_{18:2}$ | $C_{20:0}$ | $C_{20:2}$ | $C_{20:4}$ | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Nuc | Control | 0.267±0.02 | 0.324±0.01 | – | 0.256±0.02 | – | 0.435±0.01 | 0.153±0.02 | – | 3.471±0.13 | 4.905±0.18 |
| | Ischemia | – | – | 1.73±0.03 | 1.828±0.04 $p<0.001$ | 1.501±0.06 | – | – | – | 3.418±0.13 | 8.473±0.1 p<0.001 |
| Ms | Control | 0.596±0.02 | 0.056±0.01 | – | 13.29±0.09 | – | 0.977±0.04 | – | – | 6.309±0.05 | 21.23±0.04 |
| | Ischemia | – | – | 0.955±0.02 | – | 2.325±0.02 | – | – | 0.112±0.01 | 2.413±0.11 p<0.001 | 5.805±0.1 p<0.001 |

Values are expressed in mg/g dry lipid residue/ml. A dash indicates trace amounts.

Table 4. Changes of the glycolipid content in nuclei and microsomes after cerebral ischemia

| Brain fraction | | Gangliosides | Cerebrosides | Total glycolipids |
|---|---|---|---|---|
| Nuc | Control | 0.216±0.02 | 0.324±0.03 | 0.54±0.04 |
| | Ischemia | 1.363±0.05 p<0.001 | 2.006±0.05 p<0.001 | 3.368±0.04 p<0.001 |
| Ms | Control | 0.212±0.03 | 0.082±0.05 | 0.294±0.04 |
| | Ischemia | 2.503±0.04 p<0.001 | 3.372±0.05 p<0.001 | 5.875±0.04 p<0.001 |

Values are expressed in mg/g dry lipid residue/ml.

to its involvement in PI synthesis, oxidation processes, diffusion into cerebral spinal fluid or reesterification. A prominent change was the accumulation of $C_{16:1}$, $C_{18:1}$ and $C_{20:2}$ in both fractions which would contribute to membrane permeability because of the high rate of unsaturation.

**In conclusion**, our results suggest that the ischemic process causes various lipid changes in nuclear and microsomal brain subcellular structures, directed to enhancement of the functional adaptive possibilities of the brain during ischemia.

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# Stage Specific Expression of Angiotensin I-Converting Enzyme in Adult and Developing Rat Testis

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Recent studies demonstrated that testicular angiotensin-converting enzyme (tACE) is essential for fertilizing ability of spermatozoa. The aim of the present paper is to characterize localization and distribution of tACE in adult and developing rat testis. First immunoreactivity appeared in the cytoplasm of round spermatids step 8 at stage VIII of the cycle of seminiferous epithelium. Later that stage the immunostaining progressively increased and reached maximum at stages IV-VIII. Stage specific localization characterizes tACE as a marker for stages of spermatid differentiation. In the course of the first spermatogenic wave tACE is a marker for developmental stage of germ cell differentiation. Testicular ACE also could serve as a marker for germ cell depletion during experimental and pathological conditions.

*Key words:* angiotensin-converting enzyme, testis, spermatogenesis, rat.

## Introduction

ACE is well-known component of renin-angiotensin system and exists as two isoforms – somatic and testis-specific (germinal) encoded by one and the same gene. Somatic ACE is responsible for the conversion of angiotensin I to the potent vasoconstrictor angiotensin II (Ang II) and inactivation of vasodilator peptides, bradykinin. Thus, sACE is involved in the control of blood pressure and fluid-electrolyte balance [8]. In the male reproductive system sACE is localized in testicular endothelial, Leydig cells; epithelial cells of the epididymis and prostate. The enzyme is expressed transiently in human germ cell during fetal life and is constant feature of germ cell cancer. Ang II locally produced by sACE regulated steroidogenesis in Leydig cells, secretory function of epididymis and tubular contractility in the prostate. Ang II has been shown to maintain sperm motility and to stimulate capacitation [3].

Testicular ACE is transcribed by an alternative promoter in 12 intron of ACE gene. Due to transcription of 13 exon, the N-domain of tACE is unique and C domain is identical in both isoforms [3]. The substrate preference of tACE is still unknown and this

[1] N.A. and E.L. contributed equally to this study and should be considered as joint first authors.

isoenzyme does not generate Ang II. Testicular ACE has been shown to play an essential role in the control of male reproduction [4] . Dipeptidase activity of tACE was identified as factor of sperm-zona pellucida binding [1]. On the other hand, tACE acts as releasing factor for some GPI proteins anchored on spermatozoa membrane and necessary for fertilization [6]. Based on these findings, it can be suggested that tACE may serve as marker of fertilizing ability of spermatozoa.

Testicular ACE is expressed in spermatids and spermatozoa in human and mice; in rat it is hormone dependent [11]. Consisting data on the expression of tACE in rat testis are lacking. On the other hand, rat provides various experimental models for investigation of hormonal regulation of different factors involved in spermatogenesis. In this respect the current study aimed to characterize specific pattern of localization of tACE in adult and developing rat testis.

## Materials and Methods

**Immunohistochemistry for tACE.** Dewaxed and rehydrated 5 μm sections were subjected to antigen retrieval in 0.01 M Citrate buffer, pH 6 at 95°C for 5 min water bath. For endogenous peroxidase block, slides were incubated in 3% $H_2O_2$ in methanol for 5 min at RT. Then, they were blocked for 1 hour in 1.5% donkey serum in PBS. Primary antibody against ACE (1:500) was applied for 30 min at 37°C. After that goat biotinylated secondary antibody-ABC staining system was applied and liquid DAB was used as chromogen.

## Results

Immunohistochemical analysis revealed stage-specific pattern of tACE expression in postmeiotic germ cells. First faint immunoreactivity appeared in the cytoplasm of round spermatids step 8 (stage VIII of the cycle) in a round shape manner (Fig.1A, table 1). Weak intensity was found in elongating spermatids step 9 at stage IX of the cycle of seminiferous epithelium. Later that stage the immunostaining progressively increased and was located in caudally organized cytoplasm of elongating spermatids. Medium intensity of reaction was observed in spermatids step10-11 at stages X-XI of the cycle of seminiferous epithelium (Fig.1B). Immunoexpression became strong later than steps 12 of spermiogenesis (stage XII of the cycle; Fig.1C) and reached maximum in steps 17-19 (stages IV-VIII of the cycle; Fig.1D). Strong immunoreactivity was confined to residual bodies that were numerous in the lumen of seminiferous tubules at stages VIII-IX of the cycle (Fig.1B). Residual bodies were also found in the basal part of the tubules where they were phagocytosed by Sertoli cells. Flagela of late elongated spermatids step 18-19 were also strongly reactive. No immunoexpression was observed in other germ cell types (spermatogonia, spermatocytes) as well as in somatic cells (peritubular cells, Leydig and Sertoli cells).

In the course of the first spermatogenesis tACE appeared in stage-specific manner. Lack of tACE expression in the testis is due to absence of corresponding type of spermatids. Mid-pubertal testis (28-day-old) is negative for tACE as germ cell development proceeds to stage round spermatids 1-3 step (Fig.1E). In late pubertal testis (42-day-old) spermatogenesis are not completed and proceeds to elongating spermatid 16 step in stage III. Imunoreactivity is observed in all the stages with an exception of stages IV-VI (Fig.1F). Lack of reaction in these stages is due to that elongating spermatids step 17-19 did not appear yet.

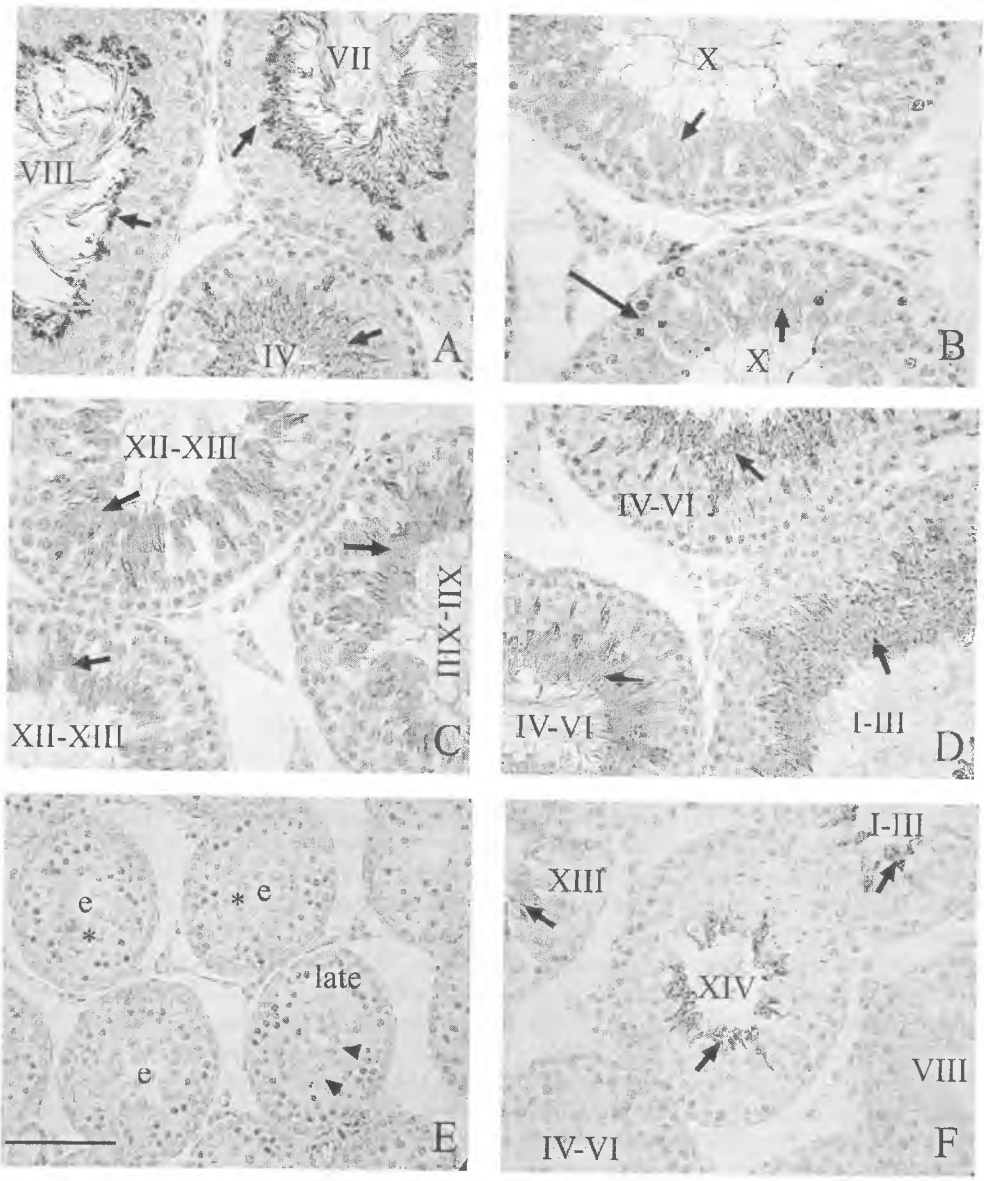


Fig. 1. Stage-specific immunoexpression of tACE in the cytoplasm of differentiating spermatids in adult (**A-D**) and developing (**E-F**) rats. Note the minimal intensity at step 8 and maximal intensity at step 19 of spermiogenesis, both at stage VIII of the spermatogenic cycle. Residual bodies are strongly positive (long arrow). Reaction product in the cytoplasm of elongating spermatids is denoted by short arrows. Negative reaction in 28-day-old rat as germ cell development proceeds to stage round spermatids 1-3 steps (asterisks) in early stages (e); late stage is identified by first and second meiotic divisions (arrowheads). In 42-day-old rat spermatogenesis are not completed and proceeds to elongating spermatids 16 step in stage III. Immunoreactivity is observed in all the stages with an exception of stages IV-VI as elongating spermatids steps 17-19 did not appear yet. Scale bar – 50 μ

Table 1. Semiquantitative evaluation of tACE immunoexpression at the stages of the seminiferous epithelium and steps of spermiogenesis in adult rats

| Stages of the seminiferous epithelium | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Steps of spermiogenesis | | | | | | | | |
| VII | | VIII | | IX | X-XI | XII-XIV | I-III | IV-VI |
| 7 | 19 | 8 | 19 | 9 | 10-11 | 12-14 | 15-16 | 17-18 |
| – | ++++ | –/+ | ++++ | + | ++ | ++/+++ | +++ | ++++ |

## Discussion

The role of tACE in male reproduction and fertilization is proved by knockout models in mice lacking ACE gene [2]. ACE null mice lacking both somatic and testicular ACE are infertile independently of normal testis weight, normal sperm count and morphology. Infertility is due to poor sperm migration in the oviduct and failure to bind zona pellucida. Mutants exhibit also low blood pressure and renal dysfunction. The expression of transgenic sACE exclusively in vascular endothelial cells of ACE-null mice restores blood pressure, but male mice remain sterile, indicated that sACE cannot substitute for tACE in supporting male fertility [5]. Interestingly, expression of testicular ACE in ACE null mice restored fertility.

The present study demonstrated stage-specific expression of tACE in the cytoplasm of male germ cells of 16-week-old normal Wistar rats. Gradual increase of immunostaining was evident from step 8 to step 19 of spermiogenesis. With one exception our results are consistent with data by S i b o n y et al. [10] in 8-10-week-old Sprague-Dawley rats. Discrepancy is related to weaker immunoreactivity in elongating spermatids at steps 15-17 compared to earlier steps. As a result gradual increased immunoexpression of tACE during spermiogenesis is not observed. In another study by L a n g f o r d et al. [7] tACE immunoreactivity in mouse testis was detected later than step 10 spermatids. The differences between our data and those by both author groups could be explained by using different antibodies against the portion common to the testicular and somatic ACE isoforms. Species-specific expression of tACE was demonstrated in human testis where reaction was found only in adluminal membranes of postmeiotic germ cells later than step 3 round spermatids [9] corresponding to step 7 round spermatids in rat.

Stage specificity of tACE localization during spermatogenic cycle characterizes tACE as a good marker for stages of spermatid differentiation. Expression of tACE starts and reaches maximum in androgen dependent stage VIII of the spermatogenic cycle that implies androgen regulation of enzyme production in postmeiotic germ cells. In this respect, future investigations involving proper experimental models for androgen ablation is needed and such studies are in progress by our group.

Localization pattern of tACE revealed the importance of elongation phase of spermatids in male germ cell differentiation with respect to gene expression and not only to morphological modifications. Expression of tACE in postmeiotic germ cells is an example for specific gene activation/translation during spermiogenesis. In the course of the first spermatogenic wave tACE is a marker for developmental stage of germ cell differentiation. Testicular ACE also could serve as a marker for germ cell depletion during experimental and pathological conditions.

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# A Case of Clear-Cell Endometrioid Adenocarcinoma Developed in an Endometrioid Cyst of the Ovary

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We present a case of a 33-year-old female with ovarian cyst verified clinically and ultrasonographically, which required surgical therapy. Cystadenoma was found on the gefrir examination. Right-sided adnexectomia was performed. The postoperative histological examination verified mixed clear-cell endometrioid adenocarcinoma originating in endometriotic cyst with atypia of the lining epithelium.

*Key words:* clear cell; endometrioid adenocarcinoma; ovarian cyst.

## Introduction

Endometriosis is an ectopically situated endometrium – glands and stroma. In cases of localisation in the myometrium it is classified as internal endometriosis (adenomyosis); when localised in other sections of the genital tract it is classified as external genital or extragenital endometriosis.

Endometriosis is a relatively common condition, found in 29% of women undergoing laparotomy [3]. It is often found in the ovaries, and its origin is associated with metaplasia of the mesothelium of the ovary into a uterine-corporal type or with the implantation of endometrial cells retrogradely carried through the uterine tube [7]. Macroscopically it manifests as cysts of varying size, called endometrioid or "chocolate" cysts.

Endometriosis rarely gives rise to malignant tumours, but it is believed to play a major role in their development in the presence of an excess of exogenous (e.g. tamoxifen) and endogenous estrogens [4, 6]. S a m p s o n was the first to describe the malignant transformation of endometriosis in 1925 [5]. Later a multitude of authors have reported malignant changes in the endometriosis in genital and extragenital places. They often make note of ovarial carcinoma associated with the endometriosis – in particular clear-cell adenocarcinoma and endometrioid adenocarcinoma. Several authors have described atypical endometriosis and have classified it as precancerocis.

In this report we describe a case of mixed clear-cell/endometrioid adenocarcinoma which originated in a endometrioid cyst.

## Case description

A thirty-three-year-old woman (S. R. B. No 1425-31/05) with two-week-long complaints of pain in the lower abdomen, stronger on the right side. A gynecologic examination revealed a tumoural formation in the region of the right ovary. An echography of the pelvis established a right-sided ovarial cyst. A laparotomy was performed. Gefrir examination showed the process in the right adnexes to be a cystadenoma. Per the diagnosis the only surgery undertaken was a right-sided adnexectomy.

A macroscopic inspection of the biopsy specimen established a monolocular cyst 10 cm in diameter, with thick, dense, and in some places fibrous yellowish-brown walls, its cavity filled with thick chocolate-like matter. A washout revealed a dense, solid knot filling more than a half of the cavity, on a wide base, with coarse imperfections and with a light-yellowish-gray sectional surface flecked with hemorrhages, necroses, and cavities with jelly-like contents. The tissue fragments of the biopsy specimen were processed and stained according to routine methodology with haematoxyllin and eosin.

During the histological examination of the sections along the wall of the cyst we discovered focal upholstery made of endometrioid epithelium – in places single-layered,

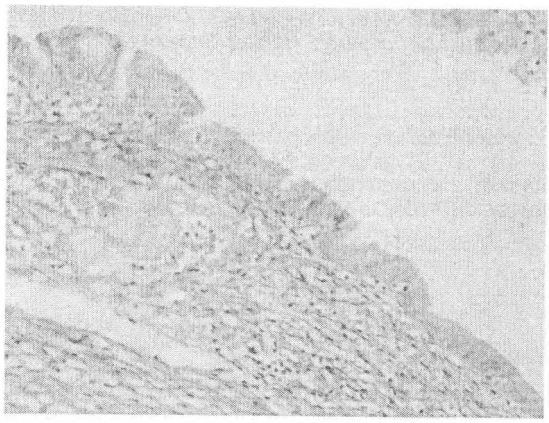

Fig. 1. Wall of endometrioid cyst lined with single-layered endometrioid epithelium and area lined with taller, stratified epithelium showing signs of cellular atypism. × 40

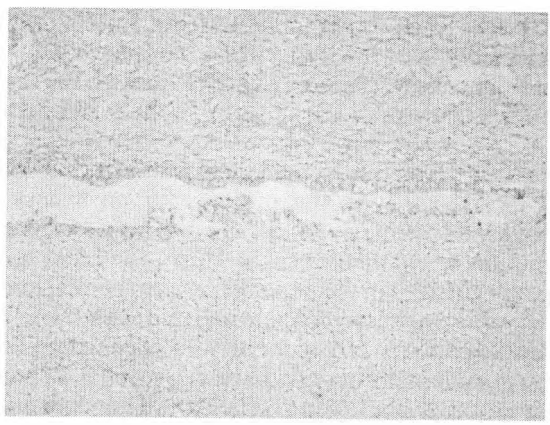

Fig. 2. Local clear cell metaplasia in the wall of endometrioid cyst. × 10

Fig. 3. Dense tumoural knot with fields of clear cell carcinoma and endometrioid adenocarcinoma. ×10

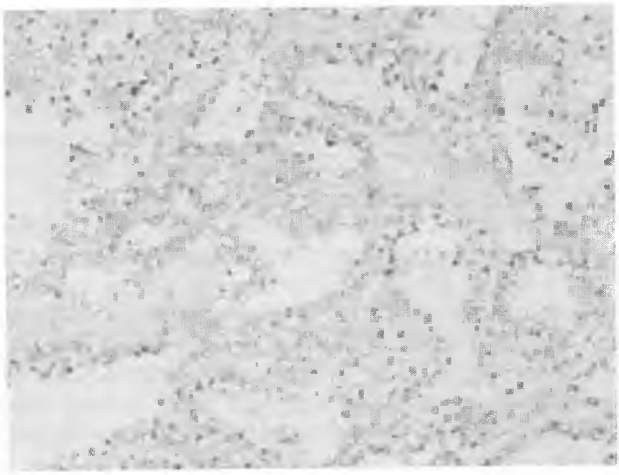

Fig. 4. Area of clear cell carcinoma. × 40

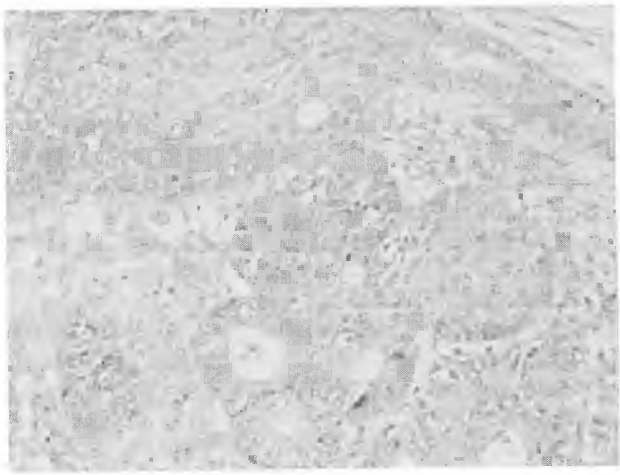

Fig. 5. Endometrioid adenocarcinoma with area of squamous cell metaplasia. Magnification x 40

in places multilayered, stacked higher and exhibiting signs of cellular atypism – hyperchromic nuclei, polymorphism, increased nucleus-to-cytoplasm ratio (Fig. 1). The endometrioid epithelial upholstery is absent in some places or is substituted by clear-cell ("thumbtack-like") epithelium, mucinous, eosinophilic epithelial metaplasia (Figs. 2, 3). In isolated short and narrow islets under the epithelium we found endometrioid stromal cells.

During the histological examination of the dense tissue knot we observed a mixing of fields with the characteristics of both clear-cell adenocarcinoma and endometrioid adenocarcinoma amidst considerable fibrous stroma. The endometrioid component has focal squamous cell metaplasia (Figs. 3, 4, 5). Biopsy verification of mixed clear-cell/ endometrioid adenocarcinoma in an endometrioid cyst with atypism of the upholstering epithelium required relaparotomy, hysterectomy with left-sided adnexectomy and omentectomy. No histological evidence for endometriosis or a malignant process was found in the material from the relaparotomy.

## Discussion

Sampson was the first to note that malignant alterations can emerge from endometriosis and proposed the following criteria for detecting them: 1) there is clear evidence of endometriosis near the tumour; 2) the histological type of the tumour suggests endometriosis as the probable origin; and 3) there is no other primary site [3, 5]. In 1990, H e a p s et al. presented 195 prior cases of malignant tumours originating from foci of ovarial or extraovarial endometriosis and also discussed 10 of their own cases. Out of 205 cases, 165 (78%) involved ovarial endometriosis, 44 (21.3%) were extraovarial endometrioses, and 4 patients had endometriosis both inside and outside the adnex [5].

Other reports based on retrospective material show that ovarial endometriosis is most often combined with clear-cell adenocarcinoma and endometrioid adenocarcinoma. The cases of endometriosis with ovarial adenocarcinoma vary between 21-73.9% (mean 38.6%) for clear-cell adenocarcinoma and from 9.6-41.9% (mean 24.4%) for endometrioid adenocarcinoma [5]. According to another source, the most common ovarial tumours associated with endometriosis are the endometrioid tumors (70%). The predominant part (90%) of carcinomas connected to extraovarial endometriosis are also of the endometrioid type [2].

The number of tissue sections in a given case of endometriosis is a factor that influences the percentage differences in reported cases. The fewer the sections made, the fewer the cases involving endometriosis [5]. In addition, the tumour may completely invade the source tissue, eliminating all histological evidence of typical or atypical endometriosis [3].

Czernobilsky and Morris investigated 192 cases of endometriosis and first described atypical endometriosis [3]. Atypical endometriosis is characterised by abnormal but not malignant cells which exhibit hyperchromasia, light to moderate polymorphism, and increased nucleus-to-cytoplasm ratio. The epithelial cells are usually tall and layered. Czernobilsky and Morris also described inflammatory atypia in endometriosis, where cells are in one row, with minimal nucleic hyperchromasia, and are adjacent to noticeable stromal inflammation [3].

The age of patients with atypical endometriosis varies between 27 and 39 years (mean 35.3 years). The attending symptom is abdominal pain. Often, endometrioid cysts are described macroscopically. In reports, atypical endometriosis is present in only 1.7% of cases of endometriosis without neoplasm and is combined very often with malignant epithelial neoplasm /predominantly clear-cell or endometrioid carcinoma [3].

Andersen et al., Kaoru et al. report cases of atypia and metaplasia of the endometrioid epithelium – in cilliar, eosinophilic, mucinous, clear-cell „thumbtack-like", squamous epithelial cells. These authors both note that cilliar and eosinophilic metaplasia are the most common types of metaplasia of ovarial endometriosis combined with ovarial carcinoma.

Recently there has been research into the biological behavior and proliferative activity of typical and atypical endometriosis with ovarial carcinoma based on immunohistochemical analysis with KI-67 [5]. KI-67 reactions with nuclear nonhistone protein manifest in the nuclei of proliferative cells during the cell cycle, with the exception of G0 and early G1 phases. Several immunohistochemical studies with Anti KI-67 monoclonal antibodies have proven a close connection between the KI-67 index and biological behavior. These reports suggest that premalignant lesions and carcinoma *in situ* have proliferative activity lower than that of carcinoma but higher than that of normal and benign epithelium. They also suggest that KI-67 can provide prognostic information in some cases of carcinoma [5, 6].

In a study by B a l l o u k et al., DNA aneuploids have been observed in 3 out of 6 endometrioid cysts with severe atypia, while histologically normal epithelium is diploid [1,5].

We believe in the appropriateness of more thorough future research aimed at determining the biological significance of metaplastic changes in the endometriosis, and of the atypical endometriosis in carcinogenesis.

## Conclusion

The case we have reported matches the criteria for malignant transformation in an endometrioid cyst indicated by Sampson; we have observed manifestations of atypism and metaplasia in the cyst epithelium; as a mixed clear-cell/endometrioid adenocarcinoma the case is especially interesting, as we could not locate other reports of mixed carcinoma in endometrioid cyst and endometriosis.

**Note:** The numbers in parenthesis correspond to the numbers of the microscope pictures on the disk.

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# Expression of Small Heat Shock Protein alpha-B Crystalline in Non-Small Cell Lung Cancer

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To evaluate the expression profile of the small heat shock protein alpha-B crystallin in NSCLC and to analyse its correlation with Ki-67 and p53 expression.
Immunohistochemistry with alpha-B crystallin, Ki-67 and p53 was applied on 25 tissue samples of patients with non-small cell lung cancer.

70% of the samples are positively stained for alpha-B crystallin. Both nuclear and cytoplasmic staining is observed. Fifty-two per cent of the tissue samples are positive for p53 and 40% were positive for Ki-67.

The small heat shock protein alpha-B crystallin is a novel biomarker that is largely expressed in non-small cell lung cancer. It is not predominantly expressed in any histologic type. There was a tendency for a more intensive nuclear staining in adenocarcinomas. Alpha -B crystalline negative cells were positive for Ki-67 and p53.

*Key words:* non-small cell lung cancer, p53, Ki-67, alpha-B crystalline.

## Introduction

Stress proteins or small heat shock proteins are synthesized under deleterious environmental conditions, that alter the protein conformation. In such conditions they are a performing a chaperoning structure, thus counteracting the formation of aberrantly folded polypeptide aggregates, and providing their renaturation during stress recovery[1, 3]. αB-crystalline belongs to the family of small heat shock proteins. In humans there are more than 10 different such proteins, but only part of them act as real heat shock proteins (Hsp 27, Hsp 22, αB-crystalline), whose synthesis is stimulated under stress. αB-crystallin exists as a homo- or heteroolygomer (with other small heat shock proteins) with a molecular mass 700-800 kDa. Under certain conditions it can be phosphorylated on three serine residues Ser 59,45,19, which provokes the disassociation of the large oligomeric complexes and the formation of smaller ones [4, 5]. The equilibrium of the two structural forms of alpha-B crystalline is closely associated with its functions as a molecular chaperone. Its oligomeric form has predominantly chaperone or antioxidant

activity, which significantly decreases under phosphorylation. It is assumed that phosphorylation increases its antiapoptotic functions [2]

It is a major component of the fibrillar aggregates, that are accumulated in cataract, desmin-associated myopathy, Alzheimer disease, Parkinson disease, Alexander neuropathy, senile systemic amyloidosis, macular degeneration, familial amyloidogenic polyneuropathy, haemodyalisis associated amyloidosis. Alongside with this it is widely expressed in many oncological diseases where it is assumed as a pathological factor, prognostic and predictive marker [6, 8]. For the first time data about high expression is reported in brain tumors (glioma multiforme, astrocytoma, oligodendroglioma). Later high expression was reported in renal cell carcinoma, thyroid carcinoma, basal-cell and metaplastic breast cancer. αB-crystalline acts as an oncogene in these carcinomas and provokes the neoplastic transformation-changed cellular architectonic, increased proliferation, repressed apoptosis, regulates invasion and metastasis [7].

Non-small cell lung cancer is among the commonest oncological diseases, and is characterized as extremely heterogenous in both histological and genetic aspects. Because of lack of data about the role of αB-crystalline in lung oncogenesis we decided to study its expression.

## Materials and Methods

Whole tissue sections of 25 patients operated on non-small cell lung cancer are studied. Patients were operated on non-small cell lung cancer during 2005-2006 and none of them has received prior chemo- or radiotherapy.

Tissue slides were deparaffinized in xylene and hydrated through graded ethanol. Endogenous peroxidase activity was blocked by a 5-min incubation in 3% hydrogen peroxide. The slides were next incubated with 10% normal horse serum for 30 min at room temperature to reduce nonspecific background staining. A primary monoclonal anti-mouse antibody – p53 (DO-7). Ki-67 (MIB-1) Dako Cytomation, Carpinteria (CA) and monoclonal anti-rabbit αB-crystalline antibody (at a 1:200 dilution with PBS) were applied for 24 hours at 2-4 °C. Secondary antibody was detected by using an antimouse horseradish peroxidase-labelled polymer secondary antibody from the LSAB (Labeled Streptavidin Biotin System) (Dako). The slides were rinsed in PBS between procedures and visualized by a 3-min incubation with diaminobenzidine (DAB). Finally, the slides were counterstained with hematoxylin. In negative control experiments, normal horse serum was used and the primary antibodies were omitted.

## Evaluation of immunohistochemistry

Immunostaining was classified as follows: 0 – lack of staining, 1 – weak staining, 2 – moderate staining, 3 – strong staining. The predominated grade of staining was determined by the percentage of cells of the same grade.

## Results

The study group consisted of 13 squamous cell carcinomas, 5 adenocarcinomas, 3 bronchoalveolar carcinomas and 4 adenosquamous carcinomas.

In four of the squamous cancers there was a lack of staining. The others showed moderate to intensive cytoplasmic staining with granular characteristic. All tissue

samples had moderate to intensive nuclear staining that varied between 50% to 100% of the counted cells. Neither the intensity of nuclear, nor the intensity of the cytoplasmic staining correlated to the grade of differentiation.

Three adenocarcinomas and two bronchoalveolar carcinomas were with intensive cytoplasmic staining. In the rest of the cases there was no staining. The nuclear staining was intensive and varied largely from 60% to 100% of the cells. In comparison to the squamous cell carcinomas the intensity and percentage of the positivity of the nuclear staining was distinctively higher.

The adenosquamous group of cancers that were only four had intensive nuclear and cytoplasmic staining and the number of positive cells was 80-100%.

In 40% (9/25) of the samples Ki-67 positivity was detected. The number of the positive cases predominated in tissue samples, where no or weak positivity was observed. The samples that turned to be positive for alpha-B crystallin were negatve for Ki-67 except one case.

P53 was positive in 52% (13/25) of the cases. Forty per cent had intensive and twelve per cent had moderate staining. No difference could be observed between the two major histologic types considering this marker.

## Discussion

Overall 70% (16/25) of the patients were positively stained for alpha-B crystalline. Both nuclear and cytoplasmic staining was observed. In adenocarcinomas there was a tendency for a higher percentage of cells with intensively stained nuclei. Fifty-two per cent of the tissue samples were positive for p53 and 40% were positive for Ki-67. None of these two markers turned to be typically expressed in the two histologic types. There was a tendency between Ki-67 positivity in alpha-B crystalline negative cases.

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# Pyoderma Gangrenosum – Morphological Challenge

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Pyoderma gangrenosum (PG) is an uncommon, ulcerative skin disease usually included in the group of vasculitic disorders. We discuss four patients with the diagnosis PG and define morphological substrate of their skin lesions. These findings we juxtapose to those reported in the literature. Based on the clinical and histopathological characteristics we make some aspirations for the pathogenesis of the disease and search the relation with vasculitis.

*Key words:* Pyoderma gangrenosum, vasculitis, neutrophilic infiltrate, histopathology.

## Introduction

Pyoderma gangrenosum (PG) is an uncommon, ulcerative skin disease usually included in the group of vasculitic disorders with distinctive cutaneous clinical characteristics first described in 1930 [3]. It is associated with underlying conditions in up to 50% of cases, the most common of which are inflammatory bowel disease, rheumatoid arthritis, and hematological malignancies [6]. The pathogenesis of PG has remained obscure even as an ever-widening array of systemic diseases described in association with it. Clinically it starts with sterile pustules that rapidly progress and turn into painful ulcers of variable depth and size with undermined violaceous borders. Course can be mild or malignant, chronic or relapsing with remarkable morbidity. PG has four major clinical types – ulcerative, pustular, bullous, and vegetative [4] and mainly affects the skin of lower limbs, but rarely atypical bullous variant seems to affect upper limbs [2]. Correct diagnosis relies on clinical signs first and is supported by biopsy for histopathology. Knowledge of the patient's history for possible underlying disease and specific investigations based on that background are necessary. Therefore diagnosis is made by exclusion of other possible disorders. No laboratory parameter for PG is available. The histopathology is nonspecific and remains speculative in most patients. The pathology includes necroses and ulceration of the epidermis and dermis, particularly in follicular zones, heavy infiltration of acute inflammatory cells at the base. In ulcerative PG the histopathological picture shows central neutrophilic abscess formation with distal lymphocytic angiocentric infiltrate. We have followed 4 very interesting patients with the rare diagnosis PG which varied both in clinical and histological picture.

## Materials and Methods

Case1 is a 45-year-old woman suffered from a painful and deep ulcerative lesion that appeared after a minor trauma on the flexural part of her right lower limb. Her medical history is complicated with chronic hepatitis.

Case 2 is a 40-year-old woman with ulcerative lesions on her forearms and hands, following successful surgery for Quervain's disease. She had pain and a moderate increase of the temperature.

Case 3 is a 28-year-old woman who developed in a short time two painful but not so deep ulcers on her left thigh.

Case 4 is a 67-year-old lady that had pustular lesions and erosions of long duration on the skin of her legs, treated with topical therapy without effect. She had also arthralgia and hypertonia.

Routine laboratory examinations, immunological investigation including anti-nuclear, antideoxyribonucleic, antineutrophilcytoplasmic and anticardiolipin (aCL) antibodies, X-ray, thermometry and Doppler ultrasound studies were done. Specific stains and cultures for fungal and bacteria were performed. Skin biopsy specimens taken from the lesions and direct immunoflourescense of all the cases were examined.

## Results and Discussion

Having in mind the clinical characteristics of the lesions, confirmed by the laboratory and histopathological findings we put the diagnosis PG in all four cases. The disease has a variety of conflicting microscopic descriptions. This is the reason we want to illustrate our morphological findings and compare them to precious descriptions.

Clinical examination of case 1 corroborated leg ulcerative lesion with deep and crusted appearance and underminded borders. Histopathological findings of skin specimen showed a dense neutrophilic infiltrate in derma and subcutaneous tissue without vasculitis. Significant upper dermal edema and erythrocyte extravasation were evident within the epidermis and dermis (fig. 1).

Histological examination of the recurrent ulcers on the hands of case 2 showed necrosis in the epidemis, fibrinoid necrosis in the middle dermis and a moderate infiltrate in the deep dermis (fig. 2). The infiltrate consisted of neutrophils and lymphocytes (fig. 3).

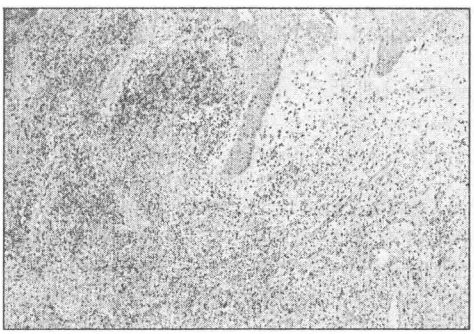

Fig. 1. Case 1 – dense neutrophilic infiltrate in derma and subcutaneous tissue, significant upper dermal edema and erythrocyte extravasation (HE, × 80)

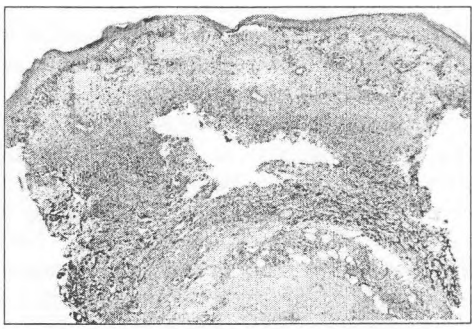

Fig. 2. Case 2 – fibrinoid necrosis in the epidermis, necrosis and moderate infiltrate in the dermis (HE, × 40)

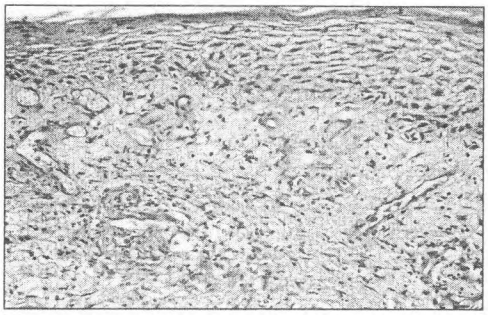

Fig. 3. Case 2 – moderate infiltrate of neurtophils and lymphocytes in the dermis (HE, × 80)

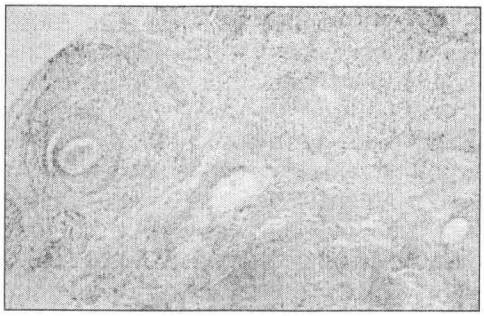

Fig. 4. Case 3 – dermal neutrophilic infiltrate and endothelial swelling without vasculitis (HE, × 80)

The ulcers of case 3 did not have underminded borders which is not typical for PG. The histological picture demonstrated dermal neutrophilic infiltrate and endothelial swelling without vasculitis (fig. 4). The patient had possitive IgGaCL and a previous spontaneous abortion. From this point of view we put also the diagnosis of Antiphospholipid syndrome (APS) and considered it as an association with PG. However, she had not any occlusion in the vessels on the histopathology.

Case 4 tended to be a pustular form of PG as the other three ones were ulcerative. Histopathological finding defined very well a subcorneal pustule with neutrophils, eosinophils and acantolitic cells. An inflammatory infiltrate of neutrophils, eosinophils and mononuclear cells were seen mainly around the vessels. Endothelial swelling was present in the dermis (fig. 5).

We reported four unique clinical cases presented within two main clinical forms– pustular and ulcerative. The histomorphological findings were quite different in the investigated biopsy's specimens. What is important is that the neutrophilic infiltrates was constant and dominated everywhere. The typical vasculitis was absent. It was difficult to demonstrate a vessel origin of the lesions and we would like to understand why PG is included in the vasculitis classification.

The histopathologic findings in lesions of PG are controversial and usually not diagnostic. Some authors studying early lesions report a primary neutrophilic infiltrate, whereas others describe a primarily lymphocytic infiltrate. Degrees of vessel involve-

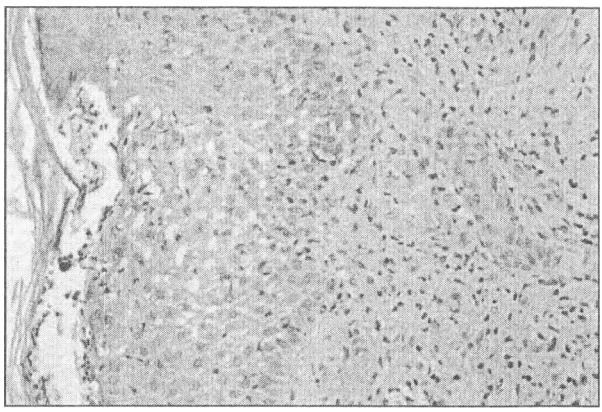

Fig. 5. Case 4 – subcorneal pustule with neutrophils, eosinophils and acantolitic cells, inflammatory infiltrate around the vessels and endothelial swelling in the dermis (HE, × 100)

ment reported vary from none to endothelial swelling to fibrinoid necrosis. Fully developed ulcers of typical PG show pronounced tissue necrosis surrounded by mild inflammatory cell infiltrate with occasional foreigh body giant cells deeper in the dermis. Subcorneal pustule and perifollicular neutrophilic infiltrate are seen in the pustular clinical variant of PG.

Regarding the major controversy concerning vascular involvement two main opinions exist [1]. Some investigators emphatically deny any involvement in the vasculature while others support it finding histologic evidence of cutaneous vasculitis, mainly necrotizing, consisting in angiocentrical segmental inflammation, endothelial cell swelling and fibrinoid necrosis of blood vessels.

Although an immune-mediated pathogenesis is suspected, a review of published reports leads to the conclusion that no consistent immunologic abnormality is found in PG patients. One of our cases is associated with chronic hepatitis and has circulated immune complexes (CIC) and another has an associated APS. An interesting possibility is that PG could be a cutaneous manifestation of APS as it has been suggested [5]. All our cases demonstrated neutrophilic infiltrate and were successfully treated with medications with an action towards neutrophils- colchicin, tetracycline, thalidomid.

The association of PG with diseases in which CIC are present (case 1), the finding of pathergy (case 1 and case 2), the overlap of PG with APS (case 3), our view of the histopathology of the lesions of all our patients and good therapeutic results have led us to two main conclusions:1. We consider neutrophils as a significant mediator of inflammation in the pathogenesis of these cutaneous lesions, and 2. We view PG as a neutrophil-mediated vascular inflammatory disorder.

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# Influence of cobalt chloride on mouse peripheral blood cells

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Cobalt chloride is shown to stimulate erythropoiesis. Our results show no visible changes in the erythrocyte morphology. On the other hand, granulocyte nuclear hypersegmentation in the peripheral blood smears of mice treated with low and/or high dose of cobalt chloride is observed when compared to the control samples. Thrombocyte aggregation can be also seen in the samples of cobalt chloride-treated mice.

*Key words:* cobalt chloride, mice, peripheral blood cells, morphological characteristics.

## Introduction

Cobalt chloride ($CoCl_2$) is a water soluble compound used for treatment of anemia. Topashka-Ancheva et al. [4] determine that consuming food containing industrial dust with cobalt induces changes in hemoglobin, hematocrit, in red and white blood cells counts. Data show that $CoCl_2$ is used by athletes for stimulating erythropoietin production [1]. Treatment of rats and mice with $CoCl_2$ protects heart and kidneys from ischemic disturbancies [2].

The *aim* of the present study is to investigate the influence of cobalt chloride on the morphological characteristics of peripheral blood cells in mice.

## Material and Methods

Adult balb/c mice were subjected to cobalt chloride treatment at daily doses of 75 mg/kg and/or 125 mg/kg. $CoCl_2$ was obtained from drinking water. After 20 days treatment mice were sacrificed, blood smears were prepared from the peripheral blood, fixed and stained with May-Grünwald-Giemsa. The morphological characteristics of the blood cells were observed on light microscope Opton.

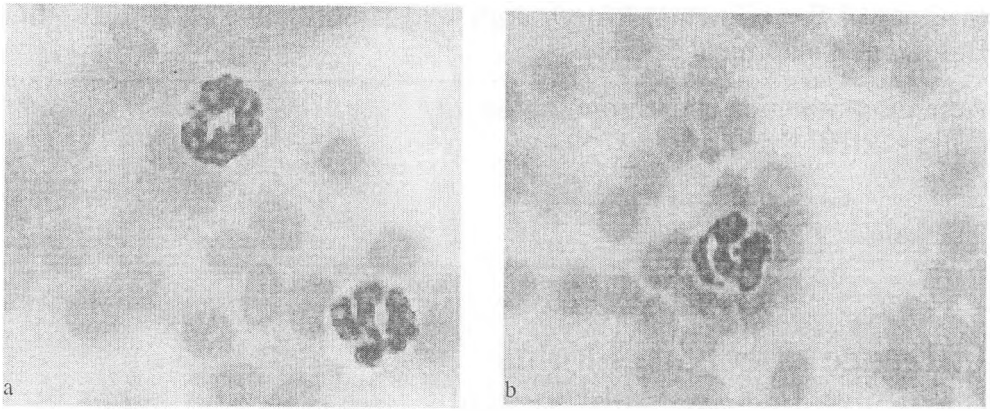

Fig. 1. Granulocyte in a peripheral blood smear – a) in a control smear and b) with nuclear hypersegmentation in smears of mice treated with 75 mg/kg and/or 125 mg/kg cobalt chloride. May-Grunwald-Giemsa staining, × 630

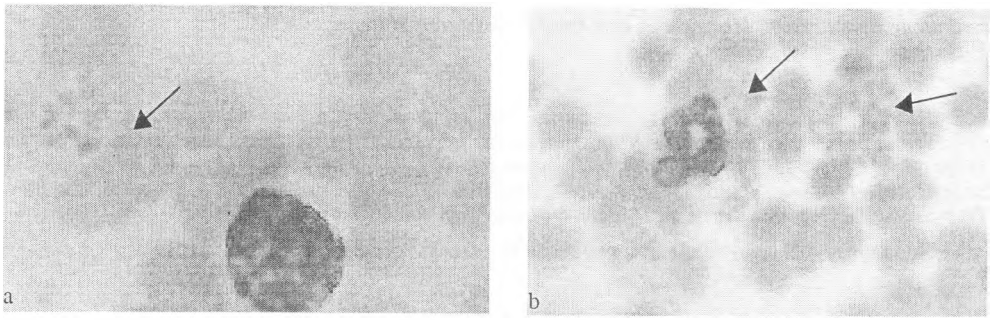

Fig. 2. Thrombocyte aggregation – a) rarely observed in control smears (see arrow) and b) large thrombocyte aggregates (arrows) in smears of mice treated with 75 mg/kg and/or 125 mg/kg cobalt chloride. May-Grunwald-Giemsa staining, × 630

## Results and Discussion

The light microscope studies show no visible changes in erythrocyte morphology of blood smears obtained from cobalt-treated mice compared to the controls. Anisocytosis is rarely observed. On the other hand, nuclear hypersegmentation of neutrophils is determined when mice are treated with low and/or high dose $CoCl_2$ (Fig.1 a, b). The nuclei seem pyknotic with compact chromatin. In smears of $CoCl_2$-treated animals thrombocyte aggregation is observed compared to the controls where there are mainly individual platelets (Fig. 2 a, b).

Although nuclear hypersegmentation is a normal phenomenon in animals [5], our studies need further investigations to determine whether the nuclei are apoptotic or with increased activity. Nuclear hypersegmentation of neutrophils is observed in cases of hyperthyroidism in humans. We have no data for the influence of $CoCl_2$ on the thyroid gland in our studies, therefore such possible affect cannot be excluded. Our results are in agreement with those of S m i t h et al. [3] showing that cobalt chloride stimulates thrombocyte aggregation.

## Conclusion

Cobalt chloride induces changes in granulocyte (neutrophil) morphology. Further studies for the ultrastructural changes are required to elucidate whether the observed nuclear hypersegmentation is a sign of apoptosis or cell activation. Quantitative analysis will be performed as well to determine the changes in red and white blood cell counts of the treated mice.

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# Relaxin-like Factor – a Marker of the Rat Leydig Cell Differentiation Status

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The cytotoxic agent ethane dimethanesulphonate (EDS) specifically destroys Leydig cells (LC) in the adult testis, followed by a complete regeneration. The process of LC renewal after EDS shows homology to the development of the adult – type LC population in prepubertal testis. After EDS treatment, the immunoreactivity for relaxin-like factor (RLF), a new marker for LC maturation, disappeared from the testis and reappeared again at the time of regeneration of the first LC. Concomitant with the increase of the LC repopulation, the number of RLF –positive cells increased. The present findings support the hypothesis that EDS-treated rats can serve as a model for studying the LC development in the prepubertal rat testis and indicate a specific role of hormonal factors like a RLF in this process.

*Key words:* RLF- EDS- testis- Leydig cells.

## Introduction

Ethane-1,2-dimethanesulphonate (EDS) is a unique testicular toxicant which selectively and temporary destroyed testicular Leydig cells (LC). Later a new population of LC regenerates, apparently from mesenchymal fibroblast-like precursors [2, 8]. The restoration of new LC population after EDS passed through the same intermediate stages occurred during normal postnatal development [8]. For this reason EDS model has been used extensively to investigate the physiological role of different hormonal and non-hormonal factors in the processes of LC regeneration and differentiation.

The Relaxin-like factor (RLF) or insulin-like 3 (INSL3) is peptide hormone, a novel member of the insulin- relaxin- insulin-like growth factor family, and seems to be localized predominantly in gonadal tissues [3]. RLF mRNA is expressed in the LC in a constitutive fashion and RLF/INSL3 thus seems to be a useful marker of LC differentiation status. The present study was aimed to establish the chronology and dynamic of RLF/INSL3 expression in the LC repopulation after exposure with EDS of mature rats.

## Material and Methods

Testes of mature Wistar rats that received single intraperitoneal (i.p.) injection of EDS (75 mg/kg body weight) dissolved in dimethyl sulphoxide (DMSO): water (1:3 v/v) were used. A second group of rats received a single i.p. injection only of DMSO: water. The animals were killed 1, 7, 14, 21 and 35 days after initial treatment (n=10 per group). The pattern of RLF/INSL3 expression in newly formed LC after EDS treatment was established using a specific polyclonal anti-RLF/INSL3 antibody (Phoenix Peptide, USA, 1:200) and high sensitive immunohistohemical polymer detection kit (Zymed, USA).

## Results and Discussion

In male mammals, RLF/INSL3 is a majority secretory product of the LC, where it appears to be expressed in a differentiation – dependent manner [3]. In the present study we found that on the 1st and especially 7 days after EDS, RLF- positive LC disappeared from the testicular interstitium consisting with the total loss of LC (Figs. 1, 2). Routine histological analysis showed that between 14 and 21 days after EDS injection a few LC with oval or spherical nuclei were observed within the intertubular space in a peritubular or perivascular position. During this period the number of LC was progressively increased and they formed large clusters usually in the vicinity of blood capillaries. According to previously reported data [1 ,9] our results revealed that the appearance of RLF/INSL3-immunoreactive cells coincided with the restoration of the LC population 14 days after EDS. On the 21st day after EDS treatment intensive immunolabeling for RLF was found in the regenerating LC (Fig. 3). 35 days after EDS destruction a larger number of RLF positive Leydig cells were seen in form of clusters corresponding with the regeneration of adult type LC population (Fig. 4). The chronology and dynamic of RLF/INSL3 expression in the present work is very similar to that seen in studies in rat postnatal development [5, 6, 7] and its pattern of expression correlates temporally with the development of steroidogenic func-

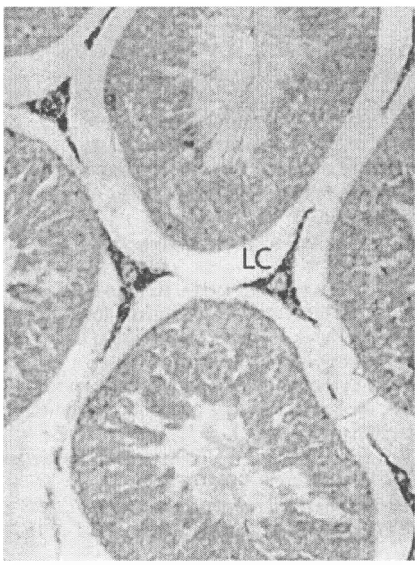


Fig. 1. Control group. INSL3 immunoreactivity in the Leydig cells (**LC**). × 200

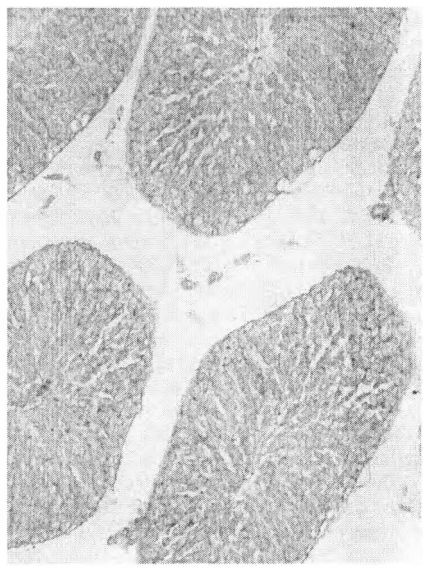

Fig. 2. 7 days after EDS. INSL3 positive cells disappeared from the testicular interstitium. × 200

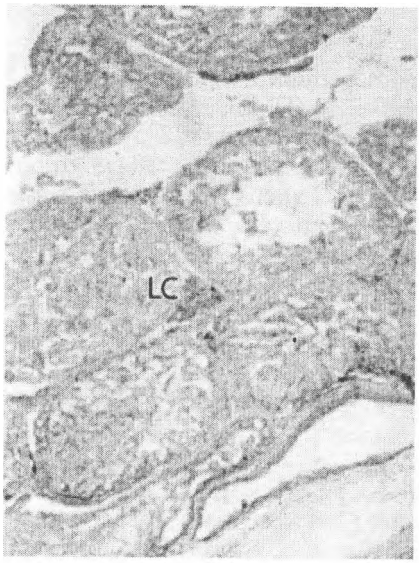


Fig. 3. 21 days after treatment with EDS. Immunolabeling for INSL3 was found in the newly formed Leydig cells (**LC**). × 200

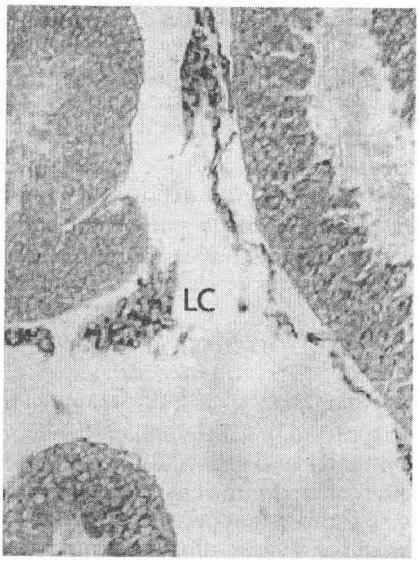


Fig. 4. 35 days after EDS. Clusters of strong INSL3 positive Leydig cells (**LC**). × 200

tion and spermatogenesis [6]. Our data are in agreement that in the mature testis, RLF expression is a good marker for adult type LC, but is weakly expressed in immature LC [4].

The results obtained are step forwards in elucidating the differentiation of adult LC in prepubertal testis and indicate a specific role of hormonal factors like a RLF/INSL3 in this process.

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# Structural and Immunocytochemical Alterations of Hassall's Bodies in Aged Human Thymus

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We investigated the structural heterogeneity and immunohistochemical profile of Hassall's bodies (HB) in human aged thymuses, obtained during cardiovascular surgery. Employing a panel of twelve antibodies, immunohistochemical methods and semiquantitative scale for detection of immunopositive cells, we observed modulation of immunoreactivity, associated with structural alterations of immunopositive HB. The results presented enrich the information about HB as antigenically distinct, functionally active, multicellular formations within the thymic medulla and raise the question of their role during age-dependent involution of the thymus.

*Key words:* Hassall's bodies, human thymus, immunocytochemistry.

## Introduction

Hassall's bodies (HB) are unique components of the thymus which provide developing thymocytes with paracrine and juxtacrine signals to ensure their proper functional maturation during intrathymic lymphopoiesis [1, 6, 7]. Although HB have been proposed to act in both maturation of developing thymocytes and removal of the apoptotic cells, their function remains an enigma [2, 4, 10]. The purpose of the present work was to verify whether age-dependent involution of the thymus influences the presence and distribution of HB, their structural heterogeneity and immunohistochemical profile.

## Material and Methods

Specimens from normal thymus ($n$=23) were obtained during cardiovascular surgery of old (aged 61-74 years; $n$=17) and young (aged 2-12 years; $n$=6) individuals. The thymuses collected showed no evident pathological disorders. The study was approved by the Ethics Committee of the Faculty of Medicine. Routine light microscopy, indirect immunofluorescence and immunoperoxidase techniques were performed according to standard protocols [5, 9]. Twelve primary (monoclonal and polyclonal) antibodies (Ab):

*Anti-Pan cytokeratin* Mo Ab (C1801, Sigma Chemical Co.), *Anti-NGF* polyclonal rabbit Ab (NGF H-20; sc-548), *Anti-TrkA* Mo Ab (p-TrkA E-6, sc-8058), *Anti-p75* Mo Ab, NGFR p75 (ME 20.4, sc-13577), *Anti-CD14* Mo Ab (UCH-M1, sc-1182), *Anti-IGF-I* Mo Ab (UBI/Biomol, Hamburg), *Anti-IGF-IR* Mo Ab (sc-N-20), *Anti-BDNF* Mo Ab (C-9, sc-8042), *Anti-bFGF* Mo Ab (UBI/Biomol, Hamburg), *Anti-CNTF* polyclonal goat Ab (R&D Systems), *Anti-EGFR* Mo Ab (clone 29.1, E-2760, Sigma St. Louis, MO), *Anti-GDNF* Mo Ab (MAB212, R&D Systems) were applied. *FITC-labeled* anti-mouse Ig (Santa Cruz Biotechnology), *Texas Red-conjugated* anti-rabbit Ig (Santa Cruz Biotechnology) as well as *ABC Staining Systems* (mouse-sc 2017 or rabbit-sc 2018) were used as secondary antibodies. Control experiments were carried out in parallel.

## Results

Aged thymuses displayed large areas of adipose tissue containing scattered islands composed of epithelial cells, lymphocytes, reticular connective tissue and HB with different morphology. Four morphological types of HB were detected in the age-involuted thymus, as compared with the young specimens (data not shown): 1. Giant HB with flaky material, lymphocytes and stromal cells in the middle (Fig. 1); 2. HB with a characteristic concentric arrangement of keratinizing epithelial cells and hyaline homogenate in the center (Fig. 2); 3. "Cellular" HB with vital cells, among which individual cells exhibited the characteristics of active secretory cells, without a degenerative center; 4. Cystically degenerated HB.

HB in the young thymus displayed intensive labelling intensity after immunostaining for CK, NGF, TrkA, p75, IGF-I, IGF-IR, CD14, bFGF, BDNF, CNTF, EGFR. HB of aged thymus preserved a strong immunoreactivity for CK, p75, IGF-IR,

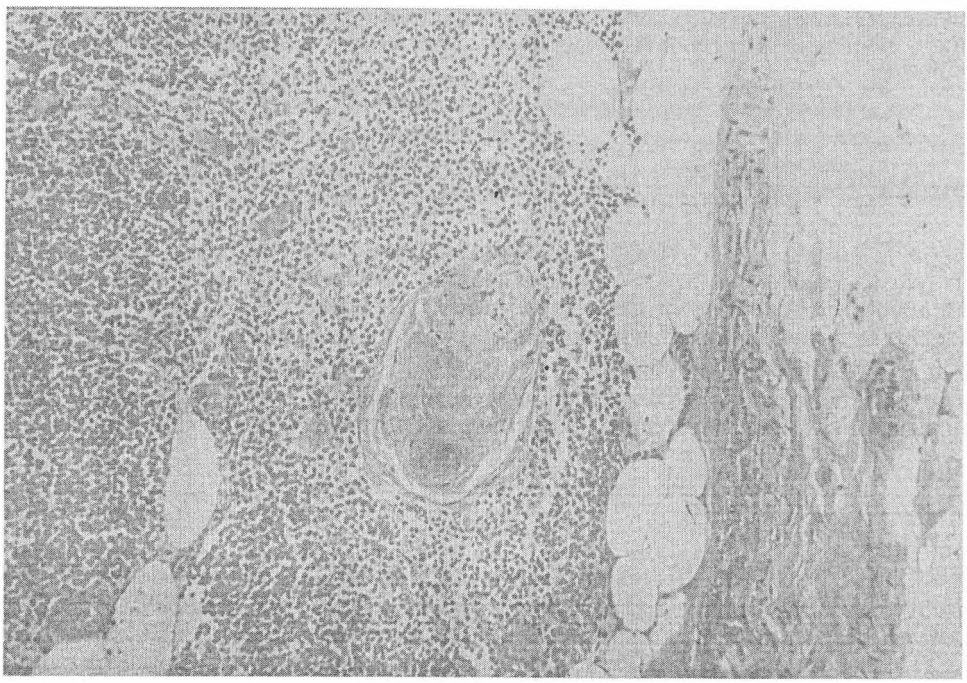

Fig. 1. Age-involuted human thymus (67-year-old male) with first type HB in the medulla. HE, × 100

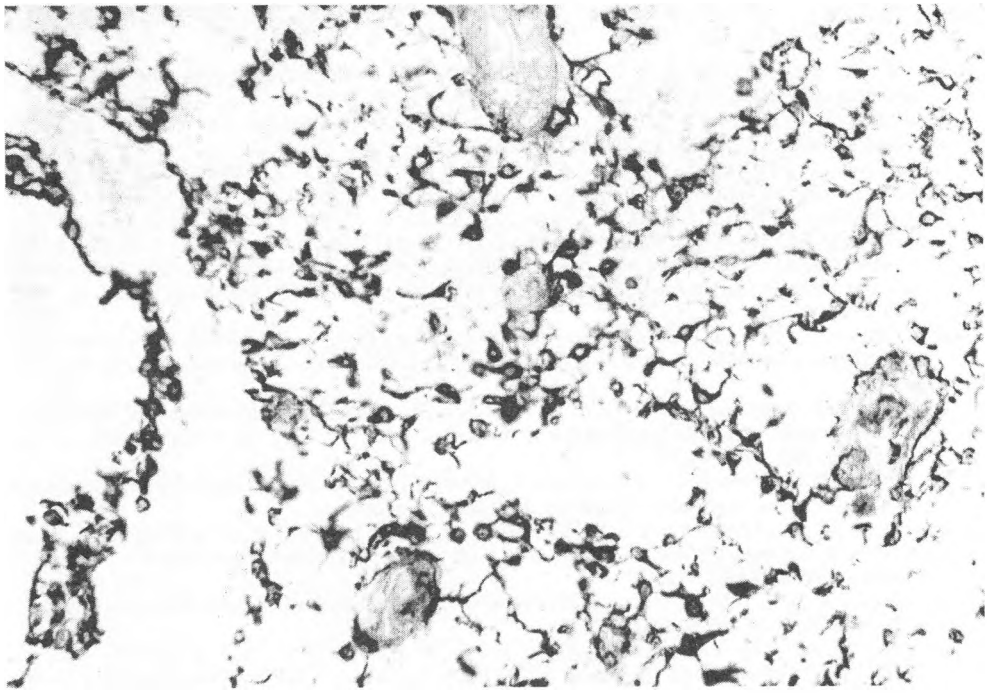

Fig. 2. Aged human thymus (68-year-old male) with strong cytokeratin immunopositive epithelial cells forming network and HB. Immunoperoxidase staining, × 400

CD14, were stained less intensely for NGF, TrkA, IGF-I, bFGF, BDNF, CNTF, EGFR and were GDNF immunonegative.

The structural components of HB displayed co-localization of IGF-I and IGF-I receptor (IGF-IR) immunoreactivity. The decreased expression of IGF-I in the aged thymus correlated with modulation of immunoreactivity of double (IGF-I/IGF-IR) positive thymic cells. In negative controls the HB were unstained.

## Discussion

The thymus undergoes age-related (physiological, chronic) involution in the course of normal ontogenetic development [2, 5]. Thymic involution is particularly important in relation to immunosenescence and its various associated diseases [2, 3]. We have previously reported presence of NGF and NGF receptors immunoreactivity in age-involuted human thymus [5] and modulation of ABH antigen reactivity in senile thymus [9] in contrast with young thymus [8]. This investigation provides new structural and immunocytochemical data for immunohistochemical profile of HB suggesting that these multicellular formations are antigenically distinct and functionally active in growth factor receptor-mediated cell signalling mechanisms during physiological thymic involution. It seems likely that HB are structures, implicated in thymocytes ontogenesis, T-cell apoptosis and possibly intrathymus negative selection throughout life.

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# Anticancer Agent Cyclophosphamide Disturbs Mice Spermatogenesis

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The anticancer agent cyclophosphamide (CY) is widely used in chemotherapy regimes. It causes severe side effects on many rapidly proliferating cells including germinal cells. In the present study the effect of CY on mouse spermatogenesis was investigated. Sexually mature BALB/c mice were injected i.p. with 200 mg/kg CY applied in a single dose or as 10 doses of 20 mg/kg each. Blood samples and pieces of testes were taken on the 1, 3, 7 and 15 days after the last treatment and preceded for RIA, autoradiographic and ultrastructural study. The obtained results showed impairment of testicular function expressed in decline of mitotic activity of spermatogonial cells, ultrastructural alterations of different germ cells, disruption of acrosome formation and increase of testosterone level, restored in 15 days after the treatment. In conclusion our results demonstrate that CY caused temporary interference of normal male reproductive system, but in high dose chronic treatment, dysfunction might be permanent.

*Key words:* cyclophosphamide, spermatogenesis, electron microscopy.

## Introduction

CY is an alkylating and antimitotic drug widely used in anticancer therapy. It is known that CY produce serious side effects especially on male reproductive system expressed as diminished sperm number and sperm abnormalities [6] . In addition CY decreases the number of mitoticaly diving germ cells (spermatogonia) [3] and damages spermatocytes. Apoptosis is one of the cell death mechanisms, responsible for the elimination of damaged cells, triggered by cytotoxic drugs as cytostatics [5]. Many studies recently have shown that the heat shock proteins play critical roles in modulating the apoptotic cascades. Two main pathways of apoptosis have been described: intrinsic and extrinsic. The intrinsic pathway involves loss of mitochondria membrane potential in response to death signals, leading to permeabilization of the outer membrane, cytochrom c and the effector caspase-3 [9]. The extrinsic pathway transduces death signals through the binding of extra cellular death ligands to their cell surface receptors and activation of procaspase 8 and caspase 3 [1]. Little is known about the action of CY on not rapidly proliferating testicular Leydig cells. We have shown prolonged inhibition of thyroxin synthesis accompanied with anormal ultrastructure of thyrocytes after acute or chronic administration of 200 mg/kg CY [7]. In the present paper we focussed on the effect of CY on mice testicular morphology and ultrastructure after acute or chronic treatment.

## Material and Methods

Forty-eight sexually mature male BALB/c mice were used. CY (Germed, Germany) was given i.p. as a single dose of 200 mg/kg b.w. or as 10 doses of 20 mg/kg each, three times weekly. Control groups were treated with saline. On the 1st, 3rd, 7th and 15th day after the last treatment the serum levels of testosterone were measured radioimmunologically. Pieces of testes were fixed in 2,5% glutaraldehyde, postfixed in $OsO_4$ and embedded in Durcupan. The observations were made on Opton 109 electron microscope.

## Results

The application of 200 mg/kg CY in acute experiment did not change the serum testosterone concentration while it was elevated significantly on the 1st, 3rd and 7th day after the 10 doses of 20 mg/kg (data not shown). In 15 days testosterone value was in the norm. On the 3rd day after application of 200mg/kg CY a strong decrease in the number of spermatogonia was registered (Fig. 1). Majority of seminiferous tubules showed normal appearance. In some the contacts between germ and Sertoli cells were disturbed. In some germ cells vacuolization of mitochondria was seen (Fig. 2A). Among spermatocytes those with normal ultrastructure were predominant. In parallel to the intact spermatids at different stages of spermiogenesis, round spermatids with disturbed acrosome formation (Fig. 2B) or disruption of acrosomal membrane in elongated spermatids (Fig. 2C) was seen. The bulk of Sertoli and Leydig cells showed normal structure. Disruption

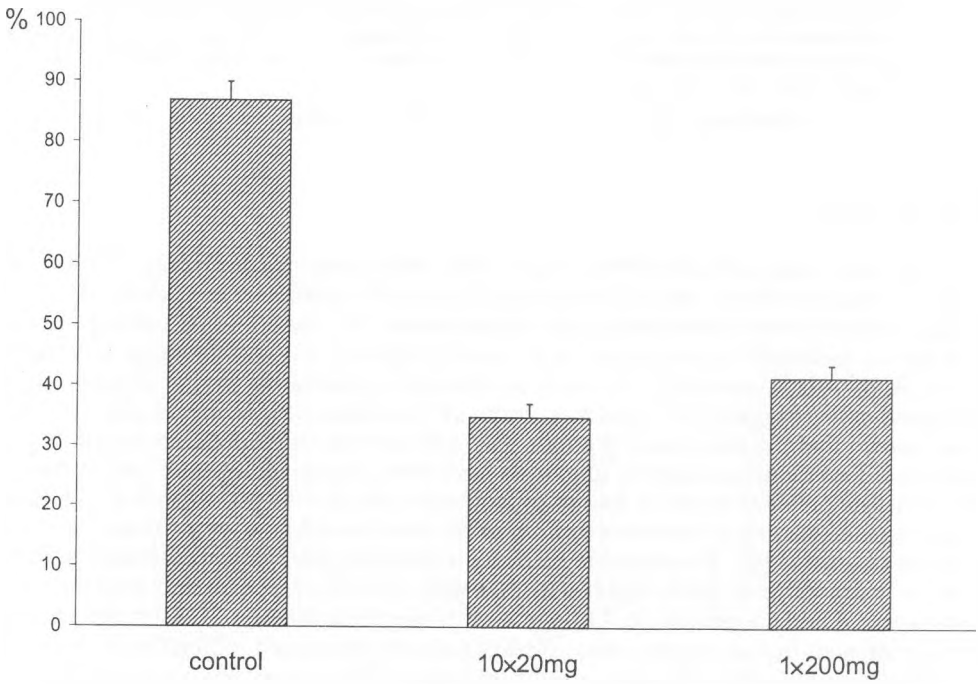


Fig. 1. Percentage of proliferating spermatogonia after acute or chronic treatment with cyclophosphamide

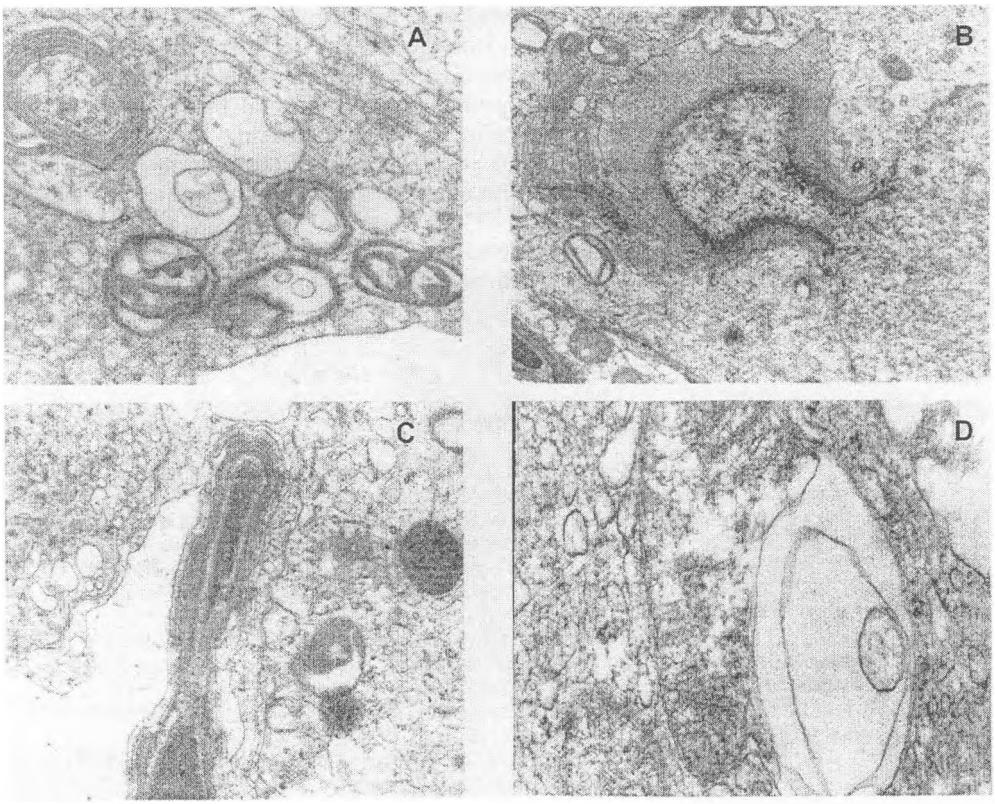


Fig. 2. Ultrastructural alterations of different testicular cell populations after treatment with cyclophpsphamide: A – Spermatogonia: vacuolization of cytoplasme and mitochondria, × 30 000; B – Round spermatid: abnormal acrosome formation. × 20 000; C – Elongated spermatid with acrosomal membrane disruption, × 12 000; D – Leydig cell: vacuolization of cytoplasme and mitochondria, × 50 000

of mitochondrial membranes and destruction of cristae were observed in some Leydig cells (Fig. 2D). On the 3rd day after injection of 10 doses 20mg/kg CY the number of spermatogonia was more decreased in comparison with the acute experiment (Fig. 1). The ultrastructure of germ and somatic cells in mouse testis at all intervals was more affected, expressed with dilatations of endoplasmic reticulum, vacuolization of the cytoplasm and abnormal acrosome formation (data not shown).

## Discussion

Our results confirm the data about the testicular failure in mice caused by the anticancer agent CY [8]. The most sensitive cells in mouse testis are proliferating spermatogonia as well as spermatids in different stages of maturation. Sertoli cells are damaged to a lesser extent while Leydig cells are less affected. It is known that spermatogonia are the most sensitive to many cytotoxic insults and therefore can serve as indicators of hazard to stem spermatogonia [4]. Treatment with 200mg/kg has shown sharp decline in total sperm counts in mouse epididymis and decrease of fertilization ability of spermatozoa [2]. Multiple CY injections resulted significant temporary elevation of serum testosterone. It is known that testosterone is a final result of variety of processes invol-

ving its synthesis, consumption and degradation. This result can be a consequence of spermatogenesis impairment and failure in metabolic control mechanisms including hypothalamo-pituitary-gonadal axis. Profound changes in mitochondrial ultrastructure in germ and some somatic testicular cells probably is a sign of intrinsic death pathway of apoptosis [1]. The permeabilization of mitochondrial membrane triggers release of pro-death molecules like cytochrome c and Smac/Diablo into the cytoplasm and leads to formation a functional apoptosomes. The next steps of signal transduction leads to cell death by activation the efector caspase-3 [9].

Our results demonstrate that CY caused temporary interference of normal male reproductive system, but in high dose treatment, dysfunction might be permanent.

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# Serotonin Producing Cells in the Small Intestine of Newborn Rats – Light Microscopic and Immunohistochemical Study

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Investigating the role of the serotonin-producing EC cells in the differentiation of the small intestine of rats we study the early neonatal period. At this stage the intestinal wall is not morphologically matured. The aim of the current study is to determine the presence and localization of the enterochromaffin EC cells in the mucosa of the small intestine of rats in the early neonatal period. Material and methods: Specimen of the small intestine of 1-day-old newborn rats is studied immunohystochemically for expression of serotonin.

The expression of serotonin in a small number of cells regularly spread along the small intestine is confirmed. The differentiated EC cells are located predominantly in the covering epithelium of the villi situated individually among the rest of the epithelium cells.

*Key words:* serotonin, small intestine, enterochromaffin cells.

## Introduction

Serotonin is one of the important neurotransmitters and signal molecules in the gastrointestinal tract [1]. Through different types of specific receptors in the smooth muscle cells, secretory cells, neural fibers it takes part in the regulation of the gastrointestinal motility, secretion and sensitivity [2]. Serotonin is produced by enterochromaffin cells that can be found in all parts of the gastrointestinal tract but their highest concentration is in its proximal sections – stomach, duodenum, jejunum.

Our immunhistochemical study of the differentiation of the enterochromaffin EC cells in the small intestine of rats focuses on the early neonatal period because in this period the intestinal wall is still morphologically immature.

The aim of the study is to determine the presence of differentiated enterochromaffin EC cells through immunohistochemical expression of their serotonin as well as their localization in the mucosa of the small intestine of rats in the early neonatal period.

## Materials and Methods

The study is carried out on fragments of the small intestine of 10 one-day-old rats. The material for the immunohistochemical study of serotonin is fixed in Buen's solution for 24 hours. Paraffin slides are investigated by the ABC method with primary antibody for MAB352 serotonin (rabbit polyclonal antibody – Chemicon USA) dilution 1:200 at 4°C for 12 hours. Serotonin is visualized as brown colored granules.

## Results

The routine study of the paraffin slides coloured with H-E staining shows that morphogenetic processes in the small intestine of the one-day-old rats are not yet complete. The intestinal villi of the newborn are high and parallel to each other. They have a thin basal part and a widening central part. The covering epithelium is columnar and single-layer. In between the villi there is a small number of shallow crypts. The muscular coat is composed of spindle shaped smooth muscle cells arranged in thick layers in different directions (Fig. 1).

The immunohistochemical study showed presence of differentiated EC cells through the expression of the serotonin they contain. The reaction is positive in a small number of cells. They are situated in the covering epithelium of the intestinal villi (Figs. 2, 3). The cells are cone-shaped. The expression of serotonin is localized in the wide basal part of the cell (Fig. 4).

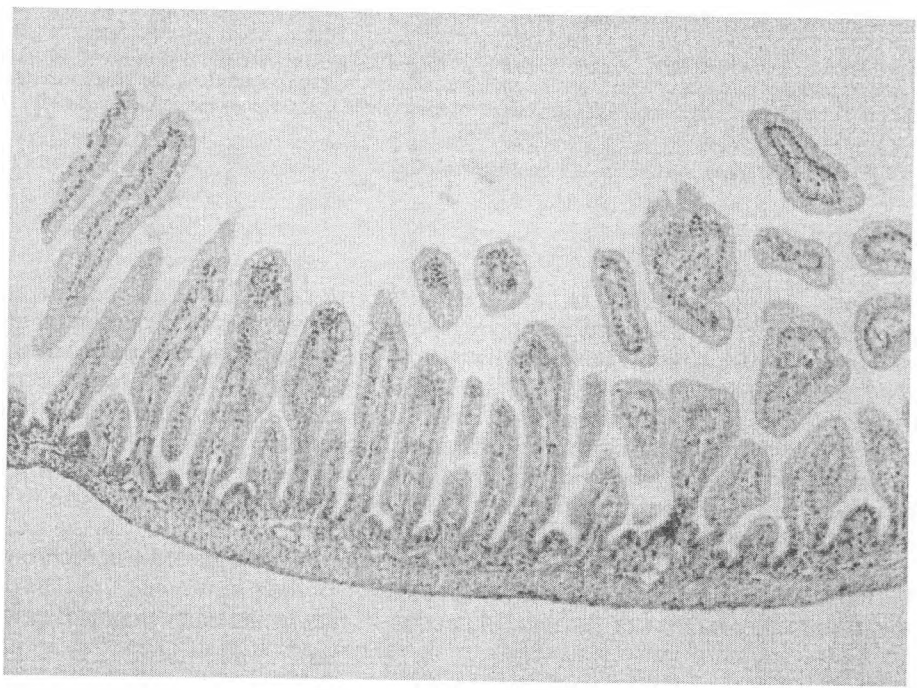

Fig. 1. Small intestine from a one-day-old rat. Parallel villi with an irregular shape. Shallow crypts of Lieberkuhn. Paraffin slides. HE, × 4.

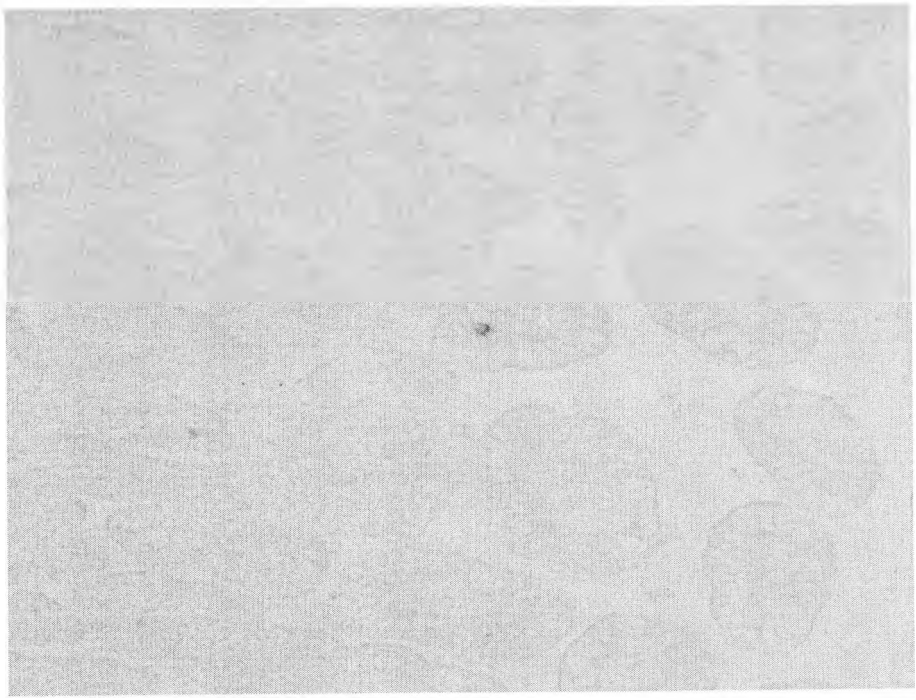

Fig. 2. Small intestine from a one-day-old rat. Transverse sections of villi. Expression of serotonin in the covering epithelium in one of the villi. Paraffin slides, × 20

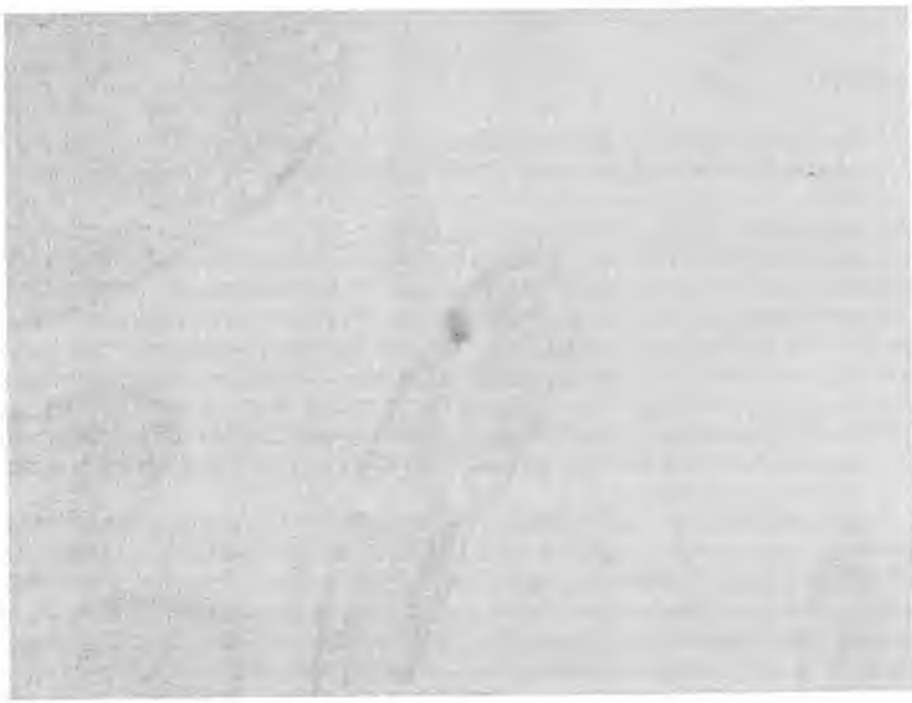

Fig. 3. Longitude slide of a villus of the small intestine of a one-day-old rat. Expression of serotonin in a cell from the covering epithelium. Paraffin slides, × 40

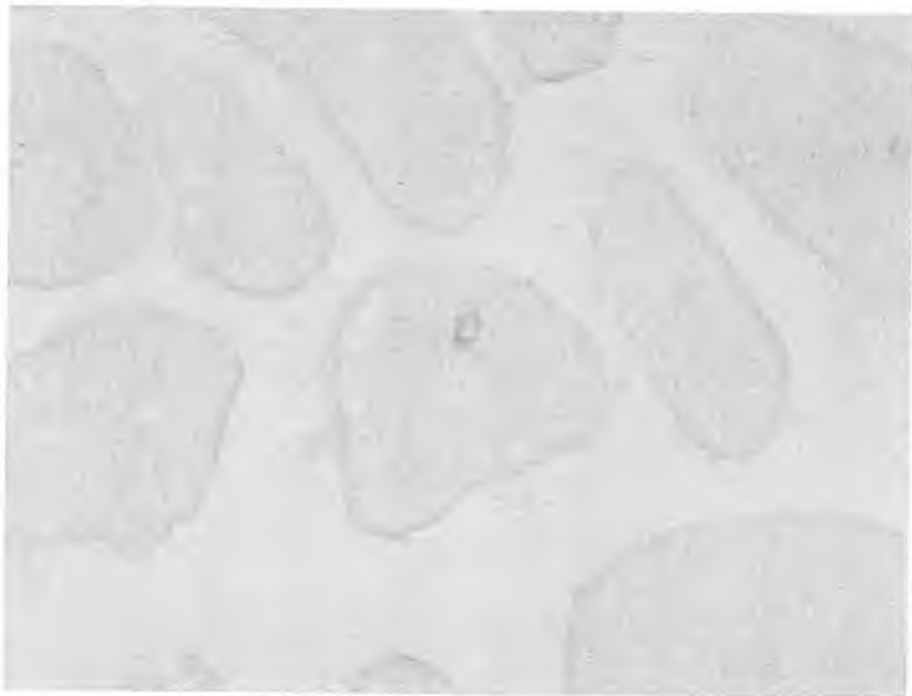

Fig. 4. Transverse section of a villus of the small intestine of a one-day-old rat. Expression of serotonin in the basal part of an EC cell. Paraffin slides, × 40

## Discussion

The adult intestinal epithelium is organized into villi, which project into the gut lumen. At the base of the villi are the crypts of Lieberkuhn which contain the stem cell compartment. That small group of proliferating undifferentiated stem cells produces the following phenotypes – absorption cells, goblet cells, enteroendocrine cells which migrate towards the nearby villi [4]. The migration of the cells along the proximo-to-disttal and crypto-to-villus gradient is influenced by alterations in the composition of the extracellular matrix via cell surface receptors of epithelial cells that interact with proteins of the extracellular matrix [3]. Studying the regenerating processes of the superficial intestinal wounds Jaladanki N. R a o et al. (1999) [5] established that the regeneration of the damaged enterocytes of the intestinal villi is carried out through a migration of differentiated intestinal crypt cells towards the villi. The factors that damage the intestinal cell differentiation lead to decreased cell migration after superficial wounding of the mucosa.

The localization of EC cells in the mature intestinal wall in adults is predominantly in the crypts. In the early neonatal period the EC cells are situated along the villi and not in the shallow crypts. This localization of the EC cells in the villi is likely connected to the intense processes of migration of enterocytes from the crypts towards the villi during this period of accelerated growth. The differentiation of these cells has probably occurred in an earlier stage of development – fatal or embryonic.

## Conclusion

Differentiated enterochromaffin EC cells are present in the small intestine of one-day-old rats. They are situated separately, in small numbers in the covering epithelium of the villi. Through an investigation of the immunohistochemical expression of the serotonin in the gastrointestinal tract from earlier stages of embryonic development the moment of differentiation of the EC cells can be established.

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# Expession of Aquaporin-2 in the Endolymphatic Sac of the Rat

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Aquaporins (AQPs) are water-specific membrane channel proteins, which have also been identified in the inner ear. AQP2 is a vasopressin-regulated member of the aquaporin gene family. Although the role of AQP2 in the kidney has been well defined, its role in hearing remains to be determined. In this study, we examined the AQP2 expression pattern in the postnatal rat endolymphatic sac (ES) using fluorescent microscopical immunostaining. AQP2 has been detected in wholemount preparations of the *saccus endolymphaticus* at cellular and subcellular level. The presence of AQP2 in the endolymphatic sac strongly suggests its functional role in the endolymph volume regulation. Future experiments may have direct implications on the pharmacological treatment of inner ear diseases associated with idiopathic endolymphatic hydrops.

*Key words:* aquaporin-2, endolymphatic sac, vasopressin, endolymph volume regulation, endolymphatic hydrops.

## Introduction

Aquaporins (AQPs) act as water-specific membrane channel proteins. They have been identified in organs with active water metabolism. The rapid movement of water across cell membranes is fundamental for the normal inner ear function. The inner ear labyrinth is divided into two distinct fluid compartments designated as perilymphatic and endolymphatic fluid spaces. The complex tubular system of the endolymphatic fluid space runs along the cochlear and vestibular parts of the inner ear. Various aquaporins have been described to be localized in the epithelia and connective tissues surrounding the endolymphatic fluid space and are implicated in the endolymph volume regulation in various anatomical sites of the inner ear [1, 2].

Of the eleven mammalian aquaporins seven have been shown to be expressed in the inner ear. These aquaporins include AQP1, AQP2, AQP3, AQP4, AQP5, AQP7 and AQP9 and demonstrate predominantly nonoverlapping cellular and subcellular distributions. All the seven aquaporins are described in the cochlea. AQP1 is found in a subset of fibrocytes in the spiral ligament near the bony capsule [2, 7], AQP2 in Reissner's membrane [5], AQP3 in a subset of fibrocytes in the spiral ligament near the basilar

membrane [2], AQP4 in the basolateral membrane of the supporting cells in the organ of Corti and inner sulcus cells [10], AQP5 in outer sulcus cells and epithelial cells of the spiral prominence [4, 6], AQP7 in Reissner's membrane and the *stria vascularis* [2] and AQP9 in Reissner's membrane and interdental cells of the spiral limbus [2].

Although the role of AQP2 in the kidney has been well defined, its role in hearing remains to be determined. AQP2, the vasopressin-regulated water channel, was initially considered to be expressed exclusively in the renal collecting duct. AQP2 has since been identified in the adult rat cochlea [5] and also reported to be expressed in the epithelium of the endolymphatic sac (ES), considered to be important in regulating the volume of the endolymph [3].

This study reports the cellular and subcellular localization of AQP2 in the endolymphatic sac of a postnatal rat.

## Materials and Methods

*Tissue preparations*

Wistar rats (postnatal day 4 (P4)) from the same litter were anaesthetized and then decapitated. Temporal bones were removed immediately and placed in cold (4°C) HHBSS (HEPES-buffered saline with Hank's balanced salt solution). *Dura mater* was gently removed from the ES and the specimen were fixed in ice-cold 4% paraformaldehyde for 30 min over ice and washed in phosphate buffered saline (PBS). The endolymphatic sacs were then removed and placed in 24-well plate with PBS.

*Immunofluorescence study*

The wholemount preparations were permeabilized in 0.5% Triton X-100 in PBS for 10 min, followed by 30 min preincubation in 4% NGS (Normal Goat Serum), 0,1% Triton X-100 in PBS. The samples were then incubated with a polyclonal rabbit anti-aquaporin-2 antibody (Chemicon) diluted 1:200 in 0,1% NGS, 0,1% Triton X-100 in PBS overnight at 4°C. Negative controls were incubated in the dilution buffer in the absence of AQP2 antibody. The reaction was visualized by fluorescein-conjugated goat anti-rabbit secondary antibody (Alexa Fluor 488, Molecular Probes), diluted 1:400 in the same dilution buffer for 60 min in the dark at room temperature. After washing with PBS the samples were counterstained with F-actin marker Alexa Fluor®568 phalloidin (Molecular Probes). The nuclei were stained with DAPI (4′-,6-diamidino-2-phenylindol, 1μg DAPI/ml PBS) for 5 min in the dark at room temperature. The wholemounts were covered with FluorSave™Reagent (Calbiochem). Microscopic analyses were made using a Zeiss Axioplan 2 epifluorescence microscope or a Zeiss 510 Meta laser scanning microscope

## Results

Immunofluorescence staining of wholemount preparations of the rat endolymphatic sac using an anti-AQP2 antibody demonstrated the presence of this water channel in the epithelial cells of the ES (Fig. 1). We used F-actin counterstaining in order to detect the cell borders.

At subcellular level AQP2 was localized in the cytoplasm and in the basolateral membrane of the endolymphatic sac cells (Fig. 2), which can be seen well on the AQP2-phalloidin merged image (Fig. 2C).

AQP2 immunoreactivity was absent in the *stria vascularis*, Reissner's membrane and the organ of Corti (data not shown).

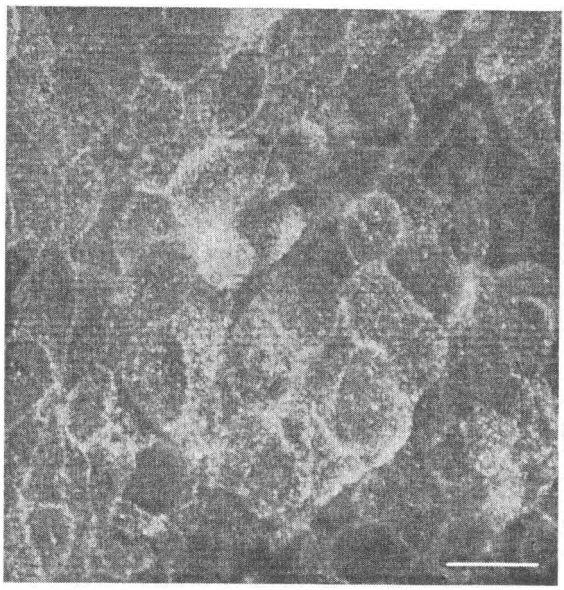

Fig. 1. Epifluorescence microscopic analysis of the AQP2 (green) expression in wholemount preparations of postnatal rat endolymphatic sac. Counterstaining of F-actin (red) serves as a marker for cellular outlines. The nuclei are labeled with DAPI (blue). Scale bar = 10 μm

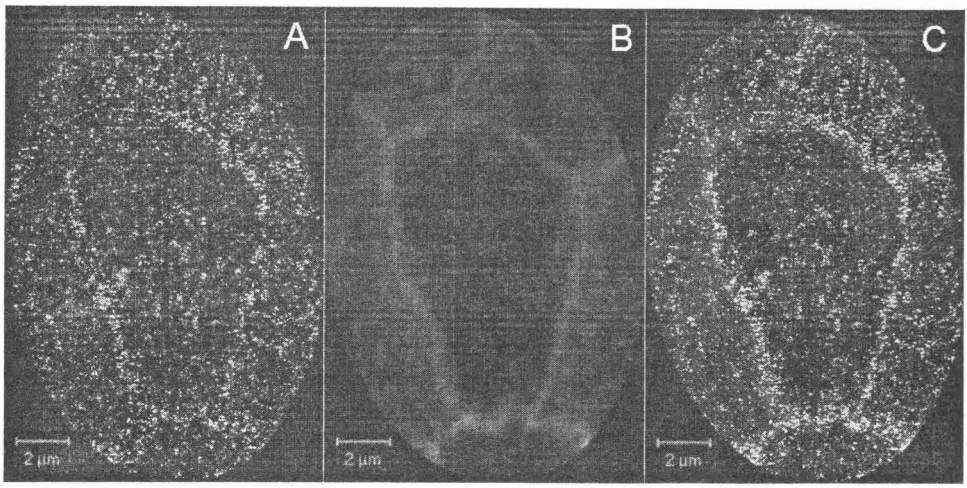


Fig. 2. Laser scanning microscopic analysis of the AQP2 (green) expression in wholemount preparations of postnatal rat endolymphatic sac. At subcellular level AQP2 (A) is localized in the cytoplasm and the cell membrane as shown in the merged image (C) of AQP2 (A) and F-actin counterstaining (B). Scale bar = 2 μm

## Discussion

This study reports the AQP2 expression pattern in the endolymphatic sac of the rat. The ES epithelium is thought to resorb the endolymph fluid. The strategic localization of AQP2 in cell types that interface with the endolymph supports its potential role in the homeostasis of the inner ear fluids, particularly as it relates to volume regulation of the endolymph. Thus, dysfunction or abnormal downregulation of AQP2 function can directly contribute to the etiology of endolymphatic hydrops, the histopathologic hallmark of Menière's disease. Menière's disease is of unknown origin. It is clinically defined by a triad of symptoms consisting of hearing loss, tinnitus and episodic vertigo. Previous studies of Menière's disease demonstrate that the vasopressin serum concentration is significantly elevated in patients with endolymphatic hydrops [9]. In the kidney vasopressin regulates water retention. This effect is mediated by the $V_2$-receptor ($V_2$-R). Upon binding of vasopressin to the receptor, AQP2 is translocated into the apical cell membrane of the collecting duct principal cells. The endolymphatic sac and other inner ear structures have been suggested as further targets for vasopressin, as based on several lines of experimental evidence, from both *in vitro* and *in vivo* studies. First, the $V_2$-R and AQP2 have been detected by various methods in the inner ear and in particular the ES [3, 5]. Second, binding of radioactive vasopressin could be demonstrated in human ES tissue. Third, vasopressin-dependent membrane turnover could be antagonized by a $V_2$-R-antagonist in rat endolymphatic sac organotypic culture preparations [3] and forth, endolymphatic hydrops could be induced by exogenous application of vasopressin in the guinea pig [3, 5, 9]. Furthermore, specific $V_2$-receptor antagonists have been shown to antagonize the effects of vasopressin *in vitro* [3] and surgically induced endolymphatic hydrops *in vivo* [8]. Future experiments may have direct implications on the pharmacological treatment of inner ear diseases associated with idiopathic endolymphatic hydrops such as Meniere's disease.

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# Expression of Small Heat Shock Proteins in Tissue Differentiation of Pig Fetuses

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Alpha crystallins are some of the major representatives of the small heat shock proteins' family. At first, they were considered organ-specific, forming the structure of the eye lens, but it is now clear that they are expressed in different tissues and organs, physiologically and under stress. Their expression is also associated with cell proliferation and differentiation.

The aim of the current study is to track out the expression of α-crystallins in some pig fetuses tissues at 20 days, 4, 6 and 8 weeks of pregnancy.

To demonstrate the presence of α-crystallins, indirect immunoperoxidase test was used, with application of obtained by us rabbit anti-alpha-crystallin serum.

The presence of the investigated small stress proteins was ascertained in differentiating tissues – mesenchyme tissue, blood tissue, muscular tissue, cartilaginous tissue, nervous tissue.

The results obtained show that the expression of alpha-crystallins in the investigated pig fetuses is realized early in the prenatal ontogenetic period, at the beginning and during tissue differentiation.

*Key words:* α-crystallins, cell differentiation, fetus, pig.

## Introduction

Under different stress conditions all living organisms react at cellular level by increasing the expression of a little number of specific genes, called heat shock genes. As a result of the activation of these genes cells synthesize stress proteins (heat shock proteins – Hsp), that are able to protect them from the effects of the altered environment conditions. It has been ascertained that stress proteins serve two major functions. First, under physiological conditions, some of them (the so called constitutive Hsp) act the role of molecular chaperones (proteins that maintain the cell homeostasis), by supporting the proper folding of other cellular proteins, protect proteins from aggregation and facilitate the renaturation of partially denaturated proteins, and second, they are synthesized in response to a number of unfavorable for the cell factors, such as high temperature, presence of free radicals, heavy metals, ethanol, ischemia, changes in pH, infectious agents, etc. Their role under such conditions is still unknown, but it is supposed to be similar with that of the constitutive Hsp.

According to their physicochemical characteristics, not by their function, they are classified into several families by their molecular weight – Hsp 110, Hsp 90, Hsp 70,

Hsp 60 and sHsp (small heat shock proteins) with molecular weight of 20-30 кD. Alpha-crystallins are referred to the family of the small heat shock proteins, and they are actually heteropolymers that consist of two types of polypeptide subunits – αA and αB, both having a molecular weight of 20-30 kD. These subunits interact with each other, forming proteins with quaternary structure, whose molecular weight varies between 400 000 and 1 100 000 kD. At first, they were considered organ-specific, as they have been isolated from the eye lens. Recently it was proved that while αA-crystalline is expressed mainly in the eye lens, αB-crystalline is found in other ocular [5] and nonucular tissues in norm [1] and pathology [3], where it probably has a protective function in the presence of stress factors.

There is no data in the specialized literature about the constitutive expression of α-crystallins during the prenatal development of the pig. Researches in that direction would help clear up the relationship between stress proteins and the processes of tissue differentiation, which suggestions are already present in some sources. According to C h a n g et al., 1995 [2], the ceramide that affects cell differentiation induces the expression of α-crystalline in NIH3T3 cells.

The aim of the current study is to trace the expression of α-crystallins in tissues of early-stage pig fetuses, when the processes of tissue differentiation are running.

## Materials and Methods

By Sephadex G-200 gel electrophoresis α-crystallins were isolated from pig eye lens extract. The column was equilibrated with the elution buffer (0,05M Tris-HCl with pH 7.5, containing 0,1M KCl, 10 mM β-mercaptoethanol) and a sample of 1 ml pig eye lens extract containing 11,8 mg/ml protein was placed upon the gel. Fractions of 2,5 ml were gathered from the elution. The presence of α-crystallins in the fractions was verified by ELISA using immune anti-alpha-crystallin serum.

Rabbit immune serum against α-crystallins was obtained by subcutaneous immunization. The first immunization was carried out using the fractions containing α-crystallins, emulgated in full adjuvant, and for the second and third – in incomplete adjuvant according to the scheme of Z i g l e r et al., 1980 [7]. Seven days after the last immunization blood sample was taken from the rabbit and the serum tested by ELISA.

The pig fetuses were separated into four groups, according to their stage of development – 20 days, 4 weeks, 6 weeks, 8 weeks. They were taken from a slaughterhouse, fixed in 10 % formaldehyde solution and embedded in paraffin. From each block 7 μm sections were cut and mounted on adhesive coated microscope slides. On each slide two sections were mounted – a control and a test one. For verifying the localization of α-crystallins, avidin-biotin-peroxidase complex method has been used in the following sequence:

– Deparaffination and dehydration of the sections in xylol and passing (them) through successively decreasing solutions of alcohol for 3 min in each, at room temperature.

– Blocking of the endogenous peroxidase with 3% $H_2O_2$ for 10 min.

– Washing of the sections in PBS and blocking of the nonspecific binding with 2,5% horse serum for 20 min at room temperature in humidity chamber.

– Incubation of test sections with polyclonal rabbit anti-alpha-crystalline immune serum (primary antibody) and the controls with PBS, overnight at 4°C in humidity chamber.

– After washing with PBS three times, the sections were incubated with biotinylated horse anti-rabbit IgG (secondary antibody) for 60 min at room temperature.

– After washing again for three times in PBS, the sections were incubated with streptavidin-HRP for 45 min at room temperature.

– The reaction was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB) as chromogen and stopped after 5 min with distilled water.

– The sections were counterstained with hematoxylin, dehydrated and cover-slipped in Canada balsam.

All steps were performed using Universal Elite ABC kit (Vectastain, Burlingame, CA 94010). For locating the presence of α-crystallins, the sections were examined with Olympus BX 40 microscope (Olympus Corp. Japan) and digital pictures were taken.

## Results and Discussion

The immunoperoxidase test revealed that α-crystallins are expressed in different tissues of the investigated fetuses.

At the age of 20 days the degree of expression in mesenchyme tissue is imperceptible. The presence of α-crystalins is observed in the developing mesenchyme tissue after the forth week. This temporary embryonic tissue is differentiated very early and consists of actively dividing cells that consequently differentiate in various directions.

The blood tissue, that is derived from the mesenchyme tissue, shows poor expression at the age of 20 days, but in the rest of the fetuses, the presence of α-crystallins in the blood cells, observed in the hemopoetic organs, as well as those in the blood vessels is marked.

In differentiating cartilaginous tissue a strong expression of α-crystallins was found. Until the age of 4 weeks the differentiation of the cartilaginous tissue has not started yet. At the age of 6 weeks expression is observed in developing cartilage, but the reaction is much stronger positive in the differentiating cartilaginous tissue at the age of 8 weeks. The cytoplasm localization of α-crystallins is clearly manifested.

The different types of muscle tissue start developing at different age. Firstly that happens with the cardiac muscle tissue, where the reaction for α-crystallins is positive in all groups. A little fainter reaction is observed at the age of 20 days, when the differentiation of the cardiac muscle cells is just beginning.

Developing skeletal muscle fibers are observed not until the age of 8 weeks, when they show strongly positive reaction. At earlier stages the differentiation of the skeletal muscle tissue has not started yet. P. T a l l o t, J. F. G r o n g n e t и J. C. D a v i d [2] have ascertained a reduction in the expression of α-crystallins in skeletal muscles of piglets over the age of 28 days, when the differentiation of the skeletal muscle fibers has been already completed.

The nervous tissue is derived from the ectodermal cells of the nervous tube, which differentiate in two directions: glioblasts (located close to the lumen of the nervous tube, which consequently differentiate to become glial cells) and neuroblasts (located peripherally, precursors of the neurons). Positive reaction for α-crystallins is observed in the cytoplasm and processes of the neuroblasts in all fetuses.

## Conclusion

On the basis of the results obtained we can conclude that the expression of the small heat shock proteins – α-crystallins is carried out early during the prenatal development. The fact that stronger expression is observed in differentiating tissues (mesenchyme, blood, cartilaginous, muscle and nervous tissues) affords an opportunity to assume that α-crystallins are connected with the processes of tissue differentiation.

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# Freezing of Preimplanted Mouse Embryos by Vitrification

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A limited number of reports are available in Bulgaria on freezing of preimplanted embryos. The aim of this study was to determine the *in vitro* survival and the viability of vitrified preimplanted mouse embryos using super-cooled liquid nitrogen. Vitrification has been the method of choice for their cryopreservation by 6.85 M glycerol. The survival rate was assessed after thawing and dilution of the cryoprotectant by 0.35 M sucrose.

Thawed embryos were cultured to the blastocyst stage. The viability was assessed by cleavage and blastocyst rates on 24 h and 48 h of *in vitro* culture respectively.

These results indicated that vitrification of mouse preimplanted embryos using 6.85 M glycerol resulted in viable expanded blastocysts and hatched blastocysts at 56 and 44 % respectively following *in vitro* embryo biotechnology culture.

*Key words:* embryobiotechnology, freezing, vitrification.

## Introduction

The biotechnology method for preimplanted embryo cryopreservation has become a useful tool for manipulating of reproductive cycle in mammals. Such interruption of embryogenesis for any period of time provides of powerful method of controlling animal reproduction. Mouse embryos are used for basic and applied research on cryobiological mechanisms. Basic and applied research has resulted in two approaches for embryo cryopreservation over the past years – convetional and vitrification respectively. During the vitrification procedures no ice forms in embryo blastomeres during freezing, storage and thawing therefore prevents lysis of the cytoplasm and membranes. On the other hand, highly concentrated cryoprotectant provokes characteristic osmotic changes in the entire volume of embryos during their dehydration and rehydration. A limited number of reports are available in Bulgaria on freezing of preimplanted embryos and most of them have described the conventional freezing procedure. Therefore systematic studies of the freezing and thawing mechanisms of the animal preimplanted embryos associated with cryoprotectants are consecutive needed.

The aim of this study was to determine the *in vitro* survival and the viability of vitrified preimplanted mouse embryos using 6.85 M glycerol and super-cooled liquid nitrogen.

## Materials and Methods

The hormonal stimulation and estrus synchronization of 7 BALBc fertile donors was done by PMSG and HCG treatment described previously. The embryos were collected at 8-12 blastomers on 3 day after mating by forceps of the oviduct. The mice were in general narcosis and the ethical principles and legal requirements for the welfare of the animals were kept at embryo recovering. The manipulating medium (MM) was Krebs-Ringer solution with 20 % fetal calf serum supplement. The freezing medium (FM) was prepared by addition of 6.85 M glycerol to MM. The embryos in a group were placed and frozen in 0.5 ml straws (IMV, Cassue, France) by direct transfer in liquid nitrogen at -196°C. They were thawed by immersing of the straws in water bath at 38°C for 1 min. For cryoprotectant diluted medium (DM) 0.35 M sucrose was added to FM. The embryos were *in vitro* cultured in 5% $CO_2$ incubator at 38°C in Dulbecko medium.

## Results and Discussion

The results of the experiments, in which collected preimplanted mouse embryos at early morula stage were vitrified then thawed and cultured *in vitro,* are summarized in Table 1.

A total of 176 mouse embryos were collected but 24 of them (Fig. 1-1) were retarded or degenerated and eliminated of the trials. The experimental 152 embryos in early morula stage of development (Fig. 1-2) with expanded blastomers and intact zona pelucida were assesed morphology normal and divided in 2 groups.

The first group of embryos was estimated as proper for the freezing program. After vitrification and following thawing procedure of 138 embryos the blastomers were contractive and zona pellucida was intact at 113 embryos (Fig. 1-3) and they were morphologically evaluated as saved. Dilution of the cryoprotectant indicates initial shrinkage and subsequent swelling to the normal isotonic volume (Fig. 1-4). A sign of zona pellucida hardening was also observed which reduced permeability of the cryoprotectant. Nevertheless the procedure decreased the survival rate and 96 embryos showed rehydration of the blastomers. The results of *in vitro* embryo culture demonstrates on 24 h and 48 h comparable saved viability and efficiency of about 56 and 44 % respectively (Fig. 1-5 and 1-6). It is also showed that gene expression and development of mouse embryos were preserved after vitrification procedure like mouse pronuclear zygotes [2].

As control group 14 of them were only cultured without applying on the freezing program. Their viability was 86% on 24 h of cultivation at blastocyst stage (Fig. 1-7) and 71% on 48 h at expanded or hatched blastocyst (Fig. 1-8) respectively.

In comparison similar results have been reported when the same protocol was applied to mouse and bovine embryos [4, 6]. On the other hand an alternative vitrification solutions and procedures have reported alternative success [1, 5, 8]. Using of ethylene glycol vitrification solution and propanediol may be especially appropriate for

Table 1. Survival and viability of vitrified preimplanted mouse embryos

| Methods | Treated embryos | Survived embryos | | Viable embryos | |
|---|---|---|---|---|---|
| | | normal after thawing (%) | normal after dilution (%) | 24 h culture *in vitro* (%) | 48 h culture *in vitro* (%) |
| Vitrification | 138 | 113 (82) | 96 (70) | 78 (56) | 61 (44) |
| Nonvitrified | 14 | – | – | 12 (86) | 10 (71) |

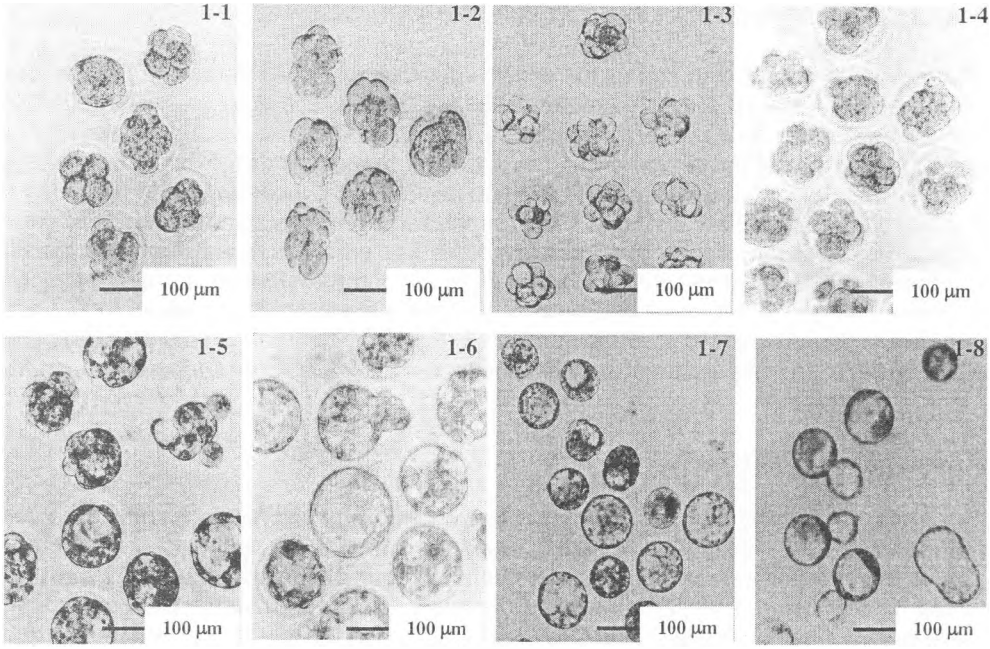


Fig. 1. Mouse preimplanted embryos exposed on vitrification and nontreated
1-1 – retarded embryos; 1-2 – normal early morula stage; 1-3 – embryos after vitrification; 1-4 – vitrified embryos after dilution; 1-5 – cultured vitrified embryos 24 h; 1-6 – cultured vitrified embryos 48 h; 1-7 – cultured nontreated embryos 24 h; 1-8 – cultured nontreated embryos 48 h

preimplanted embryos that exhibit limited permeability to glycerol [7]. Comprehensive vitrification protocol can outline its role among assisted reproductive technologies in human [3].

## Conclusion

The current research demonstrates fundamental cryobiological approach aiming prevention of the deleterious effects of chemical toxicity and intracellular freezing of the living cells. The vitrification protocol saves the survival rate and viability of mouse preimplanted embryos in authentic value. However, more precise biology experiments as well as numerous embryo species are required for determination of the optimal mammal embryos vitrification procedure.

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# Chromogranin A Expression in Diffuse Gastric Carcinoma

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Gastric carcinoma is divided into two main histological types, intestinal and diffuse type on the basis of their tendency of glandular formation. The diffuse type has been further subdivided into three types: desmoplastic, signet ring and anaplastic variant. The aim of this study was to elucidate the morphology of the acinar and ribbon-like structures, appearing in the tumor tissue of some diffuse gastric carcinoma by histochemical, immunohistochemical and electron microscopic techniques. Acinar and ribbon-like structures were found in eight cases (four desmoplastic and four mixed) from out twelve diffuse gastric carcinomas. Immunohistochemistry revealed that these structures were chromogranin A positive and they were localized in the submucosa and mainly in muscularis propria. The tumor cells in the mucosa contained PAS-positive mucus. Electron microscopically, the structures were composed of endocrine cells, which contained numerous pleomorphic or oval endocrine granules. We conclude that some diffuse gastric carcinomas acquire endocrine phenotype in the process of the gastric carcinogenesis.

*Key words:* diffuse gastric carcinoma, acinar structure, ribbon-like structure, neuroendocrine differentiation.

## Introduction

Gastric carcinoma is divided into two main histological types, intestinal and diffuse type on the basis of their tendency of glandular formation. The diffuse gastric carcinoma has been further subdivided into three variants: desmoplastic, signet ring and anaplastic variant. The intestinal type is associated with the appearance of intestinal metaplasia, whereas the origin of diffuse type gastric carcinoma is still unclear. W a l d u m et al. [9] have found ultrastructurally endocrine cells similar to enterochromaffin-like (ECL) cells in the tumor tissue of some diffuse gastric carcinoma and concluded that this carcinoma have neuroendocrine origin.

The aim of this study was to elucidate the morphology of the acinar and ribbon-like structures, appearing in tumor tissue of some diffuse gastric carcinoma by histochemical, immunohistochemical and electron microscopic techniques.

## Material and Methods

Twelve gastric carcinoma surgically resected from 2000 to 2003 years were investigated. The patients were 10 men, and 2 women. The age varied from 50 to 80 years.

*Tissue specimens*

Three or four blocks of surgically resected gastric carcinoma tissue specimens were fixed in 10% buffered formalin solution and embedded in paraffin wax. Paraffin sections were stained with HE and PAS. A histopathological diagnosis of the carcinoma was made in accordance with L a u r e n [4]. Paraffin sections (5$\mu$m thick) were processes by peroxidase-antiperoxidase technique. The primary antibody was polyclonal rabbit antibody against human chromogranin A (Code N 1535, Dako).

In seven cases electron microscope study of fresh tumor tissue was performed along with the paraffin sections.

## Results

*Histology*

All twelve gastric carcinoma had a diffuse growth. Four of them were desmoplastic, one – anaplastic and seven – mixed type. Acinar and ribbon-like structures were found in eight cases /four desmoplastic and four mixed – in desmoplastic part of the mixed variant/. They were localized in the submucosa and mainly in muscularis propria (Fig. 1).

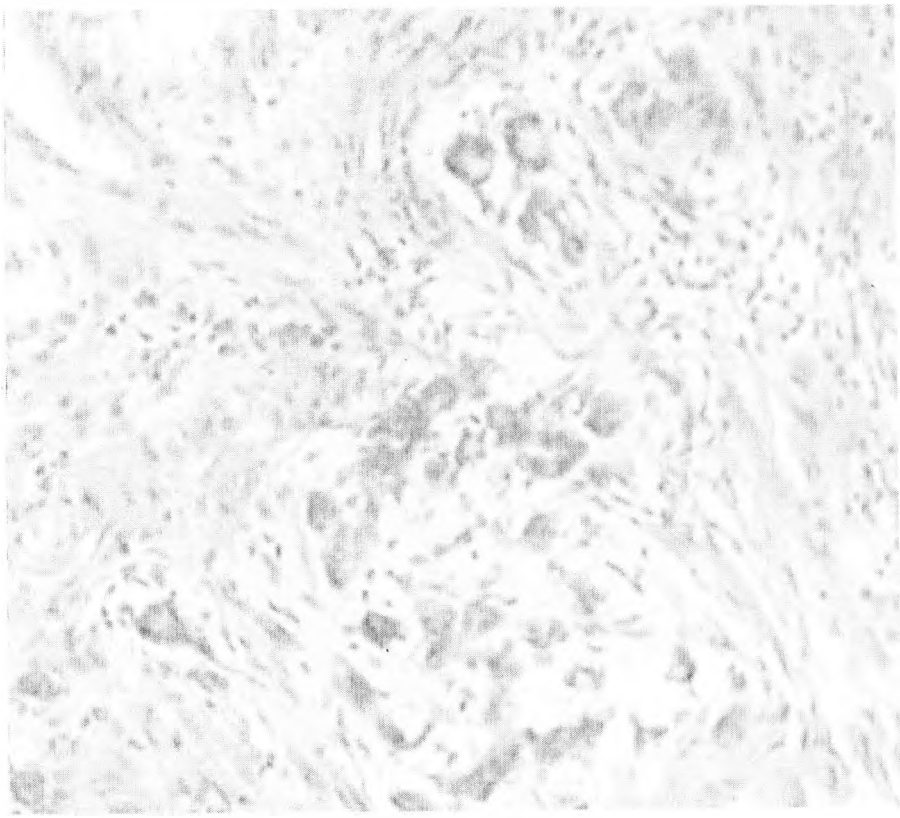

Fig. 1. Acinar structures in muscularis propria. HE, × 100

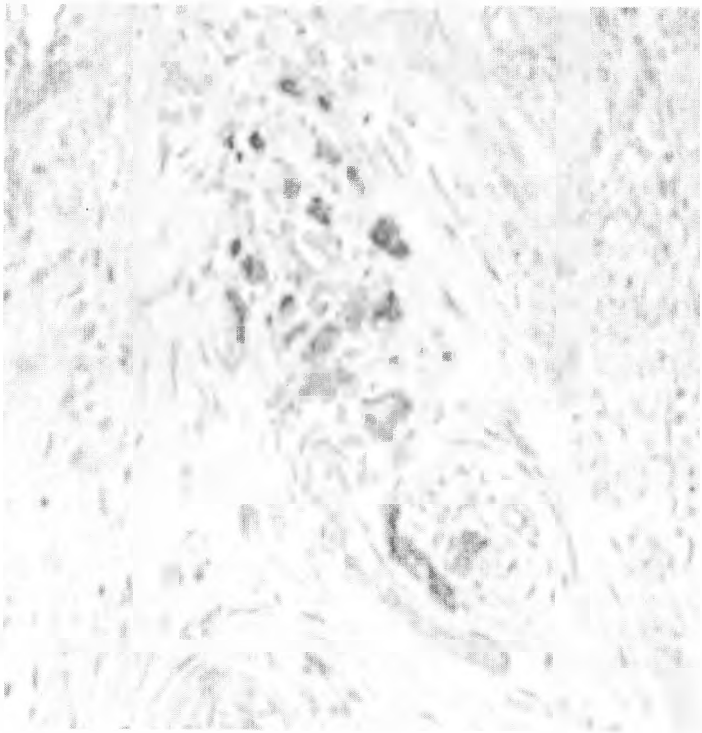

Fig. 2. Chromogranin A positive acinar structures. Immunohistochemistry, × 100

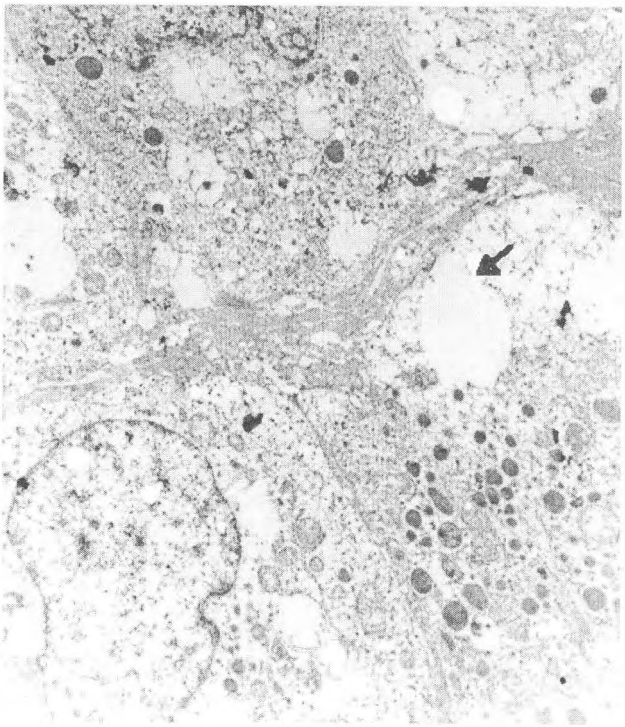

Fig. 3. The structures compose of endocrine cells with pleomorphic or oval granules and mucoid granules (↑). Electron microscopy, × 20 000

The tumor cells in the mucosa did not show glandular differentiation. They often were PAS positive. The mucus content in tumor cells localized in both submucosa and muscularis propria often was scanty and entirely absent in the acinar or ribbon-like structures.

*Light microscopic immunohistochemistry*

The most acinar or ribbon-like structures and some tumor cells were chromogranin A positive (Fig. 2).

*Electron microscopic study*

The structures were composed of endocrine cells, which contained numerous granules (Fig 3). The granules were pleomorphic or oval in shape. Some endocrine tumor cells contained electron-lucent, homogeneous mucoid granules, amongst the endocrine granules.

## Discussion

In this study, we established that the acinar and ribbon-like structures appearing in some diffuse carcinomas were neuroendocrine structures. Our results corresponded to W a l d u m et al. [9], who found ultrastructurally ECL cells in tumor tissue of some diffuse gastric carcinomas.

We found acinar or ribbon-like structures in both muscularis propria and submucosa. We did not find similar structures in the mucosa where the tumor cells were PAS positive and chromogranin A negative. Our results suggest that some tumor cells in diffuse gastric carcinoma acquire neuroendocrine differentiation in the process of the gastric carcinogenesis.

Endocrine cells have been found in all parts of the gastrointestinal tract [5, 7, 9]. Like many other cells, endocrine cells may undergo neoplastic transformation and are capable to give origin of neuroendocrine carcinoma. In well-differentiated neuroendocrine carcinoma, the endocrine cells are from one type while in poorly differentiated carcinomas they do not show to characteristic ultrastructural appearance [2]. Ultrastructurally, we found that the endocrine structures were composed mainly from well-granulated endocrine cells, which corresponded mainly to ECL cells or enterochromaffin (EC) cells. In our opinion the acinar or ribbon-like neuroendocrine structures are a way of differentiation of the tumor cells in diffuse gastric carcinoma and they do not represent dedifferentiated neuroendocrine carcinoma as considered previously [9].

We found endocrine differentiation in the desmoplastic type and in the desmoplastic part of compound type of diffuse gastric carcinoma. In the murine entero-endocrine cell line STC-1, hormone synthesis is regulated from subepitelial fibroblast cell lines by soluble factors, which inhibit the endocrine cell proliferation and modulate the expression of hormonal peptide genes [6]. It is possible that the fibroblast- or myofibroblast-like cells in sclerotic tumor tissue of diffuse gastric carcinoma do not exert similar effects.

The origin of endocrine cells in gastrointestinal carcinoma has been the subject of debate. Theories include simple entrapment of normal endocrine cells, benign proliferation of endocrine cells, and malignant transformation of two distinct stem cell lines or a single stem cell line with multiple differentiations [1, 3, 10]. The facts that: (1) the acinar or ribbon-like structures location mainly in muscularis propria; (2) the rich of granules and the correspondence of definite endocrine cell type and (3) the dual differentiation of tumor cells (amphocrine cells, which contain endocrine and mucoid granules) support the theory for a single common stem cell origin with multiple differentiations.

In conclusion, the acinar and ribbon-like structures are a manifestation of neuroendocrine differentiation in desmoplastic tumor tissue of diffuse gastric carcinoma.

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# Clinicopathological and Immunohistochemical Analysis of Gastrointestinal Neuroendocrine Neoplasms

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The distribution, the morphology and the expression of chromogranin A and synaptophysin were investigated in tumor tissue and tumor adjacent mucosa of 61 neuroendocrine neoplasms by applied of peroxidase-antiperoxidase technique. On the basis of tumor size and Ki-67, 14 (22.9%) well differentiated neuroendocrine tumors, 10 (16.3%) well differentiated neuroendocrine carcinomas and 37 (60.8%) poorly differentiated ones were identified. The distribution was as follow: stomach, 18 cases (29.5%), small intestine, 10 cases (16.3%), appendix, 16 cases (26.3%) and large intestine 17 (27.9%). The well differentiated neuroendocrine tumors (81.2%) were prevailing in the appendix while in the small intestine all neoplasms were poorly differentiated neuroendocrine carcinomas. The later were prevailing in the stomach and the large intestine, respectively 13 (72.3%) and 14 (82.4%) cases. Our results show that neuroendocrine neoplasms with a wide range of differentiation may originate in different parts of the gastrointestinal tract.

*Key words:* neuroendocrine neoplasms, chromogranin A, differentiation, distribution.

## Introduction

Neuroendocrine neoplasms comprise a family of tumors with a wide range of morphologic, functional and behavioral characteristics [3]. Their diagnostics depends on the recognition of characteristic morphologic features and on the presence of markers indicative of neuroendocrine differentiation [3, 5]. Chromogranin A and synaptophysin are the most used markers for the assessment of both normal endocrine cells and neuroendocrine neoplasms [5].

The aim of this study was to investigate the distribution, the morphology and to compare the expression of both chromogranin A and synaptophysin in tumor tissue and tumor adjacent mucosa of neuroendocrine neoplasms with a different differentiation.

## Materials and Methods

Sixty-one patients with neuroendocrine neoplasms were investigated. Clinical records for age, sex and tumor localization in the gastrointestinal tract were available. Three or four surgical specimens were collected from tumor tissue and two or three from adjacent non-tumor tissue. Paraffin sections were processed by peroxidase-antiperoxidase technique [5]. The used primary antibodies were: polyclonal rabbit anti-human

chromogranin A (Code N 1535, DAKO), polyclonal anti-synaptophysin (Syn 38, DAKO) and monoclonal mouse anti-human Ki67 Antigen, Clone MIB-1 (Code N1633, DAKO).

Every neoplasm was graded according to the World Health Organization classification [4]. The tumors were divided according to the embryonal origin of: foregut (stomach, duodenum), midgut (jejunum, ileum, appendix) and hindgut (colon and rectum) [3].

## Results

The 61 neuroendocrine neoplasms studied were divided according to their histological features into: 14 (22.9%) well differentiated neuroendocrine tumors; 10 (16.3%) well differentiated and 37 (60.8%) poorly differentiated neuroendocrine carcinomas. The neoplasms were located as follows: stomach, 18 cases (29.5%), small intestine, 10 cases (16.3%) , appendix, 16 cases (26.3%) and large intestine 17 (27.9%) . Eighteen (29.5%) cases originated in foregut, 26 (42.6%) cases in midgut and 17 (27.9%) in hindgut. The gastric neoplasms were as follows: one (5.5%) well differentiated neuroendocrine tumor, 4 (22.2%) well differentiated neuroendocrine carcinomas and 13 (72.3%) poorly differentiated neuroendocrine carcinomas. In the small intestine all neoplasms were poorly differentiated neuroendocrine carcinomas. The neoplasms of appendix were 13 (81.2%) well differentiated neuroendocrine tumors and 3 (18.8%) well differentiated neuroendocrine carcinomas. The neoplasms of large intestine were classified as: well differentiated neuroendocrine carcinomas – 3 (17.7%) cases and poorly differentiated neuroendocrine carcinomas – 14 (82.3%) cases.

*Tumor tissue.* Histologically, the well differentiated neuroendocrine tumors were strong chromogranin A positive while in the well differentiated neuroendocrine carcinomas chromogranin A was more frequently expressed in their peripheral than in the central part

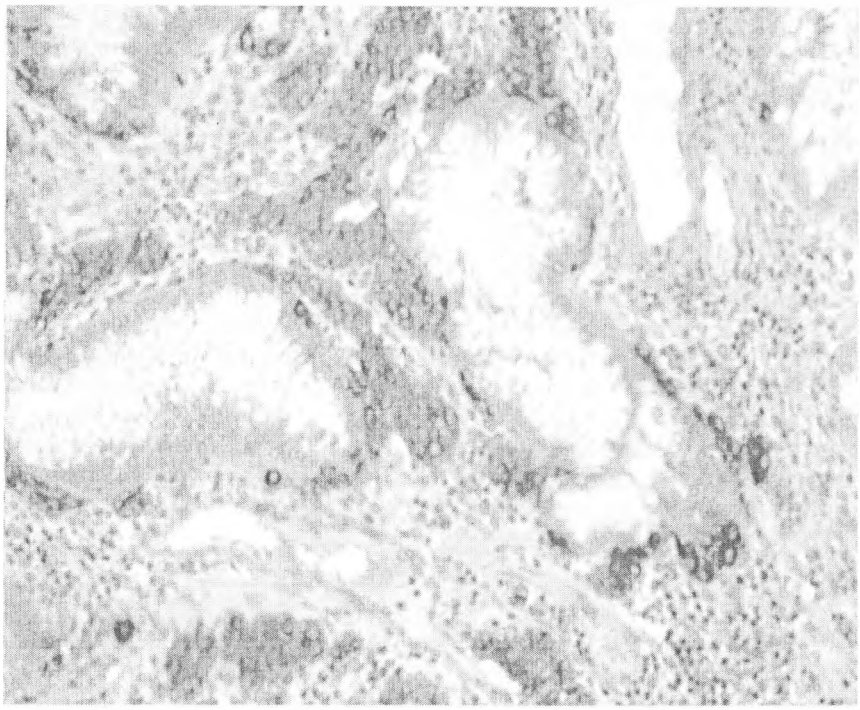

Fig. 1. Nodular hyperplasia of chromogranin A positive cells in adjacent mucosa of gastric well differentiated carcinoma. Immunohistochemistry, × 200

of tumor areas. The expression of chromogranin A was weak and localized in the peripheral part of the tumor areas in poorly differentiated neuroendocrine carcinomas. All neuroendocrine neoplasms showed weak or moderate expression of synaptophysin.

*Tumor adjacent tissue.* In the adjacent gastric mucosa in most of the cases, linear and nodular hyperplasia of chromogranin A positive cells was established (Fig. 1).

## Discussion

Our results show that the distribution of neuroendocrine neoplasms in the gastrointestinal tract is different. The neuroendocrine neoplasms originate mainly in the midgut than in the other parts of gastrointestinal tract. We established also a different differentiation of the neoplasms in midgut. The well differentiated neuroendocrine tumors were prevailing in the appendix while in the small intestine all neoplasms were poorly differentiated neuroendocrine carcinomas. Poorly differentiated neuroendocrine carcinomas were more common in both the foregut and the hindgut. Our results corresponded to others studies, which have examined both well differentiated neuroendocrine tumors and well differentiated neuroendocrine carcinoma in different parts of gastrointestinal tract [3]. In this study, we performed a comparative investigation of the neuroendocrine neoplasms with different grade of differentiations according to the site of embryonal origin of individual parts of gastrointestinal tract.

The origin and development of neuroendocrine tumors or carcinomas has been a matter of debate [5]. It has been suggested that well differentiated neuroendocrine tumors and well differentiated neuroendocrine carcinomas arise from orthotopic neuroendocrine cells of partially differentiated precursor cells while poorly differentiated neuroendocrine carcinomas originate from a stem cell [6]. Our study showed both linear and nodular endocrine cell hyperplasia next to gastric well differentiated neuroendocrine tumors and carcinomas. These results confirm the literature data [4] that the development of gastric well differentiated neuroendocrine neoplasms begins from small neuroendocrine nodules, which represent their precancerous lesions.

The origin of the poorly differentiated neuroendocrine carcinomas in the different locations and well differentiated neuroendocrine tumors and carcinomas outside the gastric mucosa is not clear. Hyperplastic lesions were not found in adjacent mucosa. So, it may be suggest that these tumors probably arise from stem cells.

We found that the expression of chromogranin A decreases in the poorly differentiated neuroendocrine carcinomas, as compared to both the well differentiated neuroendocrine tumors and neuroendocrine carcinomas. Thus, in addition of the tumor size and Ki-67 positive nuclei, the expression of chromogranin A may be used in the valuation of the differentiation of the neuroendocrine neoplasms.

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# Role of the Probiotic Biomilk on Induced Ulcerogenesis

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The effect of probiotic Biomilk in a dose of 1600 mg/kg b.w. for 30 consecutive days in Indomethacin-induced gastric ulcer in white male rats was studied. Indomethacin was subcutaneously injected in a dose of 20 mg/kg b. w. Malondialdehyde (MDA) was examined in plasma, liver and brain homogenates. Indomethacin caused the formation of numerous lesions and haemorrhages and enhanced MDA level in plasma, liver and brain tissues. The probiotic Biomilk restricted the lipid peroxidation and protected the gastric mucosa from Indomethacin ulcerogenic action. Thus it could represent a non-pharmacological alternative for the prevention and treatment of gastric ulcer in man.

*Key words:* Probiotic Biomilk, Indomethacin, malondialdehyde, gastric mucosa protection, histology.

## Introduction

A series of gastrointestinal diseases, infections are favourably influenced by the products containing original *Lactobacillus Bulgaricus* (1,4). The probiotic Biomilk is a dry lactic-acid, low-lactose product containing liver cells of *Lactobacillus Bulgaricus*.

The purpose of the present study is to establish the effect of the probiotic Biomilk in a model of Indomethacin-induced ulcerogenesis in rats.

## Material and Methods

The study covered 28 white male Wistar rats weighing 180-200 g. The experimental design is demonstrated in Table 1.

Indomethacin was chosen as an ulcerogenic agent because of its well-known ulcer-inducing and pro-oxidative action. The stomachs were taken for examination 4 hours after Indomethacin administration under ethereal narcosis. The number and surface of the mucous defects were read by means of macroscopic inspection of the stomachs. Histological sections were stained with haematoxilin/eosin (HE) and with PAS.

Table 1. Study design

| Group | Treatment | Dose, ml/kg | Duration | Number of animals |
|---|---|---|---|---|
| I | Distilled water | 10 | 30 days | 7 |
| II | Biomilk | 1600 | 30 days | 7 |
| III | Biomilk + Indomethacin | 1600<br>20 | 30 days<br>one day | 7 |
| IV | Distilled water + Indomethacin | 10<br>20 | 30 days<br>one day | 7 |

Malondialdehyde (MDA) as a marker of lipid peroxidation was estimated after the method of Porter et al. (1976) in blood plasma, liver and brain homogenates. The statistical analysis of the data was carried out after ANOVA method.

## Results

*Histological examination*

Probiotic Biomilk applied alone increases gastric mucus secretion in comparison with distilled water (Fig. 1). The application of Indomethacin causes erosions covering 2/3 or almost the whole thickness of the gastric mucosa with hemorrhages. There is no cellular infiltration at all. These defects are characterized with a greater depth and a larger surface of dissemination (Fig. 2). In the surrounding zones there is hyperemia in the vessels. The amount of mucilaginous substances is strongly reduced. In the animals treated with Biomilk in a dose of 1600 mg/kg b. w.) and Indomethacin the erosions in the gastric mucosa are more superficial and smaller in size (Fig. 3). The mucilaginous secretion in the neighbouring areas is slightly increased.

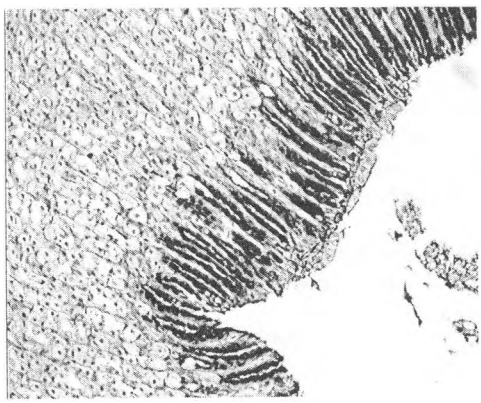

Fig. 1. Probiotic Biomilk in dose 1600 mg/kg applied alone increases gastric mucus secretion. PAS reaction, Mc Manus (original magnification 10×20)

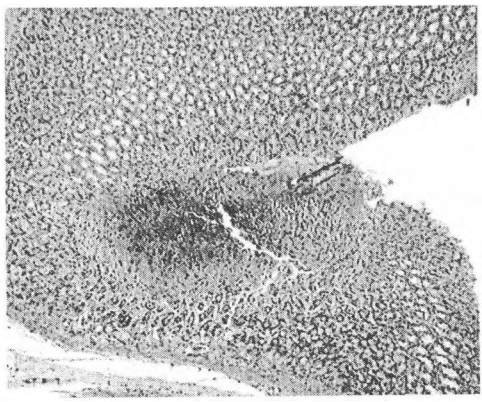

Fig. 2. Indomethacin causes erosions covering 2/3 or almost the whole thickness of the gastric mucosa with hemorrhages; the amount of mucilaginous substances is strongly reduced. PAS reaction (original magnification 10×10)

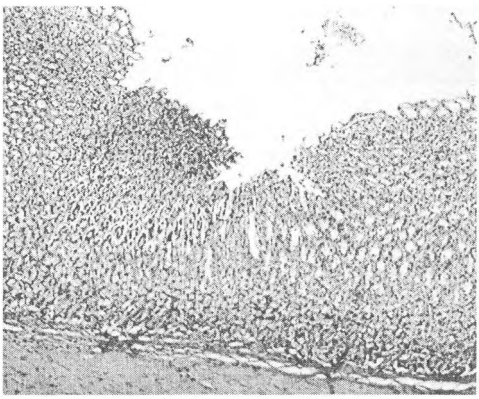

Fig. 3. The erosions in the gastric mucosa are superficial and smaller in size Biomilk in dose 1600 mg/kg + Indomethacin. Staining with HE (original magnification 10×10)

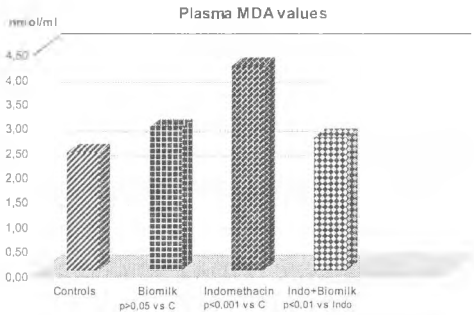


Fig. 4. Plasma MDA values

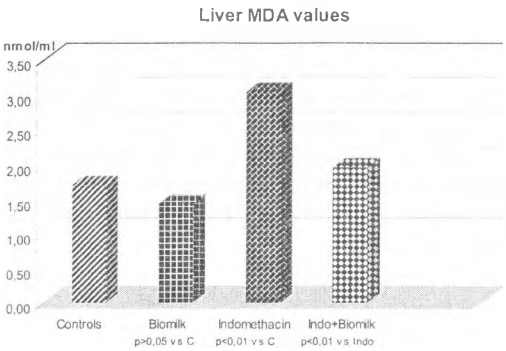


Fig. 5. Liver MDA values

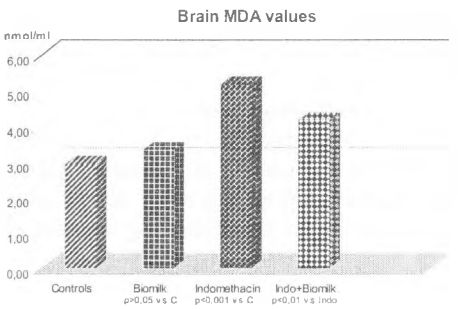


Fig. 6. Brain MDA values

*MDA in plasma, liver and brain homogenates*

MDA in plasma

In the animals treated Biomilk only, MDA plasma values did not differ significantly from these of the controls ($p>0,05$). Indomethacin treatment caused a significant ($p<0,001$) MDA increase in plasma as compared with that of the controls. MDA in plasma reduced in the group with Biomilk+Indomethacin (Fig. 4).

MDA in liver homogenate

In the animals treated with Biomilk only, MDA values in a homogenate of liver tissue did not differ significantly from these of the controls ($p>0,05$). Indomethacin treatment caused a significant ($p<0,01$) MDA levels increase in the liver homogenate as compared with that of the controls. In the liver homogenate, MDA values reduced significantly ($p<0,01$) in the animals of the group with Biostim LBS+Indomethacin when compared with these with Indomethacin only (Fig. 5).

MDA in brain homogenate

In the animals treated with Biomilk or *Lactobacillus Bulgaricus* only, MDA values in a homogenate from brain tissue did not differ significantly from these of the controls (p>0,05). Indomethacin caused a significant ($p<0,001$) MDA level increase in the brain homogenate as compared with that of the controls. In the brain homogenate, MDA

values reduced significantly ($p<0,01$) in the animals of the group with Biomilk + Indomethacin as compared with these of the group on Indomethacin treatment only (Fig. 6).

## Discussion

Indomethacin affects the mucilaginous and bicarbonate secretion of the mucosa and induces blood flow disorders [6]. Preliminary treatment with Biomilk leads to protection of the gastric mucosa. The authors relate this with the increased mucilage synthesis; with enhanced prostaglandin E2 production which is gastroprotective; with improved microflora and microecology; with receptor competition and immunomodulation in the intestinal lymphoid tissue [2,3].

Indomethacin causes MDA elevation in plasma, liver and brain homogenates that allows the assumption of oxidative stress involvement. In the group with Biomilk + Indomethacin these parameters were favourably influenced upon. One of the mechanisms of ulcerogenic action of the Indomethacin is the activation of the processes of lipid peroxidation in the tissues. It is probably that the restriction of the lipid peroxication is an element of the protective action of this probiotic. The antioxidant activity is likely determined by the number of live cells of *Lactobacillus Bulgaricus* [7].

Based on the data presented above the conclusion can be drawn that the chronic treatment with Biomilk containing *Lactobacillus Bulgaricus* posseses a clinical effectiveness concerning the Indomethacin-induced pathology of the gastric mucosa. The probiotic Biomilk could represent a non-pharmacological alternative for the prevention and treatment of ulcer disease in man.

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# Fluorescent Histochemical Localization of Dipeptidyl Peptidase IV Activity

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A novel fluorogenic substrate for dipeptidyl peptidase IV (DPP IV) – Ala-Pro-4-hydrazido-N-(4′-*iso*-butoxy)phenyl-1,8-naphthalimide (Ala-Pro-BuOPHNI) and its application for the localization of the enzyme in tissue sections of rat organs are presented. The substrate permits a precise detection of DPP IV activity *in situ* and might find various applications in the studies of the diseases for which DPP IV is a marker enzyme.

*Key words:* Dipeptidyl peptidase IV, 1,8-Naphthalimide derivatives, Fluorescent histochemical methods, Enzyme histochemistry.

## Introduction

Dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) is a membrane-bound serine protease hydrolyzing Xaa-Pro dipeptides from the $NH_2$-terminal of oligopeptides and synthetic substrates at pH optimum 7.8. The enzyme has multiple functions. It participates in the digestion of different proteins, modulates the activities of biologically active peptides and acts as a co-stimulatory protein, adhesion molecule or receptor molecule [4]. DPP IV is accepted as a diagnostic marker for thyroid carcinoma and regarded as a supporting marker for the assessment of the severity of various diseases [1].

In the present study we present a novel fluorogenic substrate for DPP IV – Ala-Pro-4-hydrazido-N-(4′-*iso*butoxy)phenyl-1,8-naphthalimide (Ala-Pro-BuOPHNI) and its application for the localization of the enzyme in tissue sections of rat organs. The substrate permits a precise detection of DPP IV activity *in situ*.

## Materials and Methods

*DPP IV substrate and inhibitor.* The substrate Ala-Pro-4-hydrazido-N-(4′-*iso*butoxy) phenyl-1,8-naphthalimide (Ala-Pro-BuOPHNI) was synthesized from 4-hydrazino-N-(4′-*iso*-butoxy)phenyl-1,8-naphthalimide (BuOPHNI) and Boc-Ala-Pro-OH (Bachem)

after TBTU-method in dimethylformamide with diisopropylethylamine as HCl-acceptor, isolated customarily and Boc-protection cleaved by HCl /dioxane [3]. DPP IV inhibitor N-(H-Phe-Pro-)-O-(4-nitrobenzoyl)hydroxylamine hydrochloride (Phe-Pro-NHONb) was synthesized according to Demuth [2].

*Animals, tissue treatment and incubation media.* Mature Wistar rats of both sexes were decapitated, peaces of different organs were extracted and frozen in liquid nitrogen. Sections (10 μm) were cut on cryocut Reichert-Jung (FRG) at –25 °C, air-dried and covered by celloidin (1% celloidin in ethanol:chloroform:aceton 3:3:4) for a minute at room temperature. The sections were incubated in 0.1 M phosphate buffer (pH 7.8) containing 0.5 mM substrate (Ala-Pro-BuOPHNI) and 2.5 mM 2,3,4-tri-methoxy benzaldehyde (TMBA) for 30-90 min at 37 °C, then fixed in 4 % neutral formalin, stained with hematoxyline and embedded in glycerol/jelly.

*Inhibitor controls.* Sections, incubated in 0.5 mM inhibitor Phe-Pro-NHONb in phosphate buffer, pH 7.8 for 45 min at room temperature were transferred to a full substrate medium, supplied with 0.5 mM inhibitor for an hour at 37°C and then treated as above.

All the preparations were studied under light and fluorescent microscopes and photographed on Konica 200 ASA colourful films.

## Results and Discussion

DPP IV substrate, developed by us (Ala-Pro-BuOPHNI) was used according to the principle in Scheme 1. The enzyme hydrolysis releases the fluorochrome BuOPHNI, which reacts immediately with the aromatic aldehyde TMBA to form an insoluble fluorescing hydrazone. The last compound precipitates on the sites of the enzyme activity and marks them by a bright red fluorescence. The substrate proposed here represents a modification of the already reported by us fluorogenic DPP IV substrate Gly-Pro-4-hydrazido-N-hexyl-1,8-naphthalimide (Gly-Pro-HHNI) [3]. Introduction of the second aromatic ring in the substrate molecule (see Scheme 1) provides a substantial decrease of the solubility of the primary reaction product (BuOPHNI is more insoluble than HHNI), which allows an increased precision of the enzyme localization. On the other hand, the elongated conjugated system provides a very bright fluorescence and the possibility to use different aromatic aldehydes as visualization agents. The histochemical method, presented here allowed us to achieve precise detection of the enzyme activity in all its known locations in tissue sections of rat organs at the lack of background noise in a short incubation time (Fig. 2). The specificity of the reaction was proved by the use of the irreversible DPP IV inhibitor Phe-Pro-NHONb. This inhibitor abolished almost fully the enzyme activity. Residual activity was observed only in the brush-borders of the small intestinal entherocytes, signified by isolated fluorescent granules (Fig. 2 – A1, B1).

Many proteases are now regarded as possible markers for the onset and progression of various malignant, immune, neurodegenerative and other diseases. Thus, development of fluorogenic substrates for the *in situ* analysis of proteolytic enzymes is considered an important tool for the study of those enzymes in normal and pathologically altered tissues. In this respect, our novel fluorogenic DPP IV substrate can find a variety of applications in diagnosis, response to therapy and prognosis of the diseases for which DPP IV is a marker enzyme.

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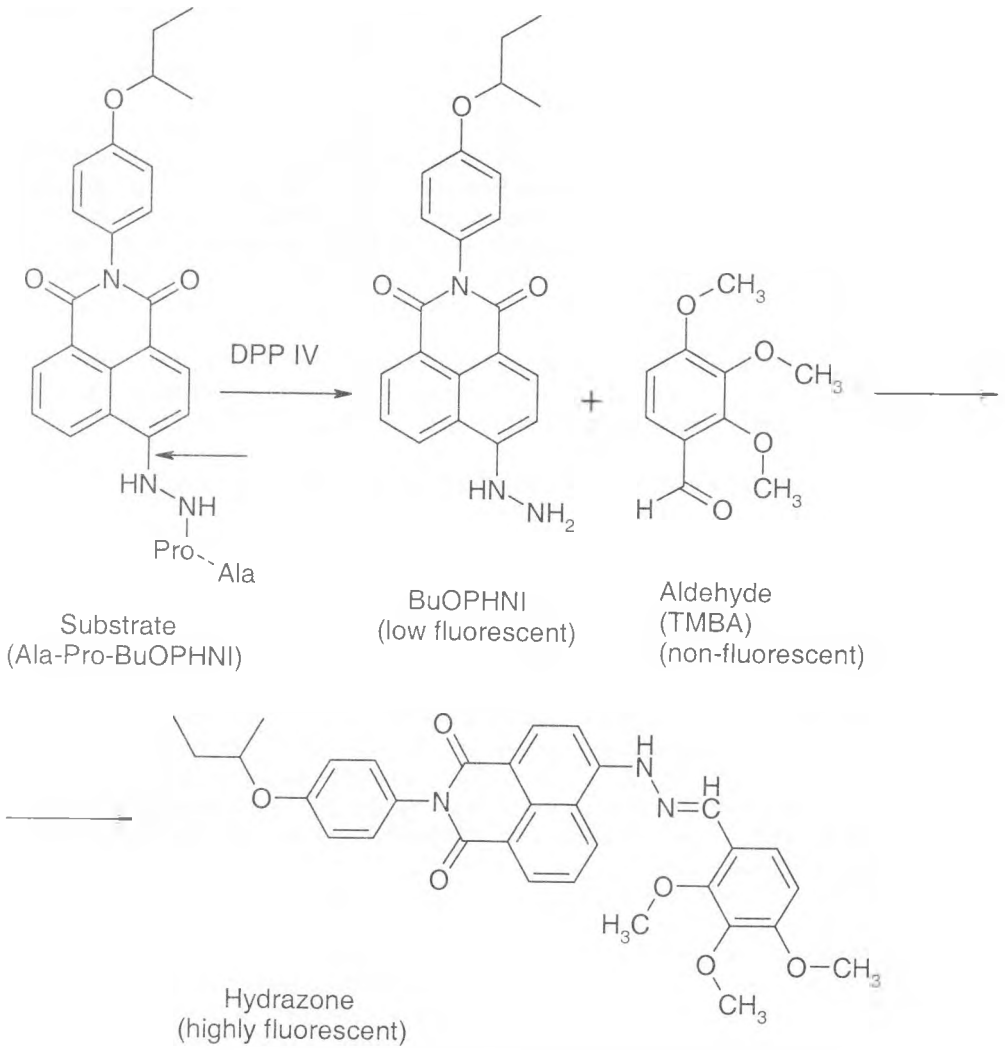


Fig. 1. Principle of the visualization of DPP IV activity

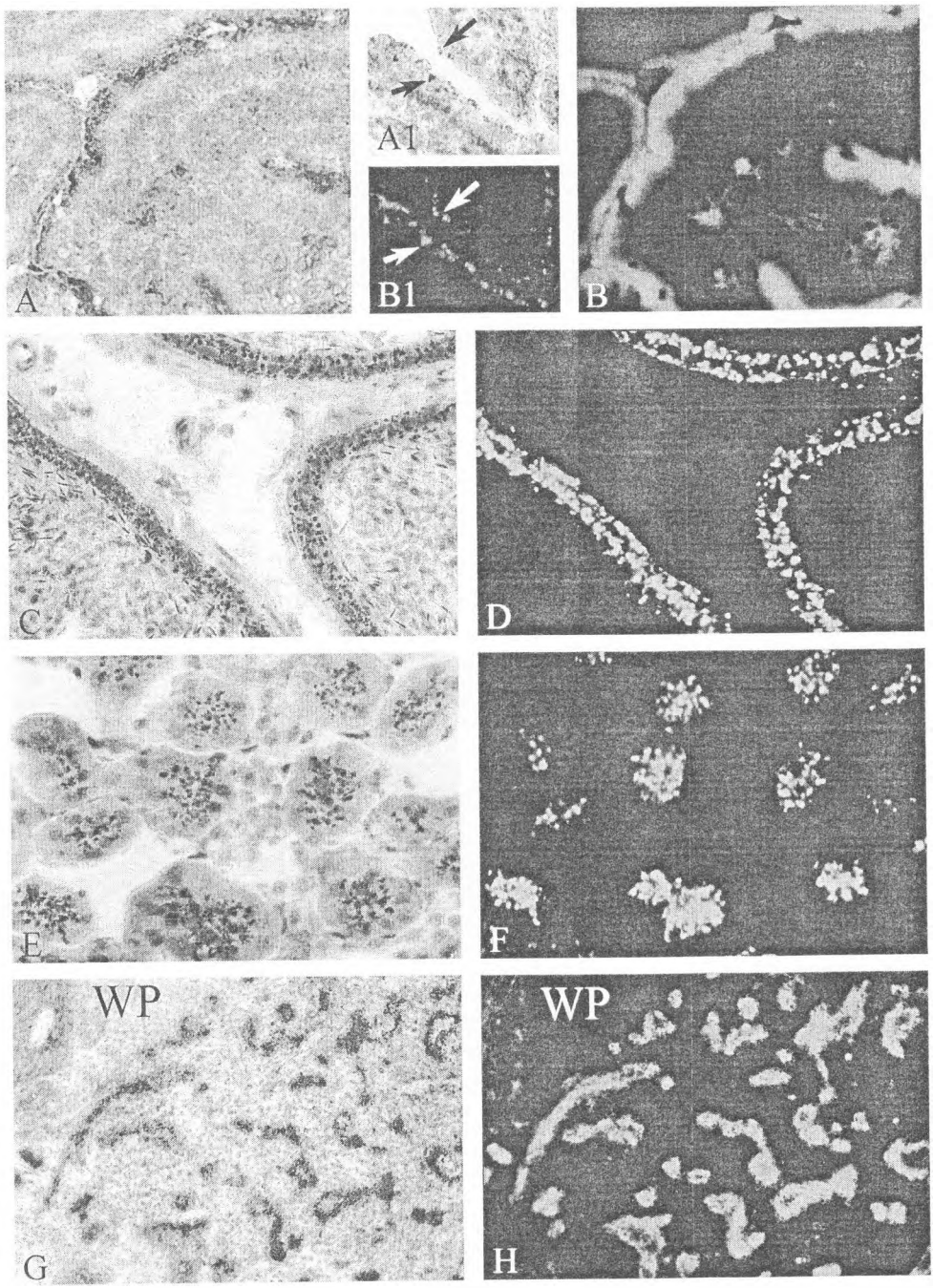


Fig. 2. DPP IV activity by the substrate Ala-Pro-BuOPHNI and TMBA in: A, B – rat intestine: reaction in the brush borders of the enterocytes, × 400; A1, B1 – inhibitor experiment in rat small intestine: a considerably decreased amount of the fluorescent reaction product in the brush border area of enterocytes (arrows), × 400; C, D – epididymis: epithelial cells of the channels are filled with the fluorescent deposits, × 400; E, F – kidney cortex: DPP IV reaction in the apical pole of the epithelial cells of convoluted tubules, × 400; G, H – rat spleen: DPP IV reaction in the red pulp veins and sinusoids; no reaction in white pulp (WP), × 160. A, A1, C, E, G – light microscopy; B, B1, D, F, H – fluorescent microscopy

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# Localisation of the Enzyme Activity of the Tissue Acid and Alkaline Phosphatases in the Prostatical Gland of Tomcat

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The acid and the alkaline phosphatases are hydrolitical enzymes whose abnormal tissue localization is indicator for prostatical pathology. Prostatical glands of seven sexually matured and healthy clinically tomcats were investigated. The animals were euthanised. Proving of the acid phosphatase by Gomori: we used frozen slices with thickness 10 μm, fixed in neutral formalin, they were put on objective slides. The slices were translocated in incubating medium and treated with Amonium sulfide, which stained them dark brown. They were included in glycerine-gelatine. Proving of the alkaline phosphatase by Gomori: the same frozen slices were transferred in incubating medium, treated with 2% cobaltous chlorate and translocated in Amonium sulfide till they get black colour. The most considerable localization of the tissue acidphosphatase activity was observed by a lightmicroscopical investigation in the apical parts of the prostatical epithelial cells, in the lumen of the glandular alveols, the basal membranes and the subcapsular zone of the gland. A remarkable expression of the tissue alkaline phosphatase in the normal prostatical gland of the tomcat was marked hystochemically in the basals parts of the epithelial cells, in the surrounding zones of the prostatical ductules, the perivascular regions and in the glandular capsule.

*Key words:* alkaline phophatase, acid phosphatase, prostata, tomcat.

## Introduction

The acid phosphatase is enzyme which catalyses in acid medium and is synthesized in the liver, the spleen, the bone marrow and in the prostatical gland. The high levels of this enzyme are indicators for prostaical pathology (cancer).

The alkaline phosphatase is also a hydrolitical enzyme, which is synthesized in the liver, the spleen, bones and in the placenta and presents normally in high levels in the growing bones and in the bile.

There are four izoenzymes of the acid phosphatase in the man. The lysosomal and the erytrocyte forms are widespread and are expressed in many cells, while the macrophagial and prostatical ones are with limited expression.

The changes of the activity of the tissue prostatical acidphosphatase are used like diagnostical and prognostical markers for the prostatical cancerogenesis [9].

There isn't any correlation between the activity of the acid phosphatase, the age of the specimen, the degree of the disease and of the prostatical cancer [3].

The izoenzymes of the acid phosphatase, in the prostatical gland of the man, vary in canceromatoses lesions, compared with the serum acid phosphatase, where it stays stable. These izoenzymes are present in the fetal and in the malignant prostate and are absent in the normal prostatical gland [11].

The ratio between the prostatspecifical antigen and the prostatical acid phosphatase is a remarkable prognostic indicator about the prostatical cancerogenesis of the man [12].

There is a decreased tissue capacity of the producing of the acid phosphatase in many malignant prostatical lesions, compared with the normal glands [7].

A tissue acid phosphatase in benignant prostatical glands is distributed in the columnar epithelial secretory cells, compared with the basal ones, where it misses [15].

The prostatical acid phosphatase is a tissue specific enzyme, whose localization is in the epithelium of the tubuloalveolar parenchyma of the ventral prostatical gland of the rat [14].

The activity of the lysosomal acid phosphatase increases after castration which is proved by an enzymohystochemical observation, compared with the secretory one, which decreases after androgenical depriviation [13].

The acid phosphatase in the complex of Golgi in the prostatical gland of the rat is different from those in the lysosomes [8].

The epithelial cells with osteoinductive potential (transitional epithelium) show a high activity of alkaline phosphatase, while these without potential- a low expression of this enzyme. There isn't such enzymohystochemical differences with the activity of the acid phosphatase in these two kinds of cells [16].

The reduction of the level of the alkaline phosphatase, in the monitoring of the disseminated prostatical cancer, is a biochemical marker for a positive prognosis [10].

In the male genital organs of the dog is found that the activity of the alkaline phosphatase is highest in the epididymis, and lower in the testis and in the prostata. The result proves, that the biggest fraction of this enzyme is secreted by the epididymis and not by the prostata [5].

The vesiculous and the bulbouretral glands show a high expression of the alkaline and acid phosphatases in the epithelium, except the alkaline one, which is expressed only in the stroma of the bulbourethral glands.There isn't any phosphatase activity in the prostatical epithelium and in the fluid [6].

There is a different localization of the alkaline and the acid phosphatases in the male genital organs (rat, rabbit, guinea pig, cat and dog ). The alkaline one is secretory, stromal, nuclear, and vascular, while the acid one is secretory and nuclear [1].

According to the other authors the reduced levels of the both enzymes are observed in malignant and benignant prostatical tissues [4].

The incomplete literary data about the localization of the enzyme expression of the prostatical tissue acid and alkaline phosphatases were a reasonable motif to make the enzymohystochemical investigation.

Our aim was to find separated tissue regions in the normal prostata of the tomcat where the activity of the investigated hydrolases is observed.

## Material and Methods

Proving of the acid phosphatase by Gomori: Prostatical glands of seven sexually matured and healthy clinically male European shorthair cats (aged between one and two years with weight 2.8 kg to 4 kg) were investigated. The animals were euthanised with 200 mg Thiopental (Biochemie, Austria) IV.

Frozen slices with thickness 10μm were used. They were fixed in 10% neutral formalin for 24 h at temperature 0-4 C° and they were put on objective slides after that.

The slices were translocated in incubating medium and were put in a termostat at 37 °C for 3 h. They were edulcorated with a distilled water, treated with Amonium sulfide for one minute which stained them dark brown and included in glycerine-gelatine [2].

Proving of the alkaline phosphatase by Gomori: The same frozen slices were transferred in incubating medium and put in a termostat at temperature 37°C for 2 h, after that they were treated with 2% Cobaltous chlorate for three minutes and translocated in Amonium sulfide till they get black [2]. The localization of the enzyme expression of the tissue alkaline and acid phosphatases were proved lightmycroscopically.

## Results and Discussion

The most considerable localization of the tissue acid phosphatase activity was found in the apical parts of the prostatical epithelial cells and in the lumen of the glandular alveols (Fig. 1). These results add the attitude [11, 14, 15] about the localization of this tissue enzyme in the prostatical gland of the man and of the rat. Probably it is a secretory form of the acid phosphatase, which is with apical and luminal fluidal expression. That confirms its presence in the ventral prostatical gland of the rat [13], the dog [1] and the man [12].

The apical localization of the acid phosphatase activity in the prostatical epithelium of the tomcat gives us a reason to suppose about its active role in the formation and in the secretion of the prostatical fluid.

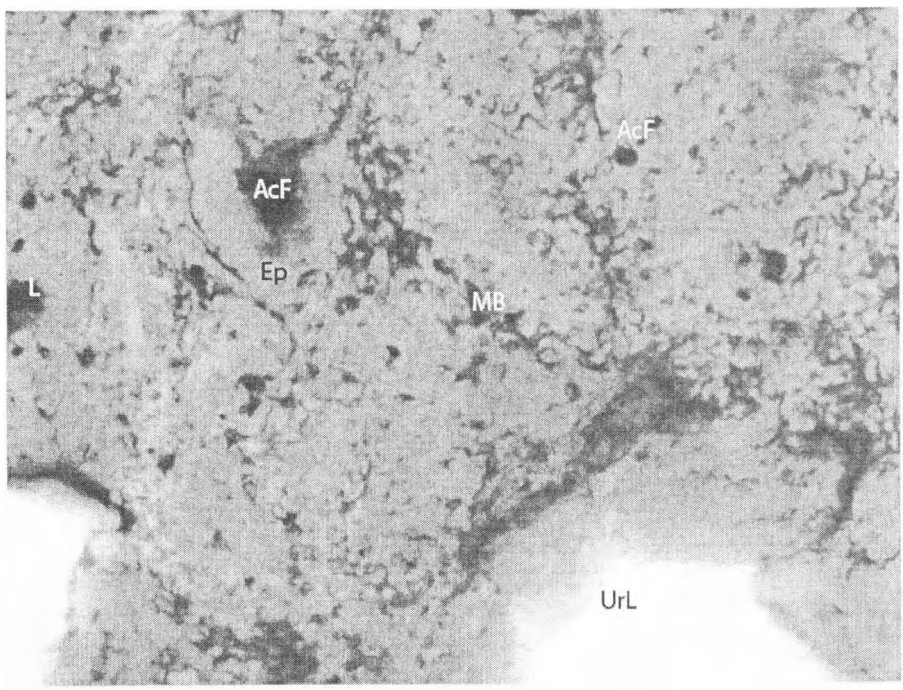


Fig. 1. Localization of the acidphosphatase activity (AcF) in the apical parts of the prostatical epithelial cells (Ep), in the basal membranes (MB) and in the alveolar lumen(L). Urethral lumen (UrL). (Bar=10 μm)

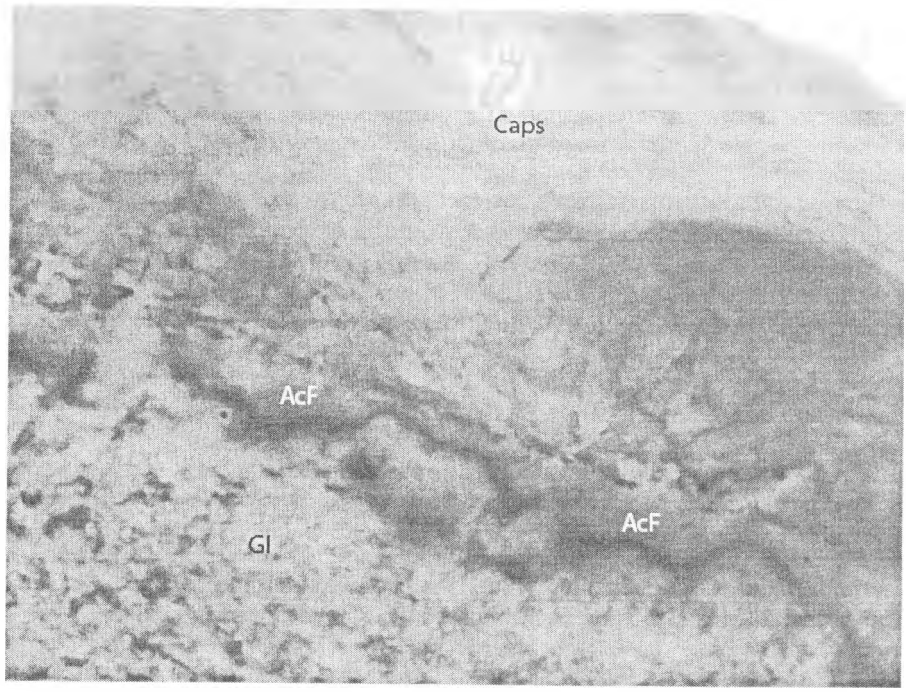


Fig. 2. Localization of the acidphosphatase activity (AcF) in the basal parts of the prostata (Caps) and in the glandular interstitium (INT). (Bar=10 μm)

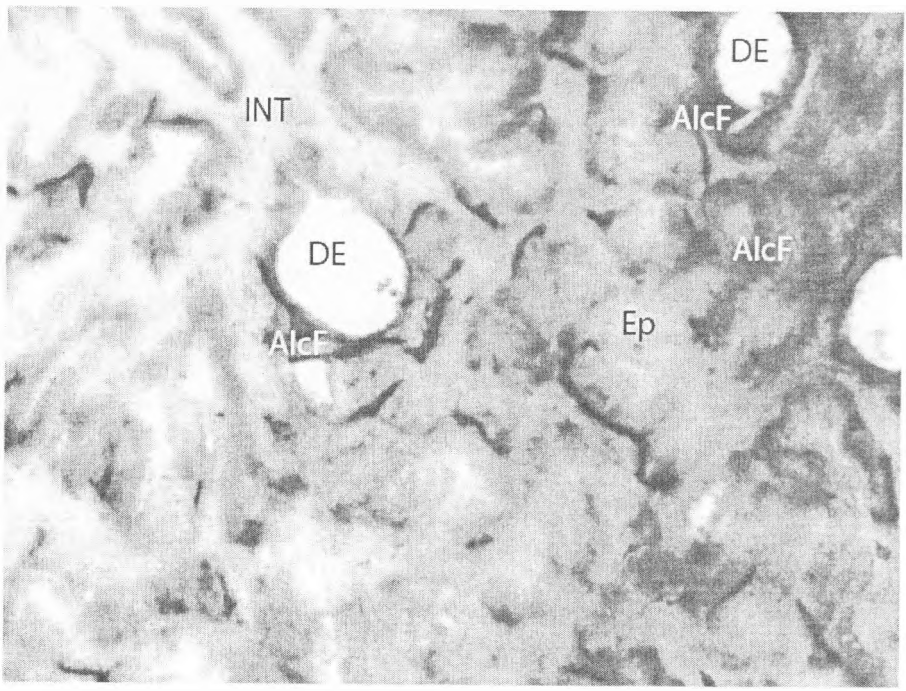


Fig. 3. Localization of the alkalinephosphatase activity in the (AlcF) in the basal parts of the prostatical epithelial cells (Ep) and in the epithelium of the prostatical excretory canals (DE). Inerstitium (IT). (Bar=10 μm)

The expression of the acid phosphatase, observed by us in the basal membranes and in the subcapsular region of the gland (Fig. 2) isn't confirmed by previous investigations [7, 8].

This distribution of the enzyme activity presumes its importance in the transport of substrates, necessary for the production of the prostatical fluid from the glandular parenchyma.

In the perivascular regions and in the periphery of the prostatical ductules, the enzyme expression is remarkable, that could be connected with the tissue collaborations between the vascular wall and the glandular part of the prostata, and between the drenaging elements and the glandular structures. The lowest acid phosphatase activity was found in the glandular stroma. (Fig. 2). That probably is connected with the different role of the interstitium about the glandular secretion-mechanical, nutritive, connecting etc.

Our results correspond with these of the man [3, 9], but they contradict with other investigations of this enzyme expression in some rodents [6]. Therefore the tissue acid phosphatase activity in the normal prosata of the tomcat shows characateristic animal species particularities, which is connected with the special glandular morphology of this specimen.

A remarkable expression of tissue alkaline phosphatase was marked in the basal parts of the epithelial cells, the periferial regions of the prostatical ductules (Fig. 3), in the perivascular parts and the glandular capsule (Fig. 4). By these results we can suppose the role of the investigated enzyme in the transport and in the processing of the secretory substrates between the parenchymal and the stromal part of the tomcat's prostata.

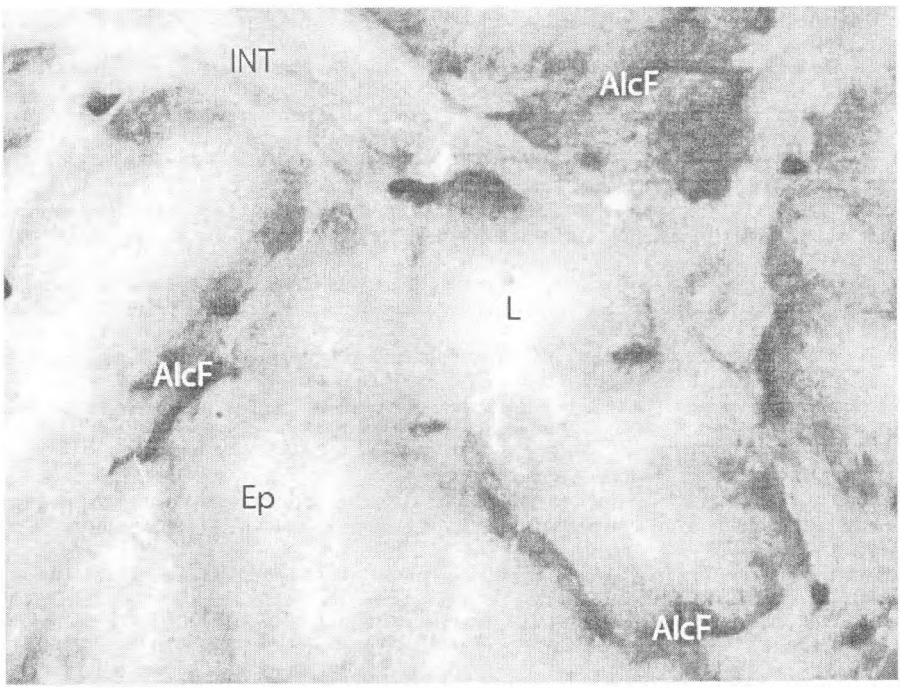


Fig. 4. Localization of the alkalinephosphatase activity (AlcF) in the basal parts of the prostatical epithelial cells (Ep). Alveolar lumen (L). Interstitium (IT). (Bar=5 μm)

The prostatical epithelial cells of the tomcat resemble the epithelial ones with osteoinductive potential which show a high activity of the alkaline phosphatase.

There wasn't any expression of alkaline phosphatase in the lumen of the glandular alveols of the tomcat, in the same of the dog there was such expression so we can suppose that it has less important role as a fraction in the seminal plasma [5].

An activity of alkaline phosphatase was found in the prostatical epithelium of the tomcat, compared with some rodents, where such activity misses [6].

The localization of alkaline phosphatase activity, which was found by us, confirms the attitude of [1], so the investigated enzyme here is with stromal, secretory, nuclear and vascular localization.

Examined tissue localization of the alkaline and the acid phosphatase in the normal prostata of the tomcat is more different than the investigations of [4, 10], which are connected with her localization in malignant and benignant prostatical lesions of the other mammals.

Therefore the results of our investigation about the tissue localization of these two enzymes can be used like a basal marker for the differentiation of the normal prostata from the pathologically transformated prostata of the tomcat.

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# Application of Lectin Cytochemistry for Differential Labelling of Surface Polysaccharides of Pathogenic Strains of *Escherichia coli*

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The study aimed to identify lectins that can differentiate between surface polysaccharide antigens – exopolysaccharide, capsular antigens and lipopolysaccharides of pathogenic strains of *E. coli*. Initially, the isolated antigens – exopolysaccharide, K and O antigens from four *E. coli* serotypes were subjected to dot-blot analysis with a panel of four lectins with different sugar specificities. After the selection of differential markers, lectin-gold labelling was performed. Changes in the surface exposure of the antigens as a result of growth temperature and differences between individual cells are described.

*Key words*: Lectin-gold, *E. coli*, polysaccharide antigens, differential labelling.

## Introduction

*Escherichia coli* pathogenic strains are adapted to survive in both external and host environments. They are capable to overcome host resistance and have the capacity to occupy different host niches, both intestinal and extraintestinal. Surface polysaccharides exposed at the surface play a variety of roles in the communication of bacteria with their environment, and changes in their accessibility may affect the interactions. Thus, the exopolysaccharide, colanic acid, when expressed protects the cells from desiccation [2]. Capsules and lipopolysaccharides (LPS) may have different impact in infection, especially with regards to serum resistance and invasion, and are considered virulence factors [6]. This determined our interest in examining the cytochemical characteristics related with the polysaccharide antigens of pathogenic *E. coli* strains that belong to different pathotypes and exploit different host habitats. This study describes an experimental approach based on differential cytochemical labelling of polysaccharide antigens with gold conjugates of glycan-binding proteins, or lectins. Lectins are characterised by specific affinities for definite mono- or oligosaccharide residues at the non-reducing termini of polysaccharides and thus, provided the structure of the antigen is known, its lectin specificity may be predicted [14]. In order to examine surface architecture, our aim was to identify lectins that can differentiate between the antigens.

## Materials and Methods

*Strains and cultivation.* The strains were chosen according to two criteria: known structure of the polysaccharide antigens and occupation of different host niches. *E. coli* O157:H-, strain A2CK, SS, represents a serotype that resides in the intestine and causes severe diarrhoea [13]. E. coli O6:K2:H1 is among the causative agents of urinary tract infections [6], and we used strain Bi7458/41 SS. Strains with K1 capsule are causative agents of neonatal meningitis [8], and were represented by E. coli O1:K1:H7 strain U5/41SS, and E. coli O7:K1, strain Bi759/41 SS. The strains were cultivated for 24 h. on nutrient agar at 20°C or 37°C.

*Choice of lectins for differential labelling.* The choice of lectins was based on the known chemical structure of the antigens. Polysaccharides were isolated after W e s t p h a l et al. [17] and Ø r s k o v et al. [11]. Lectin affinities of each antigen were tested using a panel of four lectins – concanavalin A (ConA) for mannosyl and glycosyl residues, soybean agglutinin (SBA) with affinity for galactose, wheatgerm agglutinin (WGA) for N-acetylglycosamine and sialic acids, and *Ulex europeaus* agglutinin (UEA-I) for fucose [14]. Each antigen was applied at amounts of 10, 1 and 0,1 μg on nitrocellulose disks and dot-blot was performed with 100 μg/ml of peroxidase conjugates of each of the lectins [15]. Controls included lack of antigen or incubation with the lectins in the presence of 0.2 M of the respective inhibitory sugars.

*Lectin-gold labelling.* Bacteria were collected from the agar plates and suspended in PBS. The cell suspensions were applied onto Formvar- and carbon-coated nickel electron microscopy grids. The samples were exposed for 30 min at UV light and air dried. The labeling protocol included blocking of the grid-mounted bacteria face-down on drops of 2% BSA in PBS, 2 hours incubation on drops of 50 μg $mL^{-1}$ lectin-gold in PBS (or PBS containing 0.2 mM $CaCl_2$ for ConA), and washes. The lectins were conjugated with 10 or 20 nm gold grains. The control for carbohydrate specificity included pre-absorption of the lectin-gold conjugates with 0.2 M of the respective monosaccharide prior to labelling. Negative staining was performed for 1 min with 0.5% uranyl acetate in methanol. The whole procedure was done at room temperature. Observations were made on Opton 10C electron microscope.

## Results

*Selectivity of lectin-antigen interactions.* Dot-blot experiments (data not illustrated) showed that the exopolysaccharide colanic acid interacted with SBA and UEA-I which confirmed our previous results [15]. The O157 LPS reacted with ConA and WGA. Out of the four lectins tested, the O6 LPS antigen was labeled with ConA, and the K2 capsule – with SBA only. Thus, SBA and ConA discriminated between the O6 LPS and K2 capsule. Unexpectedly, polysialic K1 capsules from both K1 strains included in the study did not interact with WGA.

*E. coli O157: temperature-related changes in lectin affinities.* When the strain was cultivated at 20°C, it expressed extracellular filamentous material that was labeled with SBA (Fig. 1a) and UEA-I, similarly to the isolated preparation of colanic acid. Cells cultivated at 37°C revealed a surface-associated intensive ConA binding (Fig. 1b). Unlike this, ConA labeling of cells grown at 20°C was by exception. No WGA labeling of native cells was observed, however short (10 min.) treatment of bacterial suspensions on boiling water bath was sufficient to expose WGA-binding sites (Fig. 1c). This was observed with all cells at both growth temperatures compared.

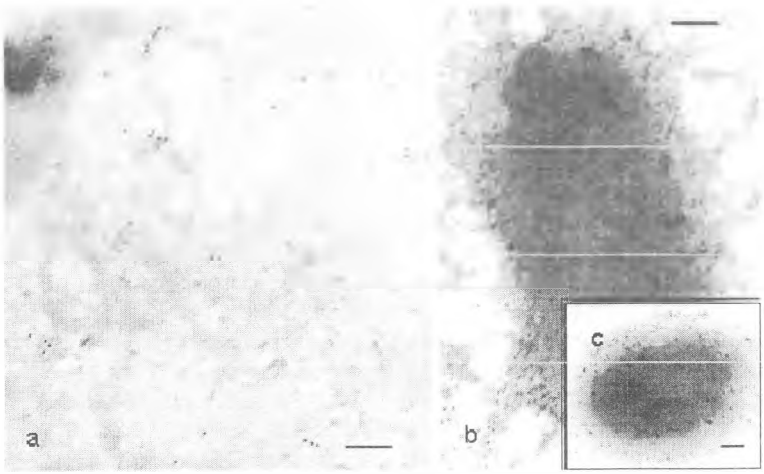


Fig. 1. *E. coli* O157
a – SBA-gold labelling of the exopolysaccharide; b – ConA-gold labelling of cell grown at 37°C; c – WGA-gold of a cell grown at 20°C and boiled for 10 min. prior to application onto the grid. Bars = 0.2 μm

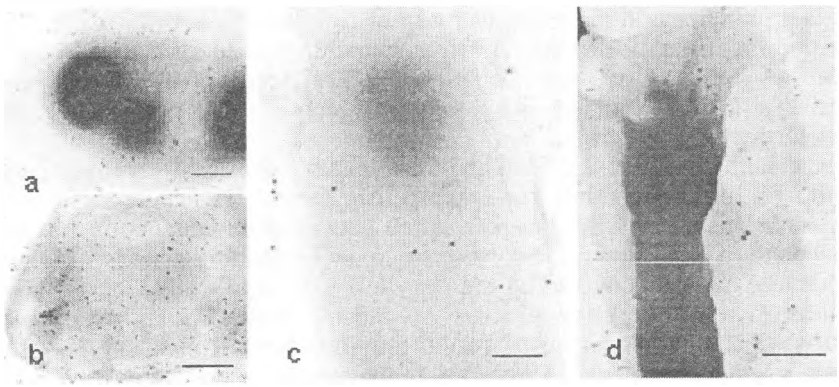


Fig. 2. *E. coli* O6:K2:H1.
(a, b) SBA-10 nm gold. (c) ConA-20 nm gold. (d) Double labelling with SBA-10 nm gold and ConA-20 nm gold. Bars = 0.2 μm

*E. coli O6:K2:H1: differential labelling of capsule and LPS* (Fig. 2). In this strain, under the experimental conditions applied SBA-binding sites were visualised all over the bacterial surface, with differences in the degree of labeling between individual cells (Fig. 2a, b). ConA labelling was not intensive (Fig. 2c), and this was also demonstrated by double labeling (Fig. 2d).

## Discussion

The present results illustrate that lectins may be appropriate tools in examination of the surface architecture of some *E. coli* strains. The interactions of the K2 capsular antigen, and the O6 and the O157 LPS antigens were as predicted by the published structures of the polysaccharides [4, 5, 10, 12, 16]. Both O6 and O157 LPS were recognized by

ConA. In addition, the WGA reactivity of the O157 LPS demonstrated by the dot blot could be due to the non-reducing N-acetylglucosamine residue outer core [1]. The outer core of the O6 LPS contains a non-reducing galactose residue [1], however preliminary dot-blot experiments showed no SBA reactivity of the O6 LPS preparation. These results did not imply any contribution of the residue to the lectin-binding characteristics of the O6 LPS. On the other hand, K2 capsule interacted with SBA in a well-expressed dose-dependent manner. Thus, it can be accepted that in *E. coli* O6:K2 SBA is a differential label of the K2 capsule. Preparations from the capsules of the two chosen K1 strains were not recognized by WGA – the lectin of choice determined by the known structure of this polysialic antigen. This could be due to antigen variation, like the O-acetylation of the K1 antigen recently commented in literature [8]. This indicated that the lectin-based approach well-applicable to the other two serotypes is inappropriate for examinations of the surface architecture of *E. coli* K1.

The four lectins were further applied to test the temperature-related changes of surface polysaccharide expression in *E. coli* O157. This serotype does not synthesize a capsule. The temperature-related difference in expression of the exopolysaccharide was predicted from literature [7, 9] and was confirmed cytochemically in the present experiments. WGA labelling, expectedly due to the outer core oligosaccharide, was insignificant in native cells independently of growth temperature, and was intense after boiling of the samples. This implies that the oligosaccharide outer core of the strain is quite inaccessible to external glycan-binding proteins in the environment such as mammalian lectins that participate in the lectin pathway of complement activation [3]. One novel observation in this study was the growth temperature-related difference in ConA reactivity of the – lack of reactivity after growth at 20°C versus a very intense labeling of cells grown at 37°C. This indicates a possible antigen variability of the O157 LPS which requires further detailed research.

The results illustrated that SBA and ConA can be used as differential labels of capsule and LPS in the uropathogenic strain *E. coli* O6:K2:H1. Under the experimental conditions in this study, SBA labeling varied between individual cells this indicating different state of exposure at the surface of the lectin-binding epitopes. However, the LPS appeared partially covered by the capsules, with only a few ConA-binding sites accessible. Given the different roles of capsule and LPS in the virulence of uropathogenic *E.coli* [6], the present results provide a cytochemical approach to examinations of changes in surface architecture of the strain. Such variations may occur under the action of biogenic factors, after in vivo passages, etc. However, since SBA cannot differentiate between the K2 capsule and colanic acid, SBA labelling of this strain is inappropriate for conditions that promote exopolysaccharide expression. Thus experiments on biofilm architecture related with exopolysaccharide accumulation on bacterial attached microcolonies should preferably use the fucose label, UEA-I, which also interacts with the polymer.

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# Influence of SCF on Enzyme Expression During Small Bowel Murine Development

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Colostrum and milk are essential for the development and growth of mammals. Among the substances present in colostrum and milk stem cell factor (SCF) has major role in gut maturation and development. The aim of our study was to determine whether SCF has influence on the expression of the enzymes alkaline phosphatase, lactase and dipeptidil peptidase IV (DPPIV). Deposition of reaction products was visualized in thin sections of frozen gut tissue. Our observations showed more vivid results on day two of explants' treatment. Presence of SCF didn't show any significant effect on the activity of alkaline phosphatase and lactase. However, in presence of SCF expression of DPP IV was increased.

*Key words*: SCF, enzyme expression, alkaline phosphatase, lactase, DPP IV.

## Introduction

Colostrum and milk are essential for the development and growth of mammals. Postnatal development and maturation of their gastrointestinal tract is influenced by supply of maternal milk. Among the substances present in colostrum and milk stem cell factor (SCF) has major role in gut maturation and development. SCF is a cytokine which binds the c-kit. It exists in two forms – cell surface bound and free (soluble) form. Its main role is to maintain the proliferation and differentiation of hematopoietic stem cells and other progenitor cells. Data demonstrates the major role for SCF in the generation of intestinal mastocytosis and the host protective immune response following parasitic infection [2]. The later factor interacting with c-kit is considered to be important for the homeostasis of epithelial barrier function in the intestinal tract [7].

The aim of our study was to determine whether SCF has influence on the expression of the enzymes alkaline phosphatase, lactase and dipeptidil peptidase IV (DPPIV) at the developing small bowel of 5-day-old mice.

## Materials and Methods

*Organ culture preparation:* Organ cultures were prepared from 5-day-old Balb/c mice from both sexes. A short segment of small intestine extending distally from the pylorus

was removed from each mouse. The segment was cut into 3 parts – duodenum, jejunum and ileum. Samples were taken according to intestinal length by P l a y f o r d et al. [6]. The explants were incubated in culture medium RPMI 1640 containing 10 % fetal calf serum with 20 ng/ml rm SCF (Immunotools /Germany) for 24, 48 and 72 hours at 37° C, 5% $CO_2$ at 100 % humidity. For enzyme check incubated explants were plunged into Tissue –Tek culture medium obtained from Sakura, USA and frozen at –25° C. Frozen tissue explants were cut by 10 μm sections on cryostat and used without fixation to determine the activity of the enzymes.

*Enzyme substrates preparation:* Alkaline phosphatase substrate was prepared by the method of Burstone. TRIS/HCl with pH 0.9, naphtol-AS-MX-phosphate and fast Blue B were used. Incubation was implemented for 8 min at 37°C, 5% $CO_2$. Specimens were post fixed in 4 % FA for four hours, washed and covered with glycerin - gelatin. Lactase substrate was prepared from 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside, Nitrotetrazolium Blue chloride and citric buffer with pH 6. Specimens were incubated for 2 hours at 37°C, 5 % $CO_2$. Specimens were postfixed in 4 % FA for 15 min, washed and covered with glycerin-gelatin. For dipeptidil peptidase IV (DPPIV) determination we used phosphate buffer with pH 7.73 and 0.3 mM solution of aldehyde piperonal. Reaction was observed on fluorescent microscope after two hours of incubation at the same conditions [4].

## Results

Small intestinal epithelium is one of the most actively renewing tissues in the body [5]. Intestinal enzyme activities are known to respond to changes in dietary composition. Studies in rats and humans suggest that adaptive mechanisms differ between species in response to altered intakes of carbohydrate and fat [3]. Colostrum is additionally a rich and concentrated source of various factors that demonstrate biological activity in vitro [1]. The ingestion of colostrum in neonatal mice has great effect on gastrointestinal tract development and influences digestive enzyme activities. Deposition of reaction products was visualized in thin sections of frozen gut tissue. The distribution of alkaline phosphatase with or without presence of SCF in the light microscope is seen in Fig.1 and Fig. 2. Our observations showed more vivid results on day two of explants' treatment. Hence we show only pictures from the second day of incubation.

Presence of SCF didn't show any significant effect on the availability of alkaline phosphatase. Explants treated with SCF and those, incubated without it showed equal distribution of the reaction product. We also compared the three parts of the small bowel

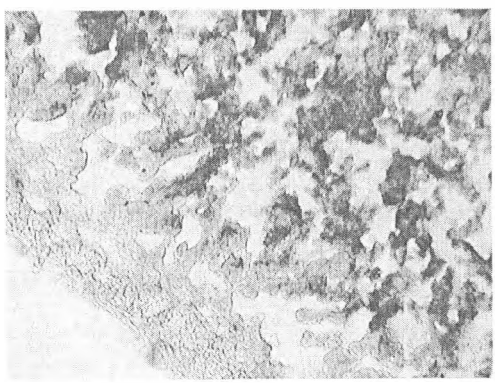

Fig. 1

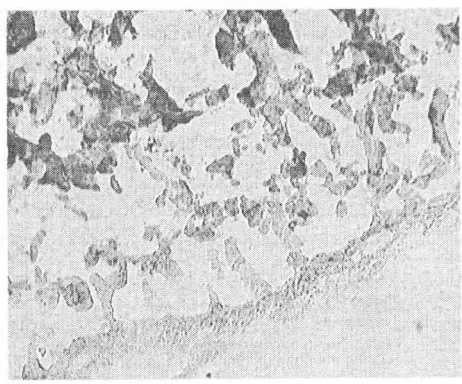

Fig. 2

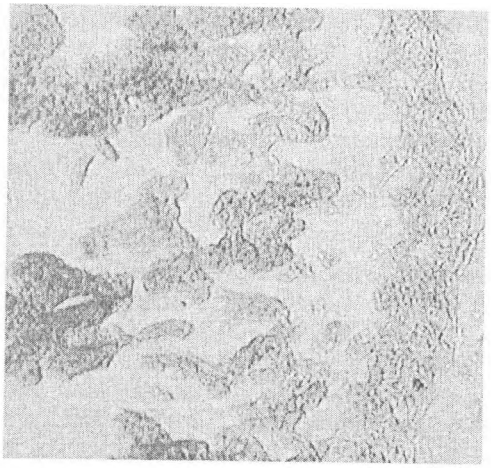

Fig. 3

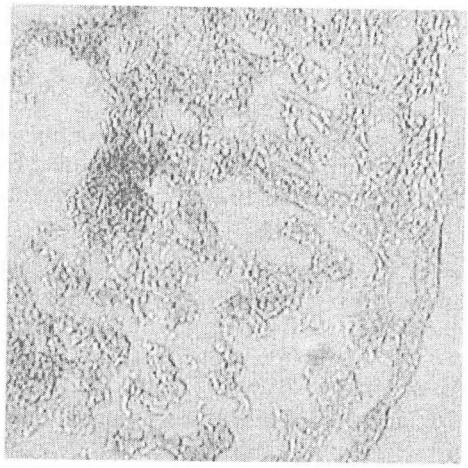

Fig. 4

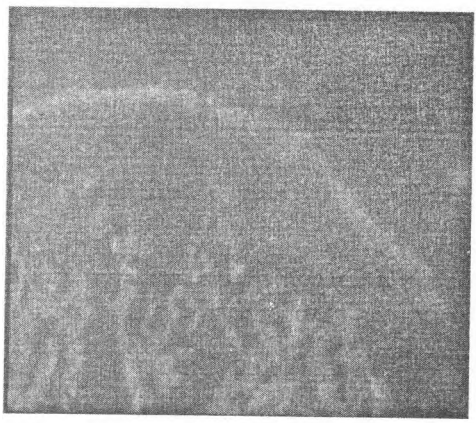

Fig. 5

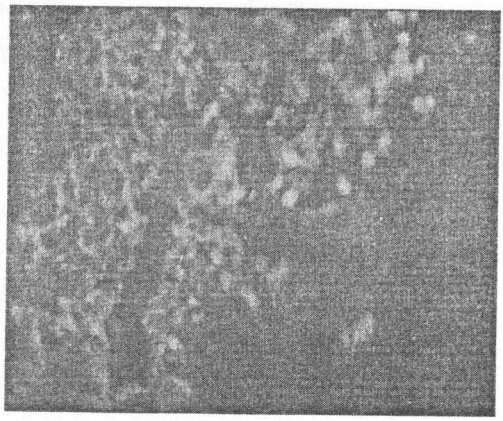

Fig. 6

(namely duodenum, jejunum and ileum) for samples treated with SCF. Staining for alkaline phosphatase was more contrasting for the jejunum and ileum.

Samples stained for lactase in presence of SCF were again more contrasting at day two of incubation. Comparing both stimulated and non stimulated samples we didn't see any significant difference in lactase's distribution (Fig. 3 and Fig. 4.). Lactase availability was lower in jejunum and ileum compared to duodenum. No lactase was found at the gut wall.

Staining for DPP IV in presence of SCF was performed by fluorescent tagging of the enzyme (Fig. 5 and Fig. 6). It showed more contrasting villi at all parts of the small intestine, comparing to non stimulated samples. Our conclusion was that in presence of SCF the expression of DPP IV was increased.

*Acknowledgements.* This work was supported by grant TKL 1609 form the Ministry of Education and Science. Bulgaria.

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# Enzyme Histochemical Expression of Lipoprotein Lipase in Canine Paranal Sinus

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The purpose of the present investigation was to study the enzyme histochemical expression of lipoprotein lipase (LPS) in canine paranal sinus (PS). The results showed an intensive enzyme reaction for LPL in the cytoplasm of most cells in all layers of the stratified squamous cornified epithelium as well as in some cells of apocrine and sebaceous glands of PS. Positive LPL expression was also observed in the stroma. In the same PS structures, groups of lipid droplets of a various size have been observed. A LPL enzyme histochemical expression together with single lipid deposits of a various size was also detected in some of large blood vessels supplying PS with blood as well as in the microcirculatory bed. There was no sex dimorphism in LPL expression. The results showed that there was an intensive enzyme histological reaction in LPS in the paranal sinus that was probably related to the function of the organ, and the pathological deviations could have an impact on the intensity of reaction. This would allow the utilization of LPL enzyme histochemical expression to be used as a marker in the diagnostics of different pathological events in PS.

*Key words*: lipoprotein lipase, paranal sinus, dog.

## Introduction

The major quantity of LPL in the body is localized in the capillary endothelium. A small amount is detected in the arterial endothelium and it is supposed to be involved in atherogenesis [16]. A subendothelial localization of the enzyme in the arterial intima is also observed. Theoretically, LPL of arterial intima could originate from circulating LPL or from the local synthesis of various cells of the intima.

Lipoprotein lipase (LPL) is the primary lipolytic enzyme, involved in the intravascular metabolism of lipoproteins [4]. This enzyme is synthesized and secreted in a catalytically active form by adipocytes and myocytes. Then, it is transported to the capillary endothelial surface. The physiological function of LPL is to hydrolyze triglycerides from chylomicrons, very low density lipoproteins (VLDL) and intermediate density lipoproteins (LDL) from the luminal side of capillary endothelium and to release free fatty acids, stored as triglycerides in the adipose tissue or oxidized for energy production in muscles [10]. The C-terminal domain of LPL is bound at a considerably higher

extent to chylomicrons and VLDL than to LDL, whereas the N-terminal domain mediates the binding to phospholipid vesicles as well as to LDL particles [7, 8]. The activity of LPL has been studied in the adipose tissue, the heart muscle, the liver, the mammary gland, and the skeletal musculature in a number of animal species and men [2, 1, 5, 3].

In the available literature, there is no detailed information about the expression and the distribution of LPL in the paranal sinus in dogs. Single reports showed traces of lipase in the apocrine cells and the secretion of sebaceous glands of this organ [11], but the type and the function of the enzyme are not still clear.

The purpose of the present investigation was to study the enzyme histochemical expression of LPL in the different structures of canine paranal sinuses and in the vessels, supplying PS with blood.

## Material and Methods

In the present study, the paranal sinuses of 7 male and 7 female mongrel dogs were used.

Immediately after the euthanasia of the dogs, specimens from PS wall were obtained. The Gomori's enzyme histochemical reaction was performed on fresh cryostat cross sections for detection of positive expression of lipoprotein lipase in PS. The reaction was based upon the Tween method consisting in the deposition of insoluble calcium soaps at the sites of enzyme activity that are further converted to lead soaps and finally, in lead sulfide precipitates. On ready preparations, the final precipitates appeared as clusters of dark-brown granules.The lipid content was detected on cryostat cross-sections by means of histochemical reaction with Sudan III (Feinchemie KG. Sebnitz, Germany) according to Daddy. Lipid deposits were stained in yellow-orange.

## Results

LPL expression was detected in the cytoplasm of most cells of all layers of the stratified cornified epithelium of the sinus. The highest number of reacted cells was observed in the basal layer and their amount decreased in the direction of stratum corneum. Clusters of brown granules with a various size were mainly observed in the apical part of glandular cells of apocrine tubules. Less frequently, enzyme activity was encountered in the basal part of secretory cells. LPL expression was established in both basal and mature cells of sebaceous glands. The expression of the enzyme was visualized as deposits of a various size in some cells of the stroma as well as in the extracellular matrix.

On serial cross-sections, we have found out that the localization of lipids in the glandular cells of apocrine tubules (Fig. 1) was the same as LPL localization in these cells (Fig. 2). Lipid deposits were also observed in the cornified epithelium, in some stromal cells, but the strongest reaction was that of sebaceous glands and their outlet duct. In the blood vessels supplying PS with blood, the pattern of LOL localization in the three vascular wall layers was irregular and appeared as single brown granules with a round shape in the subepithelial connective tissue and the endothelium of the intima, of tunica media and the adventitia. On Sudan III-stained histological cross-sections, some vessels showed single lipid deposits of a various size in the three vascular layers, reaching the intima. LPL activity was observed in the luminal surface of endothelial cells in capillaries and arterioles in the PS wall. Single reaction deposits were present in the cells of tunica media and in tunica externa of arterioles.

In this study, no sexual dimorphism in LPL and lipids expression was found out.

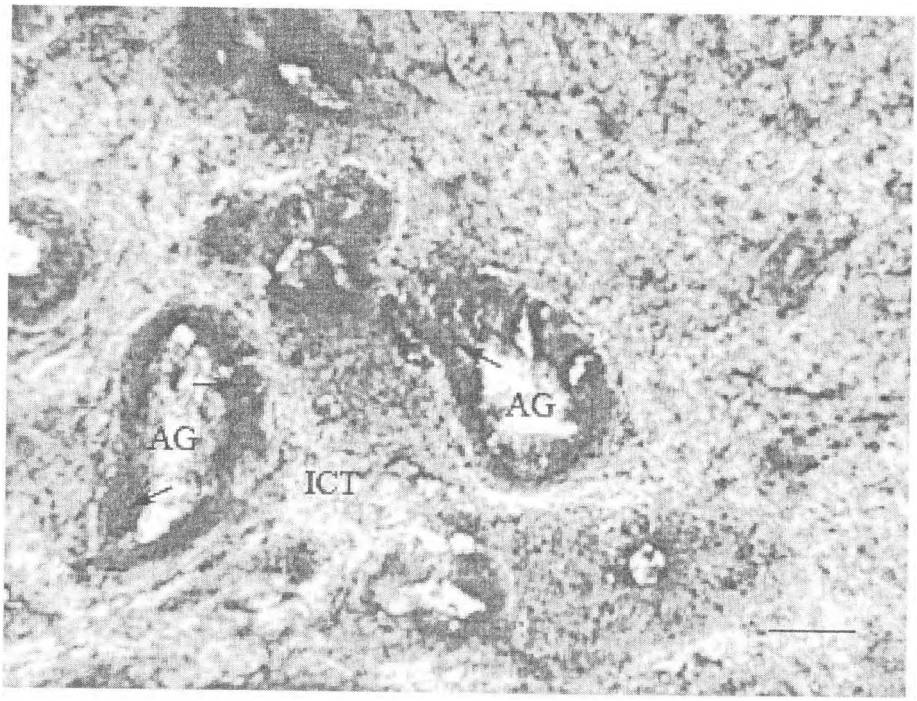


Fig. 1. Lipid droplets (arrow) in the cytoplasm of some cells of the apocrine glands (AG). ICT – interstitial connective tissue. Sudan III-H staining, × 100, Bar = 50 $\mu$m

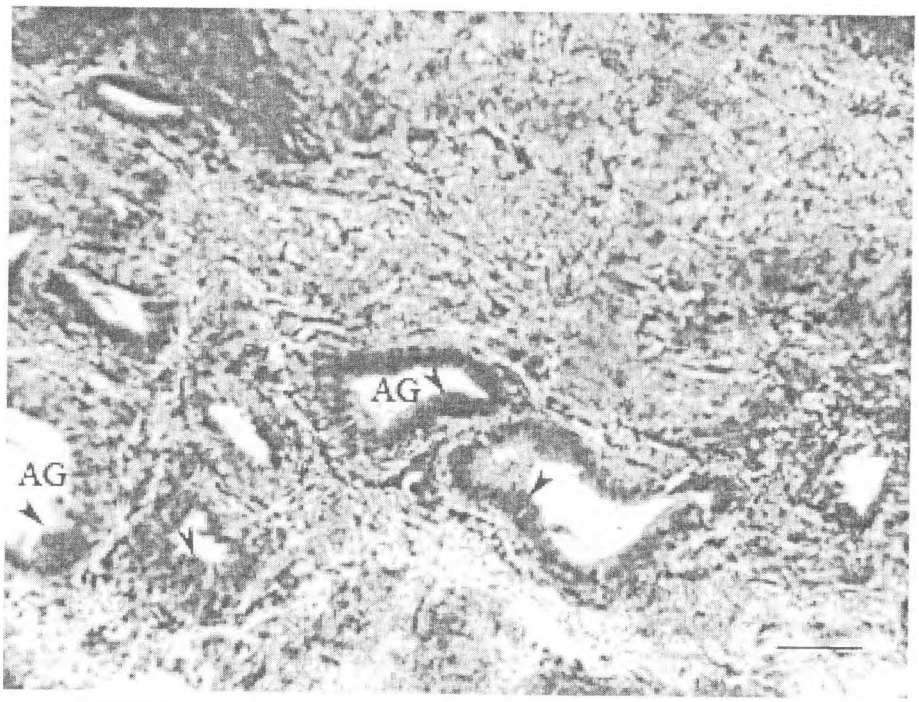


Fig. 2. Expression of LPL (arrowhead) in the cytoplasm of some cells of apocrine glands (AG), × 100, Bar = 50 $\mu$m

## Discussion

The localization of LPL in PS stroma, observed by us, could be explained by the ability of this enzyme to bind to proteoglycans of the extracellular matrix, similarly to arterial intima [9]. This could result in LDL retention in these structures [9, 12]. This retention prolongs the presence of LDL in the intimal matrix that permits the modification of these particles. LPL provokes a selective uptake of cholesterol by LDL that requires cell surface proteoglycans but is not dependent on lipoprotein receptors and LPL activity [15, 13]. It is known that LPL in the arterial intima could originate from circulating LPL or its local synthesis by various cells of the intima. R. Rumsey [14] showed that LPL increased LDL binding to fibroblasts and macrophages as well as the breakdown of LDL in them. The authors established increased triglyceride concentrations in studied cells. In their view, LPL increased the uptake of lipids and lipoproteins from the cells without a LDL receptor. This pathway, in our opinion, is important for the accumulation of lipids in LPL-synthesizing cells. The data allowed assuming that the presence of lipids in the secretion of PS apocrine glands is probably related to the regulation of its strong odour.

The observed LPL localization in canine PS could be explained assuming a synthesis of this enzyme in the studied organ. On the other side, this enzyme is able to bind both to cell surface and to extracellular matrix glucosaminoglycans. This way, LPL could penetrate through the vascular wall [6, 12] and to occur in the PS stroma.

The present study provided evidence that in canine PS, there was an intensive enzyme histochemical expression of LPL that could be associated to the primary function of the gland. This expression could be utilized as a marker in the diagnostics of various pathological processes affecting the gland.

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# Biofilm Morphology and Effects of Bacterial Cell-to-Cell Communication on Biofilm Formation by *Escherichia coli* K-12

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The study describes the morphology of biofilms formed by two *E. coli* K-12 strains as shown by reflected light microscopy and the dynamics of the bacteria-substrate contact during cultivation in liquid medium as registered by plasmon microscopy. The effects of quorum sensing on *E. coli* K-12 biofilm formation is also examined. Quorum sensing influences biofilm growth but has no apparent effects on initial attachment of bacteria.

*Key words:* microbial biofilm, autoinducers, quorum sensing, plasmon microscopy.

## Introduction

In most natural environments bacteria do not exist as solitary cells but are colonial organisms. They associate with an inert or living surface and form structures known as biofilms. Microbial biofilm is a structured community of bacterial cells enclosed in a self-produced polymeric matrix [3, 4]. There are four stages of biofilm formation: reversible adhesion, irreversible adhesion, biofilm maturation and detachment of biofilm cells [12]. Biofilm bacteria communicate with each other using chemical signal molecules, or autoinducers, excreted from the cells. This process, termed quorum sensing, allows bacteria to count the members in the community and to synchronously alter gene expression of the population [12]. Previous literature data show that many *E. coli* K-12 biofilms are poorly developed if they lack a conjugative plasmid [7, 10]. The aim of the present study is to compare the structure of two strains *E. coli* K-12 – *E. coli* W1655F+ and *E. coli* W3110F-, examine the dynamics of the bacteria-substrate contact, and characterize the effects of quorum sensing on biofilm formation.

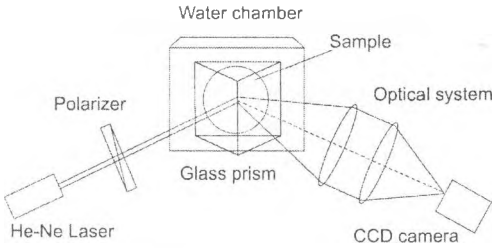


Fig. 1. Scheme of the plasmon microscope with the water chamber

## Materials and Methods

For examination of morphology, biofilms were cultivated for 24h on glass slides covered with methyl metacrilate. The observations of native hydrated biofilms were done on a light microscope with reflected light. To examine cell contact with the substratum, we used an optical plasmon sensor [2], supplied with a specially constructed cell (Fig. 1.) for real-time observation in a liquid medium. The sample slide was covered with bacterial suspension, incubated for 20 min. and washed in sterile saline prior to closing of the cell filled with M63 medium [9]. The laser beam was fixed in definite position, and serial photographs were taken at 5 min intervals.

The strains of *E. coli* K-12 used in this study were *E. coli* W1655 F+ and *E. coli* W3110 F-. The biofilm formation assay was done in 96-well microtitre plates, following two protocols. In the first experimental series 10 ml of the overnight bacterial cultures in TSB were suspended in 100 ml of M63 medium with addition of sterile supernatants of *E. coli* and *Yersinia enterocolitica,* used as sources of quorum sensing. M63 medium was supplemented with the supernatants in proportions 5:1, 5.5:0.5 and 5.8:0.2. The plates were incubated for 24h at 20°C. The planktonic cells were washed. The adherent bacteria were coloured with 0.1 % water solution of crystal violet, solubilized in 75 % ethanol and the absorbance at 620 nm was determinated by ELISA reader.

In another experiment 100 ml of the overnight bacterial cultures were placed in the wells. Quorum sensing was added to reach the concentrations described above. Cells were left to adhere for 4h at 20°C. The non-adherent cells were removed, wells were gently washed, and 120 ml of pure M63 medium were placed in each well. The plates were incubated for 24 h at 20°C.

## Results and Discussion

*Morphology of E. coli K-12 in relation with the F plasmid.* We first compared biofilm morphology of two *E. coli* K-12 strains: one F+ and another – F-. Within the 24h interval tested, the F+ strain formed thick, well-structured biofilm with well-expressed cellular masses interposed by channel-like structures (Fig. 2A). The F-strain was loosely adherent, forming thin cellular filaments organised in fractal-like pattern (Fig. 2B). This supports previous observations on the role of the F plasmid in biofilm formation [7, 10]. Reflected light microscopy to this moment had only limited application in biofilm examination [8]. One very important advantage of this is simplicity of use, and the possibility to examine morphology of the biofilm in its native hydrated state.

*Plasmon microscopy.* The setup we used for surface plasmon microscopy is shown in Fig.1. The p-polarized beam from a He - Ne laser falls onto the sample through a glass prism (index of refraction $n$=1.78). The sample consisted of 45-nm Au layer evaporated

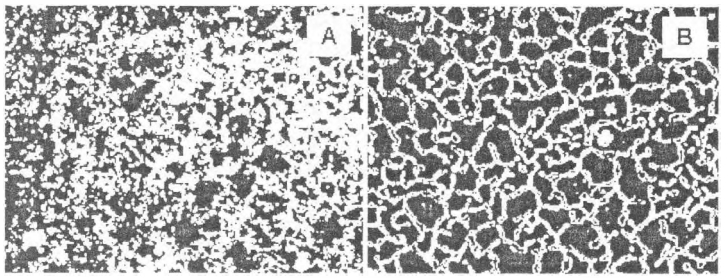


Fig. 2. Reflected light microscopy images of 4h biofilms of (A) *E. coli* K-12 W1655F+ and (B) *E. coli* W3110F-. Scale bar = 20 μm

on a cover glass ($n$=1.78) and covered with 780-nm PMMA. A 2-nm Cr layer was deposited before the Au to increase the adhesion of the gold layer. The sample was in contact with a water chamber. The optical contact between the glass prism and the sample was realized using a special immersion liquid. The sample surface was imaged onto the CCD camera by an imaging system. The plasmon sensor used is constructed so that it can visualize objects as small as 1$\mu$m [2] which exceeds previous applications of plasmon microscopy that allowed observation of large cells, e.g. neurons [6].

Strain *E. coli* W1655F+ which forms pronounced biofilms was used. Only after a short contact with the polymer substratum, numerous bacteria were attached to the slide. The illustrations (Fig. 3 A-C) show changes in cell density and distribution on a fixed point of the sample, and describe a dynamic cell-substrate interaction.

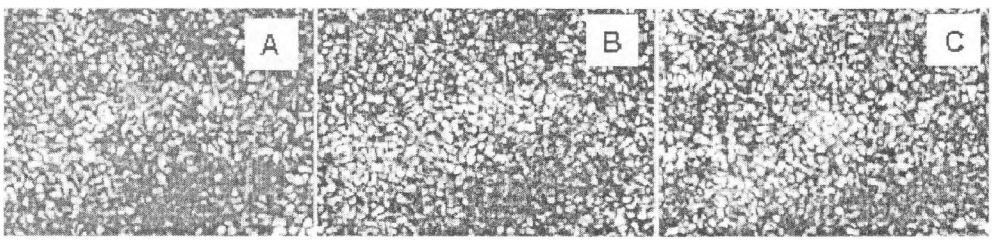


Fig. 3. Changes in the number and the position E. coli K-12 W1655F+ cells that are in a close contact with the substrate. Pictures taken with optical plasmon sensor in a fixed position of the laser beam at 30 min intervals. Scale bar = 1 μm

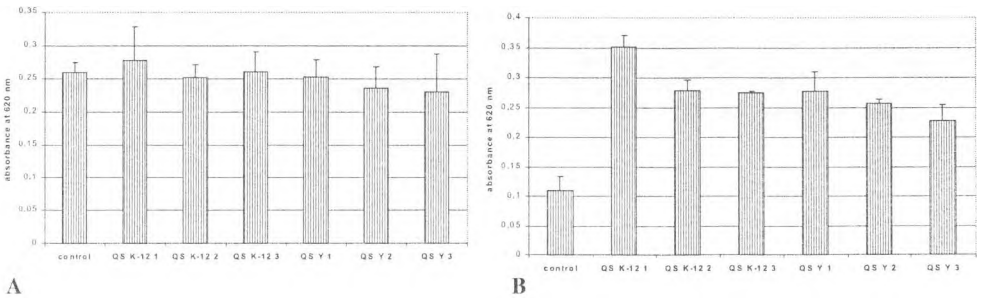


Fig. 4. Effect of QS K-12 and QS Y on the adhesion and the growth of 24h *E. coli* K-12 W1655F+ biofilm formation. Addition of 48h sterile supernatants

*Effect of quorum sensing on E. coli K-12 biofilm formation.* Further examinations of *E. coli* W1655F+ were directed to the effect of quorum sensing. As a source of signals, 48h sterile supernatants of *E. coli* K-12 (QS K-12) containing autoinducer 2 and *Yersinia enterocolitica* (QS Y) containing also autoinducer 1 were used. [1, 11]. First we examined the effect of QS K-12 and QS Y during adhesion stage of biofilm development (Fig. 4A). The obtained values of absorbance showed no statistically significancant differences between control and test groups and between test groups themselves. In another series of experiments, quorum sensing was present throughout 24 h biofilm growth (Fig. 4B). It was found that both QS K-12 and QS Y stimulate biofilm formation in a dose-dependent manner. QS K-12 has stronger effect than QS Y ($p < 0.001$).

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# The Gland of the Third Eyelid (Harderian gland) in the Broiler Chickens. I. Some Morphometrical Parameters of the Harderian gland Secretory Epithelium During the First Eight Weeks After the Hatching

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Harderian gland obtained from 90 broiler chickens originating from a commercial stock, from both genders at the age of 1, 7, 14, 21, 28 , 35 , 42 , 49 and 56 days were investigated. On the durable histological specimens the micrometric parameters of the secretory epithelial cells in the acini and in the various glandular tubules were determined by a light microscopy using a micrometer eye piece.The data was statistically processed by Student's t-test and ANOVA analysis with PC programme (StatMost for Windows, 1994) and the results were in tabular form and graphically presented. The analysis of the obtained data showed, that the height of the acinar epithelial cells from hatching to the 56th day was increased from 8.92 μm to 26.40 μm. The height of the main collecting duct epithelium increased from 9.36μm to 20.09 μm, primary secretory tubules from 12.88 μm to 24.32μm; secondary from 8.74 μm to 17.35 μm and tertiary from 5.28 μm to 11.48 μm. The results illustrated the nine – week ontogenesis of the HG- secretory epithelium. They suggest that the growth of the acinar and tubular epithelial cells in the broiler chicken's HG is one irregular, but partially cyclic processes.

*Key words:* Harderian gland, broiler chickens, morphometry.

## Introduction

*Glandula membranae nictitantis* (most commonly called Harderian) and the lachrymal gland are known to be intraorbital glands in the birds. In contrast to the mammals, in birds the better developed from the both glands is the Harderian are [5, 8, 10, 12, 13].

The complexly built system of canals and acins in the Harderian gland in the hen is covered with secreting glandular epithelium, whose cells possess different shape and size depending on their situation in the organ, so as their functional and age status. There already exists evidences for the active role of the glandular epithelium in the "assimilation"of immune bodies that got in the Harderian gland with the blood flow, or synthesized by the epithelium itself [6, 10, 5, 12, 9]. It was found, that it is possible a migration of synthesized Ig A from the tubular glandular epithelium of the chickens Harderian gland to other organs [2].It was demonstrated the presence of Ig A and Ig G

containing plasmatic cells in the Harderian gland of broiler chickens and wild fowls in Bangladesh [7]. Most of the fore quoted authors attach these abilities of the glandular cells to the secretory function of the epithelium, connecting it always with the form and size of its cells. It is an undeniable fact, that determinant for the functional and clinical sufficiency of every gland are the structural and morphometrical parameters of its secretory epithelium which are individually, functionally and age related constants.The data published in the literature is mainly about the structural peculiarities of the birds intraorbital glands. The data concerning the morphometrical parameters of the birds Harderian gland and their age dynamics is scarce, and such ones for broiler type of birds lacks at all.

Giving an account of the great significance of the epithelium of the birds Harderian gland into the provision of its functional and clinical status we aimed into this study to determine the height of the epithelial cells in all the classes of acinus and tubules of the Harderian gland of the broiler chickens from the hatching till the 8$^{th}$ week of its development using the micrometrical method.

## Materials and Methods

For the conduction of the present study was used material from the Harderian glands of 90 stock broiler chickens, which were 1, 7, 14, 21, 28, 35, 42, 49 and 56 days old. Each age group consisted of 5 male and 5 female birds. The Harderian glands were obtained after an ether narcosis of the birds, decapitation, orbitotomy and a careful detachment from the surrounding muscles and tissues [1]. The obtained 180 glands were fixated in a 10% neutral formaldehyde, the fixating mixtures of Bouen, Karnua and embedded in paraffin. From single and serial sections (5 μm) after stainings with Ehrlich's hematoxylin and eosin and a polychrome Haidenhain's Azan's staining, durable histological specimens were made [11]. The micrometrical study was conducted with a light microscope (Ergaval) and micrometricaleyepiece (Zeiss). The measurements were made over 10 ocular fields of the microscope for every slide for each of the 10 birds in the 9 age groups [3]. The obtained data was statistically processed with the Student's t-test and ANOVA with the computer software (StatMost for Windows, 1994), and the results were presented in tabular and graphical forms.

## Results and Discussion

The statistically processed results of the conducted morphometrical analysis of the height of the secretory glandular epithelium in the broiler chicken's Harderian gland during the first eight weeks of its postnatal development are presented in Table 1.

The presented in the table results shows, that the height of the glandular epithelial cells (HGEC) in the acins of the Harderian gland (HG) for the monitored 56 days period is increasing 2.95 times. From the presented data is evident, that HGEC in the acins of the Harderian gland during the second and fifth weeks (at the 14$^{-th}$ and 35$^{-th}$ day) almost do not increase or do not increase. The growth is moderate during the first, third, sixth and the eight weeks (7$^{th}$, 21$^{st}$, 42$^{nd}$ and 56$^{th}$ day) and well pronounced during the last one (49$^{th}$ day) of the studied period. It was determined an of increase of 2.18 times in the HGEC in the tertiary secretory ducts of the Harderian gland for the eight studied weeks. The results showed that there is a minimal increase of the HGEC only in two (at the 35$^{th}$ and the 42$^{nd}$ days) of the weeks of the monitored period. In the secondary secretory ducts the epithelium increases its height for 56 days with 1.89 times. It was determined from a slight to a moderate increase of the HGEC during the first, second, fifth and sixth weeks.

T a b l e 1. Harderian gland in broiler chickens (1-56 day)

| | | Height of the acinar glandular cells (μm) | Height of the main collecting duct epithelial cells (μm) | Height of the epithelial cells in the secretory ducts (μm) | | |
|---|---|---|---|---|---|---|
| | | | | I-Primary | II-Secondary | III-Tertiary |
| 1 day | | $\bar{x}$ = 8. 9265<br>S$\bar{x}$ =0. 4196 | $\bar{x}$ =9. 3660<br>S$\bar{x}$ =0. 1768 | $\bar{x}$ =12. 8865<br>S$\bar{x}$ =0. 3117 | $\bar{x}$ =8. 7450<br>S$\bar{x}$ =0. 2351 | $\bar{x}$ =5. 2800<br>S$\bar{x}$ =0. 1847 |
| | For the all group (sum of I+II+III ducts) | | | | $\bar{x}$ =8. 9705<br>S$\bar{x}$ =0. 2301 | |
| 7 day | | $\bar{x}$ = 11. 1045<br>S$\bar{x}$ = 0. 6707 | $\bar{x}$ =10. 5870<br>S$\bar{x}$ =0. 1819 | $\bar{x}$ = 15. 8895<br>S$\bar{x}$ = 0. 2879 | $\bar{x}$ = 10. 1475<br>S$\bar{x}$ =0. 2242 | $\bar{x}$ = 6. 0555<br>S$\bar{x}$ = 0. 1521 |
| | For the all group | | | | $\bar{x}$ =10. 6975<br>S$\bar{x}$ = 0. 2677 | |
| 14 day | | $\bar{x}$ =11. 7200<br>S$\bar{x}$ = 0. 7020 | $\bar{x}$ = 11. 1540<br>S$\bar{x}$ =0. 1936 | $\bar{x}$ =17. 2825<br>S$\bar{x}$ =0. 2092 | $\bar{x}$ = 11. 2035<br>S$\bar{x}$ = 0. 1924 | $\bar{x}$ = 6. 6495<br>S$\bar{x}$ =0. 1996 |
| | For the all group | | | | $\bar{x}$ = 11. 7118<br>S$\bar{x}$ = 0. 2772 | |
| 21 day | | $\bar{x}$ = 14. 2560<br>S$\bar{x}$ =0. 8270 | $\bar{x}$ = 11. 8305<br>S$\bar{x}$ = 0. 1724 | $\bar{x}$ = 18. 4965<br>S$\bar{x}$ =0. 2474 | $\bar{x}$ = 11. 7150<br>S$\bar{x}$ = 0. 1983 | $\bar{x}$ = 7. 3592<br>S$\bar{x}$ = 0. 2012 |
| | For the all group | | | | $\bar{x}$ = 12. 5236<br>S$\bar{x}$ = 0. 2929 | |
| 28 day | | $\bar{x}$ = 14. 8830<br>S$\bar{x}$ =0. 8158 | $\bar{x}$ =12. 6385<br>S$\bar{x}$ = 0. 5707 | $\bar{x}$ =18. 5295<br>S$\bar{x}$ = 0. 2621 | $\bar{x}$ =11. 9295<br>S$\bar{x}$ = 0. 2186 | $\bar{x}$ = 7. 5570<br>S$\bar{x}$ =0. 1628 |
| | For the all group | | | | $\bar{x}$ = 12. 6720<br>S$\bar{x}$ =0. 2895 | |
| 35 day | | $\bar{x}$ = 18. 7110<br>S$\bar{x}$ =0. 8482 | $\bar{x}$ =14. 1900<br>S$\bar{x}$ =0. 1905 | $\bar{x}$ =20. 4270<br>S$\bar{x}$ = 2. 108 | $\bar{x}$ =13. 4475<br>S$\bar{x}$ = 0. 2050 | $\bar{x}$ =8. 9925<br>S$\bar{x}$ = 0. 1614 |
| | For the all group | | | | $\bar{x}$ = 14. 2890<br>S$\bar{x}$ = 0. 2941 | |
| 42 day | | $\bar{x}$ =20. 4920<br>S$\bar{x}$ = 0. 7258 | $\bar{x}$ =14. 7015<br>S$\bar{x}$ = 0. 2652 | $\bar{x}$ = 22. 6680<br>S$\bar{x}$ = 0. 2188 | $\bar{x}$ =15. 7080<br>S$\bar{x}$ =0. 1941 | $\bar{x}$ = 10. 5435<br>S$\bar{x}$ = 0. 1425 |
| | For the all group | | | | $\bar{x}$ =16. 2955<br>S$\bar{x}$ =0. 3075 | |
| 49 day | | $\bar{x}$ = 24. 5190<br>S$\bar{x}$ = 0. 6898 | $\bar{x}$ = 17. 2260<br>S$\bar{x}$ = 0. 1983 | $\bar{x}$ = 23. 6280<br>S$\bar{x}$ =0. 1623 | $\bar{x}$ = 16. 5495<br>S$\bar{x}$ = 0. 1762 | $\bar{x}$ =10. 8075<br>S$\bar{x}$ = 0. 1580 |
| | For the all group | | | | $\bar{x}$ =16. 9950<br>S$\bar{x}$ =0. 3179 | |
| 56 day | | $\bar{x}$ = 26. 4000<br>S$\bar{x}$ = 0. 6116 | $\bar{x}$ = 2. 0970<br>S$\bar{x}$ = 0. 2982 | $\bar{x}$ = 24. 3255<br>S$\bar{x}$ = 0. 1980 | $\bar{x}$ = 17. 3580<br>S$\bar{x}$ =0. 1715 | $\bar{x}$ =11. 4840<br>S$\bar{x}$ = 0. 1738 |
| | For the all group | | | | $\bar{x}$ = 17. 7225<br>S$\bar{x}$ =0. 1043 | |

The primary secretory ducts has the same increase of the HGEC during the studied period – 1.89 times. Except for the forth and the seventh week (28th and 49th day), when there is not almost any growth, during the rest of the period it is from weak to moderate.

The statistical processing of the data for the growth of the epithelial cells from the threeclasses of glandular secretory ducts (the sum of the primary, secondary and tertiary tubules) in the lobule of the Harderian gland of the broiler chickens shows results similar to the one determined for every one of them. For the whole studied eight weeks period the growth of the HGEC is 1.97 times.

The secretory epithelium of the main (central) duct of the lobule in the Harderian gland of the broiler chickens for 56 days increases its height 2.14 times. Here the epithelium has a lack to moderate growth during the 7th, 35th, 49th and 56th days.

From the presented data it becomes clear, that periodes of a "relative peace"(a lack of masked growth) possesses all the structural units covered with epithelium – the acins (7, 14, 21, 28th day), the main duct (14, 21, 35 and 42th day), the primary tubules (21 and 28th day), the secondary tubules (14, 21, 28th day) and the tertiary tubules (7, 14, 21, 28th day). This "break" is generally after a period of cellular growth and continues 2 –3 weeks. Its simultaneous run was observed only in the glandular cells of the acins and the tertiary ducts (7, 14, 21, 28th day).

The detected in the literature information, with which we could compase our results, proposes only scarse data for the structural, weight and some macrometrical parameters of the Harderian gland in several bird species, not giving any data about the micrometrical structural and age peculiarities. We didn't found any data about micrometrical study of the Harderian gland in birds of the broiler type, and about their age related peculiarities.

On the basis of our results we can conclude, that the growth of the height of the acinar and tubular glandular epithelial cells in the Harderian gland of the stock broiler chickens is different in time and stage of manifestation for the different structural elements of the organ.

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# The Gland of the Third Eyelid (Harderian gland) in the Broiler Chickens. II. Histological and Histochemical Peculiarities of the Draining Duct from the Hatching to the 56 Day of the Development

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180 Harderian glands obtained from 90 broiler chickens originating from a commercial stock, from both genders at the age of 1, 7, 14, 21, 28, 35, 42, 49 and 56 days were investigated. After the broiler's narcosis, decapitation and glandular preparation using the classical methods, durable histological specimens were prepared. It was conducted a histochemical staining for polysaccharides – glycogen, mucoproteides, glycoproteides and glycopeptides using a PAS reaction. With the use of a combined staining –MAB followed by a PAS reaction, were simultaneously visualised the acid and the other mucins. The differentiation of the acid sulphated and non sulphated glycoseamminoglycans was conducted with a histochemical staining at pH 1,0 and pH 2,5.

The lightmicroscopy analysis showed that at all the studied ages, the broiler Harderian gland possessed a single draining duct, beginning from its posterior end. In the proximal and distal segment the canal wall possesses lymphocytic agregations. Along all parts of the duct in all of the age groups the epithelium is actively secreting mucosubstances of acid nonsulphated and some neutral mucopolysacharides.

*Key words:* Harderian gland, broiler chickens, draining duct, histology, histochemistry.

## Introduction

In the intraorbital glandular complex in birds, in contrast to the mammalian animal species, the dominating in size is the gland of the third eyelid, most commonly called Harderian, in honour of its discoverer. Long ago it was stated, that the Harderian gland in the birds is a place and a source of a lymphoid tissue and immune producing cells [3, 10]. Nowadays there are evidences, that the gland is a "center" for migrated through the blood flow, or a source of immune bodies [6, 11, 5, 13, 2, 7]. This scientific data makes the gland of the third eyelid in the birds a desired research biological object.

According to the nowadays accepted among the researchers of the Harderian gland classification, in the "birds kingdom" account in of over 1200 bird species, there exists

four types of Harderian glands. The domestic fowl and the galinacea bird species possess a complex tubuloacinous gland of the third eyelid.

In contrast to the well studied basic secretory structural elements of the Harderian gland – acins and different classes of secretory tubules, the draining duct of the gland is rarely mentioned in the specialized literature. Making an anatomical description of the gland in the domestic fowl, some authors mention some scarce morphological peculiarities of its draining duct [3, 16, 9 ]. In two consecutive experimental scientific studies the authors ligatures and drains the draining duct of the Harderian gland in fowls, but gives in their publications only a short description of the condition of the duct after the experiment conduction [14, 15].In the literature there is a lack of data about the microstructure of the duct. There is also a lack of information about the structural peculiarities of the draining duct of the gland in birds of the broiler type during their development.

In the present study we aimed, using the method of the light microscopy analysis, to determine the microstructural condition and some histochemical peculiarities of the draining duct of the broiler chicken's Harderian gland – from the hatching till the 56$^{th}$ day of the postnatal development.

## Materials and Methods

Harderian glands of 90 stock broiler chickens – 1, 7, 14, 21, 28, 35, 42, 49 and 56 days old were used for the conduction of the present study. Every age group consisted of 5 male and 5 female birds. The glands of the third eyelid were taken from previously anaesthetized broiler chickens after decapitation followed by orbitotomy and a careful separation from the surrounding structures and tissues [1]. The obtained 180 Harderian glands were fixated in 10% water solution of neutral formaldehid and in the fixating mixtures of Bouen and Carnoa. After embedding in paraffin with the use of a microtome (Reichert Jung, Austria) were prepared a considerable number of longitodinal and transversal sections with a maximal thickness of 6 μm. From the formalin sectios after staining with Ehrlich' s hematoxylin and eosin and a polychrome Heidenhein's Azan, durable histological specimens were made [12]. Over a part of the sections fixated in the fixating mixture of Karnua we conducted a histochemical (PAS) – reaction for demonstrating the presence of polysaccharides, glycogen, mucoproteides, glycoproteides and glycolipids [12]. Over sections fixated in the fixating mixture of Carnoa was conducted a combined staining method using Mowris's alcian blue, followed immediately by PAS reaction, for the simultaneous staining of the acid and other mucins at pH 2.5 [8]. A selective differentiating staining with Alcian blue at pH 1.0 was conducted for determinating of the sulphated glucoseaminoglycans [12], also the staining with Alcian blue at pH2,5 for the differentiation of the acid nonsulphated glucoseaminoglycans [12].

## Results and Discussion

According to the scarse literature data [16, 9 ], in the galinaceous bird species only one non pair canal leaves from the posterior end every Harderian gland (left and right), after which reaches the base of the third eyelid where drains the products generated in the gland. The examined from us large number of histological sections determined that the draining duct of the gland of the third eyelid in the stock broiler chickens leaves the gland also through its posterior pole. It begins in a different way in every bird. We came across findings, in which the duct began spontaneously among some of the last glandu-

lar lobules in the organ. In other cases some of the terminal lobules in the posterior end of the gland was "transformed" into a beginning of the draining duct.

Independently of this in what way the initial segment of the Harderian gland draining duct begins, in every age group of older chickens, its wall shows a similar structure throughaut the whole length of the duct. The duct was surrounded by a comparatively thin layer of connective tissue. The connective tissue coating was wrapped in a different thickness of adipose pillow throuhout the whale length ol the duct consistina of white adipose tissue. Although the precision of the conducted study in all age groups of birds was not determined the presence of a connective tissue capsule. With the help of the light microscopy analysis was determined in the duct tissue covering a presence of connective and white adipose tissue, well blood circulation and inervation, which can be treated as an indirect evidence for the duct functional load. Another common structural element, for which however we determined an age related dependency, were the diffuse or local concentrations of lymphocytes and lymphoid cells agregations. They were observed in the draining duct of the Harderian gland of the 4-week old broilers (28$^{th}$ day), and were present in almost every histo section of birds belonging to the last four age groups – 35$^{th}$, 42$^{nd}$, 49$^{th}$ and 56$^{th}$ day. If the draining duct of the broiler Harderian gland is conditionally divide along its length into a proximal (initial), medial (middle) and distal (terminal) parts beginning from the posterior glandular end to the conjunctiva, the lymphatic aggregations are mostly determined in the initial and terminal parts of the duct. Along with the local lymphatic aggregations along the whole length of the draining duct, were also diffuse intercellular lymphocytic infiltrations among the ducts epithelium.

Light microscopically was determined, that even in the old broiler chickens, the draining duct of the Harderian gland is with formed and activity functioning covering epithelium (Fig. 1). The epithelial cells covering the inner surface of the broiler Harderian gland draining duct varied in their shape along the ducts length – from cubic

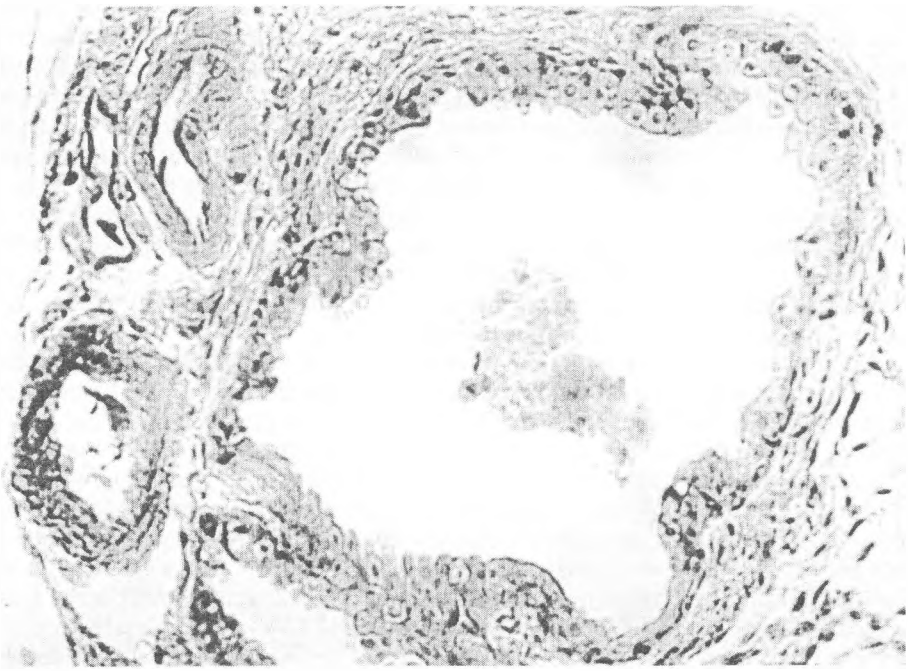

Fig. 1. Broiler chicken – first day. Transversal section from the opening segment of the draining duct, which is already covered with an actively functioning epithelium. Heidenhein's Azan, × 250

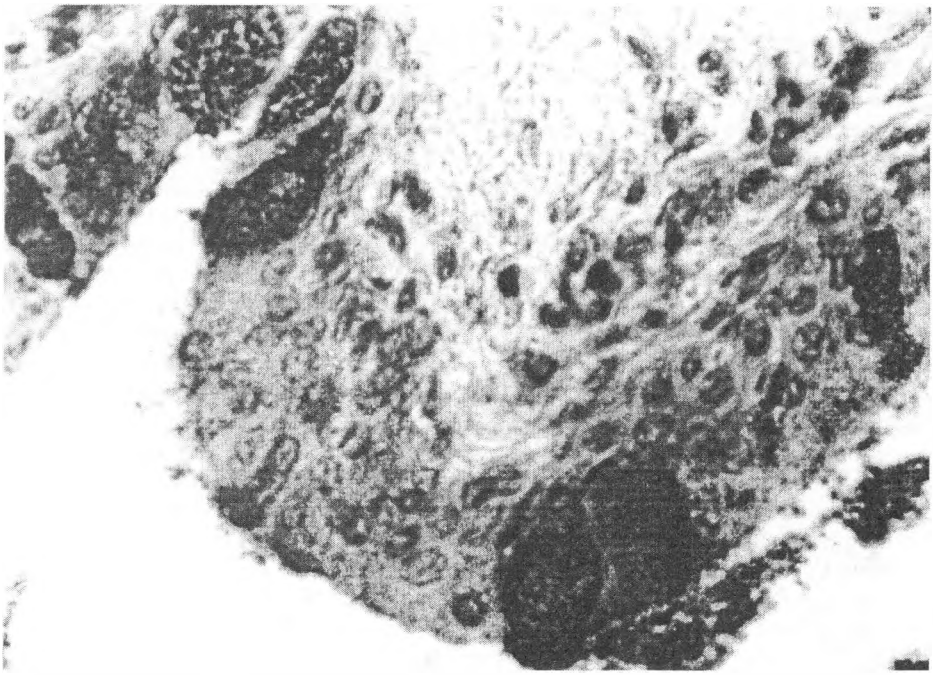

Fig. 2. Broiler chicken – 49th day. Goblet cells from the distal duct segment in different phases of secretion an secretion formation, demonstrating alcianophilie properties. MAB/PAS, × 650

to prismatic. The epithelial cells covering the inner surface of the broiler Harderian gland draining duct varied in their shape along the ducts length – from cubic to prismatic. The tipisation of the epithelium was impeded especially in the proximal and distal parts of the duct where its wall was sepeatedly folded. In the areas with folds and the spaces between them, the epithelium looked like it consisted of layers and was tipisated like a stratified. The segments of the duct with non folded, flat autline of the section surface, were covered with simple cuboidal, or simple columnar epithelium, demonstrating a secretory function. Simple cuboidal epithelium was also covering the lymphocytic aggregations, dividing them from the duct lumen. Among the epithelial cells lining the inner surface of the duct there where also goblet cells, whose quantisy was big in the proximal and distal parts, and very little in the middle segment of the duct. All the observed goblet cells were with high secretory activity.

In our study using the larger magnifications of the microscope we determinided that in all the parts of the duct, the epithelium is situated over a basal membrane in one layer of flat, mononuclear cells, whose nucleus is globe-shaped, big and light stained. The fact, that the previously described epithelium in all parts of the duct does not perform only covering functions was proved by the shown reactively with the different staining methodes. The conducted histochemical tests proved, that along the whole length of the duct surface, the epithelial cells are PAS – negative, which is an argument to treat them as mucus – secreting cells. When stained with Mowris alcian blue with pH1.0, the epithelium reacted extremlly weak, and in most areas was not detected reactivity at all. When the staining medium (Alcian blue ) is with pH2.5 – the shown reactivity was mainly medium expressed. The best pronounced reactivity was observed after a combined staining with a MAB, followed immediately by a PAS reaction (Fig. 2).

These results gives us a reason to think, that the mucosubstances secreted in the draining duct of the stock broiler chickens Harderian gland possesses a mixed composition – mainly acid non sulphated and a little quantity of neutral glucoseaminnoglycans.

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# Arrhythmogenic Right Ventricular Dysplasia – Analysis of Three Fatal Cases

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Arrhythmogenic right ventricular dysplasia (ARVD) is an underrecognized clinical entity, with unknown cause and prevalence and with a frequent familial occurrence. It is characterized by progressive degeneration of cardiomyocytes and fatty replacement of right ventricular cardiomyocytes, which causes electrical instability and sudden death. ARVD is a rare disorder, but for a short time, three cases were proven by autopsies in a small region in Bulgaria. All of them died suddenly without data for preliminary disease. In all cases, the myocardium of the right chamber was partially replaced by adipose tissue. The gross findings resembled an acute myocardial infarction and if no histology of the right chamber is performed, the diagnosis of ARVD may be easily overlooked. Proper diagnosis is important because: (i) an recessive form of ARVD is common in neighboring countries, and (ii) relatives should be informed and followed up for premorbid diagnosis.

*Key words*: sudden death, ARVD, cardiomyopathy, lipomatosis, adipose tissue.

## Introduction

Arrhythmogenic right ventricular dysplasia (ARVD), or cardiomyopathy (ARVC) is an underrecognized clinical entity, with unknown cause and prevalence and with a frequent familial occurrence (1, 2). Morphologically, it is characterized by progressive degeneration of cardiomyocytes and fibro-fatty replacement of right ventricular myocardium, causing electrical instability, ventricular tachyarrhythmias with left bundle branch block and sudden death at a young age (3, 4, 5). ARVD is a rare disorder, but for a short period of time, three cases were proven by forensic pathology autopsies in a relatively small region in the northeastern part of Bulgaria.

The aim of the present study is to analyze the autopsy findings in these cases.

## Results

Case No 1. 26-year-old man, dying suddenly during minor physical effort (No 403/2006, blood group Aβ, no alcohol in blood)

Case No 2. 42-year-old man, found dead in a toilet room at Albena resort (No 104/2007, blood group Aβ, no alcohol in blood)

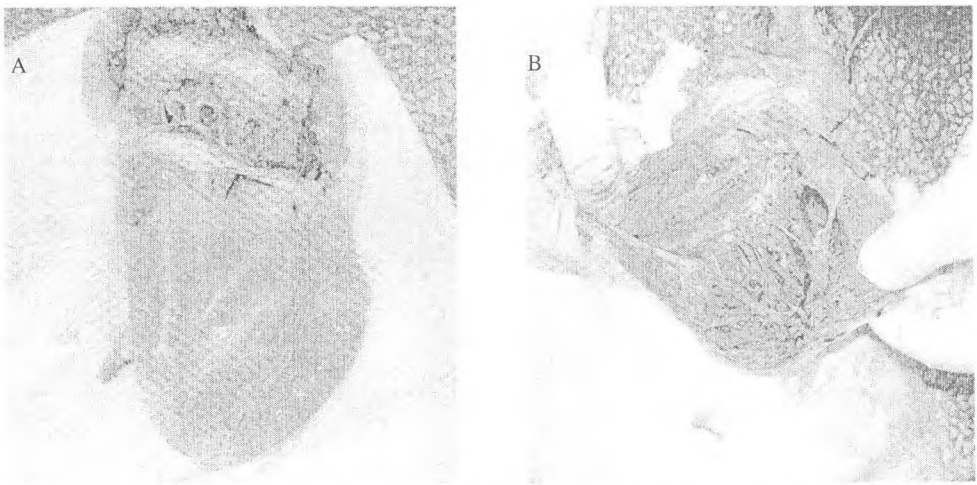


Fig. 1. Case 1, right cardiac chamber, slight dilation (A), spotty endocardial surface (B)

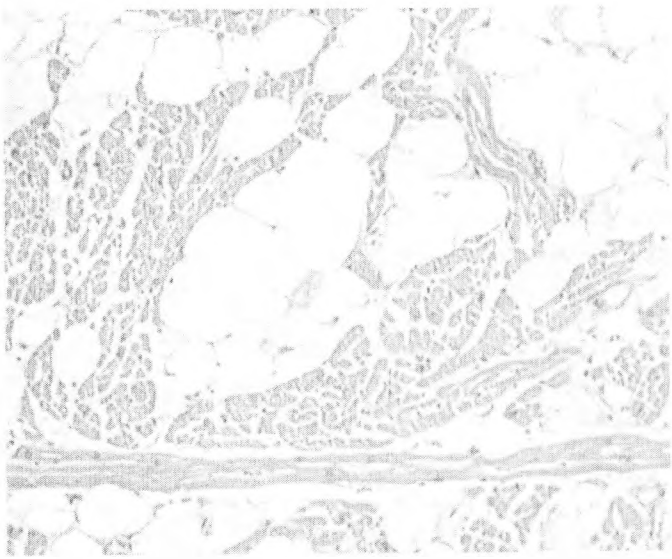

Fig. 2. Case 1, streaks of adipose tissue replace the cardiomyocytes in the right chamber. HE, × 100

Case No 3. 50-year-old woman, dying suddenly in the Outpatient clinic – Balchik (No 132/2007 – blood group Aβ, 0.4‰ alcohol in blood – subclinical level of alcohol affect). In all cases there were no data of previous complaints or disease.

The gross findings in all the three autopsy cases were consistent of signs for rapid occurring death – severe congestion in all viscera, without postmortem blood coagulation, presence of pulmonary and cerebral edema; no other significant lesions were found.

Changes in the hearts were minimal – slightly dilated cardiac chambers (in Case 1 only the right ventriculus was involved – Fig. 1 A), with spotty endocardial surface and somewhat pale and streaky myocardial cut surface (Fig. 1 B). Major arterial blood ves-

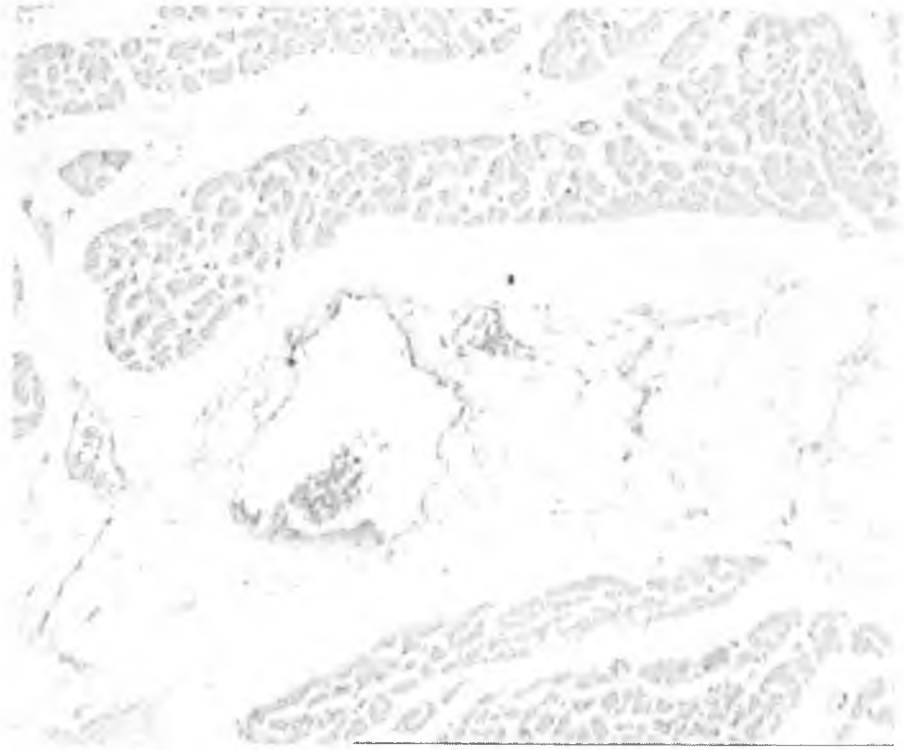

Fig. 3. Case 2, perivascular adipose tissue replacement of the cardiomyocytes in the right chamber. HE, ×100

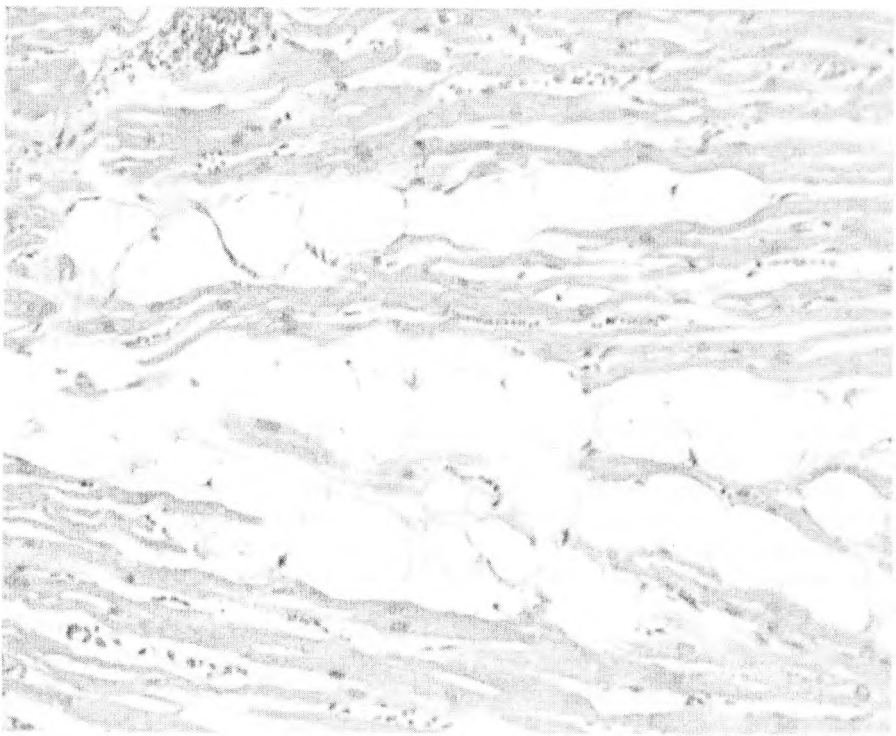

Fig. 4. Case 3, streaks of adipocyte, scattered among the cardiomyocytes in the right chamber. HE, ×100

sels showed minor degree of atherosclerotic involvement: in Case 1 – only in the aorta, in Case 2 and 3 also in the main branches of the coronary arteries without gross evidence of atherosclerotic complications, such as fissures, erosions or thrombosis.

Light microscopy findings in the myocardium of the dilated right heart chambers included increased amount of perivascular adipose tissue forming massive layered fatty infiltrates with signs of degeneration in the surrounding cardiomyocytes (Fig. 2, 3, 4). The myocardium of the left ventriculus appeared normal in Case 1; in Case 2 "wavy" cardiomyocytes were established and in Case 3 the streaks of adipose tissue involved both cardiac chambers.

## Discussion

As fat replacement of cardiomyocytes is the most essential finding in all the three autopsy cases, we consider the gross and light microscopy evidence presented above to be consistent with ARVD. All the cases studied here reveal the typical involvement of the heart without any other significant pathological changes to explain the occurrence of sudden death.

ARVD was first described about 30 years ago [6], while analyzing the causes of fatal ventricular arrytmias. Since then, a considerable progress has been made in the understanding of the clinical presentation, the morbid anatomy and the genetics of ARVD. However, a great lack of information still exists with regard to pathogenesis, premorbid diagnosis, natural history, risk stratification, and prophylaxis of life-threatening complications and prevention of death in patients with ARVD.

It is believed that ARVD is a rare condition, but since the patients usually have no severe complaints and most of them after sudden death are subjected to forensic medicine postmortem expertise, the actual incidence may be considerably higher. It is important to point out, that if no histology of the right ventricular myocardium is performed, ARVD may be easily overlooked. The three cases discussed here were initially diagnosed as suspicious of acute myocardial infarction. ARVD accounts for about 20% of sudden deaths in all individuals younger than 35 years and it was once considered a disease of the young people and athletes [4, 7, 8], but may be diagnosed in the older population [9]. The three cases discussed here belong to both age groups. Therefore, an early and accurate diagnosis followed by appropriate treatment is increasingly important for it may prevent lethal arrhythmias.

In regard to the pathogenesis of ARVD, it is suggested, that an inherited impairment of cell-to-cell adhesion (desmosomal proteins) may be the underlying pathogenic mechanism, via accelerating apoptosis of myocardial cells. Mutations genes encoding desmosomal proteins (Junctional plakoglobin, Desmoplakin, Plakophilin 2, and Desmoglein 2), have been identified in patients with ARVD. It is believed that these primary defects contribute for accelerated apoptosis of cardiomyocytes and secondary fatty or fibrofatty replacement. Since the disorder is inherited, it is of great importance not just to establish the right diagnosis on time, but also to transfer the information to the general practioners, so that genetic studies of relatives and off-springs to be carried out. The recessive form [10] of ARVD, known as Naxos disease (including also features as woolly hair and palmoplantar keratoderma) is more common in regions close to Bulgaria – the Hellenic island and Turkey.

According to the data from the literature [1, 5, 9] histological findings in ARVD may be presented by lipomatosis of the right ventricular myocardium alone, or by combination of lipomatosis/fibrosis. The observations presented here reveal no myocardial fibrosis or admixture of connective tissue alongside with the typical lipomatosis. Some

of the studies report the presence of perivascular lymphocytic infiltration in the zones of adipose tissue infiltrates [12] and consider this feature as important for the development of arrytmia. We did not encounter any lymphocytic infiltrates in our cases.

Another interesting point in the pathogenesis of ARVD is considering the synthetic and secretory activity of adipocytes. Now, it is becoming increasingly evident that adipose tissue is a multifunctional organ that produces and secretes multiple factors (**adipokines**) [13, and their references], that can act in both paracrine and endocrine fashion and thus to induce malfunction of adjacent cardiomyocytes and disturbances of the conduction system.

## Conclusions

The diagnosis of ARVD should be suspected in individuals of all ages who present with a clinical syndrome of sudden death. In all of them, histological investigation of the myocardium of the right chamber should be performed. In case, that ARVD is proved, the information has to be forwarded to the relatives and the medical specialist in concern.

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# Using Pig Carrion As an Experimental Model for a Human Body in Forensic Entomology Succession Study – a Methodology

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In the last decades, forensic entomology has begun to play an important role as an investigative procedure. For estimation of PMI, basic distribution data for the most indicator species of insects are required. It is apparent that the seasonality and species assemblage vary in different geographical areas. An initial succession study started in the suburban area of Gabrovo, Bulgaria, using pig carrion as model representing human body. The methods are described in details. It is important to introduce the basic knowledge of methods and applications of the science among all professionals involved in a death investigation. Only the good collaboration and a clear procedure can lead to a full understanding of all the evidence recorded at the crime scene and at the autopsy.

*Key words:* forensic entomology, succession study, methodology, pig, PMI.

## Introduction

Forensic entomology is the name given to the study of insects and other arthropods for use in forensic investigations. For estimation of postmortem interval (PMI), basic distribution data for the major indicator species of insects are required. It is apparent that the seasonality and species assemblage vary in different geographical areas.

A study to determine possible indicator species of insects in suburban area of Gabrovo, Bulgaria, was conducted using pig carrion as models representing human bodies. The studies were planned for each season of the year 2007 and may be repeated. The main objective of the work is to study the entomofauna of a cadaver of a white pig (*Sus Scrofa*), the period of invertebrate activity with relation to the different phases of decomposition, to determine the most indicator forensic species of insects for the season and for the region, and to prepare a reference collection of insects from this region for subsequent studies. In view of the fact that forensic entomology has provided excellent results in other countries, a first step should be taken to include these **methodologies** in Bulgaria, with the purpose and tools that may be used subsequently in legal proceedings.

**Study Site.** Study site was chosen in a suburban area of Gabrovo (almost the geographical center of Bulgaria), on a semishaded slope of a hill. The field was specially surrounded by a fence before the experiments, against occasional human or animal intrusions.

**Research Models.** The use of human corpses for field studies is illegal in Bulgaria. Pig carcasses were used in experiments because they have been widely accepted as human models for decompositional studies [3]. Pigs are similar to humans since they are omnivorous and possess a similar digestive system including their gut fauna which is important in the evaluation of the decomposition process of carcasses. The last stage of digestion in the intestinal tract of both humans and pigs occurs through bacterial action, not by autolytic enzymatic action as occurs in many other animals. Differences exist between the types of bacteria found in the intestinal tract of the pig and humans, but the end result is the production of the same gases in the gastrointestinal tract which cause refloat [7]. Pigs are relatively hairless and have skin tissue similar to that of humans, in fact it has been used in human skin grafts. C a t t s and G o f f [2] claim that a 23 kg (50 lb) pig is approximately equivalent to an average adult male torso, which is the main site of decomposition and insect colonization.

## Materials and Methods

Dead pigs, *Sus scrofa* L., were used as human models for this study, one animal for each season. The mean weight of the animals was 20-32 kg. All dead pigs were purchased from a farm near to the study site. Prior to purchase, the pigs were killed by a strong blunt force strike to the head. Each dead pig was immediately double bagged in a heavy-duty plastic trash bag and transported to the study site. Wire cage was placed over a pig body to protect it from large vertebrate scavengers. Pitfall traps were installed in the soil at the ground level, at 4 points in 1m distance from the body. Instruments: Tool box (data-loggers, containers, consumables, etc.).

**Data Collection with Meteorological Measurements.** Observations and collections were made from the day of deposition to the full decomposition of the body. Data loggers for measuring the ambient and cadaver temperatures together with air humidity were used for reference with the local meteorological station data, and for future calculating development periods of certain species at varying temperatures. In addition, every time of sample collection the ambient temperatures were measured with a digital thermometer.

**Protocols.** On day 1, the pigs were removed from bags and placed in a location on the site. There was made a stub wound in the center of the abdomen of each animal, modeling real conditions of crime, which was used for inserting a data loggers for inner corpse temperature measurement. Data were collected including pigs number (season), time of death, date, time, sample number, ambient temperature at 1.5 m high and at the ground level, inner corpse temperature, air humidity, a brief description of the weather. A collection of adult flies and other insects was made over, on and under the pig. These were placed in vials full of ethanol and labeled. Eggs of flies were placed on moistened tissue paper in vials for rearing. A part of the eggs were preserved by placing them in ethanol. After the first day, samples were made on a daily basis during first 40 days for each experiment, and every second or third day for later period. A collection of adult flies and other adult insects (and their larvae) was made over, on and under the pig. We also collected the insects from under the cadaver and from the soil, and from pitfall traps at 4

points in 1m distance from the body. They were placed in vials full of ethanol and labelled. Living samples of fly maggots from the corpse and from under it were placed in plastic containers (with perforated cups) for rearing. Another part of maggots specimens were killed as soon as possible with very hot (>80°C), but not boiling water for approximately 30 s to achieve best preservation. Afterwards they were stored in vials with ethanol. A photo and video documentation of the pigs, insects and study site was made.

**Rearing Procedures.** We stored the majority of maggots of each sample in plastic containers under controlled conditions, on a shelving in the forensic pathology district facility. The plastic containers with fine perforated lids were lined with coarse sawdust or tissue paper and filled in a part with a minced meat for feeding maggots. The maggots were checked daily. After adult emergence, the containers were placed into the freezer for a few minutes to kill the flies. Adults were put in labeled vials of 95% ethanol for future taxonomic identification. The data about the temperatures and the time of emergence were collected.

All the collecting of entomological evidence, documentation and processing were according to the standards established by the European Association for Forensic Entomology [1]. A single code was used in all of the documentation.

## Results and Discussion

The application of the entomological method to determine the time of death consists essentially of two main procedures: 1. During the early postmortem period, the estimate is based on a direct age assessment of the oldest individuals of fly larvae (maggots) that have developed on the body (minimum PMI) [4]. 2. During the late postmortem period, the estimate is based on the composition of the arthropod community as it relates to expected successional patterns. The study of arthropod succession enables scientists to associate each species or group to a well-established decomposition stage and geographical region [5, 6].

International forensic institutions already have specific entomologic laboratories to assist law officers at the death scene. Unfortunately, there are no such resources in Bulgaria and in the Balkan area, and the putting into practice of this technique is unusual and it has no practical basis. It is obvious a need of introducing forensic entomology use in investigation of death in our region.

This is a first step done for use of standard methods, on an experimental model of carrion insect succession in our region. As a result of growing practical experience, a simple protocol for collecting entomological evidence at crime scenes is to be developed. It must be according European standards for the use of forensic entomology in criminal investigations and must fit to the Bulgarian law procedures.

There is a lot of work to do in next months and years to develop a high level of competency in the field of forensic entomology for our country. But not less important is to introduce the basic knowledge of methods and applications of the science among all professionals involved in a death investigation. Only the good collaboration and a clear procedure can lead to a full understanding of all the evidence recorded at the crime scene and at the autopsy. A close interaction of the forensic entomologist with the forensic pathologist is strongly recommended.

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# Localization of Defloration Lacerations in Crescent-Shaped Hymens

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The hymen is a mucous fold that separates the vaginal entrance and the vagina itself. It could have a various shape depending on the height and the type of the free edge, the width of the opening, the number of openings etc. In this study, crescent-shaped hymens were investigated. A total of 3288 women, victims of sexual abuse were included. Out of them, 149 refused a forensic medical examination and 385 had given birth to a child. In 2.29% of the rest, crescentric hymens (*H. semilunaris*) have been observed. In 14.29% of patients with such hymens, a sexual intercourse without laceration of the hymenal membrane was possible. In cases with hymens with a single laceration, it occurred most commonly at the 6 o'clock position when the victim was in dorsal recumbency; in cases with two defloration lacerations – at 5 and 7 o'clock or 3 and 9 o'clock positions (incidence of 22.22% each) and in cases with three lacerations the commonest pattern was at 3, 6 and 9 o'clock positions (incidence 40%).

*Key words:* crescent-shaped hymen, defloration laceration, tearings.

## Introduction

The hymen was described for the first time in 1615 by Andreas Vesalius [7]. It was named after the ancient Greek god of marriage Hymenaios. The hymen is a mucous fold separating the vaginal entrance from the vagina itself. A matter of forensic medical interest is the investigation of the localization of hymenal lacerations, particularly of crescentric hymens, after defloration. In the available literature, there is no information about the localization of hymenal lacerations. The morphological traits of the hymen, important with regard to forensic medical expertise, are the shape; particularities, location and diameter of the opening; height and thickness of the free edge etc [2]. The crescent-shaped hymen looks like half-moon or horseshoe and has one eccentrically situated opening. Usually, the opening is located urethrally and the membrane – rectally. The occurrence of haematoma, abrasion, laceration, tearing of hymen during the forensic medical examination are not always indicative for a sexual abuse, but in most cases, are resulting from intercourse, bad touch, masturbation or interference with other hard blunt objects [6]. The tearing of the hymen depends on its elasticity, the size and height of its opening, the height of the free edge and its shape.

The aim of the present investigation was to determine the commonest localization of defloration lacerations of crescent-shaped hymens.

## Material and Methods

The records of 3288 female victims of sexual abuse, in 15 forensic medical units at the territory of the Republic of Bulgaria were processed. The hymenal shapes, diameter of hymenal openings, the heights of the free edge and the localization of lacerations have been determined.

The forensic medical records and expertise were processed by the documental method (Dimitrov, 2000) and the statistical processing of data was done in Microsoft Office Excel.

## Results and Discussion

From all victims of sexual abuse, 149 (4.53%) refused a forensic medical genital examination. Another 385 (11.71%) had given birth by the time of the rape and the forensic examination.

The rest 2754 (83.76 %) had a status of external genitalia with records of morphological traits of the hymen during the forensic medical examination. In 63 out of them (2.29%), crescent-shaped hymens have been registered. This prevalence is lower than that, reported by R a d a n o v [8] – 3.19%. Our data are contradictory to the affirmations of K a d a n o v et al. [5] that the most commonly encountered shape of hymen was the crescent shape.

Morphologically, according to the free edge height, women with crescent-shaped hymens were classified as with a high edge – 26 (41.27%); medium edge – 15 (24.40%) and low edge – 22 (33.35%) (Fig. 1).

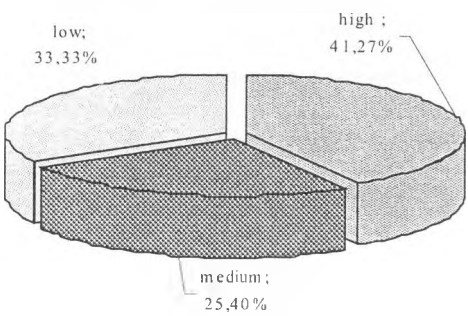


Fig. 1. Distribution of crescent-shaped hymens according to the height of the free edge

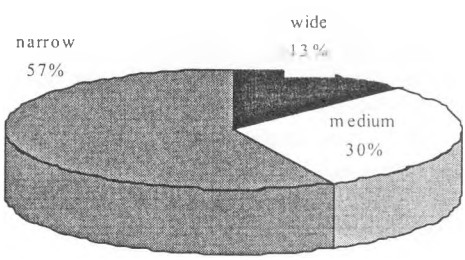


Fig. 2. Distribution of crescent-shaped hymens according to the width of the opening

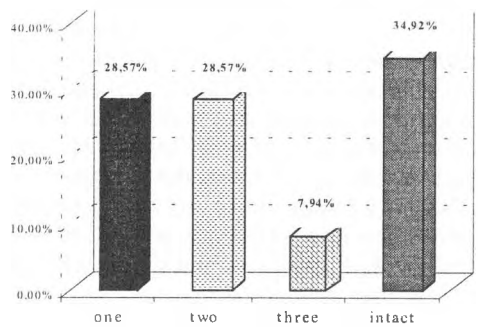


Fig. 3. Distribution of crescent-shaped hymens according to the number of lacerations

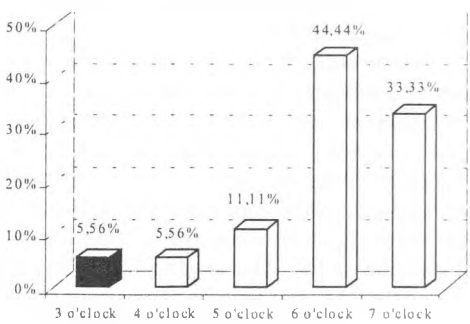


Fig. 4. Site of crescent-shaped hymens with a single laceration

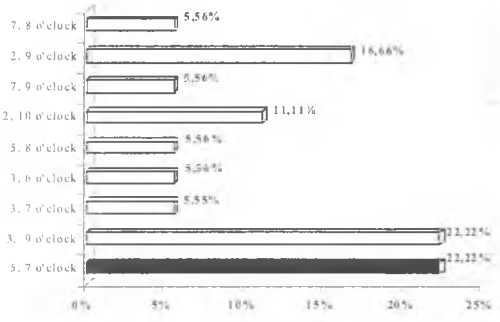


Fig. 5. Sites of crescent-shaped hymens with two lacerations

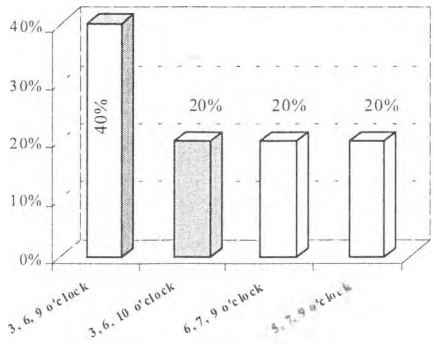


Fig. 6. Sites of crescent-shaped hymens with three lacerations

In 36 (57.00%) of cases, crescent-shaped hymens were with narrow opening, in 19 (30%) cases – with a medium opening and in 8 cases (13.00%) – with a wide opening. This distribution is presented on Fig. 2.

During the forensic medical examinations, 22 (34.92%) of hymens were intact. Defloration lacerations were established in 41 (65.08 %) female victims of sexual abuse. Out of them, 28.57% had a single laceration, the same number presented two lacerations and in 7.94% of cases – three lacerations were observed (Fig. 3). These results are inconsistent with those of Radanov (1986) who reported that in 20.45% of female rape victims with crescentric hymens, defloration lacerations have been present.

The site of single crescent-shaped hymenal lacerations was in most instances at 6 o'clock at dorsal recumbency of the patient, followed by location at 7 o'clock (33.33%). The least occurrences (5.56% each) were those at 3 and 4 o'clock positions. The distribution is shown on Fig. 4. Our data are similar to those of other authors having investigated the localization of lacerations of hymens with various shapes. H o b b s et al. [3] reported that the commonest site of hymenal laceration was at the 6 o'clock position at dorsal recumbency.

In our study, the sites of injury of crescentric hymens with two lacerations were at 3 and 9 o'clock positions or at 5 and 7 o'clock positions (with 22.22% each) at dorsal recumbency of the victim. The distribution is presented on Fig. 5. Ingram DM, et al. (2001) reported that in cases of two hymenal lacerations, their sites were at 5 and 7 o'clock positions, without specifying the shape of hymens.

Fig. 6 shows that cases with three lacerations presented localizations at 3, 6 and 9 o'clock positions.

It should be also noted that during the performed forensic medical examinations, a part of examined women with crescent-shaped hymens had had sexual intercourse without tearing of their hymens.

## Conclusions

1. In rape victims with crescent-shaped hymens with a single defloration lacerations, its site in 44.44% of cases was at the 6 o'clock position at dorsal recumbency of the patient.

2. When two defloration lacerations of crescent-shaped hymens were present, they exhibited an equal incidence of 22.22% at the 5 and 7, or 3 and 9 o'clock positions.

3. The most prevalent pattern (40%) of crescent-shaped hymens with three lacerations was observed at the 3, 6 and 9 o'clock positions.

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# Quantitative Intima-Media Relations in the Wall of the Main Blood Vessels of the Leg in Adults and Seniors

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The goal of the current study is to evaluate the dynamic quantitative changes in the wall of the main vessels (arteries, superficial and deep veins) of the leg during adulthood. Transverse sections of isolated vessels were taken from the leg of cadavers (34-88 years of age). The sections, stained with hemalaun-eosin and orcein, were measured via ocular micrometer/object micrometer system. The data was analyzed by means of descriptive statistics, correlation and graphical analysis. The increment of the intima thickness of the arteries correlates strongly with the age; in the superficial veins the intima thickness correlates only moderately to considerably with age, while in the deep veins no age dependent variation is observed. The relationships are reverse when the media thickness is observed. Generally, the intima is located evenly along the circularity of the arteries. This is established through the absence of large differences between the minimum and maximum values of the intimal thickness of the same vessel in adults under 60 years of age. After this age the intima becomes more irregular. In the superficial and deep veins the intimal thickness demonstrates large differences between minimum and maximum values. The media in the three vessel types shows more irregularities during the entire investigated age span and no age dependency.

*Key words*: vascular wall remodeling, quantitative intima-media relationship, arterial insufficiency, venous insufficiency, ontogenetic development.

## Introduction

The chronic diseases of the arterial and venous wall constitute serious health, therapeutic and economic problem of worldwide significance. A serious share of this disease group is attributed to the diseases of the lower limb vessels, causing inevitably the development of acute and/or chronic arterial or venous insufficiency. As number of aspects of the vascular pathology are cleared the interest in the normal morphometric characteristics of the vascular wall increases. Such knowledge is especially important by the development of methods for early discovery and reliable prognosis of the vascular diseases (1, 3, 4, 8) and for the needs of bioengineering by the design of adequate and reliable vascular prostheses. At the same time the morphometric parameters of the vascular wall *in vivo* and *ex vivo* are poorly studied and covered only by scarce number of studies.

Our research is particularly concerned with the main vessels of the leg. Our earlier studies [5, 6, 7, 10, 11, 12, 13, 14, 16] demonstrated that there are number of peculiarities in the dynamics of the morphological and morphometric characteristics of these vessels during the pre-, peri- and early postnatal development of men, which distinguish them not only from the vessels of other regions, but also from the proximally positioned knee and femoral segment of the lower limb. The immediate goal of this research is to depict the dynamic quantitative changes in the wall of the main leg blood vessels during adult life.

## Material and Methods

The investigation included cadaver material from 34 to 88 years old individuals, divided in three age groups (30-49y;50-69y;70-89y). Isolated segments of the following vessels were harvested from the middle and occasionally from the upper an lower third of the leg, fixed in formol-saline and embedded in paraffin:

– arteries: *a. tibialis anterior* (ata), *a. tibialis posterior* (atp) and *a. peronea* (ap);
– superficial (subcutaneous) veins: *v.saphena magna* (vsm), *v.saphena parva*(vsp);
– deep main veins: *v.tibialis posterior* (vtp).

Transverse sections were stained with hemalaun-eosin (H&E) and with orceine for elastic fibers and measured via ocular micrometer/object micrometer system. Raw data was processed and analyzed with correlation, variance, regression and graphic analysis methods [2, 9, 15].

## Results and Discussion

Variation in the average intimal thickness for both arteries and veins was found to span over the same range and is equally skewed in both vessel types:

– Intimal thickness of arteries varies between 10 μm and 340 μm, with mean value 64 μm; Interquartile range 25 μm to 68 μm with median 34 μm (Fig. 1).

– Intimal thickness of veins varies between 6.35 μm and 300 μm, with mean value 48.18 μm; Interquartile range 17.9 μm to 49.1 μm with median 32.09 μm (Fig. 2).

Arterial intima thickness shows moderate to strong correlation with age. R-squared values suggest that Age influences about 50% of the variance in the intima of the arteries. The lower value and significance of the correlation coefficient for *a. peronea* is probably due to higher interindividual variability of this artery because of genetic, developmental and haemodynamic differences (Fig. 1; Table 1).

The value of the correlation coefficients (r) and the statistical error probability (p) exclude age as factor influencing intimal thickness in the investigated lower limb veins. There is a faint tendency for increment with age, however there must be more powerful factors that determine the intimal thickness, such as genetic influences, way of life, health status etc (Fig. 2; Table 1).

The average media thickness of arteries varies over wider range than in veins. Distribution of media thickness of veins and arteries is less skewed than that of intimal.

– Average media thickness of arteries varies between 102 μm and 568 μm, with mean value 282 μm; Interquartile range 170.25 μm to 380.50 μm with median 261 μm (Fig. 3).

– In veins average media varies between 70 μm and 305 μm, with mean value 156,81 μm; Interquartile range 124.81 μm to 180 μm with median 148.75 μm (Fig. 4).

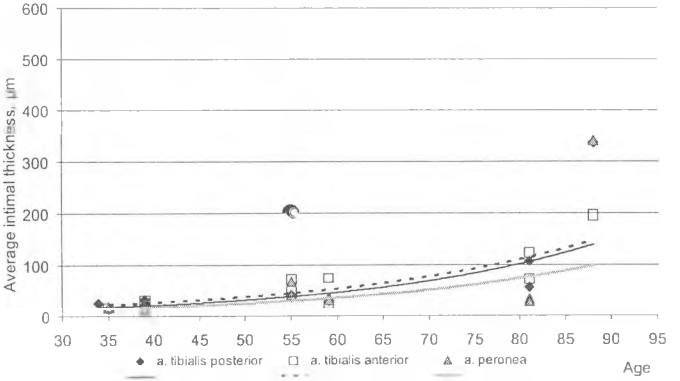


Fig. 1. Correlation of age and average intima thickness of the main arteries of the leg

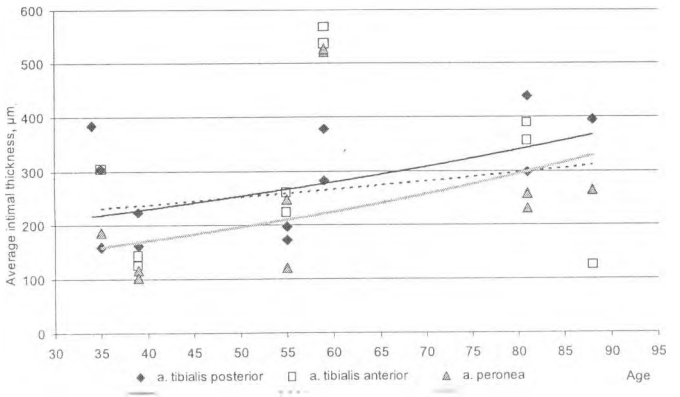


Fig. 3. Correlation of age and average media thickness of the main arteries of the leg

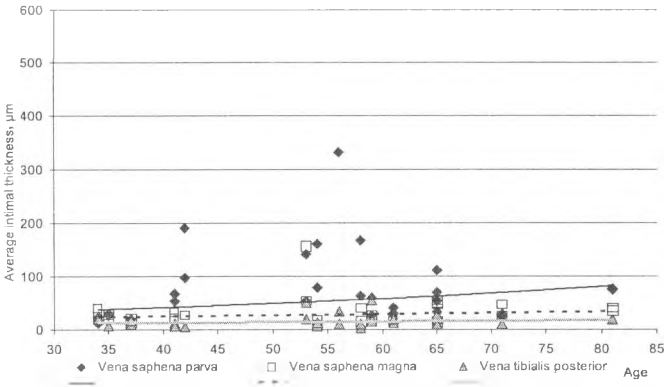


Fig. 2. Correlation of age and average intima thickness of the main veins of the leg

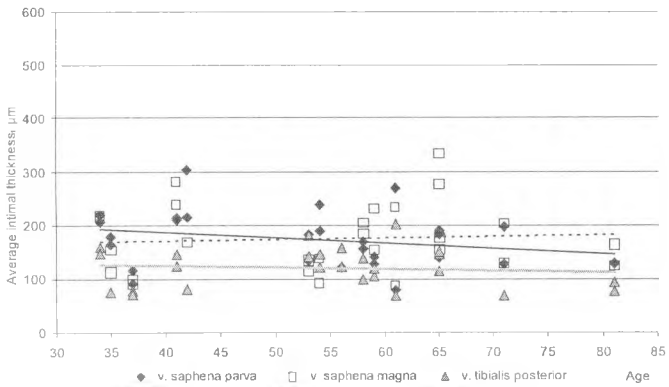


Fig. 4. Correlation of age and average media thickness of the main veins of the leg

Table 1. Coefficients of correlation of intimal thickness and age of the investigated vessels

| Vessel | *r* | *p* |
|---|---|---|
| *A. tibialis posterior* | 0.70 | **0.0112** |
| *A. tibialis anterior* | 0.84 | **0.0025** |
| *A. peronea* | 0.58 | 0.0778 |
| *V. saphena magna* | 0.16 | 0.5785 |
| *V. saphena parva* | 0.02 | 0.9251 |
| *V. tibialis posterior* | 0.08 | 0.7807 |

Table 2. Coefficients of correlation of media thickness and age of the investigated vessels

| Vessel | *r* value | *p* |
|---|---|---|
| *A. tibialis posterior* | 0.52 | 0.0801 |
| *A. tibialis anterior* | 0.19 | 0.6061 |
| *A. peronea* | 0.30 | 0.3992 |
| *V. saphena magna* | 0.004 | 0.9896 |
| *V. saphena parva* | –0.37 | 0.1392 |
| *V. tibialis posterior* | –0.15 | 0.5804 |

Correlation coefficients and statistical error probabilities suggest that age has very little to none influence on media thickness (Table 2). However there is a slight tendency for increment of the parameter in arteries and almost no change to decrement of the media in veins. This tendencies are probably connected to the age dependency of other more powerful factors that determine the media thickness in both vessel types (nutrition, way of life, pathology etc.) (Figs. 3, 4).

Comparison between minimal and maximal values measured over different regions of the same transverse section gives a very good picture of the regularity of the intima along the circumference of the vessel. Between 30 and 70 years the minimal intima of arteries remains almost constant and the increment in intima thickness over the years is mostly due to increment in size and area of the thickened parts (maximal values) of the intima. After 70 years there is shear increment in all three values of the intimal thickness in arteries (Fig. 5).

In veins the maximal and minimal intimal thickness change parallel to each other, which suggests the aforementioned impact of other individual factors on the intimal thickness in veins (Figs. 6, 7).

The intima/media index of arteries (about 0.12) is lower than that of veins (about 0.20) in the first two age groups (30-49 and 50-69 years old). In the 70+ group the index

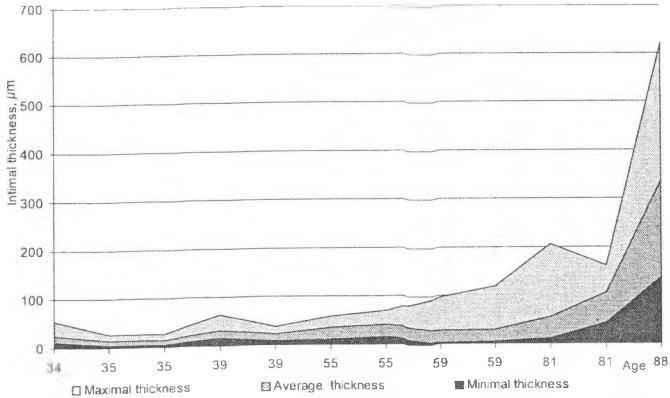


Fig. 5. Age changes in the minimal, average and maximal intimal thickness of a. tibialis posterior

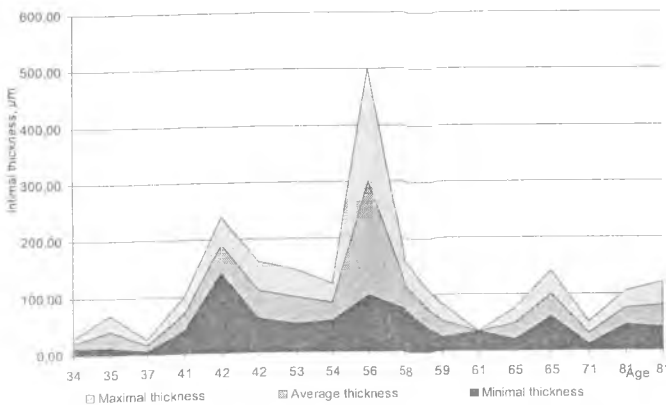


Fig. 7. Age changes in the minimal, average and maximal intimal thickness of v. saphena parva

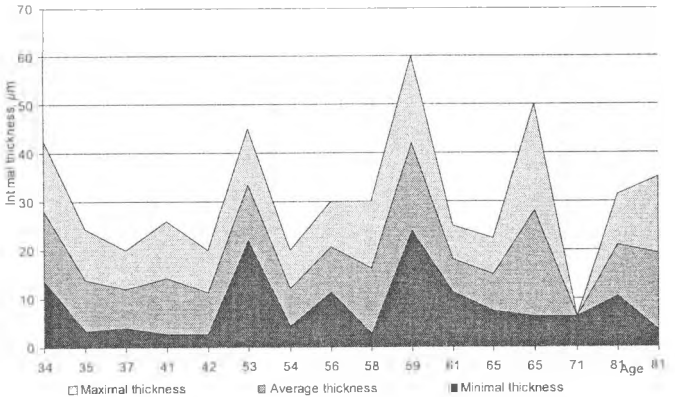


Fig. 6.Age changes in the minimal, average and maximal intimal thickness of v. tibialis posterior

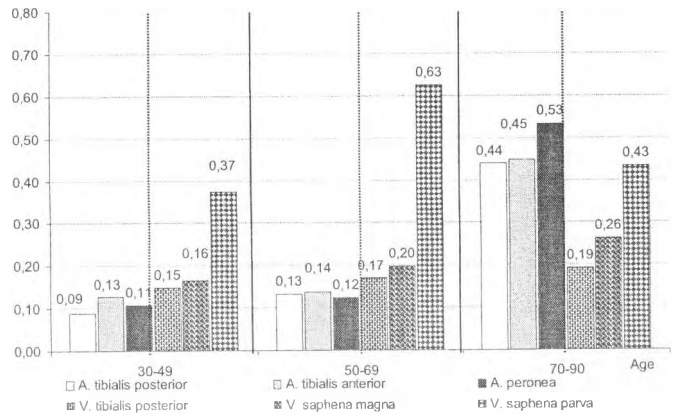


Fig. 8. Average intima/media index of the investigeted vessels

of the arteries increases at once two to three times, while the one of the veins almost doesn't change. The intima/media index for the v. saphena parva is about two to five times higher than that of other veins. A fact that probably can be explained with the peculiarities of the filo- and ontogenesis of the lower limb vessels (Fig. 8). Very interesting and deserving further analysis is the fact that the intima/media indexes are distributed in two distinct groups – below 0.20 and above 0.40.

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# Acute Bleeding at the Upper Gastrointestinal Tract – Forensic Medical and Pathomorphological Characteristics

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Autopsy cases of acute fatal bleedings of the upper gastrointestinal tract (ABUGIT) are examined. Autopsies, performed in the Department of Forensic Medicine of Medical University of Varna for a ten-year period (1998 – 2007) are characterized according to the frequency and morphological peculiarities. ABUGIT out of a total of 3619 autopsies, 21 cases (0,58%) of ABUGIT are established. They account 1,2% of sudden death cases. The average age is 52.31±2.4; 85.7% are men and 14.3% are women, in 33.3% of them alcohol in the blood is found. The distribution of the cases of ABUGIT is as follows: chronic gastric ulcer – 6 cases, erosive gastritis – 4 cases, esophageal varices – 4 cases, mucous erosions of esophagus, stomach and small intestine – 3 cases, duodenal ulcer – 2 cases, erosions of the stomach and esophageal varices – 1 case and malignant tumors – 1 case.

*Key words:* acute bleeding, upper gastrointestinal tract, forensic medical expertise.

## Introduction

Acute bleeding of the upper gastrointestinal tract (ABUGIT) represents more than 90% of the cases with bleeding from the gastrointestinal tract. On time diagnosis ensuring adequate treatment is needed [2]. In 0.9% of the cases ABUGIT causes sudden fast and fatal outcome and these cases are usually an object of forensic expertise [5], unlike the cases of chronic bleeding of the gastrointestinal tract.

The clinical appearance of ABUGIT generally includes hemathemesis, melena, anemia and shock. Specifying the cause of the acute bleeding is important for the adequate treatment and avoiding the fatal end in hospital as well as for making clear the mechanisms of death in the post mortem expertise.

The **aim** of the present study is to characterize the range and the morphological special features of cases of fatal ABUGIT, object of forensic expertise during the period 1998-2007.

## Material and Methods

The autopsy materials of the Forensic Medicine Department of the Varna Medical University for the 1998-2007 period and all cases of sudden death resulting from ABUGIT

have been observed and estimated. Expertise and histological materials from forensic autopsies have been studied and from them relevant data and characteristics for each case have been obtained.

## Results

A total of 3619 autopsies were performed in the department. The case distribution by years is shown in Table 1. The cases of ABUGIT are 21, which is 0.58% of all the autopsies. They represent 1.2% of the sudden death cases, ascertained by 1750 dead cases for the same time period (48.35% of all cases). There is a lack of specific dynamics in the case amount on a yearly basis; their number usually varies between one and three cases with the exception of the year 2004 when there were five. All of those observed have died outside hospitals. They are usually lonely people unable to or not willing to seek medical assistance. The reason for autopsy was the suspicion for criminal act and violent death since there was too much blood present at the place they died. Thus the forensic expert encounters ABUGIT at the very scene of the accident, but by inspection only he is not able to determine the origin and the cause for the accident. With the consequent post mortem investigations these questions can be answered and the cause of the death determined.

The average age of the cases is 52.31+/–2,4 years. Males predominate: 18 men and 3 women (85.7%-14.3%). Usually there is a lack of specific data for past complaints, diseases or other damaging factors. In 9 (42.8%) however, there are preliminary data of chronic alcoholic abuse.

Two of the cases were tested for alcohol in other labs and for that reason we did not have data indicating alcohol misuse. In the remaining seven cases (33.3%) the forensic-medical expertise established the use of alcohol immediately before death. In four of them the alcohol concentration was below 0.5‰ but as the alcohol in the urine was not examined it could not be judged whether this was the final phase of its elimination. In the other three cases with alcohol use, one was of light degree and the other two of average; all of them being in elimination stage. This is an important fact as the toxic alcohol influence at this stage is much stronger.

Liver cirrhosis with esophageal involvement was diagnosed in 3 cases; in 12 there were data of chronic diseases variously manifested – hypertensive disease, generalized atherosclerosis including sclerosis of coronary arteries, chronic obstructive pulmonary disease (COPD) and in one case – chronic calculus cholecistitis. However, they did not cause death but only existed in a latent state. With the rest six cases observed, other acute or chronic disease were not diagnosed.

The distribution of the cases according to the established causes for ABUGIT is as follows:

- chronic gastric ulcer – 6 cases;
- erosive gastritis – 4 cases;
- esophageal varices – 4 cases,

Table 1. Total number of autopsies and these with ABUGIT

| Year | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Total number of autopsies | 360 | 350 | 361 | 352 | 403 | 347 | 401 | 408 | 447 | 190 | **3619** |
| Persons died of ABUGIT | 2 | 1 | 2 | 2 | 1 | 1 | 5 | 1 | 3 | 3 | **21** |

- mucous erosions of esophagus, stomach and small intestine – 3 cases;
- duodenal ulcer – 2 cases;
- erosions of the stomach and esophageal varices – 1 case;
- malignant tumors – 1 case.

In the forensic medical expertise the gastric ulcer is described in a classical aspect – round, with raised edges and with thrombosed vessels at the bottom. The erosions of the stomach and esophagus are multiple superficial lesions of the mucous membrane. They have various forms, size and dark red bleeding bottom. The varices of the esophagus are of a typical shape: usually collapsed venous vessels in the distal part of the three patients with liver cirrhosis. The cause of death with these already established bleedings is the hemorrhagic shock.

However, histological investigation was not performed in all forensic medical expertise. Usually the forensic expert reports only the obvious macroscopic marks pointing to the cause of the bleeding. In most of the expertise the main changes, that caused the bleeding, as well as those that caused the death are not determined (exacerbation of the chronic ulcer, fibrinoid necrosis of the vascular wall in the bottom of the ulcer, the character of erosions, signs of decompensation of liver insufficiency, hemorrhagic shock, etc.)

## Discussion

The results show that the sudden death cases from ABUGIT represent 0.58% from all autopsies and 1.2% from those with sudden nonviolent death (48.35%). Of course these data are not absolute because part of the nonviolent death in relation to all the post mortem cases varies in a too wide range for different periods and different parts of the country: from 26.62% for the period 1924-1964 for the forensic department in the city of Sofia [3], to 44.68% for the period 1924-1975 for the region of Varna city [5] and to 54.28% for the period 1971-1980 for the whole country [4]. For this variety there might be various reasons. Not of least importance is the fact that in different periods the whole number of cases brought for forensic autopsy has been different.

The frequency of fatal acute bleeding cases from the upper gastrointestinal tract is actually much higher because autopsy cases from the hospitals are not involved here, since they are not object of forensic expertise. In their structure however, the causes for ABUGIT, determined by the forensic expertise, do not differ from those mentioned in the clinical practice (ulcer disease, erosive gastritis, bleeding esophageal varices, Mallory-Weiss's syndrome, erosive esophagitis, stress ulcer) [2]. In our material only cases of the Mallory-Weiss's syndrome and stress-ulcers are not present.

The knowledge of the causes of fatal bleeding of the upper gastrointestinal tract and their inclusion in the differential diagnosis of processes which could have similar clinical manifestation is a mandatory action for the clinical doctor as well as for the general practitioner. An example of underestimation of the main complaints and the possibility for complications with fatal bleeding is a 28-year-old man. The general practitioner did not accept as serious the patient's continuous stomach complaints, did not require specialist consult and the patient died of acute bleeding. Such kind of negligence illustrates the necessity of thorough understanding of the problem of ABUGIT, especially for the GPs who are at the front line of the public health service.

The histological investigation is an important moment in determining the causes of the bleeding and death. It should not be neglected by the forensic expert because in some cases the macroscopic picture of the changes is not completely informative and errors and inaccuracies may occur.

## Conclusion

The fatal ABUGIT represents 1.2% of the cases with sudden death, determined by forensic-medical expertise for a ten years period in the region of Varna (48.35%).

In the forensic practice, the most common causes for fatal ABUGIT are chronic gastric ulcers followed by erosive gastritis and esophagus' varices.

The structure of the cases of sudden death due to ABUGIT according to this forensic material corresponds to that observed in the clinic.

The histological investigation is obligatory in order to avoid negligence and inaccuracies in determining the causes of bleeding and the cause of death.

What is needed is recognition, precise and timely diagnosis of the illnesses which could prove a cause for the ABUGIT especially on the part of GPs.

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# Anatomy Teaching – Application of Innovation Methods in Higher Education

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*It is our duty to do something more to rouse the interest to science in young people, not only to stimulate them for professional scientific work but also to make them informed citizens* [1].

Dr Philippe Busquin
*European Commissioner for Research*

As a fundamental science, Human anatomy represents an important part of the scientific basis of medicine. It is a fundamental medical subject that requires a prolonged study, and huge terminology knowledge, which consists of an essential knowledge perimeter; terminology training; an interdisciplinarity; a suggestiveness; and specific inventory. The use of innovative didactical methods, such as discussion in small groups, didactical and role-playing games, phantoms practical education, multimedia education etc. in Faculty of Medicine of Sofia University "St. Kliment Ohridski" is presented. The innovative techniques, consisting of development and application of new pedagogical methods include new teaching technologies for education optimization, increase in effectiveness and motivation of the students. Creating a motivation for enhancing the effectiveness in the teaching process results in an increased scientific interest and creates a novel view of the medical education.

*Key words:* human anatomy teaching, innovation methods, inquiry, medical education.

## Topical Issues

A globalization in medicine and a tendency of integration of fundamental and clinical science and practice are observed lately [2]. A tendency of application of innovative educational methods as problem based education, as well as an education in small groups, distant education, continuing education through whole life etc., is recommended [2-4].

As a fundamental science, human anatomy represents an important part of the scientific basis of medicine. Topical issues regarding innovations in teaching medicine and in attaining knowledge depends on developing a system of effective teaching and education methods. That requires the learner to be placed in the middle of this process and change in teaching and education by introducing innovations to be made.

Team working requires an interdisciplinarity, high level training, and introduction of virtual training methods. By using effective didactic technologies the combined work of teachers and students enhances motivation, rouses interest for science and creates a new vision of the education as a whole. According to the specific educational benefits, modern innovation methods, like discussion in small group, didactic and role-playing games, phantoms practical education, a virtual teaching, etc. are used.

The innovations evaluate the ability of the student to analyze all factors maintaining the learning process. By introducing and experimenting with new didactic technologies, the teacher is aiming at more flexible management of educational process. It can be achieved by significant improvement of all participants in this process roles and functions.

## Results and Discussion

Human anatomy is a fundamental medical subject that requires prolonged study, and terminology knowledge, which consists of:

- essencial knowledge perimeter;
- terminology training;
- interdisciplinarity;
- suggestiveness;
- specific inventory.

Specific characteristics of anatomy teaching:

1. Information repeatedness is the most burdening without correlating with the segmentation and the levels of the results obtained.
2. The levels of the results obtained are always lower than expected even though consistent motivation efforts.
3. The visual associativeness creates achievement increase capabilities.
4. Lack of motivation may create a sense of dissatisfaction and worsen the results, so that they will not depict the efforts made and the teaching approach.

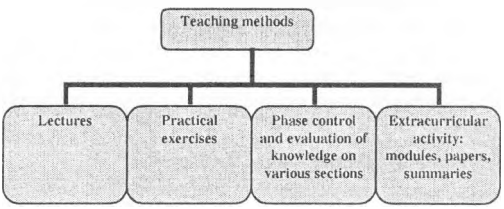



Fig. 1

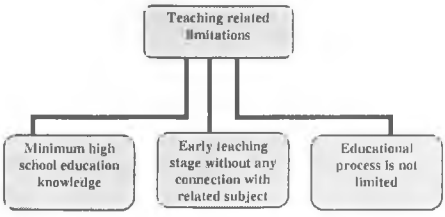
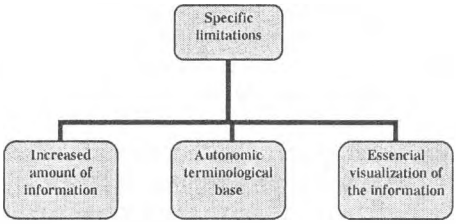



Fig. 2

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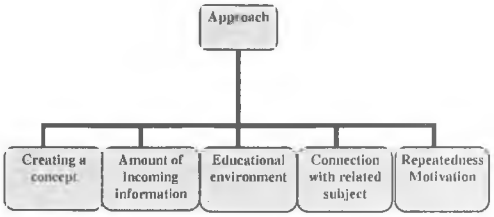


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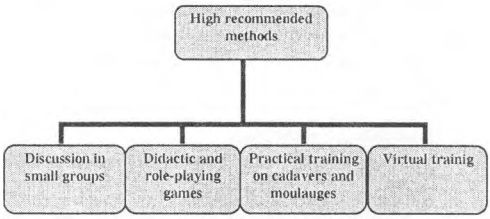


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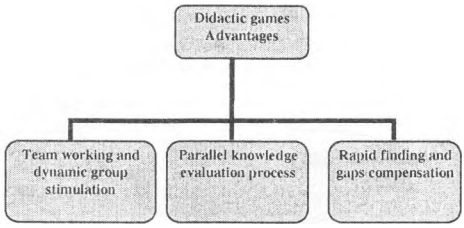


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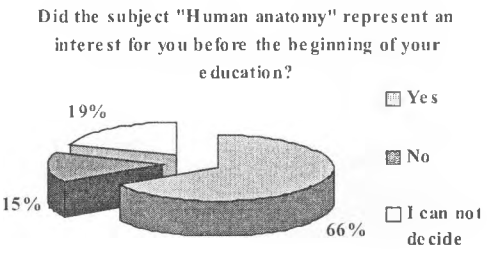


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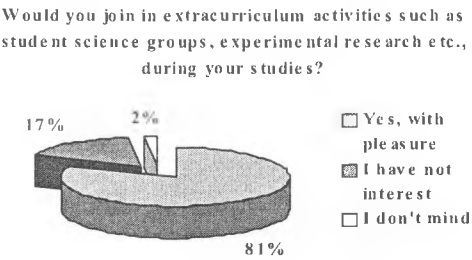


Fig. 8

For getting basic anatomy knowledge classical forms of teaching including lectures, practical exercises, phase control and evaluation of knowledge on various sections, and extracurricular activity, like modules, papers, and summaries are used (Fig. 1). However, the increase in amount of information as well as the need for an enhancement of students' motivation requires new didactical teaching methods. Due to the early stage of anatomy training during medical education, there are also some teaching related limitations (Fig. 2). Along with this, there are some specific limitations (Fig. 3).

The successful learning of anatomy science involves full analysis of some important education factors and requirements (Fig. 4).

According to the specific teaching goals, the use of innovative didactical methods such as discussion in small groups, didactical and role-playing games, phantoms practical education, multimedia and virtual education etc. (Fig. 5) leads to numerous advantages in learning (Fig. 6).

The object of the present study is an anonymous inquiry on first course students in the Faculty of Medicine of Sofia University "St. Kliment Ohridski". The questions of the inquiry are attributed to the results from innovative methods application. The motivation level and the effectiveness of human anatomy teaching are evaluated. Part of the questions is illustrated on diagrams (students' answers are presented in percents):

1. Did the subject "Human anatomy" represent an interest for you before the beginning of your education? (Fig. 7);

2. Would you join in extracurriculum activities such as student science groups, experimental research etc., during your studies? (Fig. 8);

3. In your opinion were the innovative forms of education (didactical games, role-playing games etc.) beneficial or you prefer the classical methods of education? (Fig. 9);

4. Did the innovative method selected by us (didactical games with anatomic cards) return to your expectations in the learning process? (Fig. 10);

5. Did your interest in the subject "Human anatomy" increase after application of innovative methods of education? (Fig. 11);

6. What was the time past for the applied game? (Fig. 12).

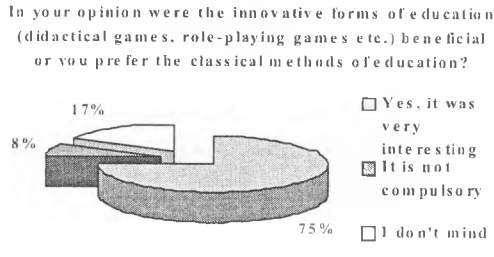


Fig. 9

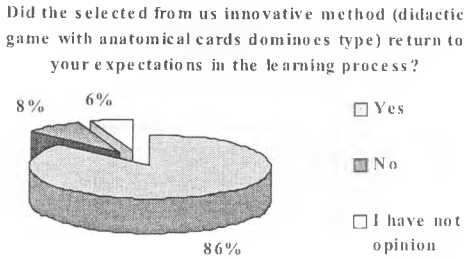


Fig. 10

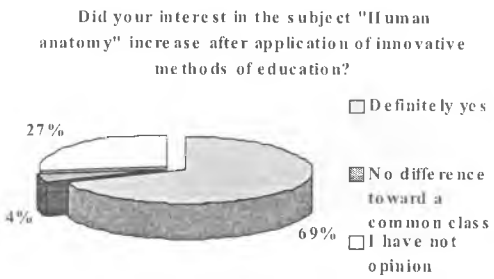


Fig. 11

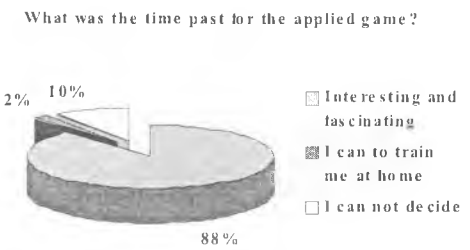


Fig. 12

The inquiry shows that the application of didactic games in the anatomy education:

- creates favorable teaching environment;
- assists for more pleasant and effective teaching;
- enhances students' willingness and motivation for active participation in teaching process;
- stimulates students' personal activity, as well as their ambitions for learning and presentation;
- stimulates the students to look for original and irregular decisions for the different situations;
- has an emotional effect;
- stimulates the thinking process;
- leads to a formation of long lasting interest and high motivation in the student and subsequently, to high quality of the education.

## Conclusion

Creating a motivation for higher effectiveness due to application of innovation approach supposes an increased scientific interest and creates a novel view of the education process for the students in the Faculty of Medicine of Sofia University "St. Kliment Ohridski" during human anatomy teaching. The innovative techniques, consisting of development and application of new pedagogical methods include new teaching technologies for education optimization, gives an untraditional approach for evaluating the learning process. This allows a rapid finding and gaps compensation, and assists for education optimization and effectiveness. This leads to better and long lasting learning of the teaching material.

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# Heterotopic Salivary Gland Tissue in the Face

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Choristoma is a nonneoplastic proliferation of histologically normal tissue that forms at an abnormal site. Heterotopic salivary tissue is a rare lesion, although most authors agree that anomalous embryologic development of salivary tissue is the main cause. It is unusual condition that occurs most frequently in various locations within the head and neck. The goal of our study is to demonstrate the prevalence of accessory salivary tissue in the face and its clinical importance. We describe 6 cases of 120 dissections with accessory salivary tissue located in the face. All 6 findings are isolated glandular tumor masses placed in the surrounding musculature. Salivary gland choristoma is located in various parts of the body and is diagnostic problem for the physicians. This ectopic glandular tissue may be a place for pathologic conditions like fistulae, adenomas, abscesses, and calculosis. In conclusion we consider that though rare this condition is important to be taken in mind during the routine clinical examination.

The aim of our study is to demonstrate by routine anatomic dissection the prevalence of ectopic salivary tissue in the face among Bulgarians and to build a classification of choristoma's localization from a review in the literature.

*Key words*: salivary gland choristoma, salivary gland adenoma, heterotopic salivary tissue.

## Introduction

Heterotopic salivary gland tissue (HSGT) consists of salivary tissue outside of the major and minor salivary glands. HSGT, also known as ectopic or choristomatous salivary gland, is unusual condition that occurs in various locations within the head and neck [7]. Additional sites have been reported and include the mandible [1], middle ear [5], pituitary [12], parathyroid [4], mediastinum [6], and rectum [13]. Other authors report rare locations of choristomas larynx [8], in the vulva [9] and the anterior chest wall [10].

## Description

Our team describes six cases of HSGT located on the face found during dissection of 120 half embalmed heads in the department of anatomy. All of the six findings were isolated glandular tumor mass situated among facial musculature. Two choristomas were placed in the labial region (Fig. 1) and 4 in buccal region (Fig. 2). The former were in the upper lip and the latter close to the oral angle (two of them) and the rest in the

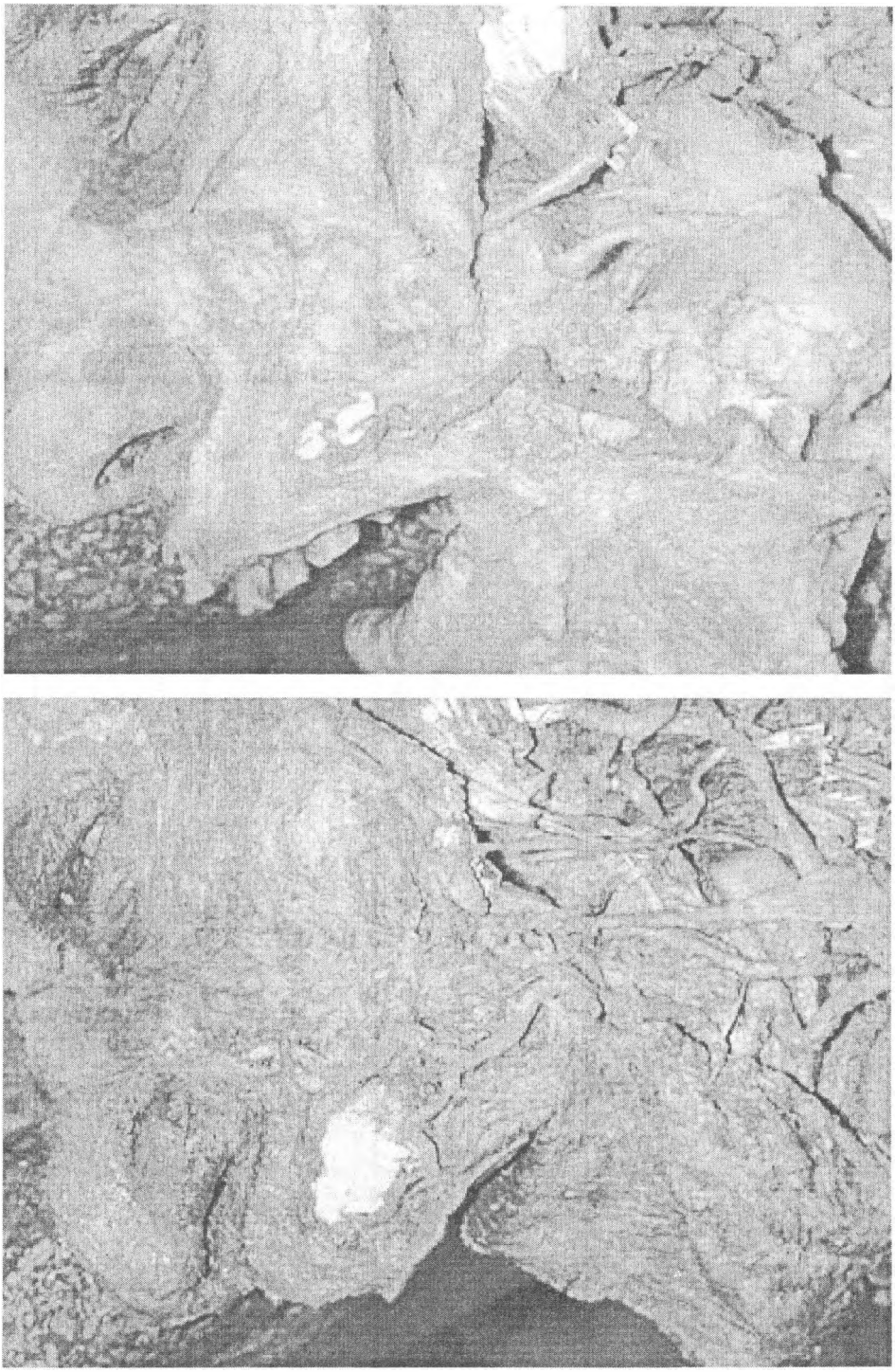

Fig. 1. Labial choristomas

Table 1. Choristomas with different location

| Location | Authors | No of Cases | Country |
|---|---|---|---|
| **Head** | | | |
| – *facial* | present study*; **3 reports | *6 от 60; **3 | *Bulgaria; **(Korea, UK, France) |
| – *intracranial* | | | |
| а) ear | 13 reports (middle ear) + 1 (external ear) | 14 | multinational |
| б) intrasellar | Schochet SS et al.; Kato et al., Tatter et al. | 78 от 2300; 2 sympt. 1 case each | Japan and USA |
| б) pontocerebellar | Rodriguez F et al.; Curry B et al. | 2 | USA |
| – *other* | 6 reports | 1 pterygopl. fossa, 2gingivae,2mandibles, 1 palatine tonsil | UK, Mexico, USA |
| **Neck** | | | |
| – *soft tissues* | *Daniel E et al.;**Youngs LA et al.; #Lassaletta-Atienza at al; ##Haemel A et al.; (7 reports) | *24; **11; #5; ##11: (7 - 1 case each) | *-**USA; #Spain; (Germany, Argentina, Korea, UK, France, USA) |
| – *organs* | 3 reports | 1 larynx, 1 pharynx and 1 parathyroid gland | Poland and USA |
| **Thorax** | | | |
| – *wall* | Shin CE et al. | 1 | USA |
| – *mediastinum* | Feigin GA et al. | 1 | |
| **Abdomen** | | | |
| – *rectum* | Weitzner S; Downs-Kelly et al. | 2 till 1983; 1 | USA |
| – *vulva* | Marwah S et al. | 1 | |

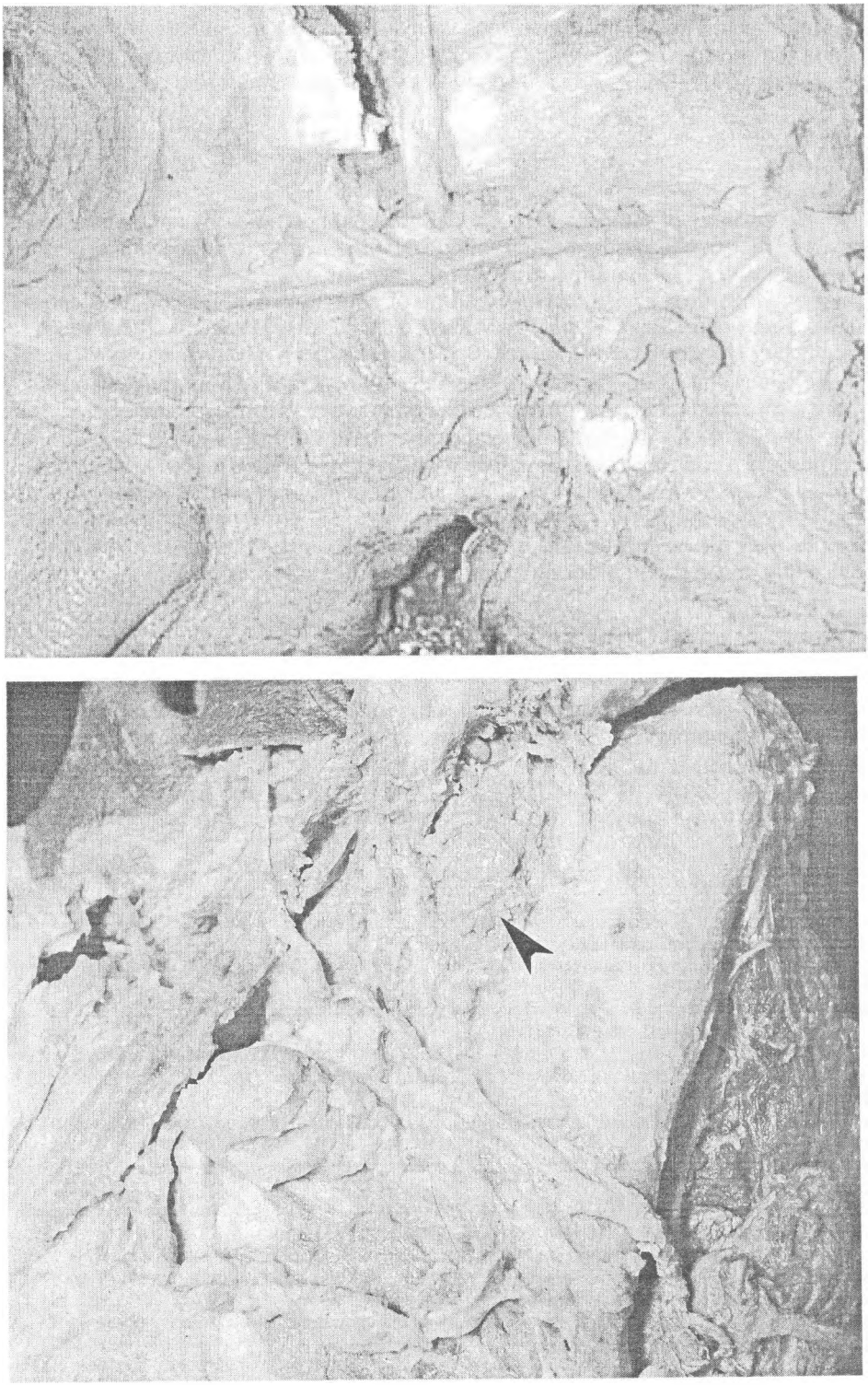

Fig. 2. Buccal choristomas

center of the cheek. The tumors dimensions varied from a few millimeters to few centimeters and the most prominent was with length 24 mm and width 13 mm. The major salivary glands of the investigated heads were with normal anatomic characteristics.

## Discussion

Although the etiology of salivary gland choristoma is not completely understood, most authors agree that an aberration in embryologic development is involved [5]. The more common location of HSGT in the head and the neck is probably due to the adjacent proximity of the structural derivates. A finding of choristomas in other places can be related to a number of factors. Generally heterotopia can occur in several different ways: a) abnormal persistence and development of a vestigial structure; b) dislocation of part of definitive organ rudiment during embryologic development; and c) abnormal differentiation of the local tissues because of their peculiar situation in malformations [11].

Salivary gland choristoma is unusual tumor mass not rarely found in head and neck. White et al. describe accessory salivary tissue in the mylohyoid boutonniere [14]. Some cases with HSGT located in mandible and gingivae are reported as the classical mandibular position is in its posterior region [2, 3]. Other investigators report choristomas with different locations in the body. All ectopic positions of choristomas may cause diagnostic problem for clinicians and radiologists (Table 1).

## Conclusion

To diagnose choristoma – adenoma, which is a benign tumor or using the morphologic term heterotopic (ectopic) salivary gland tissue, which is a typical anatomical variation with heterotopic localization, is of importance for the doctor and for the examined patient.

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*Anthropology*

# Somatotype Characteristic of Children and Adolescents from Plovdiv

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*P. Hilendarski University of Plovdiv*

The purpose of the present study is to follow the growth dynamics of the three somatotypic components in children and adolescents from Plovdiv at the age from 7 to 18 years, as well as to find the inter-sexual and inter-age differences in their somato-typological characteristic. The investigated sample of this cross-sectional study includes 888 boys and 717 girls. The Heath-Carter method of somatotyping is used.We found specific age changes in the development of the three somatotypic components. The changes in the average somatotype, with age, in both genders, are mainly expressed in coming under contiguous somatotypic categories.

*Key words:* children, adolescents, somatotype, sex, age.

## Introduction

The somatotype is an integral characteristic of the morphological status of an individual. It gives a complex assessment of body shape and structure. The changes that appear in body composition during the growth period draw the attention of modern scientists. Many of them think that there is a distinctive dependency between the characteristics which determine the somatotype, the gender and the age of an individual [2, 4, 5, 6, 7]. The purpose of the present study is to follow the growth dynamics of the three somatotypic components in children and adolescents from Plovdiv at the age from 7 to 18 years, as well as to find the inter-sexual and inter-age differences in their somato-typological characteristic.

## Material and Methods

1605 children and adolescents were measured anthropometrically (2006-2007) by the classical methods of Martin-Saller (1957), 888 boys and 717 girls at the age 7-18 from Plovdiv. Somato-typological characteristic was done through the method of Heath-Carter (1957,1990). The data was analyzed with a SPSS statistical set. The reliability of inter-sexual and inter-age differences was checked through ANOVA test.

## Results and Discussion

The differences between both genders in the growth rates of the three somatotypic components with the age growing are shown in Fig. 1.The first somatotypic component, Endomorphic, gives a notion about the development of subcutaneous fat tissue and the relative fatness in the physique of an individual. It is the lowest in the beginning of the observed period (2.80 in boys and 3.34 in girls). Up to the age of 12, values gradually increase but the differences between both genders are insignificant. In boys, it reaches its peak of growth at the age of 12 (3.76), it retains at near levels between 12-14 years ($p > 0.05$), and it decreases significantly after the age of 14 ($p<0.05$) and it changes considerably up to the end ($p > 0.05$). In girls, it reaches its peak of growing two years later – at the age of 14 (4,53) and it retains with scientifically-significant higher values up to the end ($p<0.05$). The results clearly show that at the age of 13-18 the subcutaneous fat tissue in girls is a relatively bigger part of body composition than in boys.

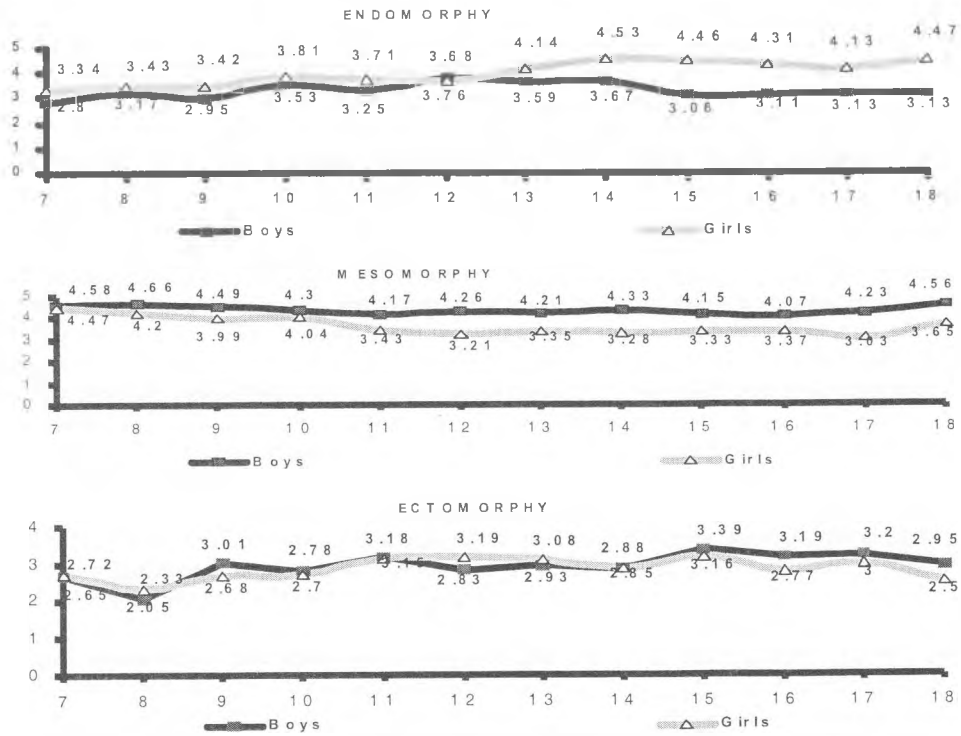


Fig. 1. Growth rates of the three somatotypic components with the age growing

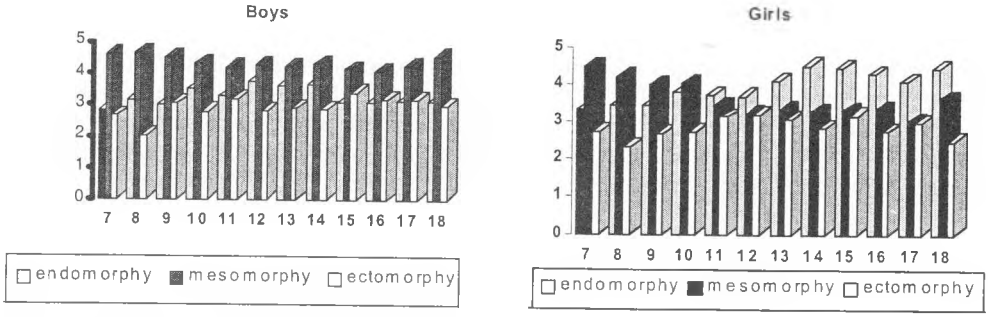


Fig. 2. Average somatotype at the different ages

The second somatotypic component, Mesomorphic, characterizes the muscle-skeletal development towards the height. In 7-year-old children, the mesomorphy has high values in both genders (4.47 in boys and 4.58 in girls). Up to 10 years in boys, the values become higher, scientifically-significant ($p<0.05$), but at the age of 10 the differences between both genders are insignificant ($p>0.05$). After this age to the end of the observed period, mesomorphy in girls falls, and in boys it retains relatively stable and scientifically-significantly higher ($p<0.05$). Hence, at the age 11-18, muscles and the bone tissue in boys develop to a considerably greater extent.

The third somatotypic component – Ectomorphic – gives a real idea about the relative lengthening of body segments and body proportion. This component shows a different variability which responds to the fluctuating changes of the height and weight during the periods of observation. The lowest ectomorphy occurs in 8-year-old children (2.65 in boys, 2.72 in girls). At the next three years (9-11 years), however insignificantly, it is higher in boys and also at the age after 14 years, in girls it is insignificantly higher during the period 11-14, i.e. the changes in the development of this component reflect the specificity of growth processes in both genders.

The summary somatotype gives total information about the body shape and composition, Fig. 2. It is noticeable that in boys mesomorphy has the highest values during the whole period of observation. The changes in its development show differences within the limits of 0.1 to 0.5 units and that reveals the stability of this component and its leading role in the common somatotype for each age. The other two components – endo- and ectomorphic – are lower than mesomorphic and they have near values at the age 9-11. This determines an average somatotype from the category – balanced mesomorphy. Between 12-14 years the significance of endomorphy in body composition in boys increases and that changes the character of somatotype – endo-mesomorphic. After the age of 14 up to the end of observation, the summary somatotype is balanced mesomorphy again. Its characteristics are well-developed bones and joints and strong muscles of limbs as well. Together with this, the moderately-developed fat tissue combines harmoniously with a moderate lengthening of body segments.

In girls the average somatotype at each age also shows specific features. Until the beginning of puberty (at the age of 10) the mesomorphic component is leading, then the endomorphic follows and that determines the endo-mesomorphic somatotype. At the age 11-12 the three components have near values and the summary somatotype becomes central. After the age of 12 the endomorphic component definitely starts dominating and determining a girls' somatotypic characteristic. In the end of the growth period, the summary somatotype in girls is meso-endomorphic. Its specific feature is its more distinguishing subcutaneous fat tissue and relatively moderate muscle-skeletal growth – a characteristic feature of women's body composition.

Table 1. Percentage distribution of the different somatotype categories in both genders

| Age | Boys | | | | Girls | | | |
|---|---|---|---|---|---|---|---|---|
| | Endo- | Meso- | Ecto- | Other | Endo- | Meso- | Ecto- | Other |
| 7 years | 0 | 70 | 10 | 20 | 9.52 | 47.56 | 9.52 | 33.33 |
| 8 years | 0.5 | 60 | 0.5 | 30 | 8 | 48 | 10 | 37 |
| 9 years | 3.51 | 59.65 | 17.54 | 19.3 | 11.48 | 37.70 | 16.39 | 34.43 |
| 10 years | 14.70 | 39.71 | 22.06 | 23.53 | 24.10 | 22.89 | 25.30 | 27.71 |
| 11 years | 10.61 | 37.88 | 30.30 | 21.21 | 23.26 | 8.14 | 38.37 | 30.23 |
| 12 years | 22.67 | 25.33 | 25.33 | 26.67 | 28.92 | 9.64 | 36.14 | 25.30 |
| 13 years | 18.29 | 32.93 | 28.05 | 20.73 | 37.04 | 7.41 | 34.57 | 20.98 |
| 14 years | 21.52 | 26.58 | 26.58 | 25.32 | 58.21 | 5.97 | 23.88 | 11.94 |
| 15 years | 9 | 38 | 33 | 20 | 45.15 | 10.26 | 28.21 | 15.38 |
| 16 years | 14.10 | 35.36 | 28.18 | 24.16 | 36.37 | 11.36 | 22.72 | 29.55 |
| 17 years | 8.79 | 39.56 | 32.97 | 18.68 | 50 | 4.55 | 21.21 | 24.24 |
| 18 years | 7.32 | 51.22 | 14.63 | 26.83 | 47.22 | 8.33 | 27.78 | 16.67 |

It was a matter of big interest for us to find the percentage distribution of the different somatotype categories in both genders – Table 1. In order to generalize the information and to have a more objective notion of body shape and structure, we combined the 13 somatotypic categories by Heath-Carter in 4 big groups: body types with leading endo-, meso- and ectomorphic component. All the other types we put in the 4$^{th}$ category where two or three components have equal share. In the early age periods 7-9 years, the majority of boys belong to somatotypes with a dominating mesomorphic component – between 50% and 70 % of the cases, the ectomorphic types are considerably less -10-17%, and the relative share of the endomorphic types is insignificant – 0-3,5%. At the age between 10 and 14 years, when growth processes are more distinguished, the percentage of mesomorphic boys reduces by half at the expense of the ecto- and endomorphic types. The changes are mainly due to the different ways of development of the morphological sections that form the somatotype, and it does not correspond to the increase of height. This determines the bigger variability of individual typological peculiarities during the puberty. After the age of 14, the fat component of body composition decreases, and that leads to reducing the percentage of boys with endomorphic body type and also to logical increase of the other types, especially the mesomorphic type.

In girls in the initial periods 7-9 years, like in boys, the mesomorphic types of structures are more – 38-48%. At the age of 10 girls are evenly grouped in the four big categories. After this age, the relative share of mesomorphic types sharply falls, while endomorphic progressively rise and dominate up to the end of the study.

On the basis of the results we received, we can draw the following **conclusions:**

1. We found specific age changes in the development of the three somatotypic components. Growth rates display a distinctive sexual dimorphism. The inter-sexual differences in growth dynamics of the endomorphic and mesomorphic components are significant.

2. The skeleton-muscle development in boys is determinant during the whole growth period, while in girls – only to the beginning of the sexual maturity. During the puberty, as well as in the late growth periods, the development of the subcutaneous fat tissue in girls is determinant for the body shape and structure.

3. With age, in both genders, the changes in the average somatotype are mainly expressed in coming under contiguous somatotypic categories: in boys – from a balanced mesomorphy, through a mixed endo-mesomorphy and again to a balanced mesomorphy; in girls – from endo-mesomorphy, through a central type to a meso-endomorphy.

4. In assessment of the relative share of the different body type categories, in both genders, we found a similarity in the prevalent type in the pre-puberty period – meso-

morphic. With the beginning of the intense growth period, some changes start and they are different in both genders: in girls, they lead to a rise in percentage of ectomorphic. and especially the endomorphic body types, while in boys – they lead to retaining the percentage of the mesomorphic types.

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# Pigmentation of the Skin, Hair and Eyes during the Growth Period between 7 and 13 Years

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The results presented constitute a part of a longitudinal anthropological study on children and adolescents from the city of Sofia. The age-related changes of the pigmentation of the skin, hair and eyes in 7- to-13-year-old children have been traced. During this period children with fair skin, brown and brown-black hair and black-brown eyes are prevalent. During the growth period the changes of the skin, hair and eye pigmentation take place with a varying intensity. The age-related differences are statistically insignificant in all three cephaloscopic features in both sexes. The sexual differences are statistically significant only with respect to the skin and only in the contingent of the 9- to 11-year old children.

*Key words:* pigmentation, cephaloscopic features, age-related alterations, sexual differences.

## Introduction

Pigmentation of the skin, hair and eyes though being genetically predetermined undergoes specific age-related changes in the postnatal period. These basic cephaloscopic features are often included in program of broad anthropological studies since they bring about to the clarification of the anthropological characteristics of a given population [2, 3, 5].

**The aim** of the present study is to trace the dynamics of age-related changes of the skin, hair and eye pigmentation at the time of growth between the age of 7 to 13 and to look for sexual differences.

## Material and Methods

A longitudinal investigation on 109 boys and 121 girls aged between 7 and 13 from three Sofia schools was carried out after the conventional methods [1, 6]. The age-related changes of three main cephaloscopic traits – skin hair and eye colour were followed and analyzed and the sexual differences were looked at. The combinations between the colour of the eyes and hair were explored in the 7- to-13-year-old children. The statistical significance of the age-related and sexual changes in the incidence of each feature separately was checked by the $\chi^2$ –test at $P < 0.005$.

## Results and Discussion

**The skin colour** has been recorded in three categories for the fair skin typical of the European race [6]: fair, light mat and mat. Between the years 7 and 13 in both sexes the fair skin is predominant its incidence being higher for the boys. Second in frequency comes the light mat skin which is higher in girls. During that period the light mat skin is more frequent at the expense of the fair skin whose incidence remains highest in all age groups in both sexes [Table 1]. The skin grows darker more slowly and evenly until the 11th year of age especially with the boys (0.9%).Between the years 11 and 13 this process is more intense especially with the boys (by 6.5 to 11%). At the age of 7 only one boy is with mat skin while in the girls such a case is found at the age of 11 years. The observed age related differences are statistically insignificant ($P > 0.05$). The sexual differences are statistically significant only in the case of the 9- to-11-year-old children ($P < 0.05$) since at that time the skin gets darker more intensively with the girls.

**The hair colour** is assessed after Fischer-Saller scale which combines the following categories: white-blond, light-blond, blond, dark-blond, brown, brown-black and reddish. Between the years 7 and 11 brown hair is predominant in both sexes, its incidence being higher with the boys. With the 12-13-year-old boys and girls the brown-black hair is dominant with very close incidence rates (Table 2). The changes in the group of the brown and brown-black hair are most intensive throughout the entire period under study. The blond hair incidence is rapidly decreased and as early as the age of 8 until the age of 11 where there is not a single boy found with blond hair. At the age of 13 this hair is only recorded in 0.8% with the girls. The dark-blond hair frequency diminishes more intensively as early as the age of 8 especially in the girls. The darkening of the blond and dark-blond hair leads to augmentation of the frequency of brown hair at this age. In the gap between 8 and 11 years of age the blondest hair undergoes slight changes and at the age of 12-13 years its incidence remains constant. The reddish hair is only found in 1 to 2 children. The age-related and sexual differences observed are statistically insignificant ($P > 0.05$).

**The eye colour** has been investigated after a 12-step scale incorporating the following groups: black-brown and brown eyes which form the category of the "dark" eyes; brown-green, green-brown and grey-brown forming the category "motley" and

Table 1. Skin colour

| Age (years) | Sex | Total | Categories | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | fair | | light mat | | mat | |
| | | | n | % | n | % | n | % |
| 7 | ♂ | 109 | 92 | 84.4 | 16 | 14.7 | 1 | 0.9 |
| | ♀ | 121 | 94 | 77.7 | 27 | 22.3 | – | – |
| 8 | ♂ | 109 | 91 | 83.5 | 17 | 15.6 | 1 | 0.9 |
| | ♀ | 121 | 91 | 75.2 | 30 | 24.8 | – | – |
| 9 | ♂ | 109 | 90 | 82.6 | 18 | 16.5 | 1 | 0.9 |
| | ♀ | 121 | 84 | 69.4 | 37 | 30.6 | – | – |
| 10 | ♂ | 109 | 89 | 81.7 | 19 | 17.4 | 1 | 0.9 |
| | ♀ | 121 | 82 | 67.8 | 39 | 32.2 | – | – |
| 11 | ♂ | 109 | 89 | 81.7 | 19 | 17.4 | 1 | 0.9 |
| | ♀ | 121 | 81 | 66.9 | 39 | 32.2 | 1 | 0.8 |
| 12 | ♂ | 109 | 82 | 75.2 | 26 | 23.9 | 1 | 0.9 |
| | ♀ | 121 | 74 | 61.2 | 46 | 38 | 1 | 0.8 |
| 13 | ♂ | 109 | 70 | 64.2 | 38 | 34.9 | 1 | 0.9 |
| | ♀ | 121 | 70 | 57.9 | 50 | 41.3 | 1 | 0.8 |

Table 2. Hair colour

| Age (years) | Sex | Total | Categories | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | blond | | dark blond | | brown | | brown-black | | reddish | |
| | | | n | % | n | % | n | % | n | % | n | % |
| 7 | ♂ | 109 | 5 | 4.6 | 16 | 14.7 | 60 | 55.0 | 26 | 23.9 | 2 | 1.8 |
| | ♀ | 121 | 9 | 7.4 | 17 | 14.0 | 57 | 47.1 | 37 | 30.6 | 1 | 0.8 |
| 8 | ♂ | 109 | 2 | 1.8 | 12 | 11.0 | 63 | 57.8 | 30 | 27.5 | 2 | 1.8 |
| | ♀ | 121 | 6 | 5.0 | 10 | 8.3 | 64 | 52.9 | 40 | 33.1 | 1 | 0.8 |
| 9 | ♂ | 109 | 2 | 1.8 | 12 | 11.0 | 62 | 56.9 | 32 | 29.4 | 1 | 0.9 |
| | ♀ | 121 | 5 | 4.1 | 9 | 7.4 | 61 | 50.4 | 45 | 37.2 | 1 | 0.8 |
| 10 | ♂ | 109 | 1 | 0.9 | 11 | 10.1 | 55 | 50.5 | 41 | 37.6 | 1 | 0.9 |
| | ♀ | 121 | 3 | 2.5 | 8 | 6.6 | 58 | 47.9 | 51 | 42.1 | 1 | 0.8 |
| 11 | ♂ | 109 | – | – | 10 | 9.2 | 54 | 49.5 | 44 | 40.4 | 1 | 0.9 |
| | ♀ | 121 | 2 | 1.7 | 8 | 6.6 | 56 | 46.3 | 54 | 44.6 | 1 | 0.8 |
| 12 | ♂ | 109 | – | – | 8 | 7.3 | 45 | 41.3 | 56 | 51.4 | – | – |
| | ♀ | 121 | 1 | 0.8 | 9 | 7.4 | 46 | 38.0 | 64 | 52.9 | 1 | 0.8 |
| 13 | ♂ | 109 | – | – | 8 | 7.3 | 44 | 40.4 | 57 | 52.3 | – | – |
| | ♀ | 121 | 1 | 0.8 | 9 | 7.4 | 44 | 36.4 | 66 | 54.5 | 1 | 0.8 |

Table 3. Eye colour

| Age (years) | Sex | Total | Categories | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | dark | | | | motley | | | | | | light-coloured | | | |
| | | | black - brown | | brown | | brown - green | | green - brown | | gray - brown | | gray | | blue | |
| | | | n | % | n | % | n | % | n | % | n | % | n | % | n | % |
| 7 | ♂ | 109 | 35 | 32.1 | 30 | 27.5 | 2 | 1.8 | 14 | 12.8 | 3 | 2.8 | 16 | 14.7 | 9 | 8.3 |
| | ♀ | 121 | 51 | 42.2 | 28 | 23.1 | 1 | 0.8 | 12 | 9.9 | 5 | 4.1 | 16 | 13.2 | 8 | 6.6 |
| 8 | ♂ | 109 | 36 | 33.0 | 27 | 24.8 | 5 | 4.6 | 12 | 11.0 | 5 | 4.6 | 14 | 12.8 | 10 | 9.2 |
| | ♀ | 121 | 51 | 42.2 | 23 | 19.0 | 6 | 5.0 | 12 | 9.9 | 6 | 5.0 | 16 | 13.2 | 7 | 5.8 |
| 9 | ♂ | 109 | 38 | 34.9 | 22 | 20.2 | 9 | 8.3 | 10 | 9.2 | 7 | 6.4 | 14 | 12.8 | 9 | 8.3 |
| | ♀ | 121 | 51 | 42.2 | 18 | 14.9 | 10 | 8.3 | 13 | 10.7 | 7 | 5.8 | 15 | 12.4 | 7 | 5.8 |
| 10 | ♂ | 109 | 34 | 31.2 | 25 | 22.9 | 9 | 8.3 | 11 | 10.1 | 9 | 8.3 | 12 | 11.0 | 9 | 8.3 |
| | ♀ | 121 | 50 | 41.3 | 19 | 15.7 | 11 | 9.1 | 12 | 9.9 | 9 | 7.4 | 13 | 10.7 | 7 | 5.8 |
| 11 | ♂ | 109 | 33 | 30.3 | 26 | 23.8 | 10 | 9.2 | 10 | 9.2 | 8 | 7.3 | 14 | 12.8 | 8 | 7.3 |
| | ♀ | 121 | 50 | 41.3 | 19 | 15.7 | 11 | 9.1 | 13 | 10.7 | 7 | 5.8 | 13 | 10.7 | 8 | 6.6 |
| 12 | ♂ | 109 | 32 | 29.4 | 27 | 24.8 | 8 | 7.3 | 12 | 11.0 | 8 | 7.3 | 14 | 12.8 | 8 | 7.3 |
| | ♀ | 121 | 49 | 40.5 | 19 | 15.7 | 12 | 9.9 | 13 | 10.7 | 7 | 5.8 | 14 | 11.6 | 7 | 5.8 |
| 13 | ♂ | 109 | 32 | 29.4 | 27 | 24.8 | 8 | 7.3 | 12 | 11.0 | 8 | 7.3 | 14 | 12.8 | 8 | 7.3 |
| | ♀ | 121 | 49 | 40.5 | 19 | 15.7 | 12 | 9.9 | 13 | 10.7 | 7 | 5.8 | 14 | 11.6 | 7 | 5.8 |

grey and blue eyes – in the category of the "light" eyes. Between the years 7 and 13 the dark eyes are prevailing in both sexes, their incidence being higher with the girls. The black-brown eyes are the prevalent ones of them and again with priority in the girls (Table 3). In the age period between 7 and 9 the dark eyes frequency drops more intensely especially with the girls (by 4.2%). This brightening is more strongly pronounced in both sexes in the group of the brown eyes. Between the age of 10 and 13 years the incidence of the dark eyes does not change in both sexes. Motley eyes rank second in frequency with exception for the 7-year-olds where the light eyes are prevailing with a predominance of the boys. The incidence of the motley eyes grows more intensely till the age of 9 in both sexes and is better pronounced in the case of the brown-green eyes (by 3.3 to 4.2%). After the age of 10 motley eyes are found with almost equal frequency in both sexes and do not undergo ostensible changes. The changes in the case of the light eyes are most insignificant with incidence remaining constant after the age of 10. The age-related and sexual changes are statistically insignificant ($P > 0.05$).

Also the combinations between the hair colour and eye coloration in the groups of the 7-year-olds and 13-year-olds have been studied and distributed into four groups: dark eyes with dark hair (dd), dark hair with light eyes (dl), light hair with dark eyes (ld) and light hair with light eyes (ll) (Fig. 1). In the group of the "light" eyes the motley ones

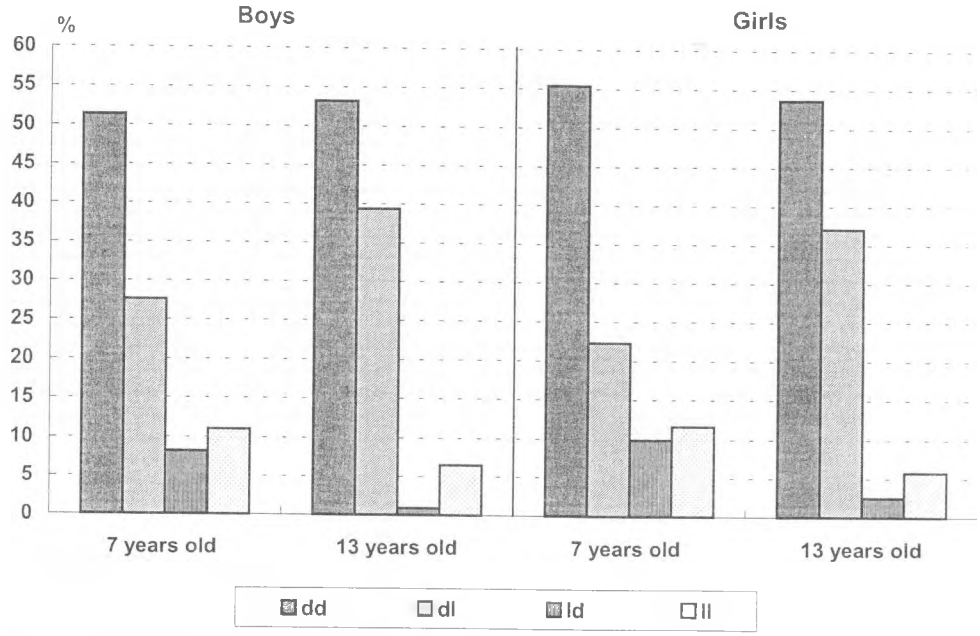


Fig. 1. Combinations of hair and eye colour

have been included. At the age of 7 years the most common combination is between dark hair and dark eyes especially in the boys. On the second place is the combination between dark hair and light eyes again with prevalence for the boys. Last ranking is the combination between light hair and dark eyes but this time with priority for the girls. In the contingent of the 13-year-olds the combinations retain their ranking positions. The incidence of the combination between the dark hair and dark eyes is very close values in both sexes. The combination of dark hair and light eyes is with priority for the boys. Last ranking in both sexes remains the combination between light hair and dark eyes whose incidence has diminished but is again with priority for the girls. The results obtained on the combinations between the hair colour and eye colour is quite close to those of V a t e v [4] about the 6-10-year old children from the towns at the beginning of the century.

## Conclusion

In the period between the years 7 and 13 the children with fair skin are predominant especially with the boys. Till the age of 11 children with brown hair are most common while at the age of 12-13 years those with the brown-black are dominant. In the 7-13-year olds most common are the ones with the black-brown eyes especially girls. At the time of growth the changes in the pigmentation of the skin, hair and eyes take place with a differing intensity. The skin grows darker more intensely between 7 and 13 in the direction from fair to light mat especially with the boys. The hair gets dark from blond and dark-blond to brown most intensely as early as the age of 8. Most intensive are the changes in the group of the brown and brown-black hair throughout the entire period under study. The brightening of the eyes occurs is with the greatest intensity between 7 and years of age especially with the girls and is with a tendency from brown to brown-green coloration.

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# Body Composition in Children and Its Relationship with Sexual Maturation Level

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278 girls and 349 boys from 8 to 17 years old were investigated in 2004-2006 in different Moscow schools. The method of canonical correlations was used to determine interactions between indices of maturation, on the one hand, and two groups of characteristics, on the other: estimations of body mass components values received with the use of equations on the basis of skinfolds measurements and bioimpedance characteristics. To estimate the role of each body mass component the regression analysis was used. Age dynamics of body fat mass in boys and girls were studied. It has been shown that for calculations of body mass components in children and teenagers equations of Matiegka (FM-M, FFM-M) and Slaughter (FM-S, FFM-S) are equally applicable. The interrelation of the level of sexual maturation and fat-free mass both in boys, and in girls is revealed. Significant sexual dimorphism was detected at interrelation level. High level of inverse relationship between the rates of sexual maturation and components of impedance analysis was found in boys.

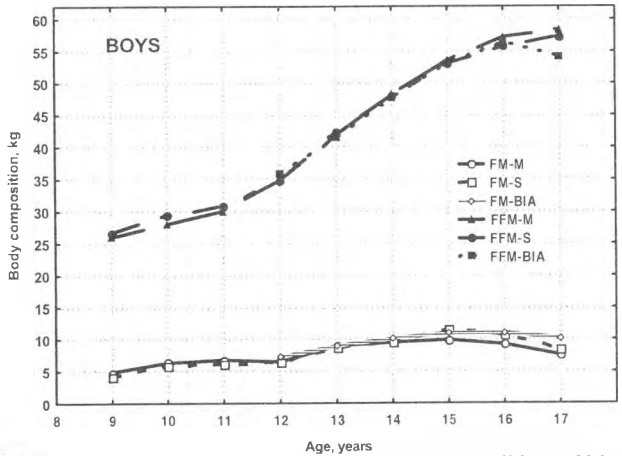
*Key words:* body composition, sexual maturation level, bioelectrical impedance analysis.

The interrelation of morphological characteristics and body mass components with puberty level in children and teenagers is discussed in many works. Various conclusions have been made [6] on the role of body mass components in morphophysiologycal reorganization of the organism during the pubertal period, and also about their direct influence on the starting rates of sexual maturation.

The purpose of the present paper is to study the relationship between body composition and sexual maturation level. The data were collected by the authors in 2004-2006 in different Moscow schools. 278 girls and 349 boys from 8 to 17 years old were investigated cross-sectionally. All children were measured by a standard anthropometrical technique [4]. For bioelectric impedance analysis a single-frequency system "ABC-01 Medass" [3] was used. It operates at a frequency of 50 kHz and a current strength of 800 μA. The estimation of body mass components was also conducted with the equations of Matiegka [1] and Slaughter et al. [2].

Age dynamics of body mass components in boys and girls are presented in Fig.1.

Evaluation of sexual maturation indices was conducted according to Soloviева [7]. Principal component analysis was applied to an estimation of the degree of sexual maturation in children and adolescents, secondary sexual characteristics presented in the form of general integrative indicator [5]. As a result, the 1st principal component is interpreted as an integrative indicator of sexual maturity.



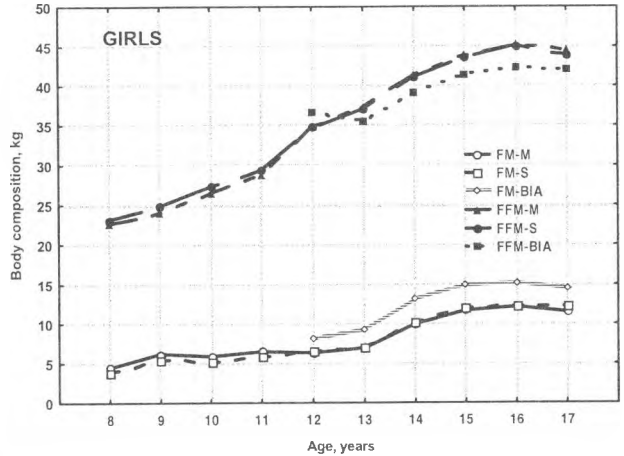
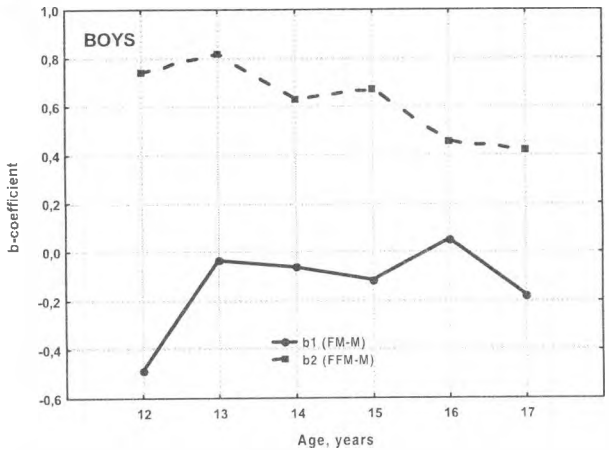




Fig. 1. Age changes of fat mass and fat-free mass in children of Moscow



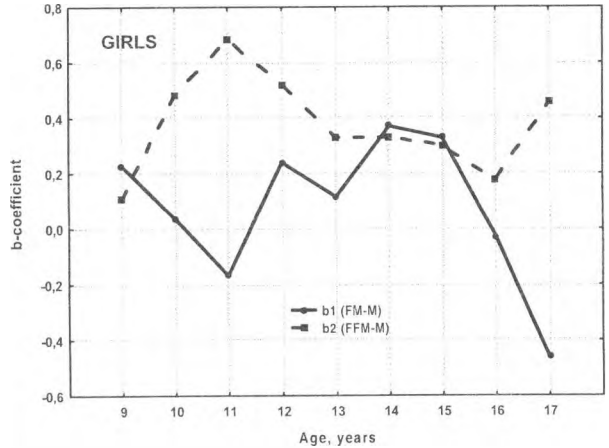




Fig. 2. Age changes of regression equation's b-coefficient: dependence of the sexual maturation level from the fat mass and fat-free mass

To reveal the presence of interrelation between the degree of sexual maturity and body mass components, and also some indicators of bioelectric resistance of the body, the method of canonical correlations was used. To estimate the role of each body mass component in the course of puberty regression analysis was used. Statistical analysis was performed with STATISTICA 6.0 software.

One of the aims of the present work was to define the degree of interdependence between characteristics of sexual maturity with two sets of traits: values of body mass components, received by different methods, and the electric resistance (impedance) characteristics, including active resistance (Rz) and reactance (Zc). The values of canonical correlations in boys and girls are shown in table 1. It has been shown that between the above-mentioned groups of characteristics there is a significant connection.

Table 1. Estimates of canonical correlations between factors of sexual maturation indices and the BIA characteristics, body composition

| Age | Factors of sexual maturation indices (PC1, PC2) | Rz, Zc, Phase angle φ | | | FM-M, FM-S, FFM-M | | |
|---|---|---|---|---|---|---|---|
| | | *R* | *p* | *Chi²* | *R* | *p* | *Chi²* |
| **Boys** | | | | | | | |
| 12 | PC1, PC2 | 0.93 | **0.024** | 14.538 | 0.72 | 0.164 | 9.1747 |
| 13 | PC1, PC2 | 0.72 | **0.000** | 28.34 | 0.85 | **0.000** | 68.815 |
| 14 | PC1, PC2 | 0.55 | **0.001** | 23.237 | 0.62 | **0.000** | 38.412 |
| 15 | PC1, PC2 | 0.49 | **0.013** | 16.116 | 0.65 | **0.000** | 28.475 |
| 16 | PC1, PC2 | 0.32 | 0.250 | 7.839 | 0.51 | **0.017** | 15.395 |
| 17 | PC1, PC2 | 0.53 | 0.568 | 4.815 | 0.48 | 0.107 | 10.443 |
| All ages | PC1, PC2 | 0.46 | **0.000** | 59.323 | 0.28 | **0.000** | 30.009 |
| **Girls** | | | | | | | |
| 9 | PC1, PC2 | | | | 0.53 | **0.045** | 12.886 |
| 10 | PC1, PC2 | | | | 0.58 | **0.013** | 16.066 |
| 11 | PC1, PC2 | | | | 0.70 | **0.003** | 19.988 |
| 12 | PC1, PC2 | 0.82 | **0.014** | 15.921 | 0.73 | **0.006** | 18.277 |
| 13 | PC1, PC2 | 0.54 | 0.393 | 6.278 | 0.54 | 0.292 | 7.325 |
| 14 | PC1, PC2 | 0.74 | 0.116 | 10.209 | 0.73 | **0.0001** | 29.602 |
| 15 | PC1, PC2 | 0.41 | 0.798 | 3.084 | 0.65 | **0.002** | 20.505 |
| 16 | PC1, PC2 | 0.49 | 0.087 | 11.047 | 0.36 | **0.524** | 5.153 |
| 17 | PC1, PC2 | 0.53 | 0.434 | 5.901 | 0.63 | **0.049** | 12.643 |
| All ages | PC1, PC2 | 0.10 | 0.968 | 1.348 | 0.30 | **0.000** | 35.285 |

To find out the character of existing connection between the level of sexual maturity and body mass components, regression analysis was performed. The following regression models were considered:

1. The 1st principal component was taken as a dependent variable (PC1), the independent variables being values of fat and fat-free mass (FM-M and FFM-M). Association between the general level of sexual maturity and body mass components was checked.

2. The fat-free mass was taken as a dependent variable, the independent variable being the 1st principal component. Association between fat-free mass component and level of sexual maturity was checked.

3. The fat mass was taken as a dependent variable, the independent variable being the 1st principal component. Association between fat mass component and level of a sexual maturity was checked.

The results are as follows:

In boys **the level of sexual maturity** have a high degree of dependency on fat-free body mass from 12 to 17 years, and low – on indicators of fat mass at all ages. Before the age of 12 years such a dependency is absent, which probably indicates that in the majority of boys at this age period secondary sexual characteristics only start to appear. The dependency of the level of sexual maturity on indicators of fat-free body mass increases during the period from 12 to 14 years, and, further, gradually decreases (Fig. 2). This may be due to the fact that during the specified period of 12-14 years the peak of longitudinal growth and the increase in fat-free body mass are observed.

An increase in fat component in the course of puberty in boys, compared to fat-free body mass is not so evident at all ages. This can explain the absence of high connection between maturation and fat component in boys. **Fat-free body mass** has high degree of dependency on the level of sexual maturity at the age from 12 to 17, which is possible to explain by greater accumulation of fat-free mass in boys during this age period. **Body fat mass** is significantly dependent on the level of sexual maturity at the age of 13 and 16 years, but estimations of $R^2$ in the regression equation are 18 and 11 % correspondingly, which indicates that the degree of this dependency is rather low.

In girls the **level of sexual maturity** has high degree of dependency on fat-free body mass in 10, 11 and 12 years, and low degree of dependency on indicators of fat mass at all ages (Fig. 2). In this case high correlation with fat-free body mass is caused by the factor that before 12 years intensive longitudinal growth and a gain of fat-free body mass is observed in girls. The values are high because in girls puberty characteristics are already expressed starting from 10 years. **Fat-free body mass** in girls has high degree of dependency on the level of sexual maturation from 10 to 12 years, and also at 14 and 15 years. **Fat body mass** in girls has high degree of dependency on the level of sexual maturation at 12, 14 and 15 years because after the achievement of puberty (approximately 12 years) in girls' organism active fat deposits start to occur.

On the basis of these results it is possible to conclude of the existence of sexual dimorphism in the level of relationships between indicators of sexual maturation and body mass composition in boys and girls. In boys no connections is revealed between sexual development and indicators of fat mass, whereas in girls the interrelation of increase in fat component and sexual maturity is traced. Differences between sexes are revealed also with the regression models. They show the connection between values of bioelectric impedance and the level of sexual maturity. In boys Rz has high degree of negative relationship with the degree of sexual maturity at 13-15 years, in girls this dependency is low at all ages.

## Conclusions

1. Age changes of the fat mass in boys and girls have different trends, which could be explained in terms of different roles of fat mass in the origin of male and female body build.

2. Differences between Matiegka's and Slaughter's methods are not significant. Thus we can use both equations for the estimation of body composition.

3. Sexual maturation level is significantly correlated with body mass components in children of both sexes at all ages. The regression analysis shows correlations between sexual maturation level and fat-free mass in boys and girls.

4. Sexual dimorphism in the level of correlation between sexual maturity and body mass components can be seen. In boys there are no correlations between sexual maturation level and fat mass. But in girls these correlations exist: fat component increases with sexual maturation.

5. The analysis of correlation between bioimpedance characteristics (Rz and Zc) with sexual maturation level shows high negative correlations in boys and smaller ones in girls.

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# Puberty and Body Composition of Plovdiv Boys and Girls

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The aim of the following research is to study the body composition of adolescents – boys and girls from Plovdiv with different rate of sexual and biological maturity. The rate of sexual development among the adolescents is determined by 3-rate on the basis of Me- percentiles among girls and on the basis of the time of appearing of the pubis and axilars hair among boys. The body composition of the studied groups (610 boys and 477 girls, aged 12-17, from Plovdiv), in the following work is given by using 22 body indexes, of which 2 total body indexes, 5 total diameters of the body, 5 circumferences, 6 skinfolds in different parts of the body and limbs and the 4 components of the body mass. The results of this study showed that during the puberty period for both sexes with the best development of the body composition are the children with early puberty, compared with children from other two groups. Body composition during the puberty undergoes essential changes. They are clearly expressed at the age of 14 for both sexes. There is sexual dimorphism in reference to the changes in the body composition during puberty.

*Key words*: early, normal and late puberty; sexual development; body composition.

## Introduction

During the puberty the hormonal changes and the fast growth lead to changes in the body composition. The total body indexes, diameters, girths are changed and of course the components of the body weight – body fats, active body mass, total water and mineral content and others. The body composition during the puberty is connected with the rate of children's biological maturity of different chronological age, too. It is announced that children with higher rate of biological maturity have better development of the components of the body composition compared to those of chronological one as well as a connection between early sexual maturity and different factors as a ь some metabolic and heart diseases, obesity, diabetes, psychococialq as well as a socioeconomical living conditionsand other [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14].

In this connection the monitoring of the changes in the body composition among the adolescents with different biological and chronological age during the puberty is exceptionally important not only for pedagogics but for medicine too for the right prevention of these diseases.

With connect to this the **AIM** of the following research is to study the body composition of adolescents – boys and girls from Plovdiv with different rate of sexual and biological maturity.

## Material and Methods

The following work is a part of a complex, transversal study of the growth processes among the children and adolescents of Plovdiv. The study is conducted transversely, during the period of 2005-2008 year. The rate of sexual development /biological maturity/ among the adolescents of the two sexes is determined by 3-rate scale – accelerated, normal and retarded development /early, normal and late puberty/ on the basis of Mepercentiles among girls, and on the basis of the time of appearing of the pubis and axilaris hair among boys. The body composition of the studied groups of adolescent, of which 610 boys and 477 girls, age from 12 to 17 years, in the following work is given by using 23 body indexes, of which two total body indexes (height, weight, and the index of the body mass), 5 total diameters of the body (biacromial, bicrystal, bithrohanterial, sagital and transversal chesr diameter), 5 circumferences – one of the body and four of the limbs (chest circumference in pause and circumferences of the limbs), 6 skinfolds in different parts of the body and limbs (under the shoulder blade, above the flank(hips), the abdomen, the armpit, the place in front of the armpit, the thigh and the place below the thigh) and the components of the body mass (absolute and relative quantity body fats, active body mass and absolute quantity of subcutaneous adipose tissue). The anthropometric indexes are given using Martin-Saller's method 1959), and the indexes and derivatives are calculated by using different formulas. The information is processed by the method of descriptive statistics, ANOVA, probit analysis, inter-group differences are estimated by the test for a multiple comparisons of Sheffe.

## Results and Discussion

The body composition of the three groups **of girls** (Table 1) – with early, normal and late puberty, shows the following peculiarities: with the highest values of the studied indexes are the girls with early puberty. They have a reliably higher weight, BMI, skin fold under the shoulder blade and components of the body weight, compared to the girls of the other groups. All the indexes for the groups of girls with normal puberty are with values above the average for the relevant age, with the exception of front – back diameter of the chest. It is with unimportantly less value of the other two groups. The girls from this group are with the highest stature, and the inter-group differences are significant only between the normally and late matured. Also the girls with late puberty are characterized with values of the studied indexes below the average. Exception is the relative part of the heaped body fats. The differences between the groups of girls with normal and late puberty are significant in reference to greater part of the studied indexes.

The Table 2 showed that among **the boys** with the best development of the studied indexes is the group of early matured which is with highest values of all the indexes. Reliable differences between the early matured and the boys with normal and late puberty can be seen for all the indexes with the exception of the thickness of the most of the studied skin folds, which is significantly higher for the late matured girls and these with normal and early puberty. The differences in the values of the studied indexes between the girls with normal development and the accelerated ones are great, also the

T a b l e 1. Statistical parameters of body measurements of the girls (Z-score values)

| Groups / Measurement | Eearly maturity | | | Normal maturity | | | Late maturity | | | Significance between groups ($p< 0.05$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Mean | SD | N | Mean | SD | N | Mean | SD | Y/N | N/L | Y/L |
| **Total body size and indices** | | | | | | | | | | | | |
| Weight (kg) | 104 | 0.41 | 1.18 | 188 | 0.11 | 0.98 | 185 | - 0.18 | 0.86 | * | * | * |
| Height (cm) | 104 | 0.13 | 1.00 | 188 | 0.18 | 0.98 | 185 | - 0.12 | 1.01 | | * | |
| BMI (kg/m$^2$) | 104 | 0.40 | 1.23 | 188 | 0.05 | 0.97 | 185 | - 0.15 | 0.86 | * | | * |
| **Body diameters** | | | | | | | | | | | | |
| Biacromial diameter | 104 | 0.20 | 0.93 | 188 | 0.07 | 0.94 | 185 | - 0.19 | 0.99 | | | * |
| Bicrystal diameter | 104 | 0.19 | 1.05 | 188 | 0.02 | 0.87 | 185 | - 0.47 | 1.05 | | | |
| Bitrochanterial diam | 104 | 0.30 | 1.12 | 188 | 0.01 | 0.92 | 185 | - 0.15 | 0.93 | | | * |
| Sagital chest diam. | 104 | 0.11 | 1.09 | 188 | - 0.05 | 0.85 | 185 | 0.00 | 1.01 | | | |
| Transver.chest diam. | 104 | 0.15 | 1.09 | 188 | 0.56 | 0.89 | 185 | - 0.87 | 0.99 | | | |
| **Circumferences** | | | | | | | | | | | | |
| Chest circum.(pause) | 104 | 0.24 | 1.01 | 188 | 0.05 | 0.97 | 185 | - 0.11 | 0.99 | | | * |
| Circumfer.upper arm | 104 | 0.34 | 1.10 | 188 | 0.08 | 0.99 | 185 | - 0.16 | 0.89 | | | * |
| Circumfer. forearm | 104 | 0.27 | 0.98 | 188 | 0.17 | 1.03 | 185 | - 0.17 | 0.94 | | * | * |
| Circumference thigh | 104 | 0.36 | 1.16 | 188 | 0.13 | 0.96 | 185 | - 0.18 | 0.90 | | * | * |
| Circumference calf | 104 | 0.34 | 1.09 | 188 | 0.11 | 0.98 | 185 | - 0.17 | 0.90 | | * | * |
| **Skinfolds** | | | | | | | | | | | | |
| SF subscapular | 104 | 0.25 | 1.05 | 188 | 0.03 | 0.92 | 185 | - 0.12 | 0.97 | | | * |
| SF suprailiac | 104 | 0.22 | 1.03 | 188 | 0.05 | 0.97 | 185 | - 0.15 | 0.98 | | | * |
| SF abdomen | 104 | 0.14 | 0.95 | 188 | 0.07 | 0.95 | 185 | - 0.10 | 1.02 | | | |
| SF biceps | 104 | 0.12 | 1.05 | 188 | 0.06 | 1.05 | 185 | - 0.10 | 0.88 | | | |
| SF thigh | 104 | 0.27 | 1.05 | 188 | 0.08 | 0.91 | 185 | - 0.19 | 1.00 | | | * |
| SF calf | 104 | 0.28 | 1.04 | 188 | 0.04 | 0.97 | 185 | - 0.19 | 0.96 | | | * |
| **Components of body weight** | | | | | | | | | | | | |
| Body fat | 104 | 0.296 | 1.12 | 188 | 0.067 | 0.95 | 142 | - 0.162 | 0.90 | | | * |
| % Body fat | 104 | 0.213 | 1.04 | 188 | 0.050 | 0.91 | 142 | - 0.145 | 0.99 | | | * |
| Fat free mass | 104 | 0.399 | 1.21 | 188 | 0.133 | 0.96 | 142 | - 0.173 | 0.91 | | * | * |
| Subcutaneous fat | 104 | 0.291 | 1.07 | 188 | 0.089 | 0.98 | 142 | - 0.188 | 0.91 | | | * |

Table 2. Statistical parameters of body measurements of the boys (Z-score values)

| Groups / Measurement | Eearly maturity | | | Normal maturity | | | Late maturity | | | Significance between groups ($p$-level 0,05) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | N | Mean | SD | N | Mean | SD | N | Mean | SD | Y/N | N/L | Y/L |
| **Total body size and indices** | | | | | | | | | | | | |
| Weight | 90 | 0,74 | 1,18 | 366 | 0,12 | 0,99 | 154 | - 0,42 | 0,75 | * | * | * |
| Height | 90 | 0,82 | 0,84 | 366 | 0,08 | 0,96 | 154 | - 0,38 | 0,93 | * | * | * |
| BMI | 90 | 0,53 | 1,23 | 366 | 0,12 | 1,02 | 154 | - 0,32 | 0,80 | * | * | * |
| **Body diameters** | | | | | | | | | | | | |
| Biacromial diameter | 90 | 0,37 | 1,15 | 366 | 0,05 | 0,94 | 154 | - 0,38 | 0,85 | * | * | * |
| Bicristalal diameter | 90 | 0,62 | 1,13 | 366 | 0,10 | 1,03 | 154 | - 0,20 | 0,73 | * | * | * |
| Bitrochanterial diam. | 90 | 0,62 | 1,18 | 366 | 0,09 | 1,01 | 154 | - 0,21 | 0,78 | * | * | * |
| Sagital chest diam. | 90 | 0,52 | 1,13 | 366 | 0,08 | 1,05 | 154 | - 0,21 | 0,77 | * | * | * |
| Transver. chest diam. | 90 | 0,56 | 1,06 | 366 | 0,06 | 1,01 | 154 | - 0,22 | 0,95 | * | * | * |
| **Circumferences** | | | | | | | | | | | | |
| Chest circum.(pause) | 90 | 0,54 | 1,15 | 366 | 0,05 | 1,02 | 154 | -0,40 | 0,77 | * | * | * |
| Circumfer. upper arm | 90 | 0,63 | 1,13 | 366 | 0,14 | 0,97 | 154 | -0,34 | 0,82 | * | * | * |
| Circumfer. forearm | 90 | 0,65 | 1,07 | 366 | 0,14 | 0,99 | 154 | -0,44 | 0,78 | * | * | * |
| Circumference thigh | 90 | 0,63 | 1,14 | 366 | 0,09 | 0,95 | 154 | -0,31 | 0,82 | * | * | * |
| Circumference calf | 90 | 0,69 | 1,14 | 366 | 0,10 | 0,96 | 154 | -0,36 | 0,85 | * | * | * |
| **Skinfolds** | | | | | | | | | | | | |
| SF subscapularis | 90 | 0,36 | 1,18 | 366 | 0,08 | 1,01 | 154 | - 0,33 | 0,69 | * | * | * |
| SF suprailiaca | 90 | 0,33 | 1,08 | 366 | 0,07 | 1,02 | 154 | -0,27 | 0,81 | | * | * |
| SF abdomen | 90 | 0,33 | 1,08 | 366 | 0,08 | 1,00 | 154 | - 0,28 | 0,84 | | * | * |
| SF biceps | 90 | 0,21 | 1,07 | 366 | 0,07 | 1,03 | 154 | - 0,22 | 0,79 | | * | * |
| SF thigh | 90 | 0,28 | 0,96 | 366 | 0,04 | 1,01 | 154 | - 0,15 | 0,92 | | | * |
| SF calf | 90 | 0,31 | 1,09 | 366 | 0,03 | 0,98 | 154 | -0,14 | 0,94 | | | * |
| **Components of body mass** | | | | | | | | | | | | |
| Body fat | 90 | 0,455 | 1,23 | 366 | 0,07 | 0,99 | 154 | - 0,34 | 0,69 | * | * | * |
| % Body fat | 90 | 0,312 | 1,07 | 366 | 0,06 | 1,00 | 154 | - 0,26 | 0,85 | | * | * |
| Fat free mass | 90 | 0,702 | 1,00 | 366 | 0,08 | 0,96 | 154 | - 0,49 | 0,81 | * | * | * |
| Subcutaneous fat | 90 | 0,428 | 1,16 | 366 | 0,06 | 1,00 | 154 | - 0,29 | 0,78 | * | * | * |

differences in the values of height, weight, BMI, diameters of the body, girths of the limbs and the relative part of the body fat are the greatest. With values of the body sizes below the average for the relevant age are the boys with late puberty. They are with reliably lowest, slightest and the smallest biacromial diameter and relative part of the body fats. The smallest inter-group differences among the boys can be seen in the values of the thickness of the skinfolds.

**The adolescents from different age groups** are characterized with specific features in their body composition. During the 12th and 13th year of their growth and development, the girls with early puberty are with higher values of the indexes of the body composition, compared to those with normal and late puberty. They are show the best development of the total body sizes, chest and limbs measurement and active body mass, the chest circumferences and circumferences of the limbs during the 12th year, as well as subscapular skinfold during the 13th year. The differences in the values of the indexes between the groups of 12-year-old girls are statistically insignificant, but significant differences are show between early and late maturity 13-year-old girls. Values below the average can be seen among 12-year-old girls matured in normal terms and among 13-year-old girls, matured in late terms.

The 14th year for girls is characterized with transformation in the body composition for the three groups due to a sudden change in the direction and values of the studied indexes. Early matured girls who up to their 13th year have the highest values of all the studied indexes slow down the growth of more features and the values reach a level below the average for the age. At this age increase the growth of the girls matured in normal terms and practically they have the highest values of these indexes, compared to the other two groups. In group of girls, matured in late terms increase the growth of shoulders and chest circumference. Significant inter-group differences among the 14 years old girls are not seen ($p>0.05$).

Between the 14th and 15th year the transformation of the body composition continues and the 15-year-old girls with early puberty are again with the highest values of the studied indexes. But they seriously decrease the growing in height and practically their height at this age is lower than the height of the late matured ones ($p<0.05$). At this age can be seen acceleration in height growth for late matured girls and the values of the index reach a level above the average. For the 15-year-old girls significant inter-group differences in the values of the other indexes of the body composition are seen mainly between early and late matured girls.

**During the period of maturity the early matured boys are** reliably heavier, higher, with bigger quantity of subcutaneous adipose tissue in the abdomen and back as well as with greater absolute quantity body fats and active body mass than the ones who matured late. At this age periods the boys with late puberty have values of all the indexes below the average with the exception of the diameter of the chest during the 12th year, some skinfolds and percent fat tissue during the 14th year.

At the age of 13 the biggest and statistically significant are the intra-group differences in the values of height, weight, active body mass and chest circumferences in pause between the boys with accelerated and retarded development. During the 14th year are show change in the body composition of normal maturity boys. The growth of the shoulder width, the transverse diameter of the chest and the chest measurement in pause, the active body mass and the absolute quantity of the body fats is retarded for the boys with normal puberty and the values of these indexes decrease below the average for this age. At this age there are significant differences between the boys who matured early and normally, as well as between them and late matured in reference to height, shoulder width and active body mass($p<0.05$). During 15th year statistically significant for all the studied indexes with exception of the skin folds in the place of abdomen and lower limb are the differences between them and the boys with late puberty.

As a result of the study of the connection between the puberty development and the changes of the body composition at this period we can do the following **CONCLUSION**:

• As a whole during the puberty period for both sexes with the best development of the body composition are the children with early puberty. Boys and girls matured in normal terms are with values of the studied indexes above the average for their age and those who matured late with values below the average. There is an exception for the 14 years old children. There are significant inter-group differences in values of the studied indexes between children at the same age.

• During the puberty the body composition undergoes essential changes. They are clearly expressed at the age of 14 for both sexes when the children with early puberty retard in their growth at the expense of normally and later matured boys and girls.

• There is sexual dimorphism in reference to the changes in the body composition during puberty. The boys react to the hormonal and other influences with bigger fluctuation in the growth speed that lead to statistically significant differences in the values of most of the studied body sizes and once again verifies the greater ecosensibility of the males.

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# Fat Patterning in Children from 3 to 17 Years of Age

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The **aim** of the present work is to characterize the age and sexual differences of Body Fat and Fat Free Mass during the growing up period from 3 till 17 years, anthropological features which are basic for the body composition assessment. The study was made after the classical methods of Martin-Saller and 1464 boys and 1468 girls were investigated. The data about stature, body weight, BMI, %BF, BF(kg), %FFM, FFM(kg) are analyzed. The results show that between 3 and 17 years girls accumulate and have constantly more quantity of Body Fats, while boys accumulate and have constantly more quantity of Fat Free Mass. The body nutritional status in boys changes considerably between 9 and 10, and 12 and 13 years of age, while in girls these changes happen one year earlier – between 8 and 9, and 11 and 12 years of age, occurring again between 13 and 15 years.

*Key words:* children and adolescents, anthropological assessment, fat patterning.

## Introduction

The anthropological assessment of body composition is an easily applied approach for the body nutritional status's evaluation and along with it about the obesity on individual and population level. Like basic patterns concerning this valuation are used mainly the following features: Body Mass Index (BMI); Body Fat (BF) and Fat Free Mass (FFM) as a relative share and absolute quantity; interrelation between waist and hip circumferences; waist circumference; interrelation between Subcutaneous Fat Tissue (SFT) quantity on body and extremities and so on. In the Bulgarian and foreign scientific literature, however, the reports concerning methodic approaches and norm's data are mainly for adults. Rarely could be found papers that assessed the body composition during growth ages, i.e. concerning the children and adolescents. The basic contemporary indicator according whose data the body nutritional status is discussed in these reports is the BMI, and only for the differentiation of overweight and obesity in children and adolescents [3]. The cut off points, recommended by the WHO, concerning the undernourishment and emaciation diagnosis were published first at 2007 [2], and we still have not come in the literature to any national or regional data about the frequency of those two types body nutritional status during the growing up period. Scantier is also the information concerning the assessment of body composition type throughout this period by means of the other indicators mentioned above, since it is missing in Bulgaria.

The **aim** of the present work is to characterize the age and sexual differences of Body Fat and Fat Free Mass during the growing up period from 3 till 17 years, anthropological features which are basic for the body composition assessment.

## Material and Methods

Two transversal investigations of children and adolescents at the age between 3 and 6 years, as well as between 7 and 14 years respectively were carried out during 1993-2001 and 2004-2007 in Sofia city covering 5 schools and 7 kindergartens. The study was made by a qualified stuff of anthropologists after the classical methods of M a r t i n - S a l l e r [4]. Object of the investigations were 1464 boys and 1468 girls at the age from 3 to 17 years, uniformly distributed into 15 age groups for both genders separately. From the detailed data collected according to the complex anthropological program, in the present work were analyzed the sexual and age differences for the following features: stature, body weight, BMI, %BF, BF(kg), %FFM, FFM(kg). The assessment of body composition was made by the two-component model of Behnke [1] that separates 2 basic components of the body weight - BF and FFM, the last one representing all fat free tissues. The relative share of BF (%BF), on which base was computed as the relative share of FFM (%FFM), so the absolute quantity of BF(kg) and FFM(kg), was determined by the regression equations of S l a u g h t e r et al. [5]. The equations are made out using the sum - subscapular skinfold plus triceps skinfold.

According to the application of these equations the investigated boys are divided into two age groups: pre puberty up to 9 years inclusive, and puberty from 10 to 17 years inclusive. The differentiation of pre puberty and puberty developmental stages in boys are made according to the studies about Bulgarian children elaborated by S t a n c h e v et al. [7] and T o m o v a et al. [8].

For every age group are computed the variation-statistical parameters of the features, as well as the sexual differences like absolute differences and relative units by the Index of Sexual Differences (ISD). The differences throughout ages are made also by the Absolute Year Alteration (AYA) and Relative Year Alteration (RYA). The statistical significance was calculated by the t-test of Student at P = 0.05.

The ISD and RYA are computed as Index Units (IU) by the submitted formula of W o l a n s k i [6] concerning the objectivity of different inter-group comparisons:

$$IU = \frac{2 \times (x_2 - x_1) \times 100}{(x_2 + x_1)}.$$

Concerning the age differences $\bar{x}_1$ is mean value of a given feature for some age and $\bar{x}_2$ – mean value of the same feature for next coming age. Concerning the sexual differences $\bar{x}_1$ is mean value of respective feature in girls and $\bar{x}_2$ – mean value of the same feature in boys.

## Results

For the objective assessment of the anthropological indicators about body composition, in the present paper we are giving also data about stature and body weight being basic parameters that characterize the human physical development.

The stature and body weight in boys increase significantly during every one year period between 3 and 17 years. In girls the stature increases significantly till 14 years and the body weight till 13 years of age. After those years the growth in girls calms down and at 17 years the growth of stature and body weight practically stops.

Even from 3 years of age on, the sexual differences are available concerning stature and body weight. Regardless of the fact that during growing up period up to 8 years these differences didn't reach statistical significance, boys are constantly higher than girls. Concerning stature between 8 and 12 years and body weight between 9 and 13 years, girls already outstrip boys, reflecting the earlier maturation in girls. After those ages the boys again are reliably higher and heavier than girls.

The data about BMI analyzed in this work are presented as general basic characterization for body nutritional status during the growing up period.

Boys and girls have relatively equal values for BMI almost through the whole period 3-17 years of age. Only at 5 years the BMI is considerably higher in boys and at 12 years – it is considerably higher in girls. In boys the BMI increases reliable from 9 till 10 years, as well as from 12 till 13 years. In girls the authentic change of body nutritional status occurred one year earlier, i.e. from 8 till 9 years and from 11 till 12 years, the change lasting from 13 till 15 years, as well.

More detailed information of body nutrition status during growth could be gotten assessing the two body weight components - Body Fat (BF) and Fat Free Mass (FFM). The relative share of BF is constantly higher in girls between 3 and 17 years, the sexual differences are statistically significant at 4 years and from 7 years of age till the end of the studied period. Reciprocally to the result above, the relative share of FFM is higher in boys during the entire period under investigation and the sexual differences are also statistically significant during the same years. The girls gain more intensively BF after 9 years, while the boys gain considerably more FFM after 13 years, which is connected with their later period of active sexual maturation. An age determined stage is established concerning the type of proportion between both basic body weight components. Periods of an intensive rise for BF and such of a FFM reduction respectively, succeeded each other. Most frequently the intervals are one year ones and rarely the change comes every second year, as for instance is the considerable % BF increment and the relative reduction of % FFM between 7 and 9 years. Except few cases the intensity of increment and reduction for both body components are similar concerning both sexes, the changes being more slightly expressed at the beginning and the end of the studied growth period. The extent of BF changes throughout ages is better underlined in girls and those of FFM – in boys.

## Conclusions

1. For the first time in Bulgaria is made anthropological characterization of body composition during the growing up period between 3 and 17 years of age on the ground of contemporary, recommended by the WHO criteria.

2. Specific sexual and age differences are established showing that:

– between 3 and 17 years girls accumulate and have constantly more quantity of Body Fats, while boys accumulate and have constantly more quantity of Fat Free Mass;

– the body nutritional status in boys changes considerably between 9 and 10, and 12 and 13 years of age, while in girls these changes happen one year earlier - between 8 and 9, and 11 and 12 years of age, occurring again between 13 and 15 years.

3. It is established that on the ground of regular, continuous increment in stature and body weight between 3 and 17 years the age changes, concerning proportion between Body Fat and Fat Free Mass, reflect the specificity in body maturation during growing up period. The intensive increment or reduction of BF goes along with reciprocally changes in FFM.

Table 1. Biostatistical data of investigated features

| Age | *n* ♂ | *n* ♀ | Stature | | | | | | Body weight | | | | | | BMI | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | ♂ | | ♀ | | Inter-sexual differences | | ♂ | | ♀ | | Inter-sexual differences | | ♂ | | ♀ | | Inter-sexual differences | |
| | | | mean | SD | mean | SD | AD | ISD | mean | SD | mean | SD | AD | ISD | mean | SD | mean | SD | AD | ISD |
| 3 | 80 | 80 | 101.20 | 4.30 | 98.99 | 3.89 | 2.20* | 2.20 | 16.33 | 2.11 | 15.48 | 1.80 | 0.85* | 5.37 | 15.91 | 1.43 | 15.76 | 1.26 | 0.15 | 0.94 |
| 4 | 80 | 80 | 107.70 | 4.13 | 106.52 | 5.39 | 1.18 | 1.10 | 18.35 | 3.09 | 17.96 | 2.68 | 0.40 | 2.20 | 15.75 | 1.83 | 15.76 | 1.48 | -0.01 | -0.07 |
| 5 | 80 | 80 | 114.66 | 4.97 | 113.91 | 4.97 | 0.75 | 0.66 | 21.06 | 3.97 | 20.01 | 2.72 | 1.04 | 5.07 | 15.92 | 1.87 | 15.38 | 1.42 | 0.53* | 3.41 |
| 6 | 80 | 80 | 121.28 | 4.93 | 120.40 | 5.16 | 0.88 | 0.73 | 23.86 | 4.26 | 22.88 | 3.93 | 0.99 | 4.23 | 16.14 | 2.04 | 15.72 | 2.04 | 0.42 | 2.64 |
| 7 | 110 | 110 | 125.79 | 5.83 | 125.85 | 5.23 | -0.07 | -0.05 | 25.84 | 4.53 | 25.55 | 4.48 | 0.29 | 1.13 | 16.24 | 1.95 | 16.06 | 2.04 | 0.19 | 1.16 |
| 8 | 100 | 101 | 131.01 | 5.93 | 130.52 | 5.13 | 0.48 | 0.37 | 28.22 | 5.01 | 27.37 | 4.51 | 0.86 | 3.08 | 16.35 | 1.97 | 15.99 | 1.96 | 0.36 | 2.22 |
| 9 | 100 | 101 | 136.49 | 5.94 | 137.56 | 6.48 | -1.07 | -0.78 | 32.39 | 6.50 | 32.88 | 7.48 | -0.50 | -1.52 | 17.27 | 2.54 | 17.23 | 2.84 | 0.04 | 0.21 |
| 10 | 100 | 98 | 142.05 | 7.14 | 142.30 | 6.45 | -0.25 | -0.17 | 36.28 | 8.68 | 35.81 | 7.44 | 0.47 | 1.31 | 17.79 | 2.92 | 17.57 | 2.86 | 0.22 | 1.26 |
| 11 | 100 | 100 | 147.89 | 6.04 | 149.20 | 6.83 | -1.31 | -0.88 | 40.16 | 7.24 | 41.15 | 9.55 | -0.99 | -2.44 | 18.28 | 2.77 | 18.32 | 3.19 | -0.04 | -0.20 |
| 12 | 97 | 100 | 152.49 | 7.97 | 155.54 | 7.22 | -3.05* | -1.98 | 43.30 | 9.12 | 47.42 | 10.95 | -4.12* | -9.07 | 18.47 | 2.65 | 19.44 | 3.44 | -0.97* | -5.11 |
| 13 | 101 | 99 | 160.48 | 8.13 | 160.11 | 5.54 | 0.37 | 0.23 | 50.67 | 10.64 | 51.08 | 11.06 | -0.41 | -0.81 | 19.57 | 3.33 | 19.87 | 4.01 | -0.30 | -1.54 |
| 14 | 99 | 101 | 167.43 | 7.84 | 162.11 | 5.53 | 5.32* | 3.23 | 54.81 | 11.02 | 50.37 | 8.13 | 4.44* | 8.44 | 19.45 | 3.14 | 19.13 | 2.72 | 0.32 | 1.66 |
| 15 | 100 | 100 | 172.64 | 6.92 | 162.77 | 6.01 | 9.87* | 5.89 | 59.64 | 12.26 | 52.99 | 8.78 | 6.65* | 11.81 | 19.92 | 3.33 | 19.98 | 3.03 | -0.06 | -0.30 |
| 16 | 119 | 120 | 175.49 | 6.19 | 163.52 | 5.29 | 11.97* | 7.06 | 63.05 | 11.99 | 54.28 | 9.47 | 8.78* | 14.96 | 20.40 | 3.23 | 20.26 | 3.13 | 0.15 | 0.72 |
| 17 | 118 | 118 | 177.70 | 5.98 | 163.84 | 5.83 | 13.87* | 8.12 | 66.49 | 12.14 | 54.49 | 9.29 | 12.00* | 19.84 | 20.99 | 3.21 | 20.25 | 2.97 | 0.73 | 3.56 |

Table 1. Continuation

| Age | *n* ♂ | *n* ♀ | %Body fat | | | | | | Body fat (kg) | | | | | | %Fat Free Mass | | | | | | Fat Free Mass (kg) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | ♂ | | ♀ | | Inter-sexual differences | | ♂ | | ♀ | | Inter-sexual differences | | ♂ | | ♀ | | Inter-sexual differences | | ♂ | | ♀ | | Inter-sexual differences | |
| | | | mean | SD | mean | SD | AD | ISD | mean | SD | mean | SD | AD | ISD | mean | SD | mean | SD | AD | ISD | mean | SD | mean | SD | AD | ISD |
| 3 | 80 | 80 | 15.60 | 3.19 | 16.01 | 2.95 | -0.42 | -2.64 | 2.58 | 0.81 | 2.51 | 0.69 | 0.08 | 2.97 | 84.40 | 3.19 | 83.99 | 2.95 | 0.42 | 0.50 | 13.75 | 1.53 | 12.97 | 1.29 | 0.78* | 5.82 |
| 4 | 80 | 80 | 14.80 | 3.56 | 16.28 | 3.55 | -1.48* | -9.50 | 2.80 | 1.25 | 2.98 | 1.01 | -0.18 | -6.24 | 85.20 | 3.56 | 83.72 | 3.55 | 1.48* | 1.75 | 15.55 | 2.01 | 14.98 | 1.90 | 0.58 | 3.79 |
| 5 | 80 | 80 | 14.84 | 4.37 | 15.85 | 3.46 | -1.01 | -6.56 | 3.26 | 2.00 | 3.22 | 1.11 | 0.04 | 1.37 | 85.16 | 4.37 | 84.15 | 3.46 | 1.01 | 1.19 | 17.79 | 2.27 | 16.79 | 1.97 | 1.00* | 5.76 |
| 6 | 80 | 80 | 15.64 | 5.53 | 16.82 | 5.40 | -1.17 | -7.24 | 3.90 | 2.29 | 4.02 | 2.07 | -0.12 | -2.97 | 84.36 | 5.53 | 83.18 | 5.40 | 1.17 | 1.40 | 19.96 | 2.55 | 18.86 | 2.20 | 1.11* | 5.69 |
| 7 | 110 | 110 | 14.58 | 5.12 | 15.98 | 4.81 | -1.41* | -9.20 | 3.95 | 2.32 | 4.25 | 2.12 | -0.31 | -7.49 | 85.42 | 5.12 | 84.02 | 4.81 | 1.41* | 1.66 | 21.89 | 2.79 | 21.30 | 2.68 | 0.60 | 2.76 |
| 8 | 100 | 101 | 15.01 | 6.05 | 17.08 | 5.35 | -2.07* | -12.90 | 4.47 | 2.86 | 4.83 | 2.23 | -0.36 | -7.74 | 84.99 | 6.05 | 82.92 | 5.35 | 2.07* | 2.46 | 23.75 | 2.87 | 22.53 | 2.89 | 1.22* | 5.26 |
| 9 | 100 | 101 | 16.56 | 6.44 | 18.46 | 6.38 | -1.90* | -10.86 | 5.69 | 3.51 | 6.46 | 3.86 | -0.77 | -12.60 | 83.44 | 6.44 | 81.54 | 6.38 | 1.90* | 2.31 | 26.69 | 3.72 | 26.42 | 4.10 | 0.27 | 1.02 |
| 10 | 100 | 98 | 15.90 | 8.96 | 19.85 | 7.49 | -3.96* | -22.14 | 6.40 | 5.71 | 7.55 | 4.43 | -1.15 | -16.47 | 84.10 | 8.96 | 80.15 | 7.49 | 3.96* | 4.82 | 29.88 | 4.47 | 28.26 | 3.96 | 1.62* | 5.58 |
| 11 | 100 | 100 | 17.14 | 8.25 | 19.72 | 6.66 | -2.58* | -14.00 | 7.40 | 4.86 | 8.61 | 4.97 | -1.21 | -15.09 | 82.86 | 8.25 | 80.28 | 6.66 | 2.58* | 3.16 | 32.76 | 3.71 | 32.54 | 5.42 | 0.21 | 0.66 |
| 12 | 97 | 100 | 16.75 | 8.40 | 21.37 | 6.99 | -4.62* | -24.24 | 7.81 | 5.73 | 10.73 | 6.01 | -2.92* | -31.49 | 83.25 | 8.40 | 78.63 | 6.99 | 4.62* | 5.71 | 35.50 | 5.31 | 36.69 | 5.95 | -1.20 | -3.32 |
| 13 | 101 | 99 | 18.95 | 9.82 | 22.57 | 7.47 | -3.62* | -17.44 | 10.32 | 7.25 | 12.20 | 6.74 | -1.88 | -16.71 | 81.05 | 9.82 | 77.43 | 7.47 | 3.62* | 4.57 | 40.35 | 6.36 | 38.88 | 5.35 | 1.47 | 3.71 |
| 14 | 99 | 101 | 16.58 | 8.06 | 23.08 | 5.72 | -6.50* | -32.78 | 9.70 | 6.62 | 11.95 | 4.72 | -2.25* | -20.81 | 83.42 | 8.06 | 76.92 | 5.72 | 6.50* | 8.11 | 45.11 | 6.77 | 38.42 | 4.55 | 6.69* | 16.02 |
| 15 | 100 | 100 | 16.42 | 8.04 | 24.06 | 6.09 | -7.64* | -37.73 | 10.60 | 8.58 | 13.14 | 5.93 | -2.55* | -21.44 | 83.58 | 8.04 | 75.94 | 6.09 | 7.64* | 9.58 | 49.04 | 5.97 | 39.85 | 4.32 | 9.20* | 20.69 |
| 16 | 119 | 120 | 16.14 | 7.88 | 24.56 | 5.95 | -8.42* | -41.35 | 10.97 | 8.03 | 13.71 | 5.59 | -2.74* | -22.20 | 83.86 | 7.88 | 75.44 | 5.95 | 8.42* | 10.57 | 52.08 | 5.74 | 40.56 | 5.21 | 11.52* | 24.86 |
| 17 | 118 | 118 | 16.66 | 7.13 | 24.68 | 5.87 | -8.02* | -38.80 | 11.74 | 7.38 | 13.86 | 5.64 | -2.13* | -16.60 | 83.34 | 7.13 | 75.32 | 5.87 | 8.02* | 10.11 | 54.75 | 6.59 | 40.72 | 4.85 | 14.03* | 29.39 |

$p \leq 0.05$ (statistical significance of inter-sexual differences)

Table 2. Growth velocity

| Age period | Stature | | | | Body weight | | | | BMI | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ♂ | | ♀ | | ♂ | | ♀ | | ♂ | | ♀ | |
| | AYA | RYA | AYA | RYA | AYA | RYA | AYA | RYA | AYA | RYA | AYA | RYA |
| **3-4** | 6.50* | 6.22 | 7.5* | 7.3 | 2.02* | 11.65 | 2.5* | 14.8 | -0.17 | -1.05 | 0.0 | 0.0 |
| **4-5** | 6.96* | 6.26 | 7.4* | 6.7 | 2.70* | 13.71 | 2.1* | 10.8 | 0.17 | 1.07 | -0.4 | -2.4 |
| **5-6** | 6.62* | 5.61 | 6.5* | 5.5 | 2.81* | 12.50 | 2.9* | 13.3 | 0.22 | 1.38 | 0.3 | 2.2 |
| **6-7** | 4.51* | 3.65 | 5.5* | 4.4 | 1.98* | 7.96 | 2.7* | 11.0 | 0.11 | 0.65 | 0.3 | 2.1 |
| **7-8** | 5.22* | 4.07 | 4.7* | 3.6 | 2.38* | 8.81 | 1.8* | 6.9 | 0.11 | 0.64 | -0.1 | -0.4 |
| **8-9** | 5.48* | 4.09 | 7.0* | 5.2 | 4.16* | 13.74 | 5.5* | 18.3 | 0.92 | 5.46 | 1.2* | 7.5 |
| **9-10** | 5.57* | 4.00 | 4.7* | 3.4 | 3.90* | 11.34 | 2.9* | 8.5 | 0.53* | 3.00 | 0.3 | 2.0 |
| **10-11** | 5.84* | 4.02 | 6.9* | 4.7 | 3.88* | 10.14 | 5.3* | 13.9 | 0.49 | 2.70 | 0.7 | 4.2 |
| **11-12** | 4.60* | 3.06 | 6.3* | 4.2 | 3.15* | 7.54 | 6.3* | 14.2 | 0.19 | 1.05 | 1.1* | 6.0 |
| **12-13** | 7.99* | 5.11 | 4.6* | 2.9 | 7.36* | 15.67 | 3.7* | 7.4 | 1.09* | 5.75 | 0.4 | 2.2 |
| **13-14** | 6.95* | 4.24 | 2.0* | 1.2 | 4.14* | 7.85 | -0.7 | -1.4 | -0.12 | -0.61 | -0.7* | -3.8 |
| **14-15** | 5.21* | 3.06 | 0.7 | 0.4 | 4.83* | 8.44 | 2.6* | 5.1 | 0.47 | 2.39 | 0.9* | 4.4 |
| **15-16** | 2.85* | 1.64 | 0.7 | 0.5 | 3.41* | 5.56 | 1.3 | 2.4 | 0.48 | 2.40 | 0.3 | 1.4 |
| **16-17** | 2.21* | 1.25 | 0.3 | 0.2 | 3.44* | 5.31 | 0.2 | 0.4 | 0.58 | 2.82 | 0.0 | 0.0 |

* $P \leq 0.05$ (statistical significance of inter-age differences); AYA – Absolute Year Alteration; RYA – Relative Year Alteration

Table 2. Continuation

| Age period | %Body Fat | | | | Body Fat (kg) | | | | %Fat Free Mass | | | | Fat Free Mass (kg) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ♂ | | ♀ | | ♂ | | ♀ | | ♂ | | ♀ | | ♂ | | ♀ | |
| | AYA | RYA | AYA | RYA | AYA | RYA | AYA | RYA | AYA | RYA | AYA | RYA | AYA | RYA | AYA | RYA |
| 3- 4 | -0.80 | -5.25 | 0.26 | 1.62 | 0.22 | 7.99 | 0.47* | 17.16 | 0.80 | 0.94 | -0.26 | -0.31 | 1.80* | 12.32 | 2.00* | 14.34 |
| 4-5 | 0.04 | 0.30 | -0.43 | -2.65 | 0.46 | 15.32 | 0.24 | 7.73 | -0.04 | -0.05 | 0.43 | 0.51 | 2.24* | 13.42 | 1.82* | 11.45 |
| 5-6 | 0.80 | 5.23 | 0.97 | 5.91 | 0.64 | 17.79 | 0.80* | 22.09 | -0.80 | -0.94 | -0.97 | -1.15 | 2.17* | 11.50 | 2.06* | 11.57 |
| 6-7 | -1.06 | -7.05 | -0.83 | -5.08 | 0.04 | 1.13 | 0.23 | 5.66 | 1.06 | 1.25 | 0.83 | 1.00 | 1.93* | 9.23 | 2.44* | 12.16 |
| 7-8 | 0.43 | 2.92 | 1.10 | 6.62 | 0.53 | 12.57 | 0.58 | 12.82 | -0.43 | -0.51 | -1.10 | -1.31 | 1.85* | 8.12 | 1.23* | 5.63 |
| 8-9 | 1.55 | 9.82 | 1.38 | 7.78 | 1.22* | 24.00 | 1.63* | 28.79 | -1.55 | -1.84 | -1.38 | -1.68 | 2.94* | 11.67 | 3.89* | 15.89 |
| 9-10 | -0.66 | -4.07 | 1.39 | 7.27 | 0.71 | 11.68 | 1.09 | 15.55 | 0.66 | 0.79 | -1.39 | -1.72 | 3.19* | 11.27 | 1.84* | 6.72 |
| 10-11 | 1.24 | 7.49 | -0.14 | -0.70 | 1.00 | 14.50 | 1.06 | 13.11 | -1.24 | -1.48 | 0.14 | 0.17 | 2.88* | 9.18 | 4.28* | 14.09 |
| 11-12 | -0.38 | -2.26 | 1.66 | 8.07 | 0.41 | 5.36 | 2.12* | 21.91 | 0.38 | 0.46 | -1.66 | -2.09 | 2.74* | 8.03 | 4.15* | 11.99 |
| 12-13 | 2.20 | 12.31 | 1.20 | 5.45 | 2.51* | 27.72 | 1.48 | 12.87 | -2.20 | -2.67 | -1.20 | -1.54 | 4.85* | 12.79 | 2.18* | 5.78 |
| 13-14 | -2.37 | -13.32 | 0.51 | 2.25 | -0.62 | -6.21 | -0.25 | -2.07 | 2.37 | 2.88 | -0.51 | -0.67 | 4.76* | 11.14 | -0.46 | -1.19 |
| 14-15 | -0.16 | -0.97 | 0.98 | 4.14 | 0.90 | 8.83 | 1.19 | 9.47 | 0.16 | 0.19 | -0.98 | -1.28 | 3.94* | 8.36 | 1.43* | 3.65 |
| 15-16 | -0.28 | -1.71 | 0.50 | 2.05 | 0.38 | 3.51 | 0.57 | 4.27 | 0.28 | 0.33 | -0.50 | -0.66 | 3.03* | 6.00 | 0.71 | 1.77 |
| 16-17 | 0.52 | 3.16 | 0.13 | 0.51 | 0.76 | 6.73 | 0.15 | 1.08 | -0.52 | -0.62 | -0.13 | -0.17 | 2.67* | 5.00 | 0.16 | 0.39 |

* $P \leq 0.05$ (statistical significance of inter-age differences); AYA – Absolute Year Alteration; RYA – Relative Year Alteration

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# A Comparison of Plovdiv and Moscow Children's Height, Weight and BMI Values

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This study compares the height, weight and BMI in children and adolescents from Moscow and Plovdiv. The results show that Plovdiv girls and boys experience an earlier growth leap than their Moscow coevals. During the period of puberty, growth rates are higher in Plovdiv's population and it associates with the higher values of BMI.

*Key words:* height, BMI, Bulgarians, Russians.

## Introduction

Ethno-territorial differences in children's growth and physical development are an object of great importance for anthropologists in Bulgaria and abroad [1, 2, 3, 4]. The various territorial conditions determine the peculiarities of growth processes and give the opportunity to assess their role in these processes. Analyzing the alterations in some anthropometrical characteristics we can draw conclusions about the determining role of the different endo- and exogenic factors in growth processes. In the present study we compare children and adolescents from Plovdiv and Moscow in order to reveal the possible ethno-territorial differences in their growth and physical development.

## Material and Methods

The subjects of observation are children and adolescents at the age of 7 to 17 years from Plovdiv and Moscow. The Russian children were studied in the years 1996-1999 and there were 1153 girls and 1152 boys. The Bulgarian children were studied in the years 2004-2006 г. and there were 1065 boys and 925 girls. The anthropometrical programmes for both excerpts include a wide range of anthropometrical characteristics, but the present study analyses the height in centimetres, done with a standard anthropometer, the weight in kilograms, read with a medical scale, and BMI proportions. The data were analysed with SPSS statistical set. The average values and standard deviations were calculated for each anthropometrical characteristic and index.

## Results and Discussion

Table 1 shows the average values of the characteristics in both ethno-territorial groups of children and adolescents, as well as the differences between them in each age group. From 8 to 12 years the average height in Moscow boys is near the 50th percentile of their Plovdiv coevals and only the 7-year-olds have an average height up to the 25th percentile, i.e. they are shorter with 2,6 cm (Fig. 1). At the age of 16 and 17 Moscow boys are insignificantly taller than their Bulgarian coevals. The highest inter-population difference is at 13, when the boys from Plovdiv are taller with 4.60 cm than these from Moscow (Table 1). This is a result from the fact that their growth rate is the highest at the age between 12 and 13, while in Moscow boys it comes a year later but they retain, though insignificantly, taller in the end of the age period.

In Moscow girls, the average height to the age of 12 is near the 50th percentile of their coevals from Plovdiv and the inter-population height difference is the highest in

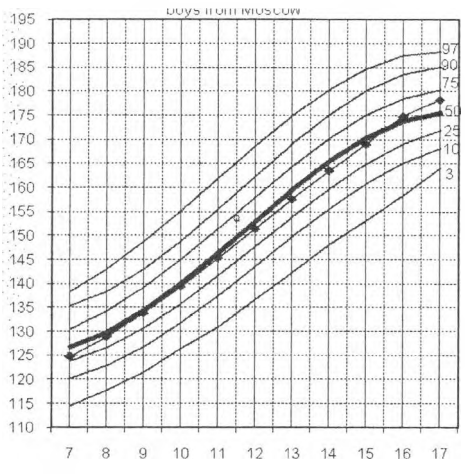


Fig. 1. Referent values of percentiles in boys from Plovdiv, compared to the 50th percentile in boys from Moscow

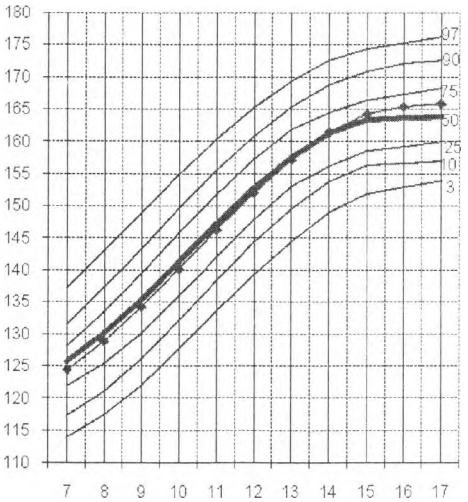


Fig. 2. Referent values of percentiles in girls from Plovdiv, compared to the 50th percentile in girls from Moscow

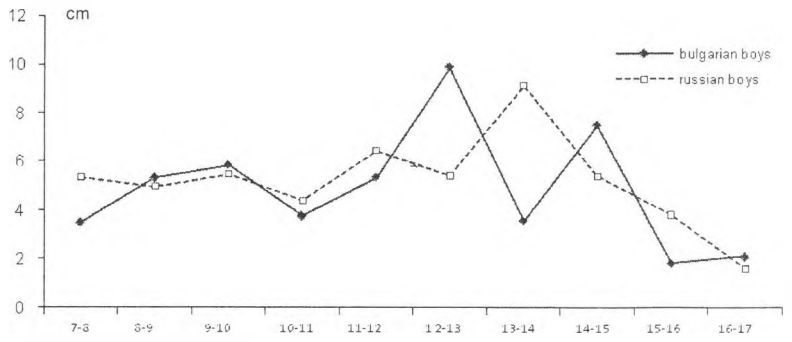


Fig. 3. Growth increments in Plovdiv and Moscow boys

11-year-olds (2 cm more for Bulgarian girls). After the age of 14 in Moscow girls, the average height is between the 50th and 75th percentile, i.e. they are significantly taller than these from Plovdiv (Fig. 2).

The growth rate cannot be assessed accurately in a transversal study, but as the both excerpts are transversal they can be compared. The growth curves are similar to the age of 12 in boys from both ethno-territorial groups, and after that the boys from Plovdiv experience a growth leap, and Moscow boys – a year later (Fig. 3). The growth rate in Moscow boys falls faster after the puberty growth leap in comparison with their coevals from Plovdiv. In girls, the growth curves are similar, taken as a whole, but in Moscow girls the growth rate is lower between the ages 8 and 10, and the growth peak is again a year later compared to these from Plovdiv (Fig. 4). The growth curve is more slanting in Moscow girls, while in these from Plovdiv, the growth rate is more concentrated between the ages of 9 and 10.

The inter-population differences in weight show that during the whole growth period Bulgarian boys are heavier than their coevals from Moscow and especially at the age of 13 (8.03 kg), while the girls from Moscow get heavier after the age of 14 (Table 1).

Table 1. Comparison of height, weight and BMI in children from Plovdiv and Moscow, at the age 7-17

| Age | Height | | | Weight | | | BMI | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Bulgarian* | *Russian* | *difference* | *Bulgarian* | *Russian* | *difference* | *Bulgarian* | *Russian* | *difference* |
| **Boys** | | | | | | | | | |
| 7 | 126.74 | 124.13 | 2.61*** | 26.72 | 24.69 | 2.03** | 16.56 | 15.94 | 0.62* |
| 8 | 130.66 | 129.48 | 1.18 | 31.81 | 27.61 | 4.20*** | 18.44 | 16.37 | 2.07*** |
| 9 | 135.98 | 134.45 | 1.53 | 32.17 | 30.75 | 1.42 | 17.30 | 16.92 | 0.38 |
| 10 | 141.83 | 139.92 | 1.89* | 37.57 | 34.01 | 3.56** | 18.51 | 17.23 | 1.28* |
| 11 | 145.59 | 144.31 | 1.26 | 38.95 | 36.75 | 2.20* | 18.24 | 17.51 | 0.73 |
| 12 | 150.93 | 150.75 | 0.18 | 45.51 | 41.92 | 3.59** | 19.77 | 18.34 | 1.43** |
| 13 | 160.77 | 156.17 | 4.60*** | 54.13 | 46.1 | 8.03*** | 20.72 | 18.76 | 1.96*** |
| 14 | 164.36 | 164.89 | 0.53 | 58.3 | 53.61 | 4.69** | 21.41 | 19.45 | 1.96*** |
| 15 | 171.65 | 170.69 | 0.95 | 63.17 | 59.18 | 3.99** | 21.71 | 20.69 | 1.02* |
| 16 | 173.68 | 174.52 | −0.84 | 65.63 | 63.20 | 2.43 | 21.71 | 20.69 | 1.03* |
| 17 | 175.77 | 176.15 | −0.38 | 67.73 | 66.29 | 1.44 | 21.94 | 21.34 | 0.60 |
| **Girls** | | | | | | | | | |
| 7 | 125.89 | 124.29 | 1.55 | 26.42 | 24.82 | 1.60* | 16.66 | 16.00 | 0.66 |
| 8 | 129.05 | 129.33 | −0.28 | 29.43 | 27.70 | 1.73* | 17.57 | 16.44 | 1.13** |
| 9 | 133.92 | 134.02 | −0.1 | 31.62 | 29.85 | 1.77* | 17.53 | 16.52 | 1.01** |
| 10 | 140.80 | 139.26 | 1.54 | 37.16 | 32.53 | 4.63*** | 18.54 | 16.63 | 1.91*** |
| 11 | 147.76 | 145.75 | 2.01* | 40.87 | 36.35 | 4.52*** | 18.55 | 16.94 | 1.61*** |
| 12 | 153.28 | 152.72 | 0.56 | 45.54 | 42.32 | 3.22* | 19.27 | 18.05 | 1.22** |
| 13 | 158.76 | 158.41 | 0.35 | 51.51 | 46.57 | 4.94*** | 20.39 | 18.49 | 1.9*** |
| 14 | 161.46 | 160.82 | 0.64 | 55.31 | 51.28 | 4.03** | 21.15 | 19.79 | 1.36** |
| 15 | 161.68 | 162.67 | −0.99 | 52.82 | 55.23 | -2.42 | 21.17 | 20.83 | 0.34 |
| 16 | 162.49 | 164.71 | −2.21** | 56.41 | 56.68 | -0.27 | 21.35 | 20.53 | 0.82 |
| 17 | 162.63 | 164.58 | −1.95* | 54.67 | 56.68 | -2.01 | 21.64 | 20.89 | 0.75 |

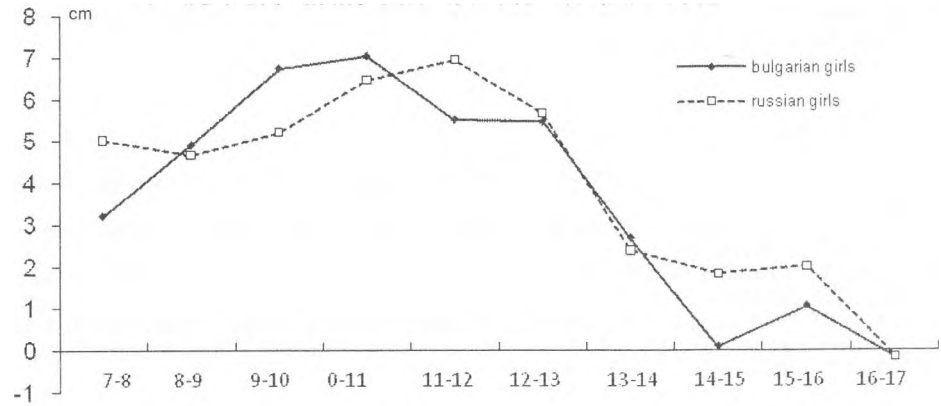


Fig. 4. Growth increments in Plovdiv and Moscow girls

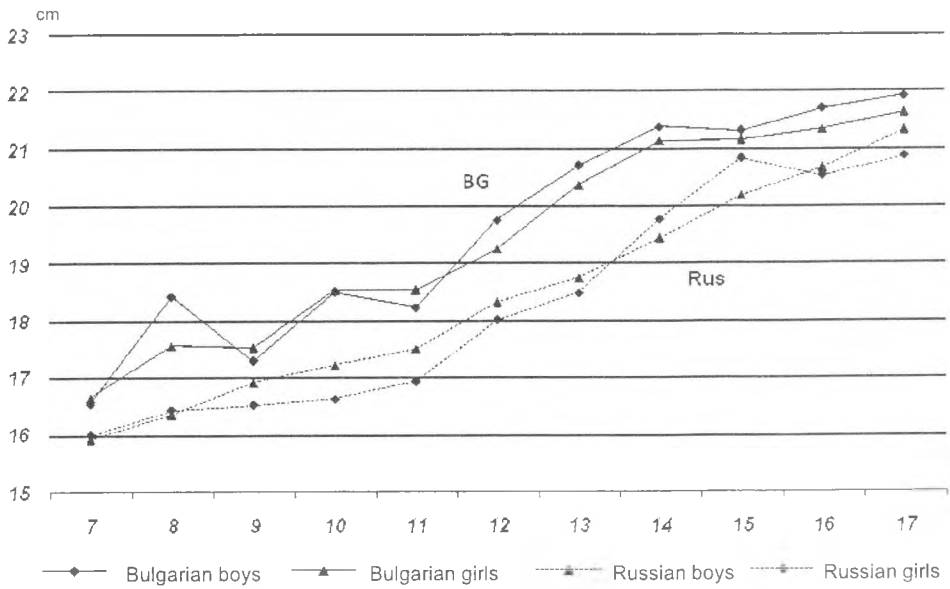


Fig. 5. Average values of BMI of Plovdiv and Moscow children

The higher values of BMI, in boys from Plovdiv at the ages 13 and 14 and in girls from 10 to 14, are associated with their heavier weight at these ages (Fig. 5).

## Conclusion

Our findings show that growth processes have territorial and ethnic peculiarities. Boys and girls from Plovdiv experience an earlier growth leap than their coevals from Moscow. The growth rate falls faster in Moscow boys in comparison with these from Plovdiv, while Moscow girls have a longer growth leap. During the puberty, growth rates are higher in the population from Plovdiv, in comparison to that from Moscow. Plovdiv children's higher BMI values are associated with the higher growth rate and the lower height when growing.

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# Age Changes in Height, Weight and Nutritional Condition in Adolescents from Novi Sad

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The height and weight are two most frequently used traits in assessing the development, nutritional and health condition of individuals and populations.

In compliance with International Biological Programme (IBP) and WHO, a transversal anthropological investigation was conducted in high schools of the city of Novi Sad in 2003. The investigation included 403 males and 462 females aged 15-18. The results indicate that males are characterised by significantly greater height and weight in all of the ages, while greater triceps skinfold thickness is recorded in females. A significantly greater body mass index after the age of 15 is recorded in males. The highest percentage of subjects are with normal nutritional condition, a lower number of them are moderately underweight or overweight, while the underweight and overweight subjects are present in the smallest number. A more adequate approach of assessing the nutritional condition includes the combined values of BMI and triceps skinfold thickness.

*Key words*: height, weight, triceps skinfold thickness, body mass index, adolescents, the city of Novi Sad.

## Introduction

Age changes of the height and weight reflect developmental changes during the period of adolescence and also clearly indicate the development of an organism and socio-economic conditions.

The height and weight are used for assessing the growth, physical and health condition of an organism [4, 2, 3, 10, 14, 13, 5]. They are also used for assessing the nutritional condition [11, 9, 12, 1, 8]. A trait is also used for assessing the nutritional condition is the triceps skinfold thickness [6, 1].

The aim of the study is to determine the body status of adolescents of the city of Novi Sad.

## Material and Method

The transversal investigation was conducted in high schools of the city of Novi Sad in 2003, in compliance with the International Biological Programme and WHO. The investigation included 403 males and 462 females aged 15-18. The height and weight

values were the basis for calculating the body mass index (BMI kg/m$^2$), as well as the triceps skinfold thickness and BMI percent. The nutritional condition assessment was obtained using BMI percent and the combined percent values of BMI and triceps skinfold thickness according to NHANES I standards [7].

The data were processed using SPSS 10 for Windows while the significance of differences was determined by t-test.

## Results

Table 1 presents the mean and standard deviation values of the height, weight, triceps skinfold thickness and BMI in relation to the sex and age. It can be observed from Table 1 that the mean of male height ranges from 176.4 cm (the age of 15) to 181.7 cm (the age of 18), while in females it ranges from 165.6 cm (the age of 15) to 167.4 cm (the age of 18), with males showing significantly greater height values in all ages ($p< 0.01$).

As for the weight, the means are in the range of 68.9 kg (the age of 15) to 78.8 kg (the age of 18) in males, and of 58.6 kg (the age of 15) to 62.2 kg (the age of 15) in females. Significant differences are observed in all ages, greater values being recorded in males ($p< 0.01$).

The triceps skinfold thikness means range from 12.0 mm to 11.9 mm in males, and from 16.7 mm to 16.4 mm in females (at the age of 15 and 18, respectively). Significantly greater values are recorded in females, in all ages.

The absolute annual increase of all of the traits is greatest between the age of 15 and 16, except for the male height, which shows the greatest increase at the age of 17.

BMI ranges from 22.1 kg/m$^2$ (the age of 15) to 23.8 kg/m$^2$ (the age of 18) in males, and from 21.3 kg/m$^2$ to 22.2 kg/m$^2$ in females (at the ages of 15 and 18, respectively). Significantly greater means are observed in males after the age of 15 ($p< 0.01$).

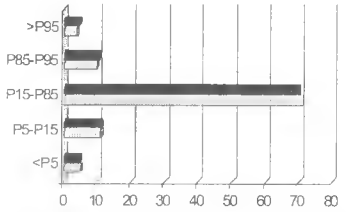
BMI distribution of males and females is given in Fig. 1.

The greatest percent of the subjects fall into the category of normal nutritional condition (P15–P85). Approximately 5.00 % are underweight ($P< 5$), and 10.00 % are moderately overweight (P85-P95).

The nutritional condition based on the combined values of BMI and triceps skinfold thickness is presented in Fig. 2.

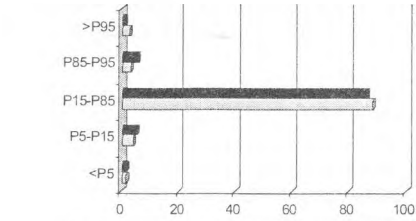
Table 1. Mean value (±SD) for body height, body weight, triceps skinfold thickness and BMI according to sex and age

| Age | Number | Body height (cm) | Absolute increase (cm) | Body weight (kg) | Absolute increase (kg) | Triceps skinfold thickness (mm) | Apsolute increase (cm) | Body mass index (kg/m$^2$) |
|---|---|---|---|---|---|---|---|---|
| | | | | Males | | | | |
| 15 | 89 | 176.4 ± 9.0 | – | 68,9 ± 13.5 | – | 12.0 ± 6.4 | – | 22.1 ± 3.7 |
| 16 | 105 | 179.1 ± 7.8 | 2.7 | 74.7 ± 13.2 | 5.8 | 12.5 ± 6.5 | 0.5 | 23.2 ± 3.4 |
| 17 | 93 | 182.2 ± 7.8 | 3.1 | 78.1 ± 14.2 | 3.4 | 11.6 ± 5.7 | -0.9 | 23.5 ± 3.7 |
| 18 | 116 | 181.7 ± 6.3 | –0.5 | 78.8 ± 12.1 | 0.7 | 11.9 ± 5.6 | 0.3 | 23.8 ± 3.1 |
| Total | 403 | 180.0 ± 8.0 | | 75.4 ± 13.7 | | 12.0 ± 6.0 | | 23.2 ± 3.5 |
| | | | | Females | | | | |
| 15 | 110 | 165.6 ± 6.0 | – | 58.6 ± 8.2 | – | 16.7 ± 5.5 | – | 21.3 ± 2.8 |
| 16 | 120 | 167.0 ± 6.4 | 1.4 | 59.8 ± 7.3 | 1.2 | 16.1 ± 4.5 | 0.6 | 21.4 ± 2.2 |
| 17 | 101 | 167.3 ± 6.2 | 0.3 | 60.6 ± 8.8 | 0.8 | 16.4 ± 4.7 | 0.3 | 21.6 ± 2.6 |
| 18 | 131 | 167.4 ± 6.6 | 0.1 | 62.2 ± 8.8 | 1.6 | 16.4 ± 5.2 | 0.0 | 22.2 ± 2.8 |
| Total | 462 | 166.9 ± 6.1 | | 60.3 ± 8.4 | | 16.4 ± 5.0 | | 21.6 ± 2.6 |




| | <P5 | P5-P15 | P15-P85 | P85-P95 | >P95 |
|---|---|---|---|---|---|
| Males | 4.47 | 10.92 | 69.73 | 10.42 | 4.47 |
| Females | 4.98 | 11.04 | 70.56 | 9.52 | 3.9 |

Fig. 1. The nutritional condition according to the percent of Body Mass Index (BMI kg/m$^2$)




| | <P5 | P5-P15 | P15-P85 | P85-P95 | >P95 |
|---|---|---|---|---|---|
| Males | 1.49 | 5.21 | 86.35 | 5.71 | 1.24 |
| Females | 1.73 | 4.11 | 88.31 | 3.03 | 2.81 |

Fig. 2. The nutritional condition according to the combined percent of BMI and triceps skinfold thickness

It can be observed that the percent of males and females that fall into the category of normal nutritional condition is higher than the percent of those that are underweight. moderately underweight, moderately overweight and overweight (Fig. 2).

## Discussion

The average height and weight recorded in high school pupils aged 15-18 in the city of Novi Sad are slightly higher than those recorded in adequate groups in Vojvodina region [4, 2, 3, 10, 14], Slovenia [13], the Czech Republic [5], Cyprus [12], the United Arab Emirates [1] and Turkey [8].

The average Body Mass Index recorded in both sexes is slightly greater than this is the case with the results obtained in Turkey [8], Croatia [11] except for 16-year-old females, and in Cyprus [12] with the exception of 15-year-old females. Except for 17-year-old girls, the females in this study are characterised by lower values than those recorded by A l - H o u r a n i et al. [1].

The nutritional condition according to NHANES I standard indicates that the number of subjects with normal values is smaller than that is the case in the study by P a v l o v i ć [9]. In our population there is a greater number of the overweight of both of the sexes, and a smaller number of the underwight males than this is recorded by O n e r et al. [8].

The percent of the adolescents with normal nutritional condition is even higher if the triceps skinfold thickness is taken into consideration, apart from BMI values. The degree of nutritional condition obtained by the combined BMI and triceps skinfold thickness is more adequate than the one obtained only by BMI values.

## Conclusion

On the basis of the obtained results, the following conclusion can be drawn:

- Adolescents in the city of Novi Sad are characterised by great height and in most of the cases of normal nutritional condition.
- The average height, weight and triceps skinfold thickness do not significantly vary during the period of adolescence.
- Males are characterised by greater height and weight, while in females greater triceps skinfold thickness is recorded.

• The nutritional condition obtained by the combined values of BMI and triceps skinfold thickness is a more adequate approach.

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# Vitamin C as a Modulator of Bone Healing

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Vitamin C is a significant antioxidant and factor in collagen synthesis. Block in collagen synthesis is the reason for widely spread in past disease scurvy. Last decades vitamin C is an object of many scientific studies over bone and connective tissue repair.

In our study we present the results of vitamin C in situ application in fracture of mice femur. We prove the role of vitamin C as a modulator of bone repair.

*Key words*: vitamin C, bone, repair, healing, collagen, fractures.

## Introduction

The role of vitamin C for human and animals is well known. It is a water-soluble micronutrient essential for human health and participates in nutrition for primates, some mammals (bats, guinea pig), birds and fish [6].

Vitamin C, also known as L-ascorbat or ascorbinic acid. Its formula is C6H8O6 and molecular weight is 176.13 g/mol (Fig. 1).

The significance of vit. C as an antioxidant is as substrate for ascorbinic peroxidase. In collagen synthesis it takes part as a cofactor and electron donor for 8 enzymes.

Human body cannot synthesize vit. C and its quantities in cell storage are limited. When there is a deficiency of that vitamin the collagen synthesis stops and scurvy occurs. The collagen without vit. C is unstable and cannot perform its function [1].

First description of scurvy was given by Hippocrates, about 400 years B.C. and first scientific attempt for explanation of the disease was made by J. Lind, surgeon in British navy, in 1753.

In 1928 the anthropologist Stefansson proves why the Eskimo do not suffer of scurvy.

From 1928 to 1933, the Hungarian research team of Joseph L Svirbely and Albert Szent-Györgyi and, independently, the American Charles Glen King, first isolated vitamin C and showed it to be ascorbic acid. The anti-scurvy compound was called Vitamin C.

From 1933 to 1937 Sir Walter Norman and Tadeus Reichstein, autonomously, succeeded in synthesizing Vitamin C. After synthesizing Vitamin C, Sir Walter Norman received the Nobel Prize in Chemistry [6, 8]. This accomplishment not only constituted

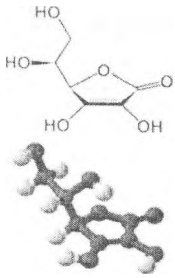


Fig. 1. Vitamin C structural formula

a valuable addition to knowledge of organic chemistry but also made possible the cheap mass production of Vitamin C for medical purposes.

1955 J.J. Burns showed that the reason why some mammals were susceptible to scurvy was due to the inability of their livers to produce the active enzyme L-gulonolactone oxidase (GLO).

Daily usage of vit. C is an object of a serious research but it's a fact that people consuming foods rich of vit. C are healthier and die rarely of chronic diseases.

It is required the daily usage of vit. C to be between 90 mg and 2000 mg.

Health organizations in different countries give similar data: Great Britain – 40 mg daily, Canada – 60 mg, United States – 60-90 mg, WHO – 45 mg daily [8].

Hipo- and avitaminosis C is characterized with disruption of mineral component of the bone. The skeleton over which calcium and phosphorus are piled is damaged. It is impossible these minerals to be adequately situated even if their intake is significant.

Bone healing is a scientific problem from decades [5]. There are several known factors which have influence over the process of bone repair. Some of them are shown on a diagram, others are age, fracture type, bone density, etc. (Fig. 2).

Continuity of the process depends on the extent of the trauma and the shortest period for initial bone healing in human is 3-4 weeks after the fracture [3, 4].

As we know bone healing process has several phases in which main part takes the periosteum. It is the source of precursor cells which differentiate later in chondroblasts and osteoblasts – the basic cells of healing bone.

First phase is the reactive phase. It begins with haematoma and ends with formation of granulation tissue of fibroblasts and aggregated cells (Fig. 3).

Next phase characterizes with formation of new bone also known as callus. Main part here has hyaline cartilage which adhere the fracture gap. The new bone is then changed by lamellar bone and the process is known as enchondral ossificatioin. Meantime collagen matrix is formed and after its mineralization the osteoblasts built new lamellar bone over its surface [2].

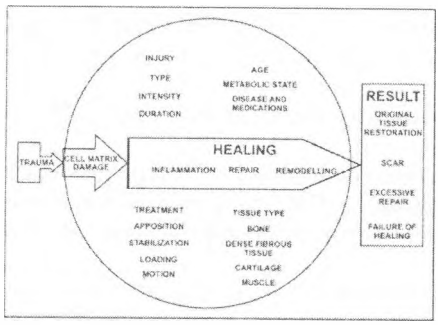


Fig. 2. Variables that influence bone healing

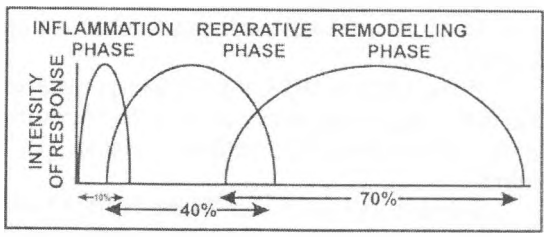


Fig. 3. Phases of bone healing process

Last phase, or remodelling, is the exchange of the trabecular bone with compact bone.

Experimental fractures are extremely difficult for standardization [1, 7]. Fracture models are made in mice, rats, rabbits, dogs, sheep, cats and calves. Although that mice and rats bones do not repeat the human model of regeneration they are used widely in orthopaedic practice [1]. The results cannot be translated directly over human and they are only orientative. Main challenge is fracture stabilization.

There are four methods of experimental fracture creation – manual, three point bending, guillotine and with osteotomies.

The evaluation of the bone healing in experimental fractures is based on several methods as fluoroscopic, histological, mechanical testing, osteodensitometric, biological markers of bone formation.

The aim of our study is to estimate the role of vit. C in bone healing process.

## Material and Methods

Our model is based over 70 experimental fractures of mice femur. We divided it into 3 groups.

First group contains 30 animals which fracture sites were injected with 1ml 30% solution of ascorbinic acid immediately after fracture. Their diet after fracture was rich of vit. C – up to 2 grams daily (Table 1).

Second group contains also 30 animals with fractures but without initial injection of vit.C in fracture site. They were only given before and after fracture occurrence, food extremely rich of vit. C. The intake was 1,5 – 2 grams per day.

Control group contains 10 mice. They have only fractured femora without any special diet. The evaluation is made by manual test and X-ray study. The days are 7th, 14th, 21st and 28th after fracture occurrence.

After dissection and visual estimation material for histology study is taken.

## Results

First evaluation was made at the 7$^{th}$ day. There were no X-ray signs for new bone formation. Manual testing showed pathologic movements.

New bone formation is seen at 14$^{th}$ day in mice treated with vit. C in difference with control group, in which this callus formation is shown after 3rd week. At the 14$^{th}$ day there is still pathologic mobility (Fig. 4).

Third week characterizes with expressive callus formation and lack of mobility of the fragments.

T a b l e 1. Material and Methods

| TOTAL 70 FRACTURES | | |
|---|---|---|
| I GROUP<br>30 Fractures | II GROUP<br>30 Fractures | III GROUP<br>10 Control |
| Vit. C<br>Injection | – | – |
| Vit. C<br>Rich Diet | Vit. C<br>Rich Diet | Normal<br>Food |

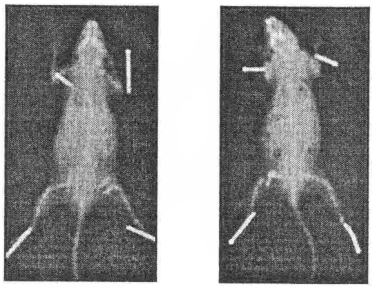

Fig. 4. Second week callus formation in Group I (left) and lack of callus formation in control group

Callus formation has bigger size in group injected with vit. C (group I) and especially in those cases with bigger dislocation of the fragments.

Group with only vit. C rich diet (group II) has more strength of bone callus but the callus is not as elastic as in control group.

After the 4th week there are not pathologic movements during manual testing. X-ray graphies show that the bone callus density is close to the trabecular bone density. There is no relation between callus size and the duration of the bone healing process.

## Discussion

Best performance of callus formation and bone healing showed mice injected with vit. C solution and fed with rich of vit. C food. In parallel with control group bone callus in mice from Group II (only vit. C rich diet) was not so tensile, but more solid.

We believe that vit. C has positive influence over bone healing and fastens the process of bone repair. It takes part in the process at the stage between first and second phase, between the first and second week after fracture. Its usage immediately after fracture occurrence, in doses up to 50 times more than normal daily intake, would fasten the process of collagen skeleton formation and bone healing.

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# Relation between Maternal Age and Stature at Child's Birth and Anthropometrical Status in Neonates

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The foetus's development depends on the parental factors, including morpho-dynamic status of the mother and father, birth order, socioeconomic living conditions, etc. The **aim** is to examine the influence of maternal age and stature on four basic anthropometrical features in fullterm babies. During 2001 a total of 219 (110 boys and 109 girls) healthy, fullterm neonates born in Sofia (38 to 42 weeks of gestation, with a body weight of more than 2500 g) were studied. Z-values of crown heel length (stature), weight, head and chest circumferences are included in the analysis. We can summarize that the maternal age and stature influence on the anthropometrical characteristic in newborn infants: the neonates from both genders, whose mothers are at the age of 20-30 years and above 170 cm in height, have biggest body sizes.

*Key words:* maternal age, maternal stature, fullterm neonates, basic anthropometrical features.

## Introduction

The formation of child in mature individual depends on harmonically interaction between exogenous (environmental, socio-economical, urbanization, etc.) and endogenous (genetic, hormonal, metabolic) factors [3, 12]. The foetus's development depends on the parental factors, including morpho-dynamic status of the mother and father, socioeconomic living conditions, etc. Some authors consider that the maternal factor, characterized mainly by age, weight and stature of mother, birth order, etc. are most important for the foetus, than the father's characteristics [4].

The **aim** is to examine the influence of maternal age and stature at child birth on four basic anthropometrical features in fullterm babies.

## Material and Methods

During 2001 a total of 219 (110 boys and 109 girls) healthy, fullterm neonates born in Sofia (38 to 42 weeks of gestation, with a body weight of more than 2500 g) were studied. The babies were examined within 24 hours after birth. The gestational age was determined according to the date of mother's last regular menstruation. The anthropometrical data were collected in the section of Neonatology at II Hospital of

Obstetrics and Gynaecology "Sheynovo". Thirty-eight anthropometrical features were measured by Martin – Saller's classical methods [6], in lying position of the child, from the right side of the body. Z-values of stature, weight, head and chest circumferences are included in the analysis. The crown heel length (stature) of the neonates is taken with the baby lying nude on a horizontal measuring table resembling a stadiometer to the nearest 0.5 cm. The infant's head is held in the vertical "Frankfurt plane". The body weight is measured with a beam balance to the nearest 0.01 kg and the circumferences are measured by plastic type to the nearest 0.5 cm.

The statistical analysis of the data is realized using statistical software for Windows - SPSS 11.0. Z-score transformation was applied to variables for easier comparison of the anthropometrical features independently of their measure units and different scales. For assessment of the influence of maternal age and stature on newborns' anthropometrical status, one-way ANOVA analyses and post hoc Tukey Honestly Significant Difference Test (HSD-test for unequal N) were applied.

According to the maternal age and stature the studied newborn infants are distributed in following groups (Table 1).

Table 1. Groups of neonates according to the maternal age and stature

| Groups / Factors | I | II | III | IV |
|---|---|---|---|---|
| Mother's age | ≤ 20 years | 21-25 years | 26-30 years | > 30 years |
| Mother's stature | ≤ 160 cm | 161-170 cm | > 170 cm | |

## Results

*Age of mothers*

The mothers in the study are aged 16-43 years.

The newborn boys of mothers aged over 26 years (III and IV groups) have values of the features above the means and boys who have younger mothers (I and II groups) have body sizes under or near to the means (Table 2, Fig. 1). The maternal age influenced significantly only the body weight and chest circumference.

The newborn girls whose mothers are aged 21-25 years (II group) have biggest body sizes (Table 2, Fig. 1). The girls whose mothers are under 20 and over 30 years of

Table 2. Differences in Z-score values of anthropometrical features in neonates according to maternal age

| Mother's age | Z-score values of anthropometrical features | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | boys | | | | girls | | | |
| | crown heel length | weight | head circumf. | chest circumf. | crown heel length | weight | head circumf. | chest circumf. |
| I group (≤ 20 y) | 0.044 | -0.247 | -0.319 | -0.235 | -0.82 | -0.983 | -0.380 | -0.655 |
| II group (21-25 y) | -0.184 | -0.268 | -0.150 | -0.190 | 0.183 | 0.331 | 0.175 | 0.291 |
| III group (26-30 y) | 0.164 | 0.284 | 0.200 | 0.129 | -0.003 | 0.026 | -0.020 | 0.039 |
| IV group (>30 y) | 0.206 | 0.355 | 0.233 | 0.504 | 0.027 | -0.254 | -0.080 | -0.279 |
| Test for multiple comparisons | – | – | – | – | I/II* | I/II* I/III* | – | I/II* |

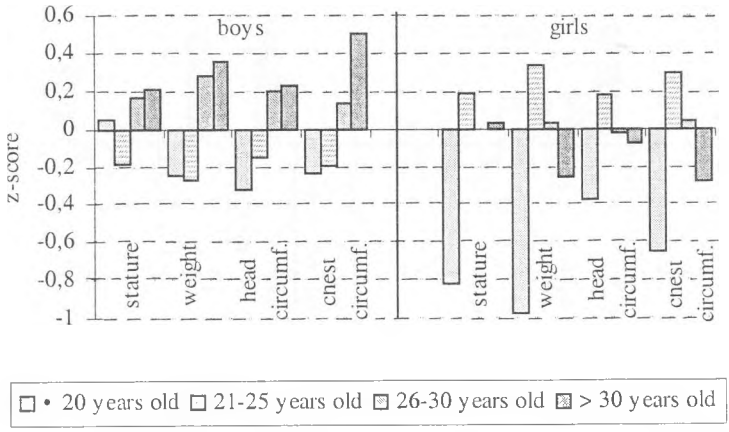


Fig. 1. Z-score values of basic anthropometrical features in neonates according to maternal age

age (I and IV groups) have values of the studied anthropometrical features lower than the means.

The pairwise multiple comparisons test shows significant differences between first and second groups of girls (with mothers younger than 20 years and mothers aged 21-25 years) in crown heel length, body weight and chest circumference. Significant differences in body weight are observed also between girls from first and third groups.

In some studies a tendency to increment of newborns' body weight and crown heel length with enhancement of maternal age is reported [1, 5, 11, 13]. Numerous authors recorded also that the younger and older mothers give birth to babies who are lighter and shorter [10, 14]. According to S e r e j s k i [9] and R o u s h a m & G r a c e y [8] mothers aged 15-18 years give birth to babies with retarded intrauterine development more frequently and consider that this maternal age could be a risk factor for low birth weight.

*Stature of mothers*

The stature of mothers varies between 150.0 cm and 184.0 cm.

The boys born to mothers under 160.0 cm (I group) and above 170.0 cm high (III group) have values of the anthropometrical features above the means, as these whose mothers are above 170.0 cm high have bigger values of the crown heel length and chest

T a b l e 3. Differences in Z-score values of anthropometrical features in neonates according to maternal stature

| Mother's stature (cm) | Z-score values of anthropometrical features | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | boys | | | | girls | | | |
| | crown heel length | weight | head circumf. | chest circumf. | crown heel length | weight | head circumf. | chest circumf. |
| I group (≤ 160) | -0.03 | 0.153 | 0.156 | 0.048 | -0.491 | -0.333 | -0.341 | -0.101 |
| II group (161-170) | -0.08 | -0.132 | -0.104 | -0.060 | 0.065 | -0.02 | 0.117 | -0.060 |
| III group (> 170) | 0.363 | 0.146 | 0.032 | 0.078 | 0.401 | 0.332 | 0.095 | 0.237 |
| Test for multiple comparisons | – | – | – | – | I/II* I/III* | – | – | – |

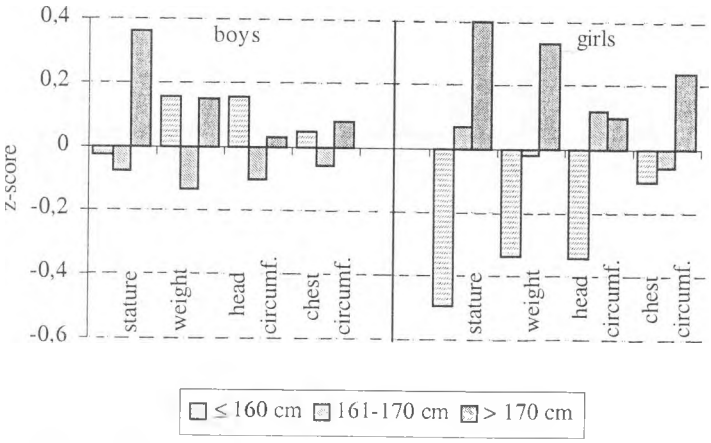


Fig. 2. Z-score values of basic anthropometrical features in neonates according to maternal stature

circumference (Table 3, Fig. 2). The values of all anthropometrical features of boys from the second group (with stature of mothers between 161.0 and 170.0 cm) are lower than the means.

The dependence between anthropometrical status of newborn girls and stature of their mothers also is stronger in these whose mothers are above 170.0 cm high (III group) (Table 3, Fig. 2). The girls of the third group have the highest values of the anthropometrical features. These values are over the mean, with exception of the head circumference ones, which are higher in the girls from the second group. As distinct from boys, the girls born to mothers with stature under 160.0 cm (I group) have values of the anthropometrical features lower and near to the means. The maternal stature influenced significantly only the crown heel length of the newborn girls.

According to the pairwise multiple comparisons test, significantly intergroup differences are observed: the girls from first group (with mothers' stature up to 160.0 cm) have significantly shorter crown heel length than girls whose mothers are taller.

The question of dependence between parental stature (mainly of the mother) and neonates' body sizes is discussed by many authors [1, 2, 7, 13]. They found that the increment of maternal stature leads to considerable increment of body weight and stature at birth. Voigt et al. [11] maintain that the mothers' stature has a twice stronger effect than the fathers' one.

## Conclusions

We can summarize that the maternal age and stature at child's birth influence the anthropometrical characteristic in newborn infants:

- The neonates' body sizes growing up with increasing of maternal age, as the boys and girls, whose mothers are at the age of 20-30 years have biggest sizes. The neonates from both genders, whose mothers are under 20 and girls whose mothers are over 30 years of age, have values of the anthropometrical features lower than the means.
- With increasing of mothers' stature (above 170 cm in height), the neonates from both genders have biggest body sizes.

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# Waist Circumference as an Indicator of Body Nutritional Status in Children Aged 3 to 6 Years

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The aim of present report is to assess the sexual and age-related differences of waist circumference in children from 3 till 6 years and to elaborate percentile curves for this age period. A sample of 640 3- to 6-year-old children (320 boys and 320 girls) living in Sofia City is used. The study is cross-sectional and carried out from June 2004 till May 2005 in 7 kindergartens. Boys have higher values for waist circumference as the sexual differences are significant in all ages excepting the 4th year. The waist circumference increase with age, but in a different manner for boys and girls. The elaborated by us curves represent the first waist circumference percentile values for Bulgarian children aged from 3 till 6 years. They could be used like auxiliary contrivance for the assessment and early identification of children who have underlined centralized distribution of fat.

*Key words:* waist circumference, children, percentile curves, sexual differences, age differences.

## Introduction

The prevalence of obesity among children increases rapidly in the world according to data published by WHO [13]. The childhood obesity is linked with an increased risk of obesity in adulthood, and thenceforward with the connected important public diseases as hypertension, diabetes, early atherosclerosis, etc. [2, 3, 6, 11].

Many epidemiological studies in adults prove the high fat centrality as heightened risk for the individual, i.e. for those individuals with excessive body fat accumulation on abdomen and waist [9].

As basic criteria for the determination of fat distribution types serves the ratios of waist circumference to other body circumferences – abdomen, hip, thigh.

Results published in the 90s show that waist circumference like an independent feature is an objective and useful indicator of fat centrality as in adults, so in children [5, 10].

Waist circumferences cut off points are already available concerning adults from both sexes, and are used for identification of individuals in health risk. In children, how-

ever, such values had to be conformed not only with sex but to age, since during the growing up period waist circumference normally increases annually.

In the worldwide literature are published age- and sex-specific reference values for waist circumference in children having different nationalities (USA, Canada, Australia, Italy, Denmark, Cuba, Spain, Great Britain, etc.), and many authors emphasizing the need of national reference values for waist circumferences to be elaborated [1, 4].

In the Bulgarian scientific literature data from anthropological investigations concerning early childhood (3-6 years of age) could be found very rarely. We have not found yet data about waist circumference in the Bulgarian children, one fact that traced out the aim of the present work.

The aim of present report is to assess the sexual and age-related differences of waist circumference in children from 3 till 6 years of age and to elaborate percentile curves concerning this age period.

## Material and Methods

A sample of 640 3- to 6-year-old children (320 boys and 320 girls) living in Sofia City is used. The study is cross-sectional and carried out from June 2004 till May 2005 in 7 kindergartens from several districts of the city.

The waist circumference measurements are taken on the level pointing the maximal hollowness of the lateral body contour, or so called in the international literature "natural waist". The measurements are taken on a naked body using non-elastic tape.

The statistical analysis is made by SPSS software for Windows, using the percentile analysis values for the 3$^{rd}$, 10$^{th}$, 25$^{th}$, 50$^{th}$, 75$^{th}$, 90$^{th}$ and 97$^{th}$ percentiles are computed concerning each sex and age group. The percentile curves are constructed and then smoothed by the Origin 7.0 software.The statistical significance of the established sexual and age differences are assessed by the t-test of Student at $P = 0,05$.

The sexual differences are evaluated in relative index units (IU) by the formula of Wolański for inter-group comparisons, named by us Index of Sexual Differences (ISD):

$$ISD = \frac{2 \times (\bar{x}_{\delta} - \bar{x}_{\varphi}) \times 100}{(\bar{x}_{\delta} + \bar{x}_{\varphi})}.$$

## Results

In Table 1 are given the biostatistical characterizations of waist circumference for each sex and age group. The waist circumference means for both sexes are shown in Fig. 1, by which the comparative assessment in inter-sexual and inter-age aspect become possible.

The waist circumference in 3 years old boys is 507,9 mm and is significantly higher compared to the one in girls – 491,4 mm. Waist circumference is always higher in boys till 6 years, as the sexual differences are significant in all ages ($P \leq 0,05$) excepting the 4$^{th}$ year. The assessment of sexual differences by the ISD shows that the differences are better underlined at the end of the studied period, i.e. in the 5 and 6 years old children (Fig. 2).

Between 3 and 6 years boys and girls have different increment of the waist circumference (Table 2). In boys the waist circumference increase most rapidly and significantly between 4 and 5 years, while in girls the increment is significant during the periods 3-4 and 5-6 years of age ($P \leq 0,05$).

Table 1. Biostatistical data and sexual differences of waist circumference (mm)

| Age | ♂ | | | ♀ | | | Sexual differences | |
|---|---|---|---|---|---|---|---|---|
| | n | $\bar{x}$ | SD | n | $\bar{x}$ | SD | Absolute differences | ISD |
| 3 | 80 | 507.9 | 28.2 | 80 | 491.4 | 29.7 | 16.5* | 3.3 |
| 4 | 80 | 519.4 | 41.1 | 80 | 510.4 | 29.3 | 9.0 | 1.8 |
| 5 | 80 | 540.4 | 54.4 | 80 | 515.9 | 32.1 | 24.5* | 4.6 |
| 6 | 80 | 556.2 | 49.7 | 80 | 537.5 | 50.6 | 18.7* | 3.4 |

* $P \leq 0{,}05$

Table 2. Year alteration

| Age | ♂ | | | | ♀ | | | |
|---|---|---|---|---|---|---|---|---|
| | n | AYA (mm) | RYA (%) | t-test AYA | n | AYA (mm) | RYA (%) | t-test AYA |
| 3-4 | 80 | 11.5 | 2.2 | 2.1* | 80 | 19.0 | 3.8 | 4.1** |
| 4-5 | 80 | 21.0 | 4.0 | 2.8* | 80 | 5.5 | 1.1 | 1.1 |
| 5-6 | 80 | 15.8 | 2.9 | 1.9 | 80 | 21.6 | 4.1 | 3.2* |

* $P \leq 0.05$: AYA – Absolute Year Alteration; RYA – Relative Year Alteration

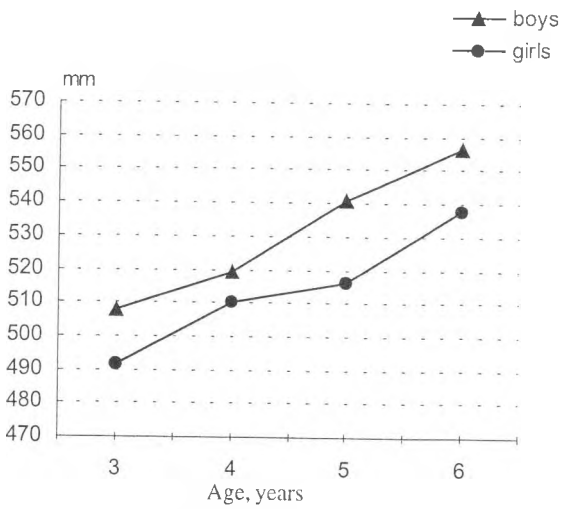


Fig. 1. Waist circumference (mm)

The presence of significant differences in waist circumference means between boys and girls, as well as the sexual differences concerning the intensity of growth during separate one-year periods determine the need of elaboration of cut off points connected with children's gender and age.

At table 3 are presented the values about 3rd, 10th, 25th, 50th, 75th, 90th and 97th percentiles for waist circumference in children from Sofia - 3-6 years old boys and girls separately, and in Fig. 3 and Fig. 4 are shown the respective suggested percentile curves.

In both sexes the waist circumference percentile values increase with age, the boys having higher values during the entire period under study.

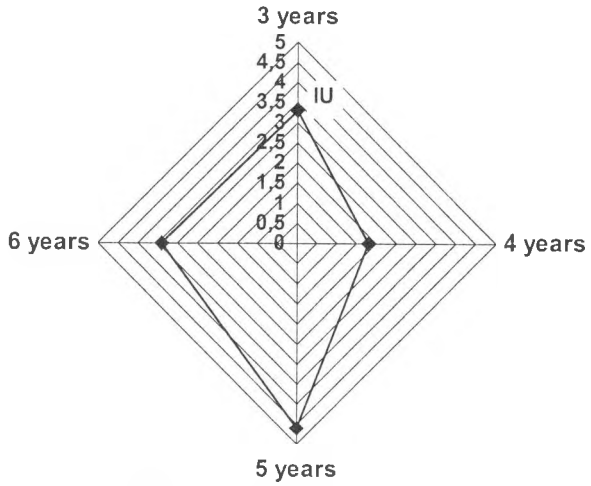


Fig. 2. Sexual differences for waist circumference after ISD data (IU)

Table 3. Percentile values for waist circumference in 3-6 years old children (cm)

| Age | ♂ | | | | | | |
|---|---|---|---|---|---|---|---|
| | $P_3$ | $P_{10}$ | $P_{25}$ | $P_{50}$ | $P_{75}$ | $P_{90}$ | $P_{97}$ |
| 3 | 46.0 | 47.1 | 48.8 | **50.5** | 52.5 | 54.0 | 57.8 |
| 4 | 46.0 | 48.0 | 49.6 | **51.5** | 54.0 | 56.0 | 60.1 |
| 5 | 49.0 | 49.5 | 51.5 | **53.0** | 55.0 | 58.0 | 69.3 |
| 6 | 50.2 | 50.6 | 52.6 | **54.5** | 57.0 | 61.6 | 66.7 |
| | ♀ | | | | | | |
| 3 | 43.0 | 45.1 | 47.5 | **49.0** | 51.2 | 53.4 | 55.0 |
| 4 | 45.4 | 47.5 | 48.6 | **51.0** | 53.0 | 55.2 | 56.5 |
| 5 | 46.4 | 47.6 | 49.5 | **51.5** | 53.0 | 55.0 | 61.9 |
| 6 | 47.7 | 48.5 | 50.5 | **52.5** | 56.0 | 60.1 | 67.9 |

Most data about waist circumference in the literature concerning children and adolescents are relevant mainly to children over 5 years. In the foreign literature available we didn't found data about the investigated by us age period (3-6 years old children). Comparison concerning one part of our data with such elaborated by authors from abroad is possible only with the data presented by McCarthy et al. [7] for British children aged from 5,0 till 16. 9 years, as well as by Moreno et al. [8] for 6.0 - 14.9 years old Spanish children (Table 4).

The data show that at 5 years of age British and Bulgarian girls have close means of waist circumference, while the Bulgarian boys have higher waist circumference compared to the British ones. In the 6 years old children, however, the Bulgarian boys and girls have higher values for waist circumference compared to their British coevals.

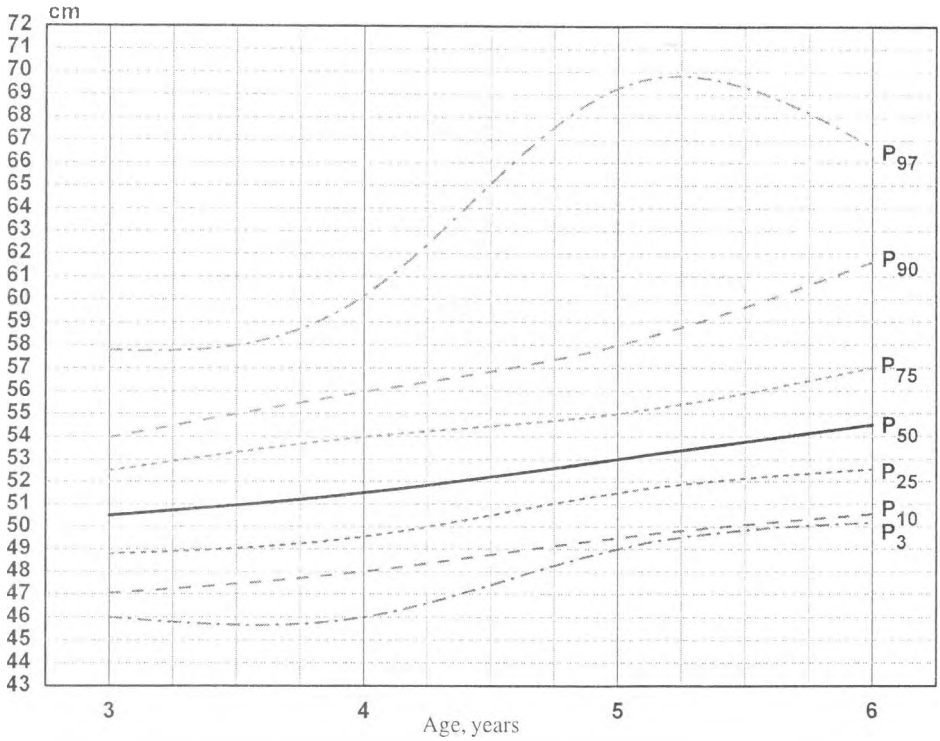


Fig. 3. Percentile curves for waist circumference in 3-6 years old boys

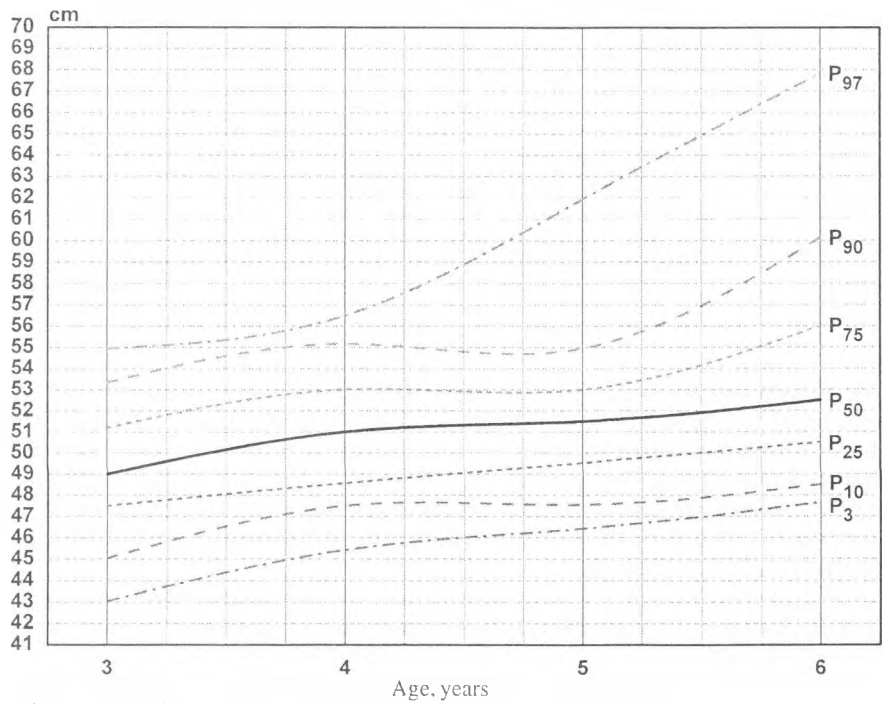


Fig. 4. Percentile curves for waist circumference in 3-6 years old girls

Table 4. Comparative data for waist circumference

| Country | 5 years old children | | 6 years old children | |
|---|---|---|---|---|
| | $\bar{X}$ boys (cm) | $\bar{X}$ girls (cm) | $\bar{X}$boys (cm) | $\bar{X}$girls (cm) |
| **Bulgaria** | 54.0 | 51.6 | 55.6 | 53,8 |
| **Great Britain** | 52.3 | 51.3 | 52.8 | 52,2 |
| **Spain** | – | – | 56.0 | 54,9 |

Comparing the data about 6 years old Spanish children, the Bulgarian ones have greater waist circumference, and the differences are better underlined in girls.

## Conclusion

The elaborated by us curves represent the first waist circumference percentile values for Bulgarian children aged from 3 till 6 years. They could be used like auxiliary contrivance for the assessment and early identification of children who have underlined centralized distribution of fat. And the subsequently require appropriate intervention could help the risk reduction of forthcoming health complications.

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# Skin Folds of Children with Type I Diabetes Mellitus

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The model of distribution of subcutaneous adipose tissue is investigated in children with type I diabetes mellitus. For this goal is determinated the skin folds thickness in 9 regions of the body: sfTriceps, sfBiceps, sfAntebrachium, sfSubscapulare, sfXcosta, sfAbdomen, sfSuprailiaca, sfThigh, sfCrus. The distribution of the subcutaneous adipose tissue is more in the periphery and not in the trunk.

*Key words*: somatotype, children, type 1 diabetes mellitus

## Introduction

Skin folds thickness is an important anthropometrical parameter for defining the model of distribution of subcutaneous adipose tissue in norm and in a range of metabolic diseases such as obesity, metabolic syndrome, etc [3]. It is particularly very useful in defining the anthropological status of people with diabetes mellitus. This procedure is relatively non-invasive. It demands simple technology and shows good applicability giving results with acceptable accuracy [1]. This study aims to determine the model of distribution of subcutaneous adipose tissue in children with Diabetes mellitus type 1.

## Material and Methods

Male and female children from the Bulgarian ethnic group with Diabetes mellitus type 1 were examined.

Girls were 80 distributed in two age groups: from 7 to 12 years and from 12 to 18 years. Boys were 90 distributed also in two age groups: from 7 to 12 years and from 12 to 18 years. 9 skin folds were sized with Harpenden skinfolds caliper using The Method of Martin – Saller: sfTriceps, sfBiceps, sfAntebrachium, sfSubscapulare, sfXcosta, sfAbdomen, sfSuprailiaca, sfThigh, sfCrus. Data about the same skin folds sized in 100 healthy boys and 100 healthy girls from the same age range was used for check that was kindly placed by the Department of Human Anatomy and Physiology of University of

Plovdiv "Paisii Hilendarski" Received results were performed using the statistical programme SPSS and Instat Degrees of significance are: low ($p \geq 0,05$), moderate ($p=0,051-0,001$) and high ($p< 0,001$).

## Results

*Boys 7-12 years (Table 1)*

The skin fold in the thigh area is the thickest one in sick and in healthy boys, but the thickness is bigger in sick boys. Statistically significant difference in the skin folds thickness in sick and healthy boys was found in: sfSubscapulare ($p=0,051-0,001$), sfXcosta ($p=0,051-0,001$), sfAntebrachium ($p=0,051-0,001$), sfCrus ($p< 0,001$) everywhere they are thicker in healthy boys with moderate degree of significance, and in the crus area the degree of significance is high. Such a tendency in precipitation of subcutaneous adipose tissue is seen also in sfSuprailiaca, sfAbdomen, sfBiceps, but with a low degree of reliability ($p \geq 0,05$). In all skin folds, values are higher in the healthy children compared to the sick ones. Ratio sfTriceps/sfSubscapulare is 1.52.

Table 1. Skin folds measurements in diabetic and healthy boys aged 7-12 years

| Variables | Diabetic patients | | | Healthy subjects | | |
|---|---|---|---|---|---|---|
| | N | Mean | SD | N | Mean | SD |
| sfTriceps | 45 | 12.29 | 4.20 | 50 | 12.94 | 4.88 |
| sfSubscapulare | 45 | 8.06 | 4.39 | 50 | 9.85 | 5.14 |
| sfXrib | 45 | 6.82 | 4.44 | 50 | 8.50 | 4.72 |
| sfSuprailiaca | 45 | 7.54 | 6.12 | 50 | 10.07 | 5.80 |
| sfAbdomen | 45 | 11.05 | 7.08 | 50 | 13.70 | 6.95 |
| sfBiceps | 45 | 6.48 | 3.09 | 50 | 7.95 | 3.44 |
| sfAntebrachium | 45 | 7.09 | 2.58 | 50 | 8.01 | 2.73 |
| sfThigh | 45 | 19.57 | 8.11 | 50 | 17.72 | 6.37 |
| sfCrus | 45 | 11.85 | 6.12 | 50 | 14.55 | 5.14 |

*Boys 12-18 years (Table 2)*

At this age, the skin folds in the thigh area are the thickest ones in both groups, but the thickness is bigger in sick boys. Statistically significant difference in the skin folds thickness in sick and healthy boys was found in sf Biceps ($p=0,051-0,001$), sfAntebrachium ($p=0,051-0,001$), sfCrus ($p< 0,001$). The degree of significance in the area of the

Table 2. Skin folds measurements in diabetic and healthy boys aged 12-18 years

| Variables | Diabetic patients | | | Healthy subjects | | |
|---|---|---|---|---|---|---|
| | N | Mean | SD | N | Mean | SD |
| sfTriceps | 45 | 11.82 | 5.96 | 50 | 11.49 | 5,43 |
| sfSubscapulare | 45 | 9.44 | 4.62 | 50 | 10.49 | 4.93 |
| sfXrib | 45 | 9.21 | 6.27 | 50 | 9.33 | 5.59 |
| sfSuprailiaca | 45 | 10.03 | 7.51 | 50 | 11.37 | 6.70 |
| sfAbdomen | 45 | 14.66 | 9.83 | 50 | 15.56 | 8.19 |
| sfBiceps | 45 | 5.61 | 3.32 | 50 | 7.25 | 3.66 |
| sfAntebrachium | 45 | 5.91 | 2.89 | 50 | 7.26 | 3.10 |
| sfThigh | 45 | 20.73 | 9.81 | 50 | 19.12 | 8.01 |
| sfCrus | 45 | 12.11 | 8.34 | 50 | 14.55 | 6.26 |

biceps and forearm is moderate and in the area of the crus, it is high. These three skin folds are thicker in healthy children than in the sick ones. In the rest skin folds, clearly pronounced tendency of differentiated distribution of subcutaneous adipose tissue is deficient in sick and healthy children. Ratio sfTriceps/sfSubscapulare is 1.25.

*Girls 7-12 years (Table 3)*

Skin folds in the area of the thigh are the thickest in both sick and healthy girls. Statistically significant difference in the skin folds thickness in sick and healthy girls was found in sfSuprailiaca ($p< 0,001$), sfAbdomen ($p< 0,001$), sf Antebrachium ($p< 0,001$), sf Thigh ($p< 0,001$). The degree of significance is high in all four skin folds. Skin folds in the area of crista iliaca, abdomen and forearm are thicker in healthy girls, but in the area of the thigh, it is thicker in the sick ones. In sfSubscapulare, sfXcosta, sfBiceps is seen a tendency for bigger distribution of subcutaneous adipose tissue in healthy children, but with a low degree of reliability ($p\geq 0,05$). Ratio sfTriceps/sfSubscapulare is 1.60.

Table 3. Skin folds measurements in diabetic and healthy girls aged 7-12 years

| Variables | Diabetic patients | | | Healthy subjects | | |
|---|---|---|---|---|---|---|
| | N | Mean | SD | N | Mean | SD |
| sfTriceps | 40 | 13.93 | 4.54 | 50 | 13.98 | 4.66 |
| sfSubscapulare | 40 | 8.69 | 3.83 | 50 | 10.83 | 4.90 |
| sfXrib | 40 | 7.58 | 3.39 | 50 | 9.22 | 4.59 |
| sfSuprailiaca | 40 | 7.82 | 4.37 | 50 | 11.21 | 5.44 |
| sfAbdomen | 40 | 11.66 | 5.22 | 50 | 14.59 | 5.79 |
| sfBiceps | 40 | 7.55 | 3.54 | 50 | 8.93 | 3.31 |
| sfAntebrachium | 40 | 6.64 | 2.16 | 50 | 8.74 | 2,73 |
| sfThigh | 40 | 24.34 | 9.26 | 50 | 18.95 | 5.67 |
| sfCrus | 40 | 14.69 | 5.32 | 50 | 15.86 | 4.67 |

*Girls 12-18 years (Table 4)*

Skin folds are thicker in girls with diabetes mellitus, with the exception of the ones in the area of the biceps and forearm. Statistically significant difference in the skin folds thickness in sick and healthy girls was found in sfSuprailiaca ($p< 0,001$), sfAbdomen ($p< 0,001$), sf Antebrachium ($p< 0,001$), sf Thigh ($p< 0.051$). In the first three skin folds, the degree of significance is high and in the last one, it is moderate. Such a tendency is found also in sfSuprailiaca. Ratio sfTriceps/sfSubscapulare is 1.33.

Table 4. Skin folds measurements in diabetic and healthy girls aged 12-18 years

| Variables | Diabetic patients | | | Healthy subjects | | |
|---|---|---|---|---|---|---|
| | N | Mean | SD | N | Mean | SD |
| sfTriceps | 40 | 19.64 | 7.27 | 50 | 16.52 | 5.57 |
| sfSubscapulare | 40 | 14.73 | 7.80 | 50 | 13.73 | 6.11 |
| sfXrib | 40 | 14.45 | 7.26 | 50 | 11.49 | 5,24 |
| sfSuprailiaca | 40 | 16.13 | 8.90 | 50 | 13.69 | 6.06 |
| sfAbdomen | 40 | 21.17 | 8.75 | 50 | 18.34 | 5.98 |
| sfBiceps | 40 | 9.88 | 4.49 | 50 | 10.03 | 3.79 |
| sfAntebrachium | 40 | 8.96 | 4.07 | 50 | 9.40 | 3.41 |
| sfThigh | 40 | 32.28 | 11.92 | 50 | 23.33 | 6.64 |
| sfCrus | 40 | 19.85 | 7.56 | 50 | 18.41 | 5,48 |

## Discussion

The precipitation of subcutaneous adipose tissue in boys at the age of 7-12 years is bigger in healthy children than in the ones with diabetes mellitus. With the exception of the thigh area, where the precipitation of subcutaneous adipose tissue is bigger in sick boys, but the degree of significance is low. Such dependency is reported by Ferrante et al. [2].

The precipitation of subcutaneous adipose tissue in boys at the age of 12-18 years is also bigger in healthy children than in the ones with diabetes mellitus. With the exception of the thigh area, where the precipitation of subcutaneous adipose tissue is bigger in sick boys, but the degree of significance is low.

The precipitation of subcutaneous adipose tissue in girls at the age of 7-12 years is bigger in healthy children than in the ones with diabetes mellitus. With the exception of the thigh area, where the precipitation of subcutaneous adipose tissue is bigger in sick boys, but the degree of significance is high.

The precipitation of subcutaneous adipose tissue in girls at the age of 12-18 years is bigger in sick ones than in the healthy ones. Tuvemo et al. /1997/ and Tillmann et al. [5] have reported such dependency. With the exception of the area of the biceps and forearm, where the precipitation is bigger in healthy girls. These differences are with a high degree of significance in triceps, X rib, thigh and moderate degree in the abdomen. Other skin folds are with a low degree of significance.

In boys and girls with diabetes mellitus, the precipitation of subcutaneous adipose tissue is higher in the periphery [upper limb] than in the trunk. Same conclusion is also drawn out by other authors [3].

## Conclusion

The skin folds in boys and girls with diabetes mellitus type I are thinner than in the healthy controls. The exception is the skin fold in the thigh area in girls of 12-18 that is thicker in sick ones. Skin folds ratio of triceps/subscapulare shows that the model of distribution of adipose tissue is in the periphery (upper limb) and not in the trunk.

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# Degrees of Blood Relationship – Review and Recommendations

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During the communist ruling genetics was among the most damaged fields of the science. Like in remaining "socialist" countries, several generations of Bulgarian medical doctors and biologists finished their higher education with absolutely insufficient knowledge in this topic. Therefore, when finding errors in this field, made by Bulgarian authors, we are inclined to excuse them, having in mind the gaps in their genetic education. Surprisingly, severe mistakes in determination of the degrees of blood relationship appeared in papers published by western authors, which could not benefit the mentioned excuse. The present work systematizes these mistakes and recommends the possible measures to avoid them in future investigations.

*Key words:* laterality, handedness, genealogical analysis, errors, recommendations.

## Introduction

In many branches of medical investigations a hereditary causes of (or at least predispositions to) different diseases are known or suspected. In such cases the drawing and analysis of genealogical trees, known as a genealogical method, are not only applied, but represent an extremely substantial approach. An important part of its application is to determine the degree of blood relationship between two subjects presented in the genealogical tree.

When applying genetic methods and meeting methodological errors, made by Bulgarian investigators, we are inclined to excuse them by gaps in their genetic qualification, due to the conditions in the former communist camp, where pseudo-scientists who had nothing to do with the real science (like Trophim Denisovich Lisenko and Olga Ivanovna Lepeshinskaya) were academicians and Stalin-prize winners and, just the opposite, great geneticists like Mendel, Morgan and Weisman were stigmatized as retrograde western scientists. However, such an excuse could not be applied towards western scientists, having been normally educated.

For many years we deal with morphological and functional asymmetries in man, the morphological one focused on the finger and palm prints (dermatoglyphics) and the functional one presented by the lateralization in sensory and motor functions (handedness, footedness, eyedness, hand clasping, arm folding, etc.). Genetic factors are con-

sidered to play a substantial role in their determination and therefore the genealogical method is undoubtedly very important in their investigation. Surprisingly, in several publications of western authors in very respectable journals we found astonishing mistakes in determination of degrees of blood relationship.

The aim of the present study is to remind the correct procedure for determination of the degrees of blood relationship, to analyze the most typical errors in this respect and to recommend how to avoid them in future investigations.

## Material and Methods

The main object of genetic investigation in the field of the functional asymmetry is the so-called familial sinistrality (FS), dealing with the non-right handed subjects among the subject's blood relatives.

We worked out in detail a quantitative method of investigation of the FS, based not only on the number of non-right handed subject's blood relatives, but considering their genealogical proximity with him. In majority of the papers on this topic, FS is considered purely qualitatively, i.e., subjects are presented as FS+ and FS-, showing and not showing familial sinistrality. Logically, the problem arises to define the genealogical proximity of relatives, which could be considered in order to determine whether the subject is FS positive or, alternatively, FS negative.

More of thirty papers devoted to familial sinistrality were used as material of the present review article. But only those of them, where the basic principles of the degrees of blood relationships are formulated ant those where the most severe errors have been found, were explicitly mentioned and cited in the References. The method was based on analysis of each of the found erroneous approaches through its comparison with the correct approach given in the classic genetic works.

## Results and Discussion

First of all, the reviewing of the literature sources showed that some terms in the field of handedness' genealogy need to be unified. For instance, the vast majority of authors include into the term "immediate family" only the subject's parents and siblings [5, 6, 7]. Others include therein parents, siblings, aunts/uncles and grandparents [12]. Third use the expressions "immediate relatives" and "immediate family members" without specifying their meaning [2].

Secondly, as indicated in the Introduction, an astonishing confusion of degrees of relationship is observed. The only correct manner to evaluate the degrees of blood relationship is to count them as consecutive steps from generation to generation. When there is a direct line proband's relative, we count these steps from the proband to his relative in ascendant direction. When a collateral line relative of the subject is concerned, we count the steps from the proband to their common progenitors in ascendant direction and then from the generation of the common progenitors back to the relative in question in descendant direction. Thus, parents and children are the only first degree relatives, while siblings and grandparents are second degree, aunts and uncles are third degree relatives, first cousins are forth degree relatives, etc. An example is presented in Fig. 1. The proband, III-9, is a female left-hander. Her parents, II-7 and II-8, are her first degree relatives; her grandparents, I-1, 2, 3 and 4, are her second degree relatives, as well as her brother III-10; and all her first cousins, III- 1, 2, 3, 4, 6, 7, 11, 12 and 13, are her relatives of fourth degree.

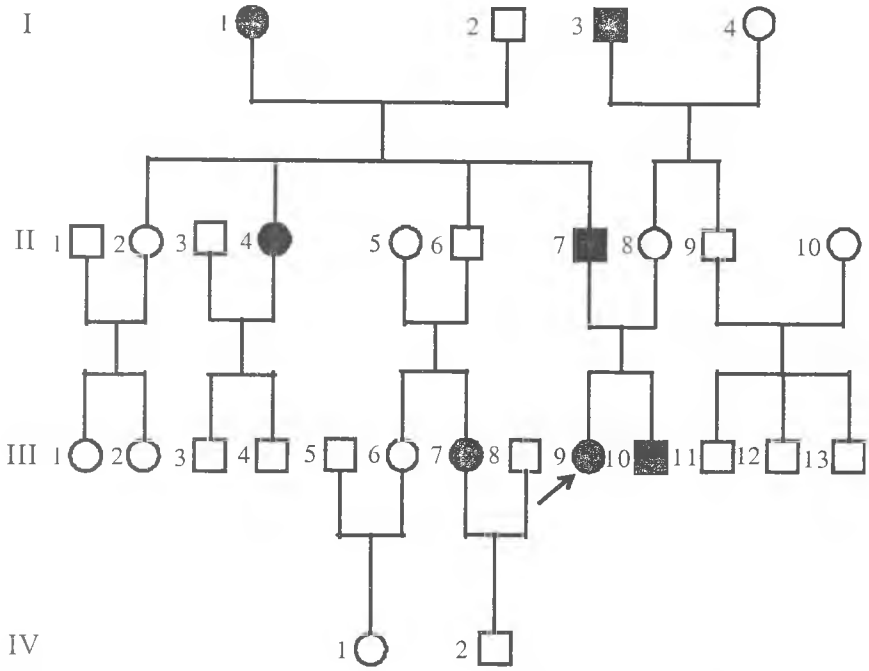


Fig. 1. Genealogical tree of one of our subjects – III-9, a left-handed female. The proband is indicated with an arrow and her non-right handed blood relatives are presented by solid symbols

In severe contradiction with these rules, McKeever and VanDeventer [9], McManus [11] and Gorynia and Egenter [4] indicated the siblings, along with parents and children, as first degree relatives. McKeever [8] and McKeever and Van Eys [10] enumerated "biologically related aunts and uncles" among the first and second degree relatives. To go the whole way, Salmaso and Longoni [13] and Cobianchi and Giaquinto [1] included among the first degree relatives grandparents, aunts, uncles and cousins! Truly, in some jurisprudences different systems to evaluate degrees of familial relationships still exist. However, one and only of them, that used in the civil law, is applicable for genetic purposes [3, 14].

## Conclusion

Inexcusable genealogical errors are met even in scientific articles. To prevent them, in the course of the medical education much more attention should be paid to (and an expressed emphasis should be put on) genetic methodology. As for the people who have been educated long ago, it is never too late to go through the classic genetic sources.

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# Consanguineous Marriages – Review and Recommendations

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The rates of the consanguineous marriages, estimated through the coefficient of inbreeding, F, vary in quite large scale in different populations. Such marriages are undesirable from genetic point of view, as far as they increase the probability of homozygous combination of rare recessive alleles and thus – the rate of recessive diseases. Three mechanisms limit these marriages – family legislation, religious wedding norms and the popular knowledge included in the common low or existing as established traditions. The author's observations are presented on the strongly exaggerated fears and prejudices of common Bulgarians concerning consanguineous marriages. Dangers arising from them are considerably overestimated by Bulgarian public opinion as compared to the legislative and religion preventions.

*Key words:* consanguinity, risks, restrictions, overestimation, Egypt dynasty.

## Introduction

Empiric observations have shown that marriages among blood relatives are connected with an increased risk of heavy hereditary pathology. Such marriages increase the probability of homozygous combination of rare recessive alleles coming from a common progenitor of both spouses and thus – the probability of recessive diseases. Therefore, in many ancient peoples some rules existed restricting sexual relationships and (more or less) official marriages between such relatives. The proximity of the relationship when marriage is prohibited varies in extremely large scale between populations, and does not correspond to their civilization level. Empirically accepted prejudices against consanguineous marriages have gradually been included into the marriage traditions, into the unwritten low, into the religious wedding norms and, much latter – into the written legislations of different countries. Of course, all these restrictions are not equally based on genetic principles and some of them are not genetically grounded at all. On the contrary, dynastical considerations and traditions (to preserve the purity of the royal blood) imposed increased rates of consanguineous marriages in many dynasties. For instance, several pharaohs of ancient Egypt who originated from marriages between extremely close blood relatives lived in good health and ruled their kingdom more than successfully.

It is our impression that Bulgarian traditions include much more negative attitude and much more restrictive marriage behavior towards consanguineous marriages as compared to the real genetic risk and to the restrictions imposed by the East-Orthodox Church and by the Bulgarian civil legislation. The aim of the present study is to compare these traditions to Orthodox-Church norms, to the written Bulgarian legislation and to historical examples.

## Material and Methods

The texts in the theological literature and in the Orthodox manuals concerning the prohibited weddings as well as the corresponding texts of Bulgarian civil legislation were systematized and analyzed from genetic point of view, mainly according to F a r r o w and J u b e r g [2] and S t e r n [3]. Analysis was made of the answers given by 3000 healthy secondary school students to the question "Do your parents have any blood relationship between them ?" with four possible options in a case of positive answer:

– first cousins;
– second cousins;
– third cousins;
– other relation (to be specified).

The author's observations on the common Bulgarian people's attitude towards consanguineous marriages are illustrated by examples from Bulgarian folklore and by the contingency of 102 readers' letters to the author with reference to his article in a daily newspaper.

The meaningless of the most restrictive marriage prohibitions is illustrated by the results of American authors and by an example from the history of ancient Egypt.

## Results and Discussion

The legislative restrictions concerning the marriages between blood relatives are presented in Family Codex of Republic of Bulgaria, article 13, paragraph 2. Marriages are prohibited between blood relatives of all degrees in direct line (i.e., someone's marriage with his/her parent, grand parent, son, daughter, grand child, etc.). As for the collateral line, marriages are prohibited between brothers and sisters (second degree), between their children (first cousins, IV degree) and other relatives up to IV degree inclusive. By the way, the last expression is completely meaningless, as far as, besides the first cousins, the only someone's "other relatives of IV degree" are the brothers and sisters of his grandparents and the grandchildren of his brothers and sisters. The age difference (two generations) evidently makes this prohibition fully superfluous.

Orthodox rules are presented in four items [1]. Points 3 concerns relationships acquired by marriage and point 4 – those acquired by baptism, so they have nothing to do with genetic relationships and with genetic risks. Point 1 is related to the direct line blood relatives and prohibits marriages between them irrespective of degree. Thus the restriction in question coincides entirely with the corresponding one of the Family Codex. Point 2 prohibits marriages between blood relatives in collateral line up to V degree inclusive. Thus, besides marriages between brothers and sisters and between first cousins, someone's marriage with the children of his or her first cousins (i.e., first and half cousins = first cousins once removed) are also prohibited. This is the only difference between the Church and the legislation restrictions.

Among 3000 subjects which we asked about an eventual blood relationship between their parents, we found one case of first cousins and four of second ones. The sample is not large enough as to determine with certainty the coefficient of inbreeding of Bulgarian people, but nevertheless the results show that probably it is among the lowest in Europe, if not in the world.

The common people attitude towards these marriages is determined by ancient prejudices and apprehensions. A lot of people is in panic fear of consanguineous marriages, how distant they may be, believing that they inevitably give rise to severe damages of the generation, to births of degenerates and monsters. Undoubtedly, during the centuries, the family environment pressure has bent unknown number of human destinies by impeding marriages between cousins which loved each other. Although not quite largely, this is reflected in the Bulgarian folklore. For instance, in a folk song from the Shumen region, a young man says to his girl-friend Denka:

"Denke le, we loved each other, Denke le, from the childhood,
Denke le, from the childhood, Denke le, up to grown.
Denke le, it was time, Denke le, to take each other.
Denke le, they made us out to be, Denke le, close relatives.
Denke le, our mothers, Denke le, two sisters in low,
Denke le, we both with you, Denke le, two first cousins !".

Two expressions make evident the strong internal protest of the speaker against the arising situation. First, instead of "it turned out that we are close relatives", the expression is used "they made us out to be...". Secondly, the statement that their mothers are sisters in low instead of the confession that their fathers are brothers evidences a hidden desire of the speaker their blood relationship to be presented as not so close as it is in fact.

Another interesting observation in this respect was made many years ago, when we published in the daily newspaper of Varna an article against the overestimation of the dangers arising from the consanguineous marriages by Bulgarian people. During the month following this publication we received 102 readers' letters with reference to it. Among them 89 (about 89%) were extremely close to each other in their meanings. The senders said that they had successful marriages and families with children and, in many cases, grandchildren. However, many years ago, they were infatuated with their cousins (most often second ones); and then their family environments (most often the parents) impeded their marriages. The senders were convinced that their life could be much happier if they had be left to follow the voices of their hearts.

Enormous differences exist between the states of the USA concerning consanguinity restrictions – from states where the marriages between brothers and sisters are the only prohibited to states where the prohibition covers even marriages between second cousins. Nevertheless, no differences were found between the rates of hereditary pathology in different states [2], which confirms once again the low importance of the legislative restrictions.

Finally, the genealogical tree (Fig. 1) of Hatshepsut illustrates the blood relationships in the 18$^{th}$ Dynasty of Egypt (1580-1350 B.C.). Hatshepsut, the only female pharaoh of Egypt, originated from a long consequence of close blood related marriages, including such ones between brothers and sisters. Her mother Ahmes was born in a family of brother and sister and married her half a brother Tuthmosis I. Hatshepsut herself was first cousin by mother and half a sister by father with her husband Tuthmosis II. Nevertheless, she lived in a good health and ruled Egypt during 21 years.

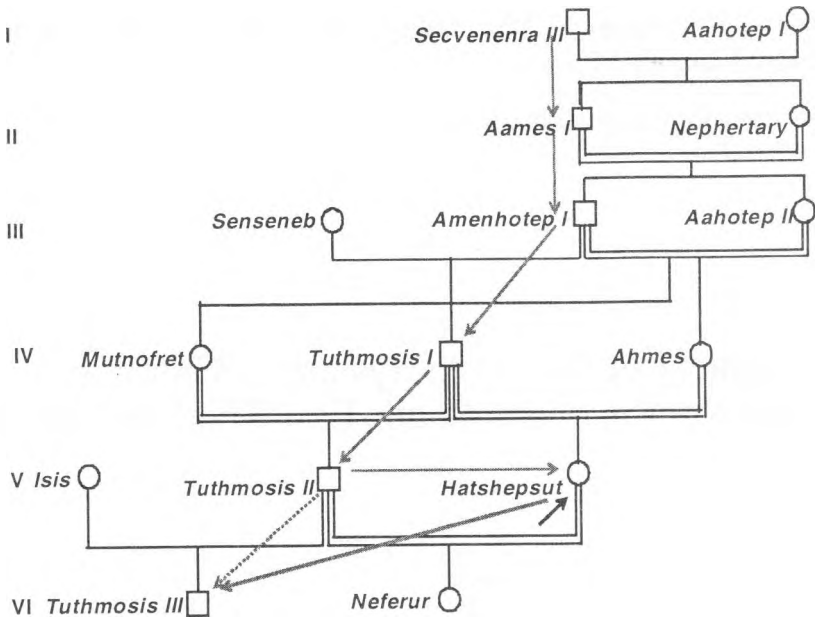


Fig. 1. Genealogical tree of Hatshepsut, Egyptian Pharaoh, from 1479 to 1458 B.C. The consanguineous marriages are presented by doubled connecting lines. The short arrow shows the proband and the long ones – the way of succession of the throne. The long dotted arrow shows a legitimate but not realized succession. After the death of her husband, Hatshepsut seized the throne and the legitimate successor Tuthmosis III took it after the mysterious disappearance (most probably murder) of his step-mother

## Conclusion

Respecting the established traditions of the Church and the legislation of the country, we will not recommend to violate them. However, the importance of the consanguineous marriages in hereditary pathology should not be overestimated. Also, marriage restrictions stronger than legislative ones should by no means be imposed to the young people.

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# Development of the Tooth System of Children from the Region of the South-East Rhodopi Mountains

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The purpose of the present study is to determine the times of cutting for the particular teeth categories in age-sexual aspect, to analyze the sequence of their cutting, as well as to determine the average age of appearing of the particular teeth categories in children and adolescents at the age from 7 to 17 years.

*Key words:* teeth, upper jaw, lower jaw.

## Introduction

Studying the development of a child's organism, it is of high importance to take in consideration the period of permanent teeth cutting, as this is the period in which an organism forms and develops. The age of cutting is a basic criterion for estimating the general physical development of a child, and together with the other factors, it is an indicator of the physiological maturity of an organism. In series of research, it has been reported the interdependency between processes of permanent teeth cutting and ossification [2], environmental effects on the age and sequence of cutting [1], the morphological features of teeth [3, 4, 5]. It is hard to find complete data about permanent teeth cutting in separate territorial groups of children. Having in mind this, we made it our aim, even though partly, to fill this incompleteness with our own research into children from the South-East Rhodopi Mountains.

## Material and Methods

The subjects of observation were 1480 children and adolescents (781 boys and 699 girls) from the municipalities Lyubimets, Svilengrad and Ivaylovgrad at the age from 7 to 17 years. The groups were formed in one-year turned ages.

We scoped and recorded the quantity of permanent teeth, as we assumed each tooth as a cut one if its crown had appeared in the gum, irrespectively of its level. The start, the end and the average age of cutting of the permanent teeth categories were deter-

mined by the methods of Danilkovich (1967). For determining the average number of permanent teeth we used a descriptive statistics, and for determining the average age and the times of cutting – non-linear probe regression analysis.

## Results

As it can be seen in Table 1, the teeth of the lower jaw develop earlier in comparison with the upper jaw and we could see it during the whole period of observation and in both genders. The process of cutting of the particular permanent teeth categories practically ends at the age of 15-16 years. The table also shows that cutting happens earlier with girls than with boys. These differences can be seen during the whole growth period, especially between the ages 10-12 when the quantity of the permanent teeth of 10-year-old girls is 17.47, and at the age of 12-23.56. In boys, a similar process can be seen a year later and the maximum addition in the total quantity of permanent teeth is between the ages of 11 and 12 – from 18.80 to 22.60.

The curves of the percentage growing of the particular permanent teeth categories show that there are periods of an active cutting and periods of delay, and it can be seen clearly with the increase and decrease of the yearly addition of the permanent teeth.

In the right half of the maxilla (Figs. 1, 2, 3, 4) in both genders, the canine tooth sprouts earliest – at the age of 7. In girls at the age between 9 and 10, this tooth can be seen in 50% of the cases and it sprouts completely at the age between 12-13 years. In boys, it happens a year later. The other categories of permanent teeth sprout during the 9th year. Although these teeth appear simultaneously, they develop at a different rate. The first premolar and the second molar have a more intensive rate of sprout in boys, and the sprout rate of the second premolar is comparatively the same in both genders. In the left half of the maxilla (Figs. 5, 6, 7, 8), there are inter-sexual differences in the beginning of cutting of the canine which cuts 2 years earlier in girls, and the first premolar which cuts a year earlier. Both teeth have a longer rate of development. The initial age of cutting of the second premolar and the second molar is the same in both genders. There are lateral differences regarding the initial age of cutting of the both premolars which cut earlier in the left half.

Table 1.Quantity of permanent teeth

| | | Permanent teeth | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Maxila | | Mandibula | | General | | | Maxila | | Mandibula | | General | |
| | | X | SD | X | SD | X | SD | | X | SD | X | SD | X | SD |
| Age | N | Boys | | | | | | N | Gyrls | | | | | |
| 7 | 52 | 3,59 | 1,84 | 4,84 | 1,70 | 8,43 | 1,77 | 51 | 3,86 | 1,92 | 5,06 | 1,34 | 8,92 | 1,63 |
| 8 | 59 | 4,37 | 1,42 | 5,59 | 0,76 | 9,96 | 1,09 | 57 | 5,38 | 1,50 | 5,75 | 0,89 | 11,13 | 1,20 |
| 9 | 93 | 5,81 | 1,35 | 6,55 | 1,19 | 12,36 | 1,27 | 76 | 6,42 | 1,13 | 7,01 | 1,49 | 13,43 | 1,31 |
| 10 | 60 | 8,51 | 2,78 | 8,53 | 2,07 | 17,04 | 2,43 | 84 | 8,36 | 1,84 | 9,11 | 1,83 | 17,47 | 1,84 |
| 11 | 68 | 9,16 | 2,44 | 9,64 | 1,08 | 18,80 | 1,76 | 74 | 9,83 | 2,41 | 10,93 | 1,64 | 20,76 | 2,03 |
| 12 | 58 | 10,74 | 2,68 | 11,86 | 2,04 | 22,60 | 2,36 | 60 | 10,98 | 2,33 | 12,58 | 1,82 | 23,56 | 2,08 |
| 13 | 63 | 12,39 | 2,47 | 12,12 | 2,66 | 24,51 | 2,57 | 57 | 13,26 | 1,97 | 13,92 | 1,89 | 26,18 | 1,93 |
| 14 | 81 | 13,23 | 1,87 | 13,85 | 2,07 | 26,08 | 1,97 | 84 | 13,44 | 1,65 | 13,94 | 1,92 | 26,60 | 1,79 |
| 15 | 61 | 13,67 | 0,17 | 13,84 | 1,09 | 27,51 | 0,63 | 82 | 13,96 | 1,51 | 13,99 | 2,03 | 27,95 | 1,77 |
| 16 | 51 | 13,97 | 0,14 | 14,00 | 0,00 | 27,97 | 0,07 | 88 | 14,00 | 0,00 | 14,00 | 0,00 | 28,00 | 0,00 |
| 17 | 52 | 14,00 | 0,00 | 14,00 | 0,00 | 28,00 | 0,00 | 68 | 14,00 | 0,00 | 14,00 | 0,00 | 28,00 | 0,00 |

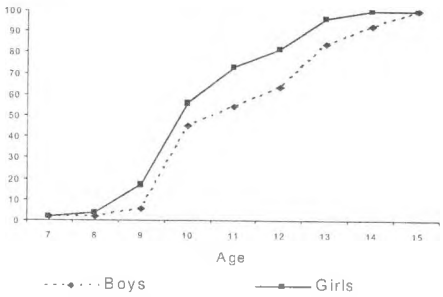


Fig. 1. Maxila dextra C

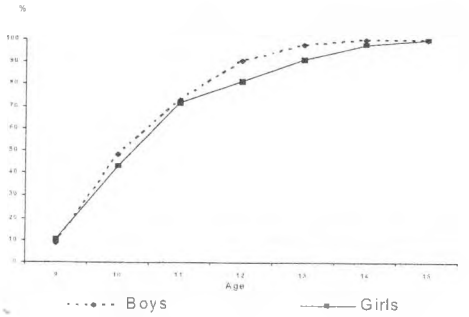


Fig. 2. Maxila dextra P1

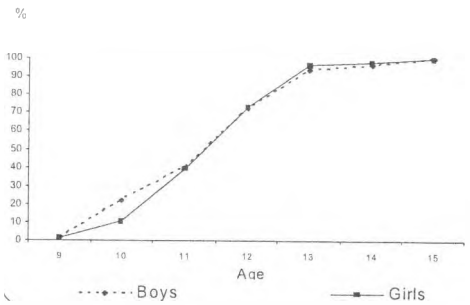


Fig. 3. Maxila dextra P2

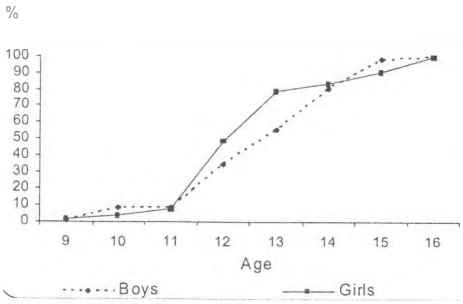


Fig. 4. Maxila dextra M2

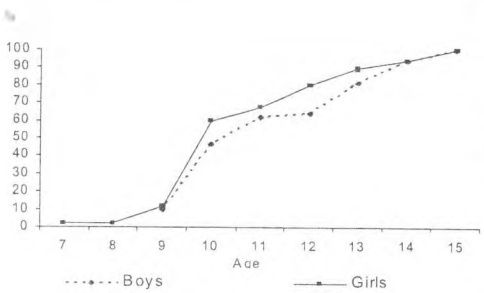


Fig. 5. Maxila sinistra C

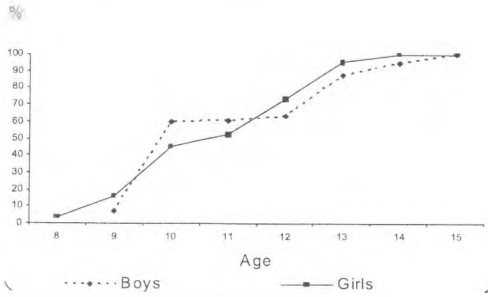


Fig. 6. Maxila sinistra P1

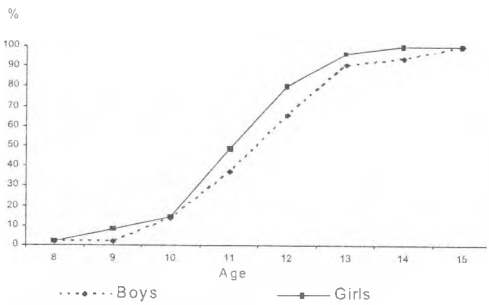


Fig. 7. Maxila sinistra P2

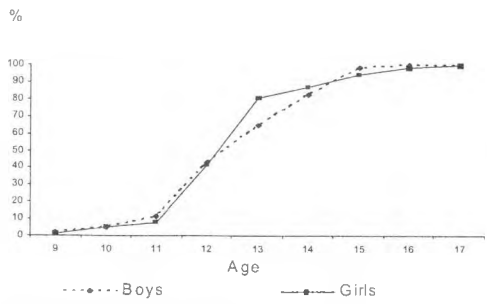


Fig. 8. Maxila sinistra M2


Fig. 9. Mandibula dextra C

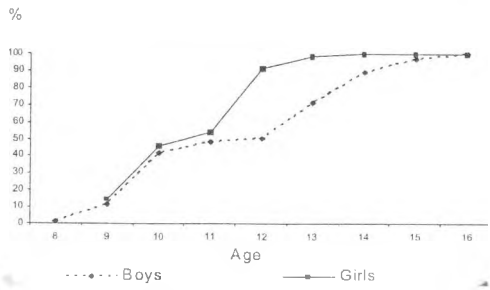


Fig. 10. Mandibula dextra P1

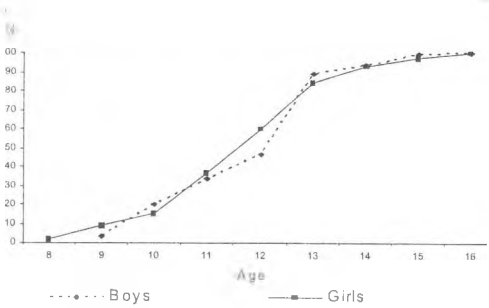


Fig. 11. Mandibula dextra P2

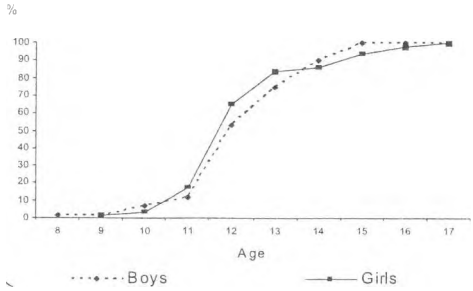


Fig. 12. Mandibula dextra M2

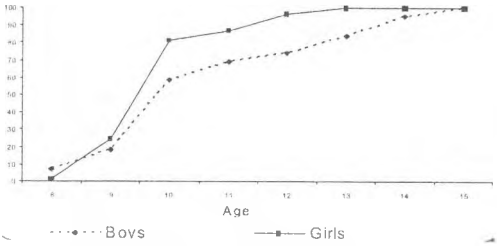


Fig. 13. Mandibula sinistra C

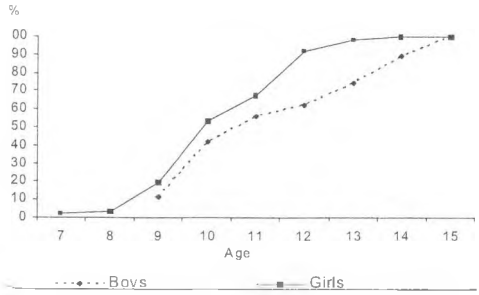


Fig. 14. Mandibula sinistra P1

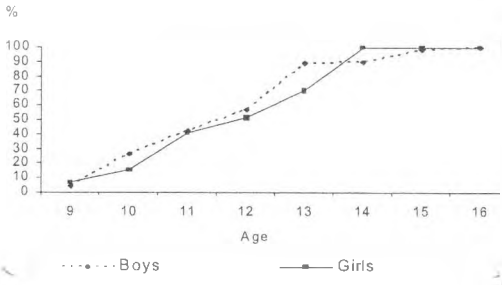


Fig. 15. Mandibula sinistra P2

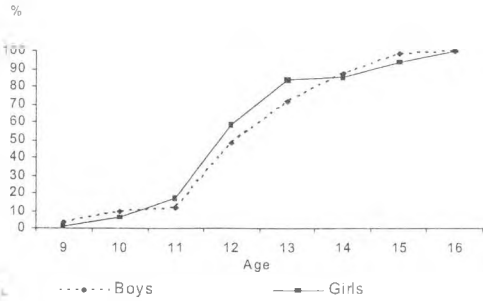


Fig. 16. Mandibula sinistra M2

| ♂ | M2,P1,C,P2 | C,P1,P2,M2 |
|---|---|---|
| | M2,P2,P1,C | C,P1,M2,P2 |

| ♀ | M2,P2,P1,C | C,P1,P2,M2 |
|---|---|---|
| | M2,P2,C,P1 | C,P2,P1,M2 |

Figs. 17, 18. The order of appearance of the teeth

In the right half of the mandible (Figs. 9, 10, 11, 12) in both genders, again the canine tooth cuts earliest – during the 7th year in 2% of the children we observed. In girls in the age period between 8 and 10 years, there is an intensive rate of cutting and it is obvious with the leap in frequency of occurrence – from 3 to 85%. In girls between the ages 12 and 13, this tooth has sprouted in 100% of the cases. In boys, the rates of cutting are longer in time and between the age of 14 and 15 it has sprouted in all boys we observed. The first premolar and the second molar cut earlier in boys, and the second premolar – in girls. There are sexual differences in the rates of cutting only with the first premolar and it has a longer rate in boys than in girls.

In the left half of the mandible (Figs. 13, 14, 15, 16) we did not find sexual differences in the start of cutting of the canine, the second premolar and the second molar. Only the first premolar cuts 2 years later in boys. There are lateral differences only regarding the start of cutting of the teeth observed and it occurs later in the left half, while their intensity of cutting is similar in both halves of the jaw.

The duration of sprout of the particular permanent teeth categories is different in both genders. In boys, the longest period of growing is this of canines and the first premolar; the second premolar has the shortest time of cutting. In girls, the longest period of cutting is this of both premolars; the second molar of the maxilla has the shortest time of sprout, while of the mandible – the canine.

The order of appearance of the teeth in both jaws (Fig. 17, 18) shows that in boys in the mandible and in the right half of the maxilla the canines sprout earliest, then it follows the first premolar, while in the left half the second premolar is the first followed by the canine. In the right half of the mandible, the second molar sprout earlier than the second premolar, while in the left half it is the contrary. In girls, there can not be seen an asymmetry in the sequence of sprout of the permanent teeth in the two halves of the maxilla. In the right half of the mandible the canine sprouts first, followed by the second premolar, while in the left half the first premolar sprouts first and then the canines.

Analyzing our results, we can assume that throughout the observed age period in both genders the average number of permanent teeth is bigger in the lower jaw compared with the upper jaw and it is bigger in girls in comparison with boys. The sequence of sprout of the particular permanent teeth categories shows lateral and inter-sexual differences. The duration of the cutting period of the teeth we observed is shorter in girls in comparison with boys because of the more intensive rate of cutting in them. The times and the average age of cutting of the teeth we observed fluctuate depending on the gender and it is different for the particular permanent teeth categories. In both genders, between the age of 15 and 16 all categories of permanent teeth cut.

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# Palm Flexion Creases in Bulgarians

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Normally on the palm there are three major flexion creases: Plica flexoria pollicis, Plica flexoria transversa proximalis, Plica flexoria transversa distalis.The type and frequency of the abnormal flexion creases in representative group of 1160 healthy males and 1268 healthy females from 116 settlements in Bulgaria are investigated. Highest is the frequency of males and females having normally situated flexion creases. The frequency of the established abnormal flexion cresses in healthy Bulgarians is low. It is 10.20% in males and 6.71% in females. The results obtained about abnormal flexion crease *type I* are in unison with the data elaborated by some authors concerning other European populations.

*Key words*: dermatoglyphics, palm flexion creases, healthy Bulgarian men and women.

## Introduction

Normally on the palm there are three major flexion creases existing already at birth: Plica flexoria pollicis (Pfp), Plica flexoria transversa proximalis (Pftp), Plica flexoria transversa distalis (Pftd). It is well known that the flexion creases developed at the same time with papillary ridges during the 3rd fetal month and could be distinguished in the 12-months fetuses. According to H. C u m m i n s, C. M i d l o [4] the flexion creases are not elements of the dermatoglyphics. H. K u m b n a n i [8], however, minds that "...the flexion creases are the integral part of palm and are even most important landmarks of the dermatoglyphics." We agree with the attitude of K u m b n a n i [8] since in different genetic and hereditary diseases are found abnormal flexion creases – fourth transverse palmar crease, the so-called "simian crease"; Sydney crease; their different variations and transitional forms. Concerning abnormal flexion creases there are various definitions and classifications [1, 3, 4, 7, 11, 16 and others].

The aim of the present study is to define the type and frequency of the abnormal flexion creases in healthy Bulgarians.

## Material and Methods

Object of the investigation are the dermatoglyphic prints taken from both hands of 2430 Bulgarians from both genders (1160 males and 1268 females) at the age 30-40 years living in 116 settlements of the country.

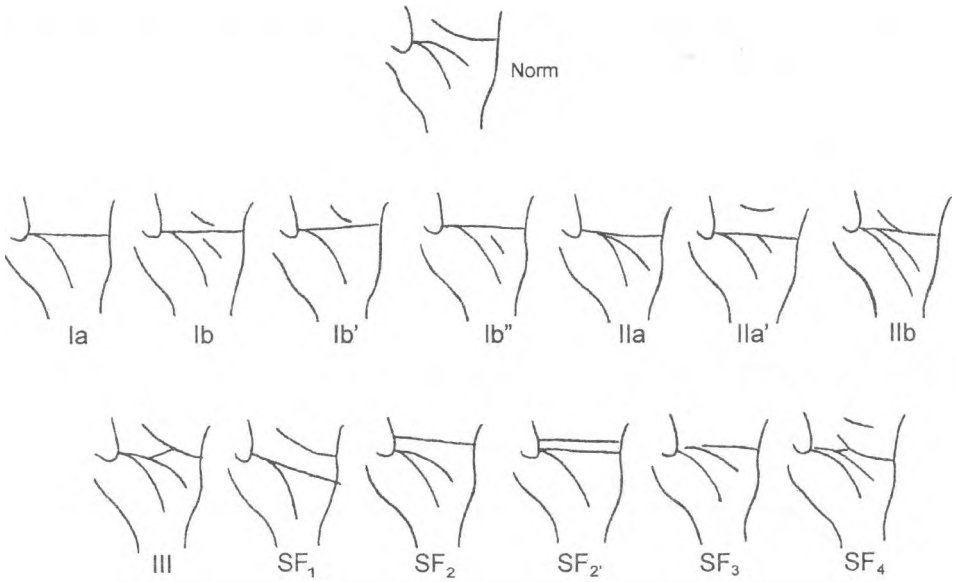


Fig. 1 Scheme of the different types of palm flexion creases (after W e n i n g e r, N a v r a t i l [16] added by us)

The determination of abnormal creases is made using the scheme of M. W e n n i n g e r, L. N a v r a t i l, [16]. The authors distributed the fourth finger crease into two forms: classical crease (Ia) and such one having also fragments from the proximal and distal creases. They defined also the transitional and peculiar forms. For our investigation we added into their scheme some new forms (Fig. 1).

## Results

The data presented in Table 1 show that highest is the frequency of males and females having normally situated flexion creases (type N) on both hands. The common frequency concerning all abnormal forms of flexion creases in males is 10.20% and in females – 6.71%.

From the abnormal forms in males and females most frequent are the flexion creases of the summed *type II* (IIa + IIa′ + IIb). Next by frequency for males is the *type SF* ($SF_1 + SF_2 + SF_{2'} + SF_3 + SF_4$), while for females it is the *type I* (Ia + Ib + Ib′ + Ib″) (Fig. 2).

Combinations of the flexion creases type on right and left hand are computed and compared, as well. Most are the individuals by which is observed a normal situation of the flexion creases on both hands (males 85.18%; females 89.44%). The other combinations distributed between right and left hand are pretty rarer. One and the same abnormal creases' type on both hands are found in 3.19% of the males and 1.02% of the females, while different types abnormal creases on both hands are found in 2.41% of the men and 1.81% of the women (Fig. 3).

Some forms of abnormal flexion creases found in the investigated by us contingent Bulgarians are established in Figure 4.

The results obtained in the present investigation and being representative for the Bulgarian population about abnormal flexion crease *type I* are in unison with the data

Table 1. Frequency of the types of palm flexion creases (%)

| Type | Males $n = 1160$ | | | Females $n = 1268$ | | |
|---|---|---|---|---|---|---|
| | Hand | | | Hand | | |
| | right | left | both hands | right | left | both hands |
| Norm | 89.24 | 90.34 | 89.79 | 93.61 | 92.98 | 93.29 |
| Ia | 0.17 | 0.69 | 0.43 | 0.55 | 0.32 | 0.44 |
| Ib | 1.12 | 0.95 | 1.04 | 0.95 | 0.95 | 0.95 |
| Ib' | 0.78 | 0.25 | 0.52 | 0.08 | 0.32 | 0.20 |
| Ib'' | 0.25 | 0.35 | 0.30 | 0.24 | 0.39 | 0.32 |
| IIa | 0.43 | 0.43 | 0.43 | 0.16 | 0.39 | 0.28 |
| IIa' | | 0.17 | 0.09 | | | |
| IIb | 2.41 | 2.24 | 2.32 | 1.97 | 1.88 | 1.92 |
| III | 1.72 | 1.47 | 1.59 | 0.47 | 0.79 | 0.63 |
| $SF_1$ | 0.35 | 0.25 | 0.30 | 0.47 | 0.47 | 0.47 |
| $SF_2$ | 0.43 | 0.35 | 0.39 | 0.32 | 0.32 | 0.32 |
| $SF_{2'}$ | | 0.09 | 0.04 | | | |
| $SF_3$ | 1.55 | 1.21 | 1.38 | 0.47 | 0.24 | 0.35 |
| $SF_4$ | 0.52 | 0.52 | 0.52 | 0.32 | 0.63 | 0.48 |
| Other | 1.03 | 0.69 | 0.86 | 0.39 | 0.32 | 0.35 |
| Abnormal flexion creases - total | 10.76 | 9.66 | 10.21 | 6.39 | 7.02 | 6.71 |

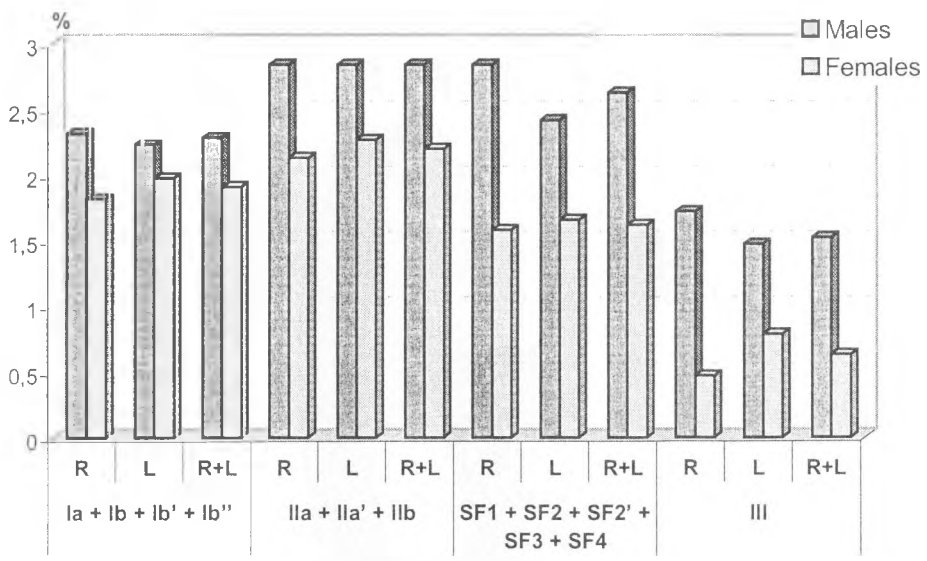


Fig. 2. Frequency of different types abnormal flexion crease (%)

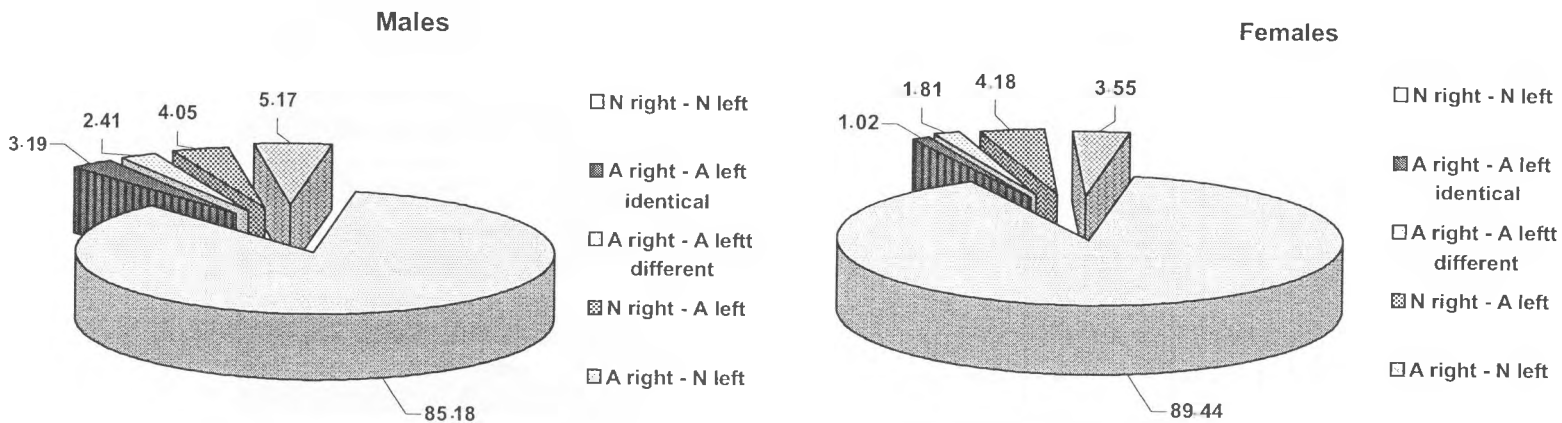


Fig. 3. Frequency of the type's combinations of flexion creases on right and left hand (%)

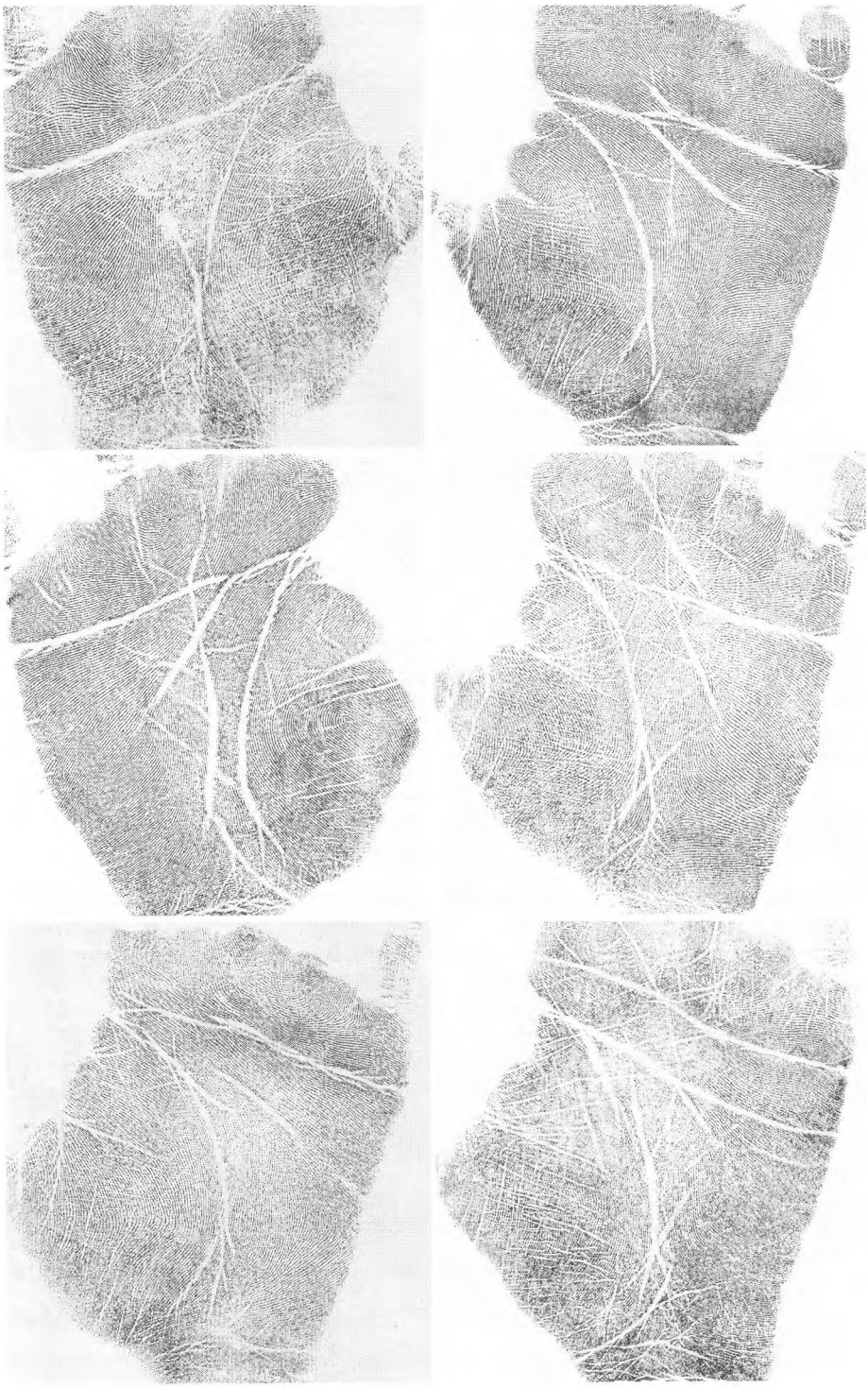

Fig. 4. Examples of different abnormal flexion creases from the investigated contingent

Table 2. Frequency of the type I (Ia + Ib) of palm flexion creases in other European populations

| Population / Study | Gender | Number | Percent |
|---|---|---|---|
| Romanian | Males | 521 | 5.37 |
| M. Weninger, 1953 | Females | 557 | 2.69 |
| Austrian | Males | 316 | 4.11 |
| M. Weninger, L. Navratil, 1957 | Females | 270 | 1.85 |
| Romanian | Males | 2535 | 3.59 |
| M. Dumitrescu, 1964 | Females | 2659 | 1.99 |
| French | Males | 696 | 4.02 |
| M. Lestrange, 1966 | Females | 952 | 1.47 |
| Czech | Males | 300 | 1.49 |
| V. Hajn, 1984 | Females | 300 | 1.16 |
| Czech | Males | 400 | 0.75 |
| V. Hajn, A. Gasiorowski, 1995 | Females | 400 | 0.75 |
| Pole | Males | 300 | 1.16 |
| V. Hajn, A. Gasiorowski, 1995 | Females | 300 | 0.83 |
| Bulgarian | Males | 413 | 1.58 |
| S. Tomjova, 2000 | Females | 403 | 1.09 |
| Bulgarian | Males | 1160 | 2.29 |
| the present study | Females | 1268 | 1.91 |

elaborated by some authors concerning other European populations [5, 6, 9, 14, 15, 16]. From Table 2 could be seen that in males the frequency falls within the limits from 0.75% till 5.37%, and in females – from 0.75% till 2.69%. In our investigation the tendency remain the same, i.e. the frequency of this type abnormal crease in males to be a little bit higher.

## Conclusion

The frequency of the established abnormal flexion cresses in healthy Bulgarians is very low. Our investigations, as well as such ones made by foreign authors concerning the palmar flexion creases in patients having different inborn and hereditary diseases show a high frequency of the abnormal types being relative to the patients [2, 10, 12, 13,14]. That's why we would like to recommend a detailed clinical, biochemical and genetic study concerning patients to be carried out when diversions from the normal palm flexion creases were established. In this way an early diagnostics, as well as the question weather a given disease has acquired or inherited etiopathology could be realized.

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# Anthropometrical Investigation of Bone Remains from Medieval Church and Necropolis in the Countryside Selishte, Village of Novosel, Shumen Region (10th-15th Centuries AD) (preliminary report)

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The aim of the present work is to characterize anthropometrically the bone material from the medieval church and necropolis in countryside Selishte, Novosel village, Shumen region (10th-15th Centuries AD) and to make a comparison with the data for other medieval necropolises in the same region. The research includes 50 individuals. The methods of Martin-Saller, Y. Yordanov, Alekseev-Debetz are applied. The data are statistically processed. After the formula of Trotter-Gleser the mean stature for both genders comes under the category "tall". From the metrical characterization made of skulls belonging to the buried near to village Novosel, is ascertained that facial measurements in both genders, as well as cerebral features in males come mainly under the category "middle", and concerning females - cerebral sizes predominantly belong to the category "large". The comparison of the four medieval necropolises shows that basic differences assign to the buried from necropolis near to village of Sechishte.

*Key words:* medieval necropolis, anthropometrical characterization, sexual differences, index characterization.

## Introduction

The monument locates in the countryside Selishte, which lays North East to the village of Novosel, Shumen region. The excavations are made during the period July-August 2006. The necropolis is dated from 10th Century till 15th Century AD according to the archeological data [6]. Totally 68 graves of the necropolis are unearthed, 41 of them are fully investigated and the rest – partially destroyed: 11 graves are localized but they are still not revealed. The burials were made according to the Christian tradition (Figs. 1, 2). Frequently are the cases found in dislocated anatomical order and missing parts of the skeletons by reason of pits overlap (Figs. 3, 4). Concerning three of the cases is found 2 skeletons in each of the graves – mother with child (Fig. 5) and two children (Fig. 6).

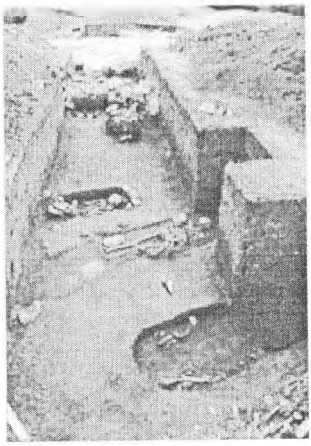

Fig. 1. Scheme of Christian burials

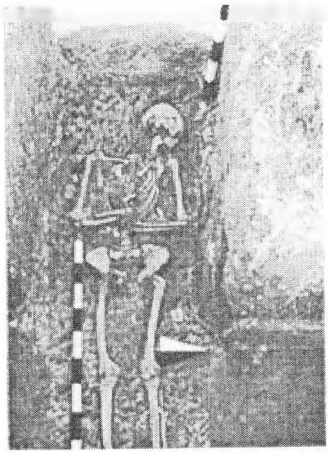

Fig. 2. Christian grave No 13 belonging to a male individual (Adultus)

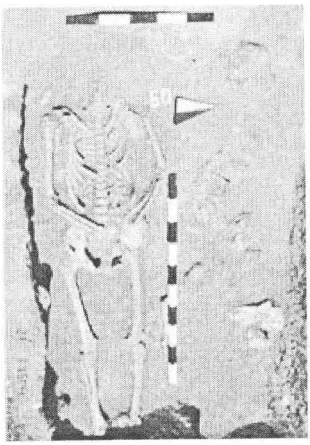

Fig. 3. Missing skull of the skeleton belonging to an adult male from Grave No 60

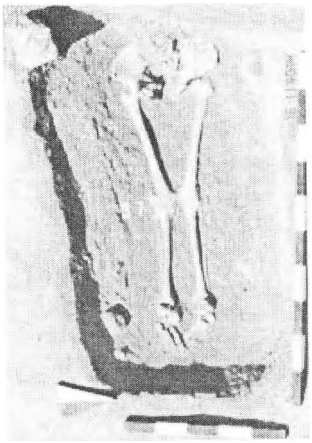

Fig. 4. Missing parts of the skeleton belonging to an adult female from Grave No 37

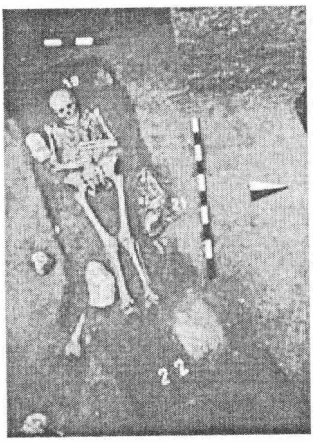

Fig. 5. Burial pit with two individuals – female (Maturus) in anatomical order (Grave № 19) and a child (Infans I) in dislocated anatomical order (Grave No 21)

Fig. 6. Burial pit (Grave No 48) – two children (Infans I)

Table 1. Demographic structure of the buried individuals in the medieval church and necropolis in countryside Selishte, near to Novosel village, Shumen region (10th-15th Centuries AD)

| | Age group | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Infans I | Infans II | Juvenilis | Adultus | | | Maturus | | | Senilis | | |
| | | | | ♂ | ♀ | ♂ ♀ | ♂ | ♀ | ♂ ♀ | ♂ | ♀ | ♂ ♀ |
| Total number | 11 | 2 | 1 | 7 | 15 | 3 | 3 | 8 | – | – | – | – |

The **aim** of the present work is to characterize anthropometrically the bone material from the medieval church and necropolis in countryside Selishte, near to the village of Novosel, Shumen region (10th-15th Century AD) and to make a comparison with the data for other medieval necropolises in the same region.

## Material and Methods

The research includes 36 adults (10 individuals from male gender, 23 individuals from female gender, 3 individuals with undetermined gender), 1 juvenile, 2 children from second infant age and 11 children from first infant age, individuals from the senile age are absent (Table 1).

The analysis in the present work includes only the adults.

The methods of Martin-Saller [1], Y. Yordanov [7, 8] and Alekseev-Debetz [5] are applied.

The small number of skulls in the group of males and females does not enable to apply the classical variation statistics – submitted are the prime mean value and variation breadth.

The stature is assessed on the basis of long bones' extremity length by the formulae of Pearson [2] and Trotter-Gleser [3].

The sexual differences are assessed by Mann-Whitney U test and by the Index of Sexual Differences (ISD), calculated on the formula of Wolański [4] for relative intergroup differences:

$$ISD = 2 \times [(\bar{x}_{males} - \bar{x}_{females}) \times 100] / (\bar{x}_{males} + \bar{x}_{females}).$$

The t-criterion of Student at $P<0.05$ is used to determine the authenticity of the established sexual differences.

It is made also comparison with data from other medieval necropolises in North East Bulgaria, which data are published by Y. Yordanov et al.: Village of Trustenik, Rousse region [10]; Village of Batin, Rousse region [9]; Village of Sechishte, Novi Pazar municipality [11].

## Results and Discussion

The **stature** ranges from 164.9 to 178.3 cm (after the formula of Trotter-Gleser) and from 158.0 to 170.0 cm (after the formula of Pearson) concerning male individuals, and for the female ones – respectively from 151.4 to 170.0 cm and from 144.4 to 159.8 cm. In males the mean stature is 173.0 cm after the first formula and 165.4 cm after the second one, in females – accordingly 161.8 and 153.9 cm. The difference between both genders is 11.2 cm after the first formula, and after the second – 11.5 cm (the differences are statistical significant). After the formula of Trotter-Gleser the mean values come under the category "tall" (in accordance with the categories of Martin [1] for

European population), and after the formula of Pearson – respectively under the category "middle".

The comparison with the data concerning another three medieval necropolises shows that the buried individuals near to the village of Batin from both genders have highest stature. The mean stature of males in the present investigation is very close to this one concerning male individuals from the necropolis near to the village Sechishte, and in females – the stature approaches this one concerning the investigated individuals from the village of Trustenik.

*Anthropometrical skulls' characterization of the buried individuals near to the village of Novosel*

According to the mean values of **absolute cerebral skull's features** the male individuals have "middle" cranial length and breadth, and "large" minimal frontal breadth. Concerning the female skulls, all three measurements belong to the category "large".

About the **facial part of cranium for male individuals** the mean values come under the following categories – "small" upper face height, ocular breadth and height; "middle" nasal breadth and height; "middle" maxilloalveolare length and breadth; "large" palate breadth. The characterization of mandible bone is: "middle" bigonial breadth; "very large" length; "middle" height and "small" breadth of the branch.

Concerning the **facial part of female skulls** the characterization is as follows: "middle" upper face height, "small" ocular breadth but "middle" height; "middle" nasal breadth and height; "small" maxilloalveolare length but "large" breadth; "large" palate breadth; "large" bigonial breadth, and "very large" mandible length; "middle" height and "small" breadth of ramus mandibulae.

We have found statistical significant differences between both genders only for the mandible branch height, as well as for the ocular height.

*Angular features of facial skull's part*

Concerning individuals from the female gender, four basic facial skull angles are measured, while in males it was possible to measure only the mandibular angle. For both genders the mean values of measured angles belong to the category "middle".

*Sexual differences*

To determine not only the extent but also the direction of sexual differences we have calculated the Index of Sexual Differences (ISD). These differences are largest for the mandible branch height followed by the maxilloalveolare length and stature. The margin is narrowest for the cranial length, and concerning cranial breadth any difference is missing. With priority for female individuals are only four features, from which ones the ocular height and mandibular angle have biggest considerable differences.

*Comparison between indices of skulls from the four medieval necropolises*

Comparing the indices of *brain skull's part concerning both genders* is established that the basic differences could be related to the skulls of buried individuals in the necropolis near to the village of Sechishte – being "brachycran" and "hypsicran", while the skulls from the rest three necropolises are "mesocran" and "orthocran".

The comparison between mean values of the indices for *facial skull's part* shows that **males** from all necropolises come under the same categories. Exceptions are observed only for the orbital index in the skulls from village of Sechishte, as well as for the maxilloalveolar index concerning the necropolis near to Trustenik.

As about jaw index the **female skulls** pertaining to the four necropolises fall into the same category like male ones – namely "orthognat". According to the values for maxilloalveolar index all female skulls come also under one and the same category – "brachyuran". Differences between the four necropolises are observed for the nasal index and like in males – for the orbital index.

## Conclusion

*Regarding buried individuals near to the village of Novosel:*

– after the formula of Trotter-Gleser the mean stature for both genders comes under the category "tall", and after the formula of Pearson – respectively under the category "middle";

– according to the metrical characterization of skulls, the facial measurements in both genders, as well as the cerebral features in males come mainly under the category "middle"; and concerning females the cerebral sizes predominantly belong to the category "large";

– the most significant sexual differences for the skull's features are established in the mandible branch height which is bigger in the male individuals.

*Comparison of data about the four medieval necropolises from North Eastern Bulgaria:*

– for both genders the mean values of stature fall into the category "high";

– the index characterization of cerebral and facial skull's part by categories shows that the basic differences could be related to the buried individuals in the Moslem necropolis near the village of Sechishte.

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# Bilateral Asymmetry of *Os Zygomaticum*

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The aim of the study is to perform a comparative quantitative assessment of the manifestations of bilateral asymmetry of *os zygomaticum* on bone material. A total of 125 male crania from the ossuary at the National Museum of Military History, Sofia, were studied. The crania belong to adult individuals aged between 20 and 43 years. Two linear features were measured bilaterally – breadth of *os zygomaticum* and height of *os zygomaticum*. Scopicly for both sides were investigated presence and number of *foramen zygomaticofaciale* and form *of processus frontalis ossis zygomatici*. The asymmetry assessment in the investigated male crania shows that the left zygomatic bones are higher and wider compared to the right ones. The asymmetry manifestations for the investigated scopic features are described as well.

*Key words:* asymmetry, *os zygomaticum*, *foramen zygomaticofaciale*, form of *processus frontalis ossis zygomatici*.

## Introduction

In the human skull as in the other parts of the skeleton may be found bilateral asymmetry manifestations as well. Due to the key role of *os zygomaticum* in the structure and the aesthetical appearance of the face, the evaluation of its bilateral asymmetry is of significant importance [1, 4, 5].

The aim of the study is to perform a comparative quantitative assessment of the manifestations of bilateral asymmetry of *os zygomaticum* on bone material.

## Material and Methods

A total of 125 male skulls from the ossuary at the National Museum of Military History, Sofia, were studied. The skulls belong to adult individuals aged between 20 and 43 years who died in the wars during the period 1913-1917.

Two linear features were measured for both sides – breadth of *os zygomaticum*, using the method of A l e k s e e v - D e b e t s [3] and height of *os zygomaticum*, feature introduced by us. Height of *os zygomaticum* represented the distance between the standard anthropometric landmarks – *zygomaxillare* and *frontomalare-orbitale*.

Scopicly were investigated presence and the number of *foramen zygomaticofacialis* and the form of *processus frontalis ossis zygomatici* on both sides of the skull. The latter was recorded as straight, protruding and presence of *tuberculum marginale* [6]. In order to record the manifestations of asymmetry in scopic features, 16 combinations of different numbers of *foramen zygomaticofacialis* and 9 combinations of the three forms of *processus frontalis ossis zygomatici* either at the right or left side were used.

The data obtained were statistically processed through variation and alternative analysis (SPSS, version 13.0). The reliability of bilateral differences recorded for metric features was verified through Student's t-test at $p<0.05$. Quantitative assessment of the bilateral asymmetry was performed using Wolanski's index for intergroup comparison. In this case, the index was used to determine the manifestations of bilateral asymmetry and is referred to as Index of Asymmetry (IA):

$$IA = \frac{2.(x_1 - x_2).100}{x_1 + x_2},$$

$x_1$ – value of the feature to the right; $x_2$ – value of the feature to the left.

The sign of the resulting IA value designates the direction of bilateral asymmetry; "–" shows asymmetry at the left (metric priority in favour of the left side), and "+" shows asymmetry at the right (metric priority in favour of the right side).

The degree of manifested bilateral asymmetry was assessed as slight, moderate and strong, using percentile analysis of the data for IA. The border values were set at $P_{25}$ and $P_{75}$.

## Results

### *Metric characterization*

The data obtained from the biostatistical analysis of the linear features, borderline values for assessment of the asymmetry degree and percentage distribution are given in Table 1, Table 2 and Table 3.

Breadth of *os zygomaticum*. Data about the mean value for this feature at the right (53.13mm) and at the left (53.22mm) shows that there is presence of left-side asymmetry as a whole. Breadth of *os zygomaticum* at the left is 0.09mm greater than the value at the right. In the investigated cranial series, 26.84% of the skulls did not exhibit bilateral asymmetry for this feature. Total frequency of crania with right-side asymmetry (34.14%) is accounted with 4.88% more rarely compared to this whit left-side asymmetry (39.02%). Assessment of the asymmetry degree shows that slight asymmetry is

Table 1. Biostatistical data of the investigated linear features of *os zygomaticum*

| Measurements of *os zygomaticum* | Right | | | | | | Left | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *n* | mean | min | max | SD | Sx | *n* | mean | min | max | SD | Sx |
| Breadth of *os zygomaticum* | 123 | 53.13 | 40.00 | 63.00 | 3.89 | 0.35 | 125 | 53.22 | 43.00 | 63.00 | 3.67 | 0.33 |
| Height of *os zygomaticum* (zm-fmo) | 125 | 42.79 | 36.00 | 51.00 | 2.80 | 0.25 | 125 | 43.03 | 36.00 | 51.00 | 2.81 | 0.25 |

Table 2. Borderline values for assessment of the

| Measurements of *os zygomaticum* | Slight (IU) | Medium (IU) | Strong (IU) |
|---|---|---|---|
| Breadth of *os zygomaticum* | $x - 0.66$ | 0.67 – 3.32 | $3.33 - x$ |
| Total height of *os zygomaticum* (zm-fmo) | $x - 1.08$ | 1.09 – 3.31 | $3.32 - x$ |

Table 3. Frequency distribution of symmetry and asymmetry manifestations

| Measuremcuts of *os zygomaticum* | Left-side asymmetry (%) | | | Symmetry (%) | Right-side asymmetry (%) | | |
|---|---|---|---|---|---|---|---|
| | strong | moderate | slight | | slight | moderate | strong |
| Breadth of *os zygomaticum* | 13.82 | 25.20 | 0.00 | 26.83 | 0.00 | 22.76 | 11.38 |
| Total height of *os zygomaticum* | 16.00 | 26.40 | 1.60 | 23.20 | 0.80 | 24.00 | 8.00 |

not recorded for both sides. Moderate left-side asymmetry (25.20%) is more frequent than moderate right-side asymmetry (22.76%). Strong bilateral asymmetry in favour of the right side is recorded for 11.38% of the skulls, and in favour of the left side – for 13.82%.

Height of *os zygomaticum.* In accordance with the mean value of this feature there is presence of left-side asymmetry, respectively 42.79 mm on the right side and 43.03 mm on the left side. Height of *os zygomaticum* at the right is 0.24 mm less compared to that on the left side. Symmetry for this feature is observed in 23.2% of the investigated skulls i.e. the values are equal for both sides. Total frequency of crania with right-side asymmetry of *os zygomaticum* is 32.80% and is with 11.20% more rarely accounted compared to this whit left-side asymmetry (44.00%). Assessment of the asymmetry degree shows that slight asymmetry in favour of the left side (1.60%) is two times more common than of the right side (0.80%). Moderate asymmetry is established more frequently for the left side (26.40%) compared to the right side (24.00%). Strong asymmetry was again two times more frequent in favour of the left side (16.00%) compared to the right side (8.00%).

*Scopic characterization*

*Foramen zygomaticofaciale* is located on the front surface of *os zygomaticum*, through it passes a branch of *nervus zygomaticus – ramus zigomaticofacialis* and blood vessels. As normal anatomical variations, *foramen zygomaticofaciale* may be either absent of present in the form of several separate foramina.

In the investigated cranial series, the anatomical variations established for this scopic feature ranged from total absence of *foramen zygomaticofaciale* to the presence of three separate foramina. In general, the highest percentage of skulls has a single *foramen zygomaticofaciale* on both sides (47.58% to the right and 48.00% to the left). Presence of two facial zygomatic bone foramina at the right (26.61%) is found to be 2.42% more frequent than total absence of a foramen (24.20%). On the left side in 28.00% of the skulls *foramen zygomaticofaciale* is absent and is represented by two separate foramina in the 17.60%. Presence of three foramina on the right side is recorded for 1.61% of the skulls, while on the left side the percentage is comparatively higher – 6.40%.

In accordance with the 16 possible combinations of different numbers of facial zygomatic bone foramina for both sides the assessment of frequently distribution shows that the highest percentage of skulls are with bilateral presence of single facial zygomatic bone foramen (30.65%) (Table 4). Bilateral absence of *foramen zygomaticofaciale* is registered for 14.52% of the examined skulls, followed by the combination of two foramina at the right and one at the left – 12.10%. For 11.29% of the skulls, presence of a single foramen at the right and absence of a foramen at the left is established, while 9.68% of the skulls exhibits bilateral presence of two foramina. Absence of *foramen zygomaticofaciale* at the right and presence of a single one at the left is recorded for 5.65% of the skulls, while its absence on the right side and two foramina on the left

Table 4. Different combinations of number of *foramen zygomaticofaciale* on the right and left sides.

| Combination № | Number of *foramen zygomaticofaciale* | | % |
|---|---|---|---|
| | Right | Left | |
| 1 | 0 | 0 | 14.52 |
| 2 | 1 | 1 | 30.65 |
| 3 | 2 | 2 | 9.68 |
| 4 | 3 | 3 | 0.81 |
| 5 | 0 | 1 | 5.65 |
| 6 | 0 | 2 | 3.23 |
| 7 | 0 | 3 | 0.00 |
| 8 | 1 | 0 | 11.29 |
| 9 | 2 | 0 | 1.61 |
| 10 | 3 | 0 | 0.00 |
| 11 | 1 | 2 | 4.03 |
| 12 | 1 | 3 | 1.61 |
| 13 | 2 | 1 | 12.10 |
| 14 | 3 | 1 | 0.00 |
| 15 | 2 | 3 | 4.03 |
| 16 | 3 | 2 | 0.81 |

side are established for 3.23% of the skulls. The combinations of one *foramen zygomaticofaciale* at the right and two at the left and the combination of two at the right and three at the left are established in equal percentages – 4.03%. Presence of two facial zygomatic bone foramens at the right and absence of such at the left is as common as presence of one foramen at the right and three at the left – 1.61%. The combination of bilateral presence of three facial zygomatic bone foramina and the combination of three foramina on the right side and two on the left side are most rarely established – 0.81%. In the investigated cranial series, no cases of the following three possible combinations are established: absence of *foramen zygomaticofacialis* at the right and presence of three foramina at the left; presence of three foramina at the right and absence at the left; presence of three foramina at the right and one at the left.

Descendent formula for the combinations of different number of *foramen zygomaticofaciale* on both sides.

2 (30.65%) >1 (14.52%) >13 (12.10%) > 8 (11.29%) > 3 (9.68%) > 5 (5.65%) > 11 (4.03%)=15 (4.03%) > 6 (3.23%) > 9 (1.61%)=12 (1.61%) > 4 (0.81%)=16 (0.81%) > 7 (0.00%)=10 (0.00%)=14 (0.00%)

Lateral margine of *processus frontalis ossis zygomatici* is the insertion for *lamina superficialis fasciae temporalis*, one of the two layers of the fascia covering *musculus temporalis*. Depending on the extent of protrusion of the insertion of the fascia, the form of *processus frontalis ossis zygomatici* was scopicly classified as straight (smooth), protruding or presence of *tuberculum marginale*.

In the investigated cranial series, the protruding form of *processus frontalis ossis zygomatici* is established to be most common bilaterally (56.45% on the right side and 57.60% on the left side), followed by the form represented as a *tuberculum marginale* (42.74% on the right side and 41.60% on the left side). The straight form of *processus frontalis ossis zygomatici* is least common (0.81% to the right and 0.80% to the left).

The assessment of frequently distribution of the 9 possible combinations resulting from the three forms of *processus frontalis ossis zygomatici* in both sides shows that bilaterally the protruding form is most common (50.81%) (Table 5). Bilaterally developed *tuberculum marginale* is established for 35.48% of the crania, followed by the combination of presence of a *tuberculum narginale* on the right side and protruding form of the lateral margin of the frontal processus of the zygomatic bone on the left side

Table 5. Different combinations of form of *processus frontalis ossis zygomatici* on the right and left sides.

| Combination № | Form of *processus frontalis ossis zygomatici* | | % |
|---|---|---|---|
| | Right | Left | |
| 1 | straight | straight | 0.81 |
| 2 | protruding | protruding | 50.81 |
| 3 | *tuberculum marginale* | *tuberculum marginale* | 35.48 |
| 4 | straight | protruding | 0.00 |
| 5 | straight | *tuberculum marginale* | 0.00 |
| 6 | protruding | straight | 0.00 |
| 7 | *tuberculum marginale* | straight | 0.00 |
| 8 | protruding | *tuberculum marginale* | 5.65 |
| 9 | *tuberculum marginale* | protruding | 7.26 |

(7.26%). Protruding form at the right and presence of a *tuberculum marginale* at the left are recorded for 5.65% of the crania, while bilaterally straight form of *processus frontalis ossis zygomatici* is observed for 0.81%. No cases of the other four possible combinations of different forms of *processus frontalis ossis zygomatici* either at the right or left side are established in the investigated cranial series.

Descendent formula for form of *processus frontalis ossis zygomatici* on both sides.

2 (50.81%) > 3 (35.48%) > 9 (7.26%) > 8 (5.65%) > 1 (0.81%) > 4 (0.00%)=5 (0.00%)=6 (0.00%)=7 (0.00%).

## Conclusions

In accordance with the metric features asymmetry assessment shows that in the investigated male crania the left zygomatic bones are higher and wider compared to right ones.

In both metrical features the moderate asymmetry is the most common.

The scopic characterization shows that more frequently are found the following variations of different number of *foramen zigomaticifaciale*: bilateral presence of single foramen, bilateral absence of foramen, presence of two foramina at the right and one at the left side, presence of a single foramen at the right and absence of a foramen at the left. In the investigated cranial series, the protruding form of *processus frontalis ossis zygomatici* and presence of *tiuberculum marginale* are established to be most common bilaterally.

The obtained data enrich the metrical and scopic characterization of cranium facial part. These data can be used as important information for distinction of different pathological changes of *os zygomaticum* from normal anatomical variations in this bone.

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# Bilateral Asymmetry of Human Clavicle (Osteological Investigation)

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The aim of the study is to make a comparative assessment of the manifestations of asymmetry in lengths, diameters, circumference and heights of clavicle and to seek for sexual differences in direction and degree of established asymmetry. A total of 136 clavicles (36 right and 36 left clavicles of male skeletons; 32 right and 32 left clavicles of female skeletons) were investigated. All investigated clavicles belong to adult individuals. Ten metric features were measured. It is established, that the left clavicles in both sexes are longer than the right ones, but the right clavicles are more massive and more curved, compared to the left ones.

*Key words*: clavicle, anthropometric features, asymmetry.

## Introduction

The metrical characterization of pair bones in human skeleton contributes the manifestations of bilateral asymmetry in human body to be established. Scarce data in the specialized literature on asymmetry in the clavicle determine the aim of this study – to make a comparative assessment of the manifestations of asymmetry in lengths, diameters, circumference and heights of clavicle and to seek for sexual differences in the direction and degree of established asymmetry.

## Material and Methods

The anthropological investigation was done on osteological material from archaeological excavations. A total of 136 clavicles (36 right and 36 left clavicles of male skeletons; 32 right and 32 left clavicles of female skeletons), belonging to adult individuals, were investigated. The sex and age were determined previously by metric and scopic features of cranium and postcranial bones described by R. Martin – K. Saller [5], V. P. Alekseev [9], etc.

The anthropometric investigation was done mostly after the classical methods of R. Martin and K. Saller [5] and V. P. Alekseev [9]. Ten metric features were measured and, for eight of them, the numbers assigned by Martin are marked in brack-

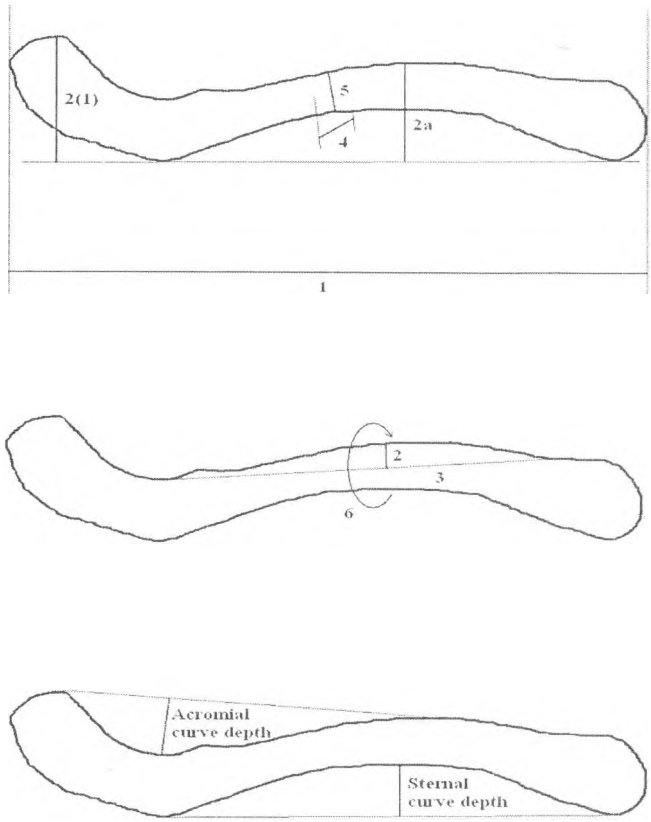


Fig. 1. Measurements of clavicle – greatest clavicle length (1), length of corpus curve basis (2), middle vertical diameter (4), middle sagital diameter (5), middle circumference (6), height of corpus curve (2), height of corpus curve (2a), acromial end curve (2(1)), sternal curve depth, acromial curve depth

ets (Fig. 1). Both heights of corpus curve were marked conditionally as first height of corpus curve (2) and second height of corpus curve (2a). The sternal curve depth and acromial curve depth are introduced by us.

The metrical data were statistically analyzed using SPSS version 13.0. The reliability of established bilateral asymmetry was verified by the U-test of Mann – Whitney at $P<0.05$. The quantitative assessment of the bilateral asymmetry was made using the relative index of Wolanski for inter-group comparisons [8]. The index in this study is used for determination of the asymmetry and is called Index for Asymmetry (IA):

$$IA = \frac{2.(x_1 - x_2).100}{x_1 + x_2},$$

$x_1$ – mean value of the feature in right clavicle; $x_2$ – mean value of the feature in left clavicle.

The degree of bilateral asymmetry was assessed by percentile analysis according to the data of IA. The border values were set at $P_{25}$ ($P_{25}$ = 0.62 IE) and $P_{75}$ ($P_{75}$ = 2.49 IE). The bilateral asymmetry is slight at values of IA less than 0.61 IU, the asymmetry

is moderate at values between 0.62 IU and 2.48 IU, and it is strong at values more than 2.49 IU. The positive sign of IA shows right-side asymmetry and the negative one – left-side asymmetry.

## Results

The main biostatistical results of the study are given in Table 1.

*Length features of clavicle*

Both length features of the clavicle, which are measured, give general information on the clavicle size. After the results obtained for first feature, the left clavicles of male and female skeletons have greater lengths than the right ones. The bilateral differences are statistically significant for female clavicles only. The greater length of left clavicles in both sexes observed in the present study confirms the results of many authors (T e r r y [7], S i n g h and G a n g r a d e [6], J i t and S a h n i [2] and K a u r et al. [4]). However, this is refused by B i l o d i et al. [1], which results show greater length for the right clavicles. The length of corpus curve basis also has greater values in left clavicles of both sexes. The bilateral differences for this feature are not statistically significant. According to the border values of IA for determination of the asymmetry degree, the asymmetry of greatest clavicle length is moderate in both sexes. The length of corpus curve basis shows strong bilateral asymmetry in the clavicles of male and female skeletons.

*Diameters and circumference in the middle of the clavicle*

The three measured features in the middle of the clavicle characterize the massiveness of the bone. The middle vertical diameter is with greater values in right clavicles of male and female skeletons. The middle sagital diameter also has greater values in right clavicles of male skeletons. However, the left clavicles of female skeletons have greater sagital diameter, in comparison with the right ones. The mean values of middle circumference show right-side asymmetry in both sexes. The circumference of right clavicles is greater by 0.78 mm in male skeletons and by 0.28 mm in female skeletons. The bilateral differences are not statistically significant for any of these three features. The greater diameters and circumference in the middle of right clavicles are also observed by J i t and S i n g h [3] and B i l o d i et al. [1]. According to IA data, the right-side asymmetry for these three features in male skeletons is moderate. The middle vertical diameter and the middle circumference in female clavicles show right-side asymmetry as well, but the middle sagital diameter is with left-side asymmetry. According to the degree of asymmetry, both diameters in female skeletons belong to the slightly asimmetric features and the circumference – to the moderately asymmetric features.

*Height features of clavicle*

The three height features measured characterize the clavicle curve. The first height of corpus curve is greater in left clavicles of both male and female skeletons. The second height of corpus curve has greater values in right bones of both sexes. The values of acromial end curve are greater in the right bones of male and female skeletons, respectively by 0.71 mm and by 0.81 mm. This shows that acromial end of right clavicles of both sexes is more curved than the acromial end of left ones. The right-left differences for these three features are not statistically significant. According to the values of IA, the strongest left-side asymmetry in both male and female clavicles is found for the first height of corpus curve. ("strong" degree). The second height of corpus curve in both sexes is with positive sign of IA i.e. it is with right-side asymmetry – by "moderate"

Table 1. Asymmetry indicators of male and female clavicles

| No | Feature | Male ($n$ = 36) | | | | | Female ($n$ = 32) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Asymmetry indicators | | | | | Asymmetry indicators | | |
| | | $\bar{x}$ right | $\bar{x}$ left | Absolute difference | U-test | IA | $\bar{x}$ right | $\bar{x}$ left | Absolute difference | U-test | IA |
| 1 | Greatest clavicle length (1) | 147.28 | 149.58 | -2.30 | 0.321 | -1.55 | 132.00 | 135.19 | -3.19 | 0.038* | -2.39 |
| 2 | Middle vertical diameter (4) | 11.10 | 10.96 | 0.14 | 0.602 | 1.26 | 9.48 | 9.44 | 0.04 | 0.842 | 0.50 |
| 3 | Middle sagital diameter (5) | 12.63 | 12.38 | 0.25 | 0.570 | 2.00 | 10.56 | 10.58 | 0.02 | 0.882 | -0.15 |
| 4 | Middle circumference (6) | 39.53 | 38.75 | 0.78 | 0.368 | 1.99 | 33.69 | 33.41 | 0.28 | 0.745 | 0.84 |
| 5 | First height of corpus curve (2) | 7.58 | 7.82 | -0.24 | 0.659 | -3.07 | 7.14 | 8.09 | -0.95 | 0.182 | -12.51 |
| 6 | Length of corpus curve basis (3) | 92.60 | 95.03 | -2.43 | 0.203 | -2.59 | 85.19 | 87.67 | -2.48 | 0.121 | -2.87 |
| 7 | Second height of corpus curve (2a) | 29.97 | 29.72 | 0.25 | 0.905 | 0.84 | 26.09 | 25.94 | 0.15 | 0.665 | 0.60 |
| 8 | Acromial end curve (2(1)) | 32.82 | 32.11 | 0.71 | 0.252 | 2.18 | 28.75 | 27.94 | 0.81 | 0.310 | 2.87 |
| 9 | Sternal curve depth | 18.61 | 18.93 | -0.32 | 0.498 | -1.70 | 17.31 | 17.42 | -0.11 | 0.788 | -0.63 |
| 10 | Acromial curve depth | 13.53 | 13.51 | 0.02 | 0.959 | 0.10 | 11.97 | 11.91 | 0.06 | 0.995 | 0.52 |

* statistically significant differences at the $P<0.05$.

degree in the male skeletons and by "slight" degree in the female ones. IA values of acromial end curve show that this feature is with comparatively strongest right-side asymmetry. In accordance with asymmetry intensity, the acromial end curve in male clavicles belongs to the moderately asymmetric features and this in female clavicles – to the strongly asymmetric ones.

*Clavicle curve depths*

Both clavicle curve depths, which are measured in this study, show that the sternal curve depth is greater in the left clavicles of both sexes. However, the acromial curve depth has greater values in the right bones of male and female skeletons. This result shows that the sternal curve is deeper in the left clavicles and the acromial curve – in the right ones. The right-left differences for both depths are not statistically significant. IA values of sternal curve depth show right-side asymmetry of "moderate" degree in both sexes. According to the border values for determination of asymmetry intensity, the asymmetry of acromial curve depth is slight right-side one in female skeletons, while in male skeletons can be assumed that the asymmetry of this feature is missing, i.e. there is symmetry.

*Comparative assessment for asymmetry profiles of both sexes* (Fig. 2)
A summary idea of asymmetry in size and form of the human clavicle is given by the graphically presented asymmetry profiles based on IA data from all ten investigated features.

Six of all clavicle features in the male skeletons are with right-side asymmetry and four – with left-side one. A right-side asymmetry is accounted in five metric features of the female clavicles and a left-side asymmetry – in the other five.

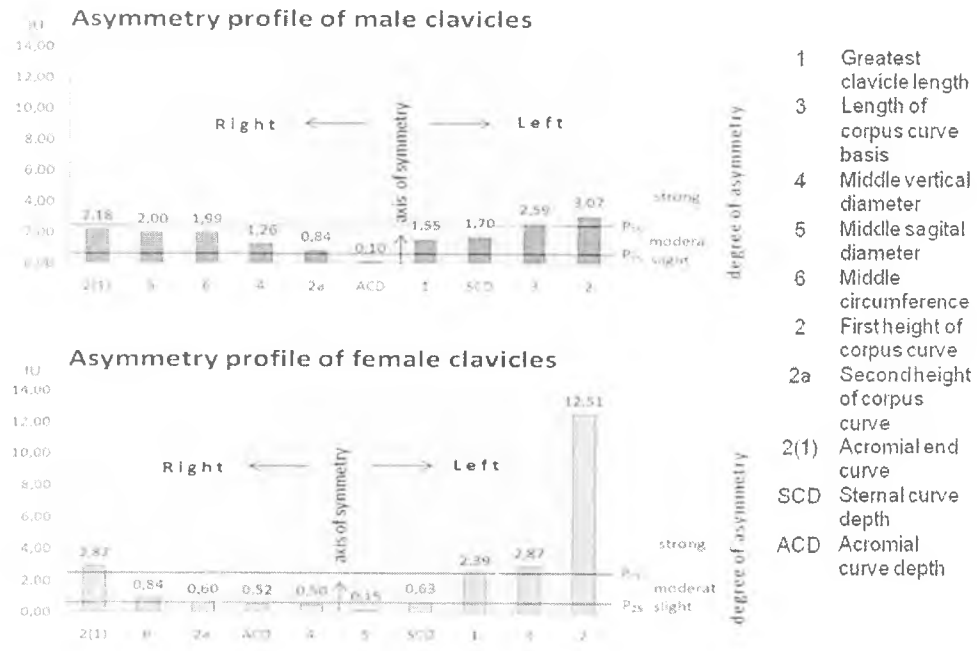


Fig. 2. Asymmetry profiles of male and female clavicles. The features with right-side asymmetry are ranked in descending line and those with left-side asymmetry – in ascending one

The features with moderate asymmetry predominate in the male clavicles and this degree of asymmetry is accounted for 7 features – 5 with right-side and 2 with left-side asymmetry. More features in the female clavicles have slight asymmetry (4 features), 3 of the features are with moderate asymmetry and 3 – with strong one.

According to the direction of asymmetry, the sexual differences are found for one feature only and it is the middle sagital diameter. It is with right-side asymmetry in the male clavicles and with left-side one – in the female clavicles. According to the degree of asymmetry, it is found that right-side asymmetry in male clavicles is mostly of "moderate" degree, while in female bones – of "slight" one. Among the features with left-side asymmetry, the asymmetry is "moderate" and "strong" in the clavicles of both sexes.

The comparative assessment of the asymmetry profiles of both sexes shows that the strongest right-side asymmetry in male and female clavicles is established for the acromial curve depth, and the strongest left-side one – for the first height of corpus curve. The asymmetry for these two features is stronger in the female clavicles in comparison with the male ones.

## Conclusions

The left clavicles in both sexes are longer than the right ones, but the right clavicles are more massive and more curved, compared to the left ones.

According to the direction of asymmetry, the sexual differences are found for the middle sagital diameter only, as in male skeletons it is greater in the right clavicles and in female ones – in the left clavicles.

According to the degree of asymmetry, the strongest asymmetry in male clavicles is the left-side asymmetry of first height of corpus curve and length of corpus curve basis. The strongest asymmetry in female clavicles is the left-side asymmetry of first height of corpus curve and length of corpus curve basis also and the right-side asymmetry of acromial end curve.

The general assessment of the asymmetry manifestations shows that clavicles of male skeletons as a whole are more asymmetric than those of female ones.

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*Review articles*

# Research Concepts and Contributions of Acad. Assen I. Hadjiolov for the Development of Morphology in Bulgaria on the Occasion of His 105th Anniversary

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The development and consolidation of morphology in Bulgaria is firmly fused with the name of Acad. A. I. Hadjiolov. He is one of the founders of the Bulgarian morphological scientific school. Acad. A. I. Hadjiolov is one of the most prominent representatives of morphological science and one of the leading scientists in the field of the biological and medical thought in our country. This year the scientific community celebrates the 105th anniversary of the birth of the outstanding Bulgarian and world-known scientist and active public figure Acad. Assen Ivanov Hadjiolov

*Key words*: morphology, histochemistry, luminescent microscopy, electron microscopy.

This year the scientific community celebrates the 105th anniversary of the birth of the outstanding Bulgarian and world-known scientist and active public figure Acad. Assen Ivanov Hadjiolov. He is one of the most prominent representatives of morphological science and one of the leading scientists in the field of the biological and medical thought in our country. The development and consolidation of morphology in Bulgaria is firmly fused with the name of Acad. Assen Hadjiolov. He is one of the founders of the Bulgarian morphological scientific school.

He was born on January 6th 1903 in the village of Shirokovo (Rousse region). He has graduated from the People's State Men's Secondary School "Konstantin Fotinov" in the town of Smolyan (1920) and Medical Faculty of Sofia University (1926). He has also attended studies in philosophy and psychology at the Sorbonne in Paris (1926-1927). He has also made his PhD degree in the sciences in Lyon (1929). He has been

elected and nominated consecutively as follows: assistant-professor at the Chair of Histology and Embryology at the Medical Faculty in Sofia (1928), associate professor (1930), full professor (1945), corresponding-member at the Bulgarian Academy of Sciences (1947) and full member-academician (1952).

In his over 1000 papers, monographs and textbooks important studies on the histochemistry of lipids, the proper luminescence of cells and tissues, cytobiology and histobiology of the sexual, connective, blood, nervous and epithelial tissues have been highlighted. Acad. A. I. Hadjiolov has developed the concept of the tissue organoid; he has offered a histobiological classification and systematization of the tissues and organs, etc. These are well-known not only in Bulgaria, but throughout the world as well. He is the author of a number of textbooks and monographs in cytology, histology, embryology and haematology. In the field of morphological sciences acad. Asen I. Hadjiolov has numerous and original contributions with international recognition. He has profoundly developed the concept of the "Morphological metabolism of lipids". Acad. A. I. Hadjiolov has also created and developed a theoretical concept of the structure and evolution of cells up till their integration in the so-called "tissue organoid". His ideas on the classification of the tissues are also of importance. He has attached to the accepted and recognized four classical tissues in histology (epithelial, connective, muscle and nervous tissues) yet another two – the sexual and blood ones.

Acad. A. Hadjiolov is the founder of luminescent microscopy in Bulgaria. In the field of the luminescent analysis of the cells and tissues he has published as early as 1930 a monograph named: "A study on the actinoluminescent properties of the tissues by filtered ultraviolet rays. The light of Wood and its application in histology and medicine". He has isolated from frog's skin three fractions pterine (ranopterines) and has proved their effect on the proliferation and differentiation of haemopoietic cells.

For the first time in literature in 1966 in collaboration with J. Jordanov et al. he has described the ultrastructural organization of the so-called vitellin body of Balbiani in the ooplasm of chicken oocyte as complex of a centrosomes, mitochondria, and Golgi vesicles cisterns.

Of importance are also his investigations on the action of ionizing irradiation on sexual tissue. They bring about to the revealing at the cellular level of the differing degrees of radio-sensitivity at the different degrees of radio-sensitivity at the different stages of spermatogenesis and oopoiesis. Also provoking an interest are his studies in 1977 conducted in collaboration with A. Manina, E. Zaprianova, A. Boyadjieva et al. on the adaptational – compensatory reaction of the brain upon irradiation [5].

The tributes of Acad. Hadjiolov in the area of studies on the blood, sexual and nervous tissues reviewed in his profound works: "On the sexual and blood tissues" (1932); "Historical and the theoretical survey of the blood tissue" (1941); "Textbook in histology and microscopic anatomy" (1946 - 1958); "Fundaments of haematology" (1950); "Methodological Importance of the Tissue and Organ Biology" (1959); " The Problem of monocytes and macrophages from the point of view of organ biology" (1961); " An attempt for a physical-chemical and colloidal theory of cariokinesis with respect to the phylogenesis of the cell and tissues" (1961), etc.

His investigations carried out in the period 1958-1968 in collaboration with N. Damova at al. on the nervous system pertain mainly to the embryonic development of the cerebral hemispheres in the human. They contribute to the establishing of certain dependencies and facts in the morpho- and histogenesis of the hemispheres.

For the first time in the Bulgarian individual the system of the erythrocyte glutamatepyruvate transaminases has been characterized by A. I. Hadjiolov, E. Yaneva et al. in the period of 1979-1986 for the separation of which an own method has been devised. A number of other novel methods created by A. I. Hadjiolov et al. have been

introduced in practical application. Methods for histochemical detection of lipids in the cells and tissues by hydrotropic solutions of lipid dyes based on new principles have been created by A. I. Hadjiolov et al. The hydrotropic method created by A. I. Hadjiolov et al. The hydrotropic method of Hadjiolov has been adopted in the world histochemical practice and is reviewed in most of the histochemistry manuals. In 1972 A. I. Hadjiolov has created together with E. Zvetkova a fluorescent-cytochemical method for nuclear fluorochroming with berberin-sulphate after DNA-denaturation. This method based on the differing intensity of light and morphology of the eu- and heterochromatin in the bone marrow allows for a good differentiation between the mature normal cells and both the immature and neoplastic ones. They have also described a fluorescent – cytochemical method with acridine yellow for proving the acidic glucosaminglycans and epithelial mucines on crystal sections. This method is fast and widely used in practice [1]. His studies in the fields of history and popularization of science are also of contributing nature.

Acad. A. I. Hadjiolov was not a highly knowledgeable scientist but also a long-years profound teacher and good organizer of science in our country. He created the Bulgarian morphological school with dozens of disciples and followers. During his 40-year long teaching career he has been supplying deep histological knowledge on the structure of the organism and its alteration in norm and pathology to his thousands of students and PhD graduates. With his legacy is also connected the building and development of major research and teaching scientific institutions. The scientific and staff work contributions of Acad. A. I. Hadjiolov are also very significant as a long-standing Head (Chief) of the Chair in Histology and Embryology at the Medical Faculty (Department) of Sofia University (1930-1968). In 1947 acad. A. I. Hadjiolov together with acad. D. Orahovetz have laid the foundations of (have found) the Institute of Experimental Medicine at the Biology Department of the BAS. Acad. A. I. Hadjiolov has been elected Deputy-Director (1952-1953) and Head (Chief) of the Section of Histology, Embryology and Experimental Medicine (1947-1953). He is the founder and first Director of the Institute of Morphology at the Department of Biological and Medical Sciences at the Bulgarian Academy of Sciences in Sofia and has been elected Head of the Department of comparative embryology in the same institute (1953-1974). Under his guidance the Institute of Morphology – BAS has grown to be the leading scientific institution in our country, developing at the international level a number of up-to-date significant trends in the fields of morphology and anthropology.

He has taken an active participation in a number of academic managerial activities being elected as follows: Deputy Scientific secretary of BAS (1950-1953); Scientific Secretary of BAS (1953-1956) and Chief Scientific Secretary of BAS (1955-1956).

For his merits as a brilliant organizer and outstanding scientist he has been elected deputy-chairman of the Bulgarian Doctors' Union and Chairman of its Sofia branch. He is also founder of the Union of Scientists in Bulgaria and its first Chairman (1944-1990). He was also elected Chairman of the newly created Association of the Bulgarian anatomists, histologists and embryologists (1959-1967) [2, 3, 4, 6].

Acad. A. I. Hadjiolov is a long-standing member of the Editorial Board at BAS (1956-1973), "The Journal of BAS" and "Nature", "Praemedicus", etc.

With his broad vision on the organization and development of science and morphology he has authoritatively represented Bulgarian science and BAS at numerous international forums and meetings.

The high estimate given by our and the world scientific community is expressed in his election as first Chairman and founder of the Union of Scientists and the Bulgarian association of anatomists, as member of the National Commission and Chairman of the Department of Physical-Mathematical and Natural Sciences at UNESCO, correspon-

ding-member of the Department for Space Biology at the International Association in Space science, corresponding-member of the International Academy in the History of Science in Paris, member of the Board of the International Association of the Francophonic Anatomists, etc.

The recognition for his significant scientific contributions abroad is also his election as an honorary member of the Hungarian Academy of Sciences, Doctor Honoris Causa of the Budapest Medical University, corresponding member of the Yugoslav Academy of Sciences and Arts in Zagreb, honorary member of the Czechoslovak Medical Association "Jan Purkinje", etc.

For his great scientific merits in the field of histology he has been conferred prestigious titles and awards: "Laureate of Dimitrov Award" (1951),"Merited Scientific Activist" (1963), twice bearer of the "Cyrill and Methodius" order, etc.

Acad. A. I. Hadjiolov passed away on June 1st 1994 in Sofia.

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# The Contributions of Corr. Member G. Galabov for the Development of the Neuromorphology in Bulgaria on the Occasion of His 90th Anniversary

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Corr.-member Georgi Galabov was one of the most outstanding Bulgarian neuromorphologists of international renown and of an extraordinarily broad professional scope, huge humane virtues and creativity. He is one of the founders of the Bulgarian neuromorphological school.

*Key words:* neuromorpholgy, histochemistry, histoautoradiography, electron microscopy, regeneration

90 years ago corresponding member of Bulgarian Academy of Sciences Georgi Galabov was born on November 7th 1918 in the village of Yavorovo (Plovdiv region) – one of the most outstanding Bulgarian neuromorphologists of international renown and of an extraordinarily broad professional scope, huge humane virtues and creativity.

He has graduated from secondary school in Plovdiv (1937) and the Medical Faculty (Department) of Sofia University in 1943. He has worked as General Practitioner in the village of Raikovo and IInd Divisional hospital in the village of Orisare (Smolyan region). He has also been elected and nominated later assistant professor at the Chair of Anatomy of the Human at the Medical Faculty in Sofia (1944); associate professor (1950); full professor (1960); corresponding – member of Bulgarian Academy of Sciences (1977) [10, 11, 12].

With great dedication and professional expertise corr. – member G. Galabov has put great amounts of efforts in the teaching activity in the Chair of Anatomy of the Higher Medical Institute since 1944 till the end of his life. He was a revered tutor, teacher and guide in science for a number of generations of Bulgarian students in medicine, stomatology, Ph.D.- students, nurses, etc. corr.-member G. Galabov is one of the founders (creatots) of the Bulgarian neuromorphological school.

Applying great skills and insight he devoted all his efforts for raising neuromorphology in Bulgaria to the world level. Corr.-member G. Galabov has authored over 150 papers dedicated to important and up-to-date medico-biological problems in the fields of neuromorphology, anatomy and popularization of science. Corr.-member G. Galabov is the author and co-author of a number of textbooks in

anatomy : "Short Textbook in Anatomy of Man" for nurse and obstetrical schools (with D. Kadanov); "Defining the situation and direction of the organs and parts of the human body"; "Short textbook in anatomy of man"; "Anatomy of man for Students of the Sports Academy " (with Sp. Morov and R. Kossev); "Introduction to the anatomy and general Anatomy" (with D. Kadanov). Anatomy and Physiology of man. A textbook for students in a teacher`s training colleges. (with T. Gotsev); Anatomy of man. A textbook for students in medicine and stomatology (with V. Vankov); Repetitorium anatomicum (with V. Vassilev) etc. [5, 11].

At the bottom of the entire scientific activity of corr. – member G. Galabov lies the idea of restoration of the link between the two parts of the spinal cord upon its severing, an extraordinarily important issue affecting the destiny of thousands of disabled individuals.

Corr.-member G. Galabov and the team led by him has a number of original tributes to the neuromorphology and regeneration of the CNS. Important relationships between the neurons, the glial tissue and capillaries in the spinal cord have been established.

In collaboration with corr.-member M. Davidov in 1973 he has published the monograph "Lysosomen und lysosomale Enzymen im Zentralnervensystem der Ratta" devoted to the lysosomes in CNS [2].

He has also studied at the ultrastructural level with TEM the distribution of a number of hydrolases and has done the first description with TEM of a number of other enzymes in CNS.

The works of corr.-member G. Galabov dedicated to the study of reactivity and regeneration of the nervous system are also of original nature. He has studied in detail the axonal reaction after severing the peripheral nerves.

The works of corr.-member G. Galabov on the trans-synaptic changes in various parts of CNS after a cross – cut of the spinal cords or separate tracts are also of great interest. He has established differences in the morphology and metabolism of the structures situated over and under the point of cut of the spinal cord.

In 1971 together with prof. K. Ichev and prof. S. Manolov he has published an original paper devoted to the changes in the ultrastructure of the blood vessels of the spinal cord after its transversal section [6].

In collaboration with prof. Chuchcov in 1971 he has performed in – detail histoautoradiographic study on the synthesis of DNA in the spinal cord after cutting of plexus brachialis [7].

In 1983 his monograph co-authored by assoc. – prof. R. Dimova dedicated to the morphology of the glial cells in CNS was published [8].

His works on the macro anatomical investigations and the studies of the morphology of separate organs are also of importance.

Corr.-member G. Galabov has described in detail the adaptational capacities of the organism, the peculiarities of metabolism of the different cell types as well as the tracts and connections between the different parts of the nervous system.

The study on the conductive tracts in CNS allowed corr.-member G. Galabov to make important discoveries. He has proved that a part of the long dendrites of the motor – neurons in the spinal cord cross into the contralateral side and are incorporated in the composition of the corresponding front radices (roots).

He has reported for the first time on the presence of cortico-nigral, cortico-bulbar and cortico-subthalamic tract in the brain of certain mammals.

The data from the investigations carried out in collaboration with prof. V. Vassilev on the biomechanics of the lumbar portion of the spine are of great practical value [9].

Together with prof. K. Uzunov and prof. A. Palov by the help of TEM the cell characteristics and synaptic organization of the medial thalamic nucleus in the cat brain have been established [3].

The methodological contributions of corr.-member G. Galabov are also of importance. He has created an original method for histochemical detection of the acid 5 – nucleotidase; a method for the visualization of the enzyme acetylcholine - esterase; a method for extramedullar recovery of the lesion by implantation of an intercostals nerve from the intact segments of the proximal portion into segments of the distal portion, etc. [1, 4]

His activity as editor of a number of periodicals, monographs and textbooks published in Bulgaria is also very significant.

As a long-standing, respected and highly valued manager (organizer) of science and public health in our country he has actively participated and contributed to the building of several large research units in Bulgaria. In 1942 he has founded the Chair of anatomy at the higher Sports School hose head he was till 1963. Since 1965 he was elected head of the Chair of Human Anatomy at the Medical faculty in Sofia. In the same year he has founded the Central Laboratory in Regeneration at BAS and was elected its director. From 1959 till 1966 he served as Scientific Secretary of BAS. In the period 1966-1970 he was elected Rector of the Medical Academy (Higher Medical School) – Sofia. He has actively taken part in and contributed to the founding of the Medical Faculty in Plovdiv.

His role as Chairman of the Medical Scientific Association in Bulgaria (1973) and as Chairman of the association of the anatomists, histilogists and embryologists in Bulgaria (1971-1982), etc. is also of great value.

A lot of efforts and energy he has devoted as Counsellor of Direction "Higher Education of CSAC" (Committee for Science, Arts and Culture) – (1948-1951); Head of Department "Science and Education" at the Ministry of Public Health (1950-1951).

For his great merits for the development of neuromorphology in Bulgaria and his commitment in his scientific-organizational activity he has been conferred the public distinctions "Merited Scientist" (1970); "People's scientist"(1982) and "Laureate of Dimitrov Prize" (1982). He has also been awarded the order of the "People's Republic of Bulgaria" I$^{st}$ degree (1968); the "Cyrill and Methodius" order I$^{st}$ degree (1960), etc.

Corr.-member G. Galabov passed away on August 6$^{th}$ 1982 in Sofia.

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