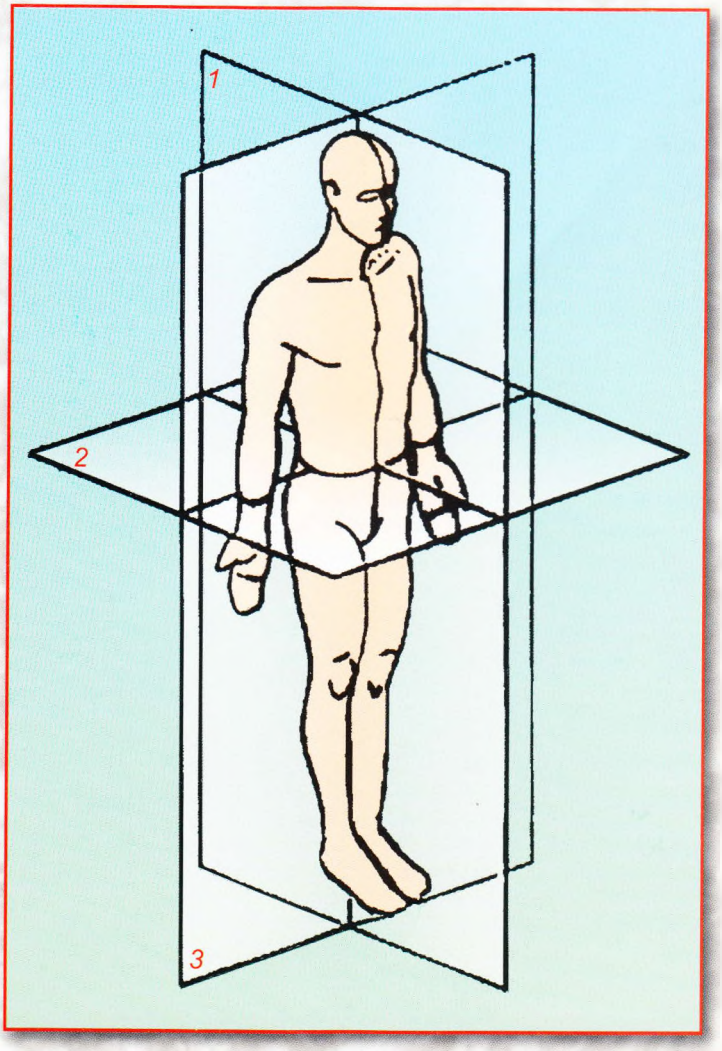
Acta morphologica et anthropologica (16)



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Morphology

Neurotransmission in the Human Carotid Body: Focus on the Role of Dopamine and Histamine in Hypoxic Chemoreception

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The carotid body (CB) is the only chemoreceptor sensitive to systemic hypoxia in humans. Its physiological action is regulated by multiple neurotransmitters, including several biogenic amines. Evidence to date shows an involvement of dopamine as an inhabitory modulator of the chemoreception in man. Histamine, released from glomus cells, has recently been considered a putative transmitter in hypoxic chemosensitivity in rats. In the present study, we investigated the expression of markers for histamine metabolism, transport and corresponding receptors in the human CB and revealed an expression of histidine decarboxylase, synaptosome-associated protein of 25 kDa, vesicular monoamine transporter 2, and histamine receptors 1 and 3 in virtually all chemosensory cells within the glomera. By contrast, dopaminergic traits (tyrosine hydroxylase, vesicular monoamine transporter 1 and D2 receptors) were only detected in a subset of glomus cells. Our data show that histamine, along with dopamine, plays an important role in the chemosensory function in humans.

Key words: carotid body, dopamine, histamine, hypoxia, human.

Introduction

The carotid body (CB) is a major arterial oxygen sensor that plays essential roles in the blood gas and pH homeostatic control, initiating an appropriate respiratory and cardiovascular response to hypoxia, hypercapnia and acidosis. It is a small paired organ strategically positioned at the bifurcation of each common carotid artery. The CB consists of two main cell types: neural crest-derived type I (also called glomus) chemosensory cells, which contain secretory granules, and type II (or sustentacular) cells, which are supporting glial-like cells [5] and recently pro-

posed to be CB stem cells [13]. These two cell types are juxtaposed and together make up small clusters called glomeruli or glomoids. On the other hand, glomus cells are synaptically connected to the nerve endings of petrosal ganglion neurons, thus ensuring the transmission of the chemosensory information from peripheral arterial chemoreceptors to the central nervous system. The efferent limb of the chemoreceptor reflex arc is formed by solitary axons projecting to the respiratory control centers, distributed in a ponto-medullary respiratory network. They control the coordinated contractions of the abdominal, thoracic and laryngeal respiratory muscles.

It has been proposed that several transmitter candidates are released upon hypoxia by the glomus cells of the CB in different animal species. In their turn, the neurotransmitters also contribute to the modulation of glomus cell function via autoreceptors. However, there are differences in species regarding the expression of various transmitters and their corresponding receptors in the CB which may result in variations of chemosensory signalling. On the other hand, since the CB is not fully developed at birth, plasticity-induced neurochemical changes may occur later in life [4]. As a result, the change in neurotransmitter or receptor profiles in the CB during maturation may cause altered CB responses to hypoxia [3]. Moreover, as human infants seem particularly vulnerable to hypoxic and hypercapneic episodes during sleep, cellular alterations in peripheral chemoreceptors resulting in altered chemosensitivity may be one of the factors contributing to a higher incidence of sudden infant death syndrome in premature newborns [4].

Biogenic amines are considered to be primary messengers in the junctions between glomus cells and nerve terminals [5]. In particular, dopamine is considered an important inhabitory modulator of chemoreceptor activity in most mammalian species; previous research has shown that in man it plays a significant role in ventilatory adaptation to hypoxia [2, 7, 8, 12]. Nonetheless, with the exception of one report about the localization of tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis, in glomus cells and nerve fibers in the human CB [10] the full biochemical machinery for dopamine storage and release, as well as specific dopamine receptors, have not been localized there so far. Recently, histamine has also been implicated in hypoxic chemosensitivity in rats [9, 11]. Its actions are mediated by at least four G-protein-coupled receptor subtypes encoded by different genes referred to as H1-H4. In this study we have investigated the chemosensory traits in the human CB of different ages with a particular focus on the role of dopamine and histamine in hypoxic chemoreception.

Materials and Methods

The experiments were carried out on human CB samples obtained at routine autopsies from nine patients of both sexes. Their age ranged from 4 months to 76 years and the time elapsing before tissue fixation did not exceeded 48 h. The carotid bifurcations were excised, both CBs were immediately dissected out, specimens were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 and tissue blocks were embedded in paraffin, cut at 5 μm thick sections and subsequently processed for ABC (avidin-biotin-horseradish peroxidase complex) immunohistochemistry. Briefly, following antigen retrieval in 10 mM citrate buffer, pH 6.0 in a microwave oven, the sections were preincubated in 5% normal goat serum to avoid nonspecific staining and treated with ABC blocking kit (Vector Laboratories Inc., Burlingame, CA, USA) to block unspecific biotin. Afterwards, they were incubated in a humid cham-

ber overnight at 4°C with primary antibodies against histidine decarboxylase (HDC; Progen Biotechnik GmbH, Heidelberg, Germany), histamine (HIS; Sigma, St. Louis, MO), human histamine 1 receptor (H1R; Acris Antibodies GmbH, Hiddenhausen, Germany), histamine 2 receptor (H2R; Alpha Diagnostics, San Antonio, TX), histamine 3 receptor (H3R), histamine 4 receptor (H4R; both from Abcam Ltd., Cambridge, UK), vesicular monoamine transporter 1 (VMAT1) and vesicular monoamine transporter 2 (VMAT2; both from Phoenix Pharmaceutical Inc., Belmont, CA), rabbit polyclonal antiserum to dopamine D2 receptor (D2R; BIOTREND Chemikalien GmbH, Köln, Germany), mouse monoclonal antibodies to TH (LOXO GmbH, Dossenheim, Germany), dopamine (Abcam) and synaptosome-associated protein of 25 kDa (SNAP25; SMI, Lutherville, MR). After rinsing in phosphate buffered saline, the sections were reacted with the respective secondary antibody, biotinylated goat anti-rabbit IgG or goat anti-mouse IgG (both from Dianova, Hamburg, Germany) and then the ABC-complex (Vectastain Elite Kit; Vector) was applied. After color development the sections were coverslipped with Entellan through alcohols and xylene. Finally, the specimens were examined and photographed with a Zeiss research microscope.

The specificities of antibodies used and control staining applied in this study have been described in detail previously [9, 11].

Results

Immunoreactive for dopamine cells were distributed throughout the human CB of different ages and characteristically appeared as cell clusters. In particular, a subset of dark glomus cells in both immature and mature CB was immunoreactive for TH, the catecholamine synthesizing enzyme, as well as for the dopamine molecule (Fig. 1A, B). Likewise, relatively few type I cells, some of them TH-containing, were also immunopositive for the other dopaminergic traits, i.e. VMAT1, transporting catecholamines and SNAP25, an important component of the neuroendocrine exocytosis apparatus, that was localized on nerve fibers within and around the glomic lobules in the CB (Fig. 1C-F). Conversely, the immunohistochemical experiments demonstrated immunoreactivity for D2-dopamine receptor in a much greater number of glomus cells in comparison with TH-containing cells in both infantile and fully developed CBs (Fig. 1G, H).

In general, the distribution of histaminergic traits and the intensity of immunostained cells in the juvenile CB was essentially the same as that of the adult CB. Using antibodies directed against histamine itself and against HDC, the enzyme necessary for histamine synthesis, we identified a large number of histaminergic cells in both the immature and mature CB, typically aggregated in cell clusters (Fig. 2A, B). In addition, almost all glomus cells were immunoreactive for VMAT2, which is highly specific for histamine (Fig. 2C, D). Our results also showed that relatively more type I cells within the glomera of different ages expressed H1 (Fig. 2E, F) and H3 (Fig. 2G, H), but not H2 and H4, histamine receptor proteins. No immunoreaction to any of the tested antigens was detected in the tissues when normal serum instead of a primary antiserum was used (not shown).

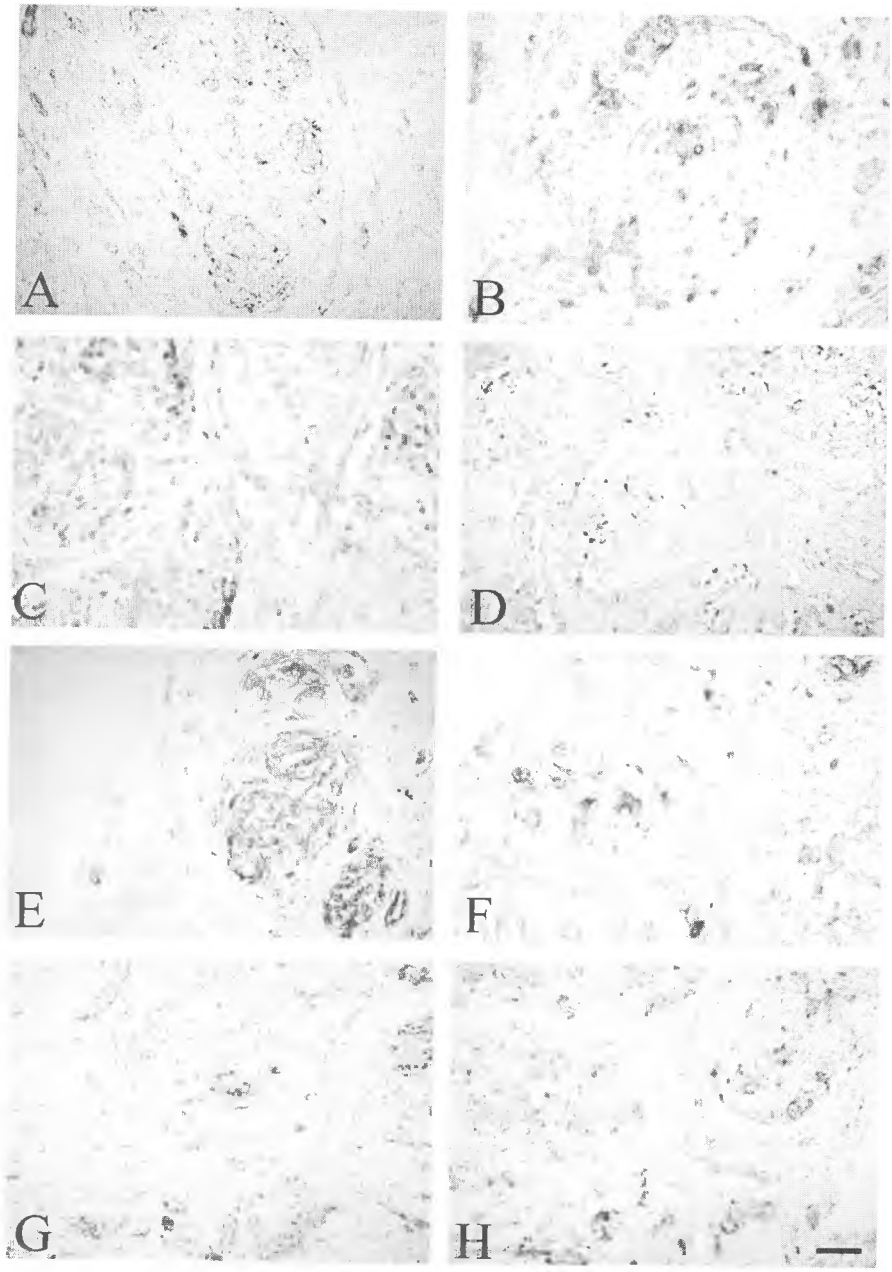

Fig. 1. Expression of dopamine and dopaminergic traits in the infantile (**A, C, E, G**) and adult (**B, D, F, H**) human CB. Immunohistochemical staining for dopamine in the immature (**A**) and mature (**B**) CB. Note that only a few glomus cells are immunoreactive with no age differences in their number and intensity of staining. (**C**) and (**D**) show the VMAT1 immunoreactivity in a subset of type I cells in childish and adult CB, respectively. (**E, F**) SNAP25-immunopositive glomus cells and nerve fibers within and around the glomic lobules are also observed. Relatively more numerous glomus cells contain D2 receptor protein (**G, H**). Scale bars = 100 μm

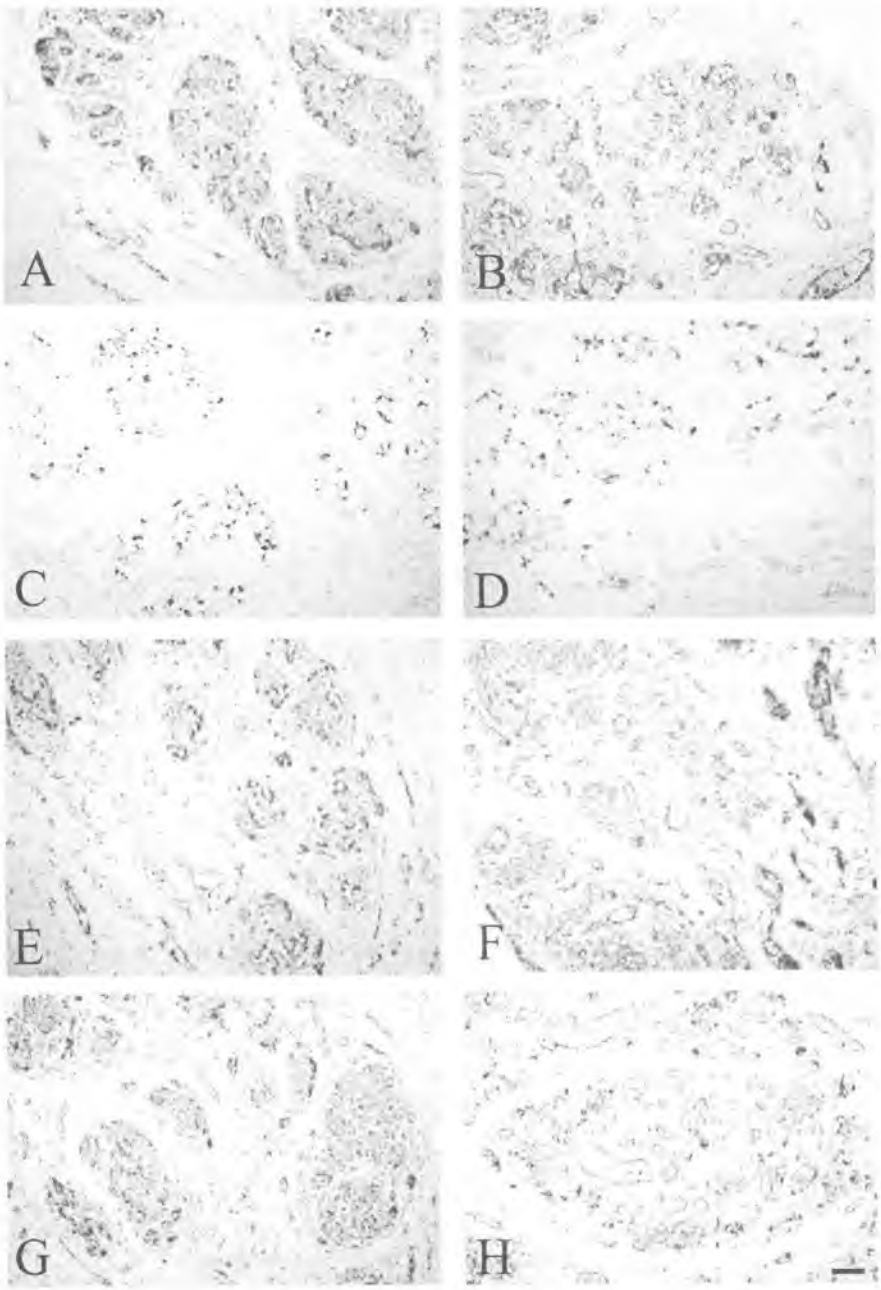

Fig. 2. Expression of histamine and histaminergic traits in the infantile (**A, C, E, G**) and adult (**B, D, F, H**) human CB. Microphotographs at low magnifications showing the presence of histamine in the immature (**A**) and mature (**B**) CB. A vast majority of glomus cells in the glomeruli exhibit strong immunoreactivity for histamine molecule with similar distributional patterns. (**C, D**) Also, most of the histamine-containing cells are intensely VMAT2 immunostained. A large number of glomus cells are abundantly endowed with H1 (**E, F**) and H3 (**G, H**) receptors. Scale bars = 100 μm

Discussion

The results of our study provide the first immunohistochemical evidence that glomus cell, regardless of their postmortem structural changes [6, 14], express all the biochemical components for biosynthesis, storage and release of dopamine and histamine upon hypoxia as well as the existence of certain specific receptors at the presynaptic and/or postsynaptic levels in the human CB. Nonetheless, the distributional patterns of dopaminergic and histaminergic traits do not differ with age, indicating that aminergic profiles of human CB glomus cells are not age-dependent.

Investigations of CBs in many different species, during various stages of development, have led to the conclusion that dopamine is a likely primary transmitter in the CB, because it meets most of the necessary criteria for such a role including biosynthesis and storage of dopamine, as well as Ca^{2+}-dependent release triggered by hypoxia. Our present findings on the expression of dopamine, its components of exocytotic apparatus and dopamine receptors allow for more definitive characterization of dopaminergic profiles of glomus cells involved in hypoxic chemosensitivity. Moreover, expression of inhabitory, hyperpolarizing presynaptic D2 autoreceptors on the glomus cells confirm that dopamine may serve as an inhibitory modulator of the transmitter(s) responsible for afferent sensory activity upon hypoxia (see [2, 8], and references therein). However, though dopamine has already been found to be the major amine at birth [1] we were not able to prove the postnatal developmental enhancement of dopaminergic traits and changes in oxygen responsiveness, reported by Gauda and Lawson [3]. Thus, dopamine does not seem to be directly involved in the maturational processes of CB oxygen sensitivity in man.

On the other hand, several lines of evidence suggest that histamine can be more essential than dopamine in hypoxic transmission during postnatal development in humans. Firstly, radioenzymatic and immunohistochemical evidence points out that storage of histamine in the glomus cells exceeds that of dopamine more than 10-fold [9]. Secondly, here we show that histaminergic traits tend to be expressed in virtually all glomus cells of young and adult humans. Thirdly, our data also demonstrates that a substantially greater number of chemoreceptor glomus cells are richly endowed with histamine H1- and H3 receptors. It is likely that signal transmission of the human glomus cells may be differentially modulated at the presynaptic level by histamine through excitatory H1 and inhibitory H3 autoreceptors.

In conclusion, it can be inferred that histamine and dopamine are important transmitters in hypoxic chemosensitivity in man acting via certain corresponding receptors (H1, H3 and D2, respectively). Furthermore, the changes in their levels may play important roles in the maturation of the physiological function of carotid chemoreceptors in response to hypoxia in humans.

Acknowledgements. This work was supported by the Alexander von Humboldt Foundation through the resumption of a fellowship (grant 1015945 to NL) and by the Deutsche Forschungsgemeinschaft through a predoctoral fellowship to SR in the Graduate School 333 (Biology of human diseases). We thank Astrid Tiefenbacher and Marlies Rauchfuß for their excellent technical assistance.

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Aspartylglucosaminidase Activity in Rat Central Nervous System — a Histochemical Study

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Aspartylglucosaminidase (AGA, EC 3.5.1.26) is involved in the final stages of glycoprotein hydrolysis in the lysosomes. Its genetically determined deficiency causes the lysosomal storage disease aspartylglucosaminuria (AGU), clinical symptoms of which include progressive psychomotor retardation, diminished communication skills, grotesque facial appearance and skeletal abnormalities, i.e. a number of symptoms pointing out a substantial decline in neuronal function. In this paper, the enzyme distribution in rat central nervous system (CNS) is studied by a newly synthesized artificial substrate — β-Asp-2-naphthylamide used after a simple simultaneous azo-coupling procedure. This method reveals for the first time a high AGA activity in neurons of various regions in rat brain (cerebrum, cerebellum, medulla oblongata) and spinal cord.

Key words: aspartylglucosaminidase, aspartylglucosaminuria, enzyme histochemistry, central nervous system.

Introduction

Aspartylglucosaminidase (glycosylasparaginase, AGA, EC 3.5.1.26) is a lysosomal amidase hydrolyzing the N-glycoside bond between L-asparagine (Asn) and N-acetyl-D-glucosamine (GlcNAc) in the core of N-linked glycoproteins. The enzyme requires free α-amino and α-carboxyl groups of Asn and is less specific towards the carbohydrate moiety attached to it [7]. Thus, AGA is responsible for the very final step of N-glycoproteins digestion in lysosomes. The enzyme has been shown to possess a surprisingly high pH optimum of 7.6 [2]. The discrepancy between lysosomal localization of AGA and its alkaline pH optimum is not explained yet. Genetically determined AGA deficiency leads to a most common lysosomal storage disease that directly involves glycoprotein metabolism, named aspartylglucosaminuria (AGU). AGU is connected with piling up of non-degraded N-acetylglucosaminyl-L-asparagine (GlcNAc-Asn) within lysosomes and its excretion with the urine [11]. Clinical symptoms of AGU are usually developed in the late puberty and include progres-

sive psychomotor retardation, diminished communication skills, grotesque facial appearence and skeletal abnormalities [8]. AGU patients have a relatively long survival — up to 45-50 years of age. Presently, AGU is studied using experimental models of targeted disruption of mice AGA gene (knock-out mice). AGU mice exhibit similar pathophysiology as human patients. Very specific characteristics of those mice brains at autopsy are the enlarged lysosomes in the CNS and loss of Purkinje cells in the cerebellar cortex [5]. Thus, both clinical symptoms and morphological characteristics in the experimental model of AGU point out that AGA might be present in the neurons of CNS. Biochemical experiments of Conchie and Strachan [3], however, have shown that in rat and mouse brain AGA activity is comparatively low. No histochemical study of the enzyme distribution in the CNS of laboratory animals has been performed so far.

The aim of the present study was to develop a specific synthetic substrate for AGA and using it, to study the enzyme activity distribution in the CNS of Wistar rats. The results are expected to help in elucidation of AGA importance for neuronal function and to be useful for the studies of animal models of AGU.

Materials and Methods

Synthesis of AGA substrate — β-Asp-2-naphthylamide. TFA (Asp-NA) and AGA inhibitor - 5-diazo-4-oxo-L-norvaline (DONV). The substrate was synthesized using the ordinary DCC (dicyclohexycarbodiimide) method [1]. In brief, equimolar amounts of 2-naphthylamine (NA, Aldrich), Boc-Asp(OH)-OtBu (Novabiochem) and DCC (Fluka) were mixed in dry tetrahydrofurane for 3 hours at room temperature. Then, the reaction mixture was extracted with ethylacetate and the product — Boc-Asp(2-NA)-OtBu was isolated by solvent evaporation in vacuum. The Boc- and OtBu-protective groups were cleaved simultaneously with trifluoroacetic acid (TFA) for two hours at room temperature and the substrate — Asp-NA was precipitated with diethyl ether as TFA-salt. The AGA specific inhibitor — 5-diazo-4-oxo-L-norvaline (DONV) was synthesized precisely as described by Handschumacher et al. [6].

Animals and tissue treatment. Adult Wistar rats of both sexes were decapitated under deep anesthesia. Cerebrum, cerebellum, medulla oblongata and cervical part of the spinal cord were extracted and fixed in formol-Calcium for one hour at 4°C. Then, the samples were washed in modified Holt's solution (15 % sucrose, 1 % gum arabic) for 36 hours at 4°C and frozen in liquid nitrogen. Serial sections (10 μm) of cerebral cortex, cerebellar cortex, medulla oblongata at the level of hypoglossal nerve and of the cervical region of spinal cord were cut on cryotome Reihert-Jung 2800 (Germany), mounted on gelatinized glass slides, air-dried and covered by 1% celloidin (Fluka) in acetone : diethyl ether : ethanol 4:3:3 for a minute at room temperature.

Visualization of AGA activity and inhibitor controls. Sections were incubated in a substrate solution consisting of 0.3 mM substrate (Asp-NA) and 1.8 ml freshly hexazotized pararosaniline (Merck) in 0.1 M sodium acetate, pH 7.0 for 8 hours at 37°C. The sections were transferred to fresh incubation solution at the fourth hour of incubation. Then, they were post-fixed in 4 % neutral formaline overnight, stained with haematoxyline according to the standard procedure and embedded in glycerol/jelly. Control sections were treated as above, but the incubation medium was supplied with 200 μM AGA inhibitor — DONV.

The examination was made in an Opton IM 35 microscope.

Results and Discussion

AGA possesses the unique ability to cleave off the N-glycoside bond between L-Asn and N-acetylglucosamine in N-linked glycoproteins to give L-Asp and an unstable 2-acetamido-2-deoxy-D-glucopyranosylamine, which hydrolyzes non-enzymatically to N-acetyl-D-glucosamine and ammonium ion (Fig. 1A). The enzyme recognizes L-Asn moiety and acts on its β-amide bond but is not specific towards the carbohydrate chain attached to it. Although the enzyme is classified as an amidohydrolase, its amidase activity is low and even hydrolysis of its natural substrate progresses slowly [4]. Thus far, only one synthetic substrate for AGA — β-Asp-methylcoumarylamide (Asp-AMC) has been synthesized and used for the biochemical analyses of AGA activity in blood samples and cultivated fibroblasts [9]. The enzyme cleaves the amide bond to release a fluorescent compound — aminomethyl coumarine (AMC). Even though the fluorescent analyses are very sensitive, low amidase activity of AGA imposes a problem of a very prolonged incubation time — at least 6 hours are needed for the test. Nevertheless, this test is now extensively used for the diagnosis of AGU [9, 10]. Asp-AMC substrate is not suitable for histochemical studies since the final reaction product of enzyme hydrolysis — the AMC compound is water-soluble.

In the present paper we describe the synthesis of another artificial substrate for AGA - β-Asp-2-naphthylamide (Asp-NA), which can be used for histochemical investigations of the enzyme activity. This substrate possesses a substantial similarity to the natural AGA substrate and is to be used according to the most common histochemical principle of simultaneous azo-coupling and azo-dye formation (Fig. 1B). Its rate of hydrolysis by AGA is also low and the incubation time needed for the

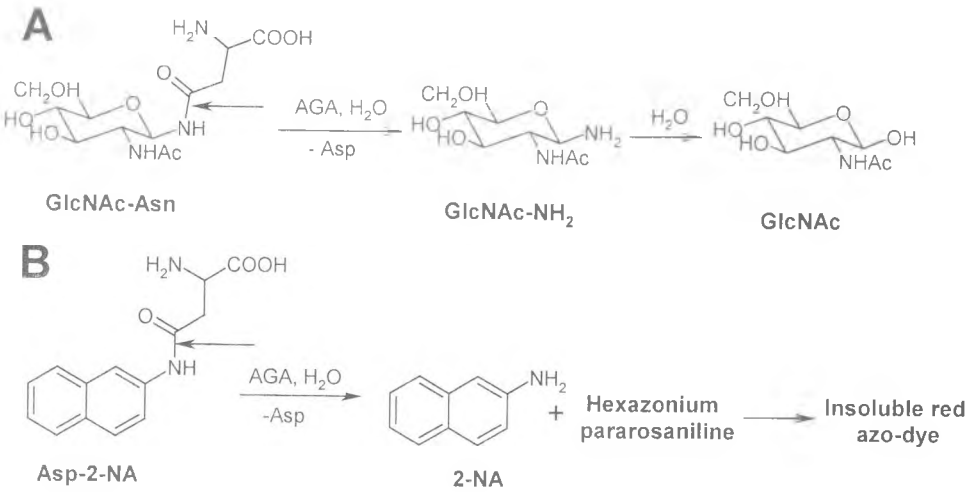

Fig. 1. A: AGA hydrolysis of its natural substrate. The enzyme hydrolyzes N-glycosylic bond in β-N-acetyl-D-glucosaminyl-L-asparagine (GlcNAc-Asn) to give aspartic acid and 2-acetamido-2-deoxy-β-D-glucopyranosylamine (GlcNAc-NH_2); the last compound is later hydrolyzed non-enzymatically to N-acetyl-D-glucosamine (GlcNAc) and ammonium ion; B: Histochemical principle for the visualization of AGA activity. The newly synthesized AGA substrate — Asp-2-NA has a substantial structural similarity to the natural AGA substrate and permits to visualize the enzyme activity according to a common principle of enzyme histochemistry — the simultaneous azo-coupling with diazonium salt to a deeply colored azo-dye

visualization of the enzyme activity is about 8 hours including at least one change of incubation solution. Using our novel AGA substrate we studied the enzyme distribution in the CNS of adult Wistar rats. Preliminary biochemical studies have shown that AGA has a moderate activity in the human brain [4] and comparatively low activity in rat and mouse brains [3]. These studies, however, could not show AGA activity distribution throughout neurons and glial cells of various regions in rat CNS. In our experiments, we found out that AGA has a very high activity in the cell soma of cerebral cortex neurons, Purkinje cells of cerebellar cortex, neurons of the cerebral nuclei of hypoglossal nerve and motor neurons in the cervical part of spinal cord (Fig. 2). The specificity of the enzyme reaction was confirmed by experiments with AGA specific inhibitor DONV. Control sections of all the parts of CNS had no non-specific precipitations. These results gave us grounds to conclude that the enzyme is present and very active in some neurons of the studied parts of rat CNS. This outcome could be expected since genetically determined AGA deficiency leads to symptoms pointing out a substantial impairment of neuronal function. However, the clinical signs of AGU usually start to appear at late juvenile or even adult age. So, the disease follows a very slow progression and it would be interesting to know how late in the pathogenesis of the disease the enzyme begins to be active in the rat CNS. The tracing of AGA activity in the rat CNS during development is going to be performed soon in our laboratory.

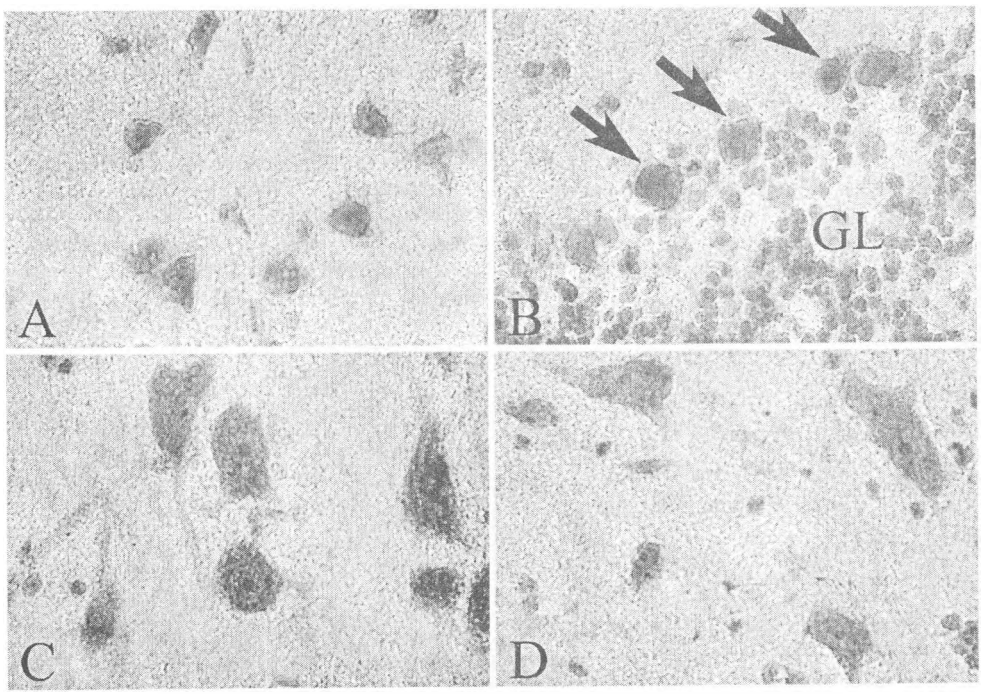

Fig. 2. AGA activity in different parts of rat CNS. A —cerebrum— high enzyme activity in the cytoplasm of cortical neurons; B — cerebellum — Purkinje cells are heavily stained for AGA (arrows), whereas Bergmann's glia and cells in the granulose cell layer (GL) are negative for the enzyme; C — medulla oblongata — neurons of the cerebral nuclei of hypoglossal nerve are highly AGA-positive; D — cervical part of spinal cord — strong reaction for AGA in the cytoplasm of motor neurons. A-D: originally × 400

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Light and dark epithelial cells of the rat choroid plexus

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In the present study were carried out ultrastructural investigations of the light and dark epithelial cells of the rat choroid plexus during development. 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 of the rat choroid plexus 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.

Key words: light and dark epithelial cells, rat choroid plexus, development.

Introduction

The choroid plexuses are specialized highly vascular anatomical structure which protrudes into the lateral ventricle, as well as in the third ventricle and fourth ventricle. The surface of the choroid plexus consists of numerous villi each covered with single layer of epithelial cells surrounded by vascular connective tissue cells [3, 10, 11]. These cells are generally considered to be modified ependymal cells with epithelial cell characteristics and referred to as choroidal epithelial cells.

Plexus choroideus participates in the formation of cerebrospinal fluid (CSF) and in the transportation of the substances from the blood, to the CSF and vice versa [13]. As a secretory source of vitamins, peptides and hormones for neurons, the choroid plexus provides substances for brain homeostasis [5]. Most blood vessels in the plexus choroideus are wide-calibers (approximately 15 μm) capillaries with thin fenestrated endothelial walls and bridging diaphragms overlying the fenestrations [9]. Light and dark choroidal epithelial cells were identified by Wislocki and Ladman [17] and they suggested that the difference in the cell density reflected different stages in the secretory cycle of the choroidal epithelium. Arginine vasopressin (AVP) decreases CSF formation rate and choroidal blood flow, and AVP also increases by more than twofold the number of dark epithelial cells and possibly dehydrated but otherwise morphologically normal choroid epithelial cells in adult rat choroid plexus [6].

Development of the choroid plexus has been studied with light microscope by Goldmann [4], Weed [16] and Kappers [7], with the transmission electron microscope by Tennyson and Pappas [15] and Davis, Lloyd and Milhorat [2], and scanning electron microscope by Chamberlain [1].

The purpose of this paper is to describe light and dark epithelial cells of the rat choroid plexus during development.

Materials and Methods

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 [18] and by intracardial perfusion [8]. The choroid plexuses were embedded in Durcupan and examined with light microscope Carl Zeis Jena and JEOL JEM 1200EX transmission electron microscope.

Results and Discussions

The most essential structural elements of the brain ventricles are developed before birth. The light and dark epithelial cells of the rat choroid plexus are present from aged 17 days postconception to 22 months. In our investigations of the rat choroid plexus we established the three periods of the development.

The light and dark epithelial cells possess through pseudostratified, low columnar and cubic during the first period of the development (17 days postconception — 30 days postnatum).The epithelial cells have electron-light cytoplasm with many glycogen granules and scanty cell organelles, concentrated at the apical part. There are many cytoplasmic protrusions, short and fine microvilli in the apical part of the epithelial cells. The most marked ultrastructural changes of the epithelial cells are many cytoplasmic protrusions, filled with glycogen, many vacuoles, granular endoplasmic reticulum and mitochondria with unformed cristae. The concentration of glycogen in the rat choroid plexus epithelial cells increased to 5 days postnatum and decreased at the 15 days postnatum. Similar ultrastructural changes are observed in the mouse choroid plexus epithelial cells [14]. 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. The main difference between *dark* and *light* epithelial cells was the density of the cytoplasm, nuclei and matrix of the microvilli (Fig. 1). Dark cells were uniformly denser than the light cells. A slightly increased concentration of osmiophilic droplets in the cytoplasm was observed in the dark cells. Observation of the plasma membranes of dark and light cells revealed membrane continuity and no differences in membrane structure. The dark cells have a much darker cytoplasm but organelles are generally similar to those of the light cells except that the dark cells seem to contain more ribosomes and rough endoplasmic reticulum. The microvilli of the dark cells are much thinner, and seem to be longer, than those of light cells.

On the basis of the ultrastructural 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 light and dark epithelial cells are cuboidal. The nuclei of the epithelial cells are rounded, located basally and have homogeneous chromatin. The large numbers of mitochondria are present, concentrated at the apical ends of the cells. These ultrastructural changes are evident for increased choroid

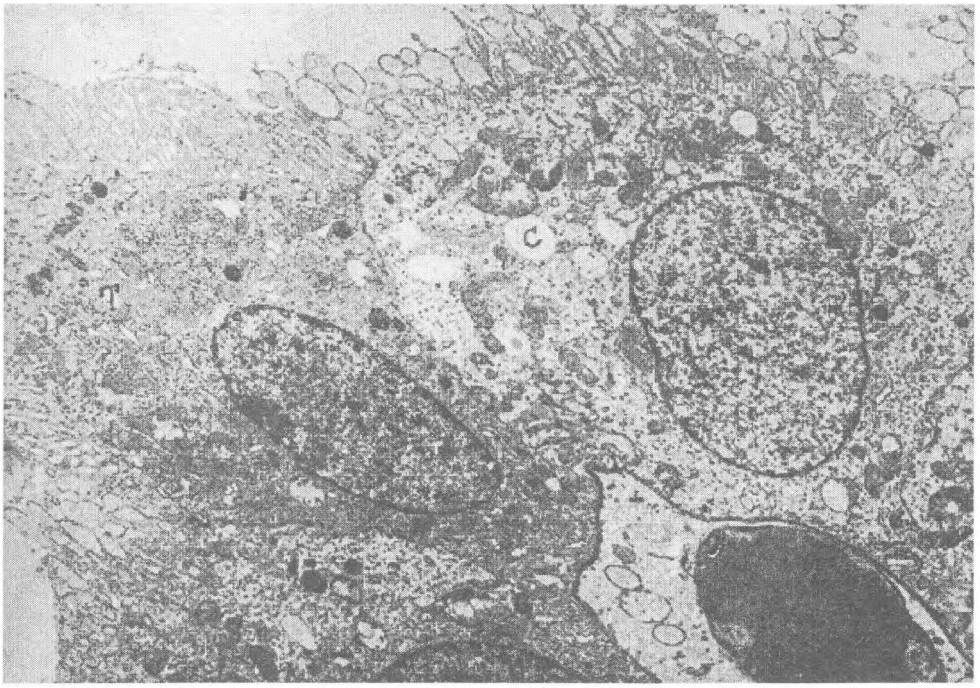

Fig. 1. Dark (D) and light (L) epithelial cells of the rat choroid plexus 45 days postnatum. The dark epithelial cell has more electron-dense cytoplasm and contains more polyribosomes and granular endoplasmic reticulum. × 3000

plexus functions of secretion, absorption and transport of the substances, which are necessary for cerebrospinal fluid homeostasis.

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. 2). The main morphological changes noted with age suggest a decrease in efficiency of choroid plexus cells in old age [5]. From morphometrical analysis of the rat choroid plexus during development in previous your investigations 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 [12]. The relative part of the dark epithelial cells increased during the whole period of development and after the age of 13 months remains higher (61.97%) than the relative part of the light epithelial cells (38.08%). This tendency concurs with ultrastructural data of decreased functional activity of the choroid plexus with age, and may be correlated with the age changes of the rat choroid plexus epithelial cells.

Conclusion

Plexus choroideus performs a multiplicity of functions for the central nervous system. Changes of the epithelial cells of the rat choroid plexus suggest that dark and light cells are modulations of the same basic cells with possible functional differentiation starting from fetal period of development through adult, and extending into terminal physiological stages.

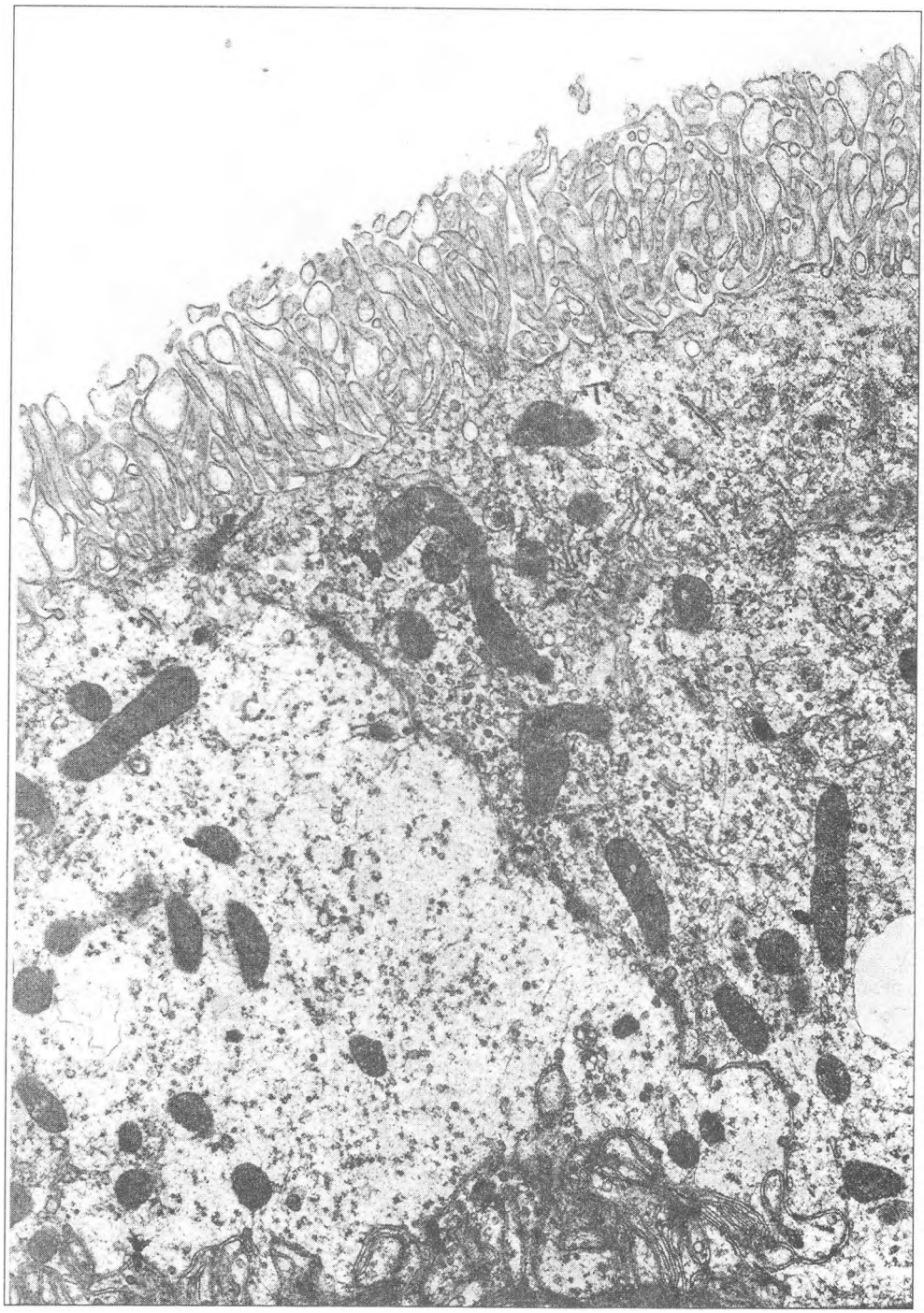

Fig. 2. Light (L) and Dark (D) epithelial cells of the rat choroid plexus aged 22 months. The cytoplasm of the dark epithelial cell contains more polyribosomes and granular endoplasmic reticulum. × 5000

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Ultrastructural characterization of Sertoli cells of Salmonidae from Ohrid Lake during the spermatogenetic cycle

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Ultrastructural characteristics of Sertoli cells of Salmonidae from Ohrid Lake during the spermatogenetic cycle have been analysed. Sertoli cells being an integral part of the seminiferous lobules underwent considerable changes, which influenced their cytomorphological features. The degenerative changes of Sertoli cells were manifested by an extreme vacuolisation, mitochondria in degeneration with widened crysts and thickened matrix, disorganised ER, autophagosomes, "myeline-like" structures and lysed cytoplasmic regions. The above mentioned changes were followed by karyopycnisis, complete degeneration and delamination of cells from the wall of the seminiferous lobules, lysis and detritus formations (Sertoli necrotic material) in the lumen of the lobules. The aim of this paper is special research of the ultrastructural characteristics, i.e. the changes on a level with testes which happen in the postspawning period in the two species of *Teleostei* of Ohrid Lake, Ohrid trout (*Salmo letnica* Kar.) and Ohrid belvica (*Salmothymus ochridanus* Steind.). The postspawning period is emphasized in *Teleostei* in this relatively short period, when one reproductive cycle finishes and the following has to start, on a level of testicular parenhyma very important histological changes are going on which give special histological identification, i.e. in the postspawning period there is a complete reorganization of the testes.

Key words: Sertoli cells, testes, Salmonidae, Ohrid Lake, spermatogenesis, ultrastructural characteristics.

Introduction

The number of authors having described the structural and functional characteristics of Sertoli cells in different Teleostei species is noticeable (Billard; Nicols & Graham; Gresik et al.; Dimovska et al.) [1, 10, 4, 2]. However, literature data about the changes in the postspawning period in different species of Teleostei, i.e. changes which occur immediately after the spawning, and even later, are less (Billard; Tavciovska-Vasileva; Tavciovska-Vasileva & Dimovska) [1, 12, 16]. The studies about

the annual reproductive cycle in natural and experimental condition in Salmonidae are also relatively (Hurk et al.) [6]. The Sertoli cells were analysed in the period after the spawning when their phagocytotic role was remarkable (Hurk et al.) [6]. The lack of literature data concerning the testis (Tavciovska-Vasileva & Dimovska) [16], especially the Sertoli cells as somatic components of the seminiferous lobules of testes of the two species of Salmonidae from Ohrid Lake (Tavciovska-Vasileva; Tavciovska-Vasileva & Rebok) [13, 14, 15, 17, 18, 19], has motivated this research. On the other hand, the two species of Salmonidae from Ohrid Lake were chosen as an object of research because of their big economic significance for the Ohrid Lake and due to the fact that they represent a relic and endemic species of this lake.

Material and Methods

Testes of 100 sexually mature male Salmonidae, i.e. 50 sexually mature male of Ohrid trout (*Salmo letnica* Kar.) and 50 sexually mature male of Ohrid belvica (*Acantholinqua ohridana*) caught in the Ohrid Lake were analysed by electronic microscopy. Small parts of testes, 1-2 mm, were used. The material was prepared using the following procedure: immediately after obtaining tissue specimens, they were fixed in 3% glutaraldehyde and then conserved in 0, 1 M phosphate buffer for 12 hours. After adequate fixation, the material was submitted to postfixation in 1% osmium tetraoxide (OsO_4). Further, the material was washed in phosphate buffer, dehydrated in series of acetone and uranil acetate, and then dehydrated in dry acetone. The tissue sections were infiltrated with Durcopan ACM mixture, mixture of acetone-Durcopan, Durcopan No 1, Durcopan No 2, fit in Durcopan No 2 and polymerised. For the ultrastructural analysis, ultrathin sections of 40-60 ηm were prepared using glass knives, on Reichert-Yung "Ultracut" ultramicrotome, installed on cooper nets and contrasted with uranyl acetate and lead citrate. The sections were observed on Tesla BS 500 and OPTON (Zeis) EM 109 electronic microscope. The microphotographs for electronic microscopy were obtained on Agfa Scientia EM Film 23056/6,5 × 9 cm, ORWO NP 20 panchromatic 120, Kodak 120 and made on Agfa papirtone Paper P1-3.

Results

In the period after the spawning the most important changes in testes of Salmonidae occurred on the level of Sertoli cells, being in the structure of seminiferous lobules as their somatic components. Compared to the period before spawning in which Sertoli cells were characterised with squamous appearance, as the process of involution of seminiferous lobules continued, in the period after the spawning they gradually lost the squamous form, increased their dimensions and acquired polymorphic nuclei. The presence of lipid vacuoles of different sizes was evident in their cytoplasm, especially well seen on ultrathin sections (Fig. 1). At an ultrastructural level a nucleus with prominent nucleolus could be seen in the Sertoli cells' cytoplasm (Fig. 2). On the surface of the nucleus there was a nuclear cover (Fig. 3). Mitochondria with lamellar and tubular crysts (Fig. 4), vesicles of SER and lysosomes could be observed (Fig. 5). Also, at an ultrastructural level, the cell membrane between the adjacent Sertoli cells (Fig. 6), the basal lamina of the seminiferous lobules themselves (Fig. 7), as well as interdigitations between the Sertoli cells were clearly noticed (Fig. 8). One of the functions of Sertoli cells is phagocytosis of the sperm residues. The pre-

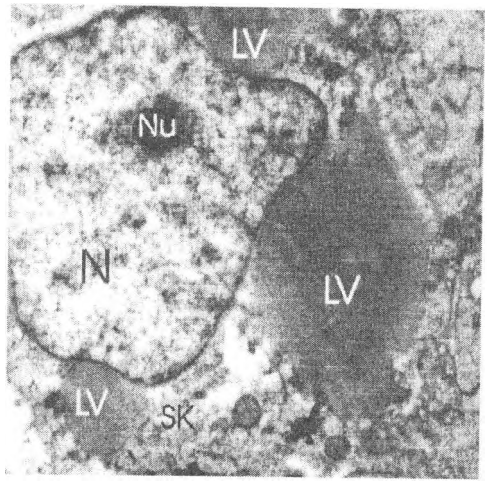

Fig. 1. A part of Sertoli cell (SK) with well seen nucleus (N) and nucleolus (Nu), presence of big lipid vacuoles (LV). Ultrathin section (× 7000)

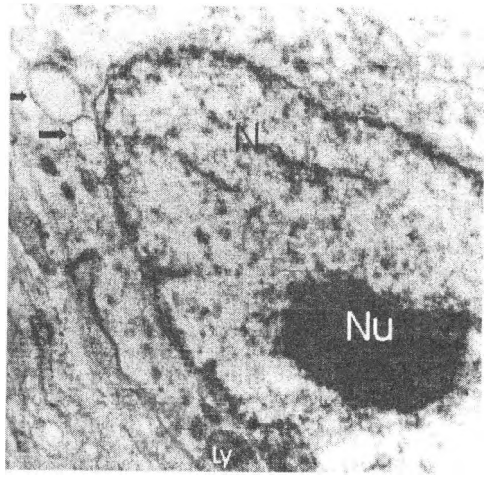

Fig. 2. A part of cytoplasm of Sertoli cell with well visible nucleus (N), prominent nucleolus (Nu), vesicles of SER (black arrows) and lysosomes (Ly). Ultrathin section (× 12 000)

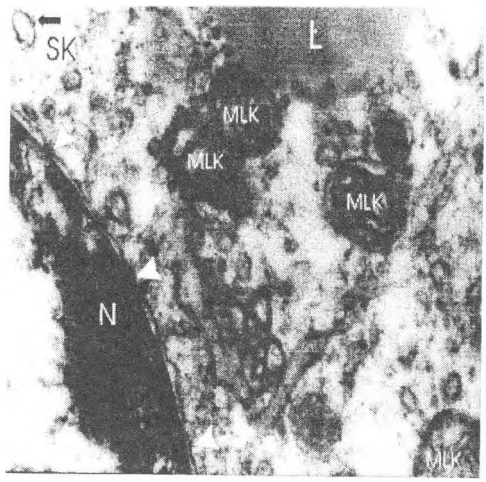

Fig. 3. Cytoplasm of Sertoli cell (SK) with mitochondria with lamellar crysts (MLK), vesicles of SER (black arrow), lipid droplets (L) and nucleus (N) with nuclear membrane on its surface (white arrow). Ultrathin section (× 20 000)

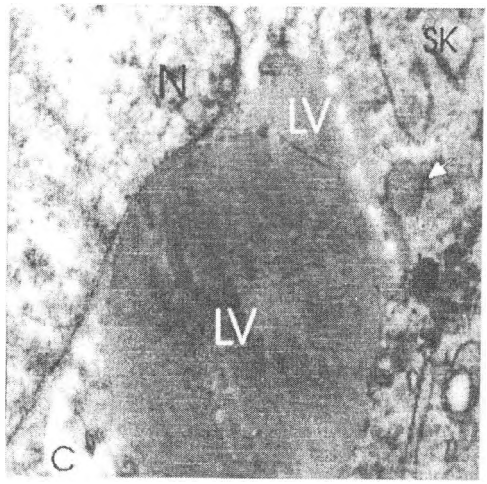

Fig. 4. A part of nucleus (N) and cytoplasm (C) of Sertoli cell (SK) with big lipid vacuoles (LV), mitochondria with tubular crysts (white arrow). Ultrathin section (× 12 000)

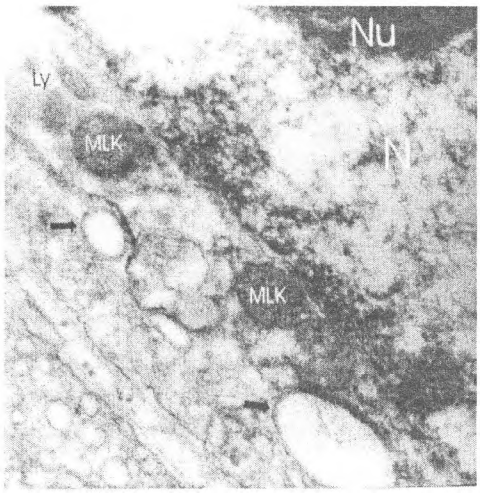

Fig. 5. A part of Sertoli cell with well visible nucleus (N), prominent nucleolus (Nu), mitochondria with lamellar crysts (MLK), vesicles of SER (black arrows) and lysosomes (Ly). Ultrathin section (× 20 000)

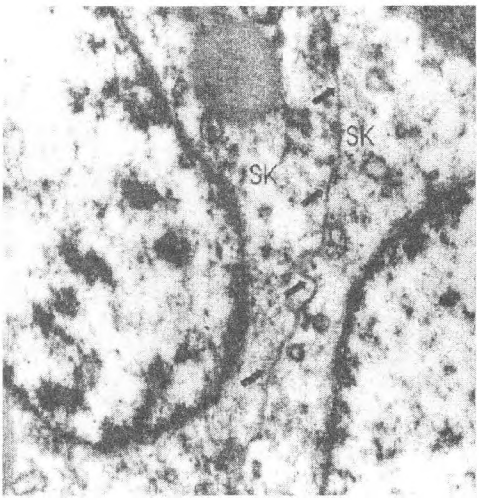

Fig. 6. Clearly visible cell membrane (black arrows) between two adjacent Sertoli cells (SK) Ultrathin section (× 12 000)

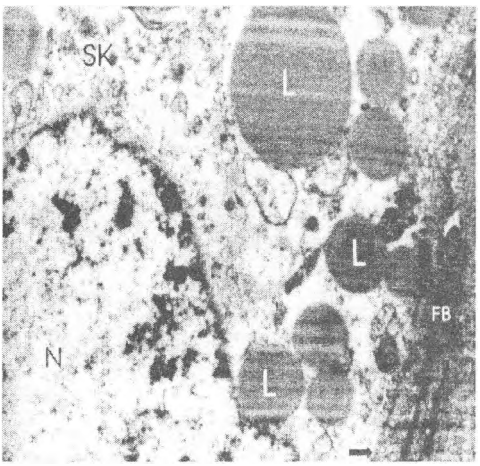

Fig. 7. A part of Sertoli cell (SK). Presence of lipid droplets (L) with different size and well visible nucleus (N). The basal lamina of the lobule (black arrow) and presence of one fibroblast (FB) near the basal lamina are visible. Ultrathin section (× 7000)

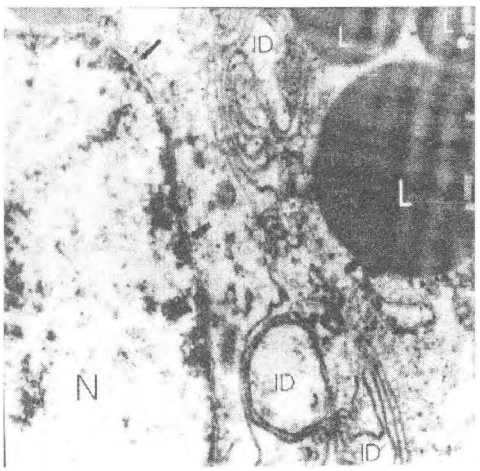

Fig. 8. Interdigitations (ID) between two adjacent Sertoli cells, lipids (L) in the cytoplasm and prominent nucleus (N) with well seen nuclear membrane (black arrows). Ultrathin section (× 12 000)

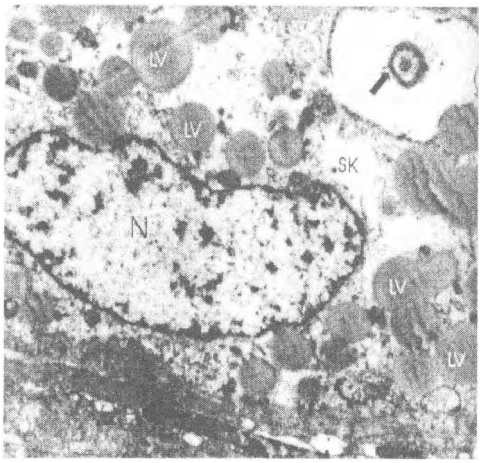

Fig. 9. A part of cytoplasm of Sertoli cell (SK) with well seen nucleus (N) and lipid vacuoles (LV) of different size. Presence of transversally cut fragments of flagellumes of sperm residues (black arrow) Ultrathin section (× 4400)

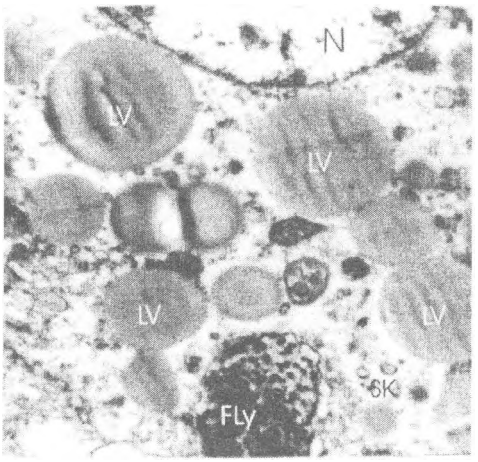

Fig. 10. A part of Sertoli cell cytoplasm (SK) with phagolysosomes (FLy) with sperm residual material. Presence of lipid vacuoles (LV) of different size and a part of nucleus (N) of the Sertoli cell are also visible. Ultrathin section (× 12 000)

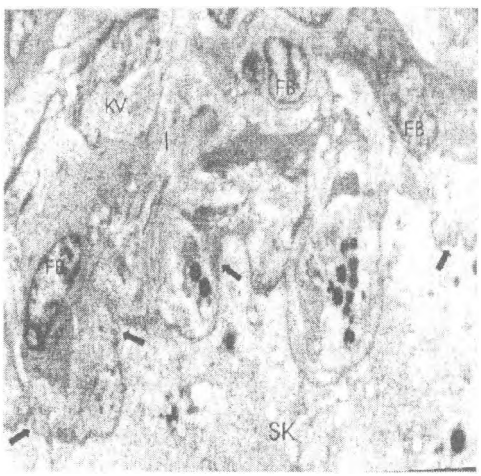

Fig. 11. Well distinguished interstitium (I) with fibroblast (FB) and colagenous fibers (KV). A part of Sertoli cell (SK) cytoplasm in degeneration, is seen, as well as the basal lamina (black arrow) of the lobule. Ultrathin section (× 3000)

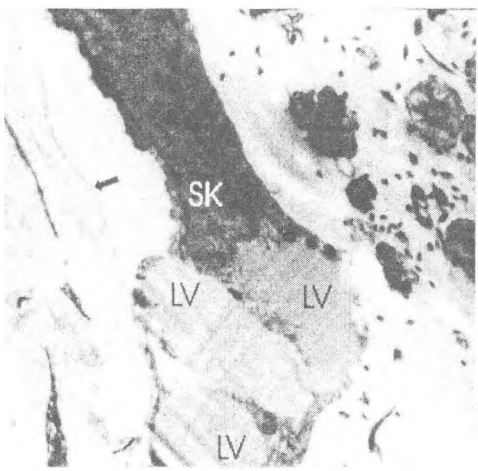

Fig. 12. Sertoli cell (SK) in degeneration. Presence of lipid vacuoles (LV) in the cytoplasm and separation of cytoplasm from basal membrane (black arrow) are visible. Ultrathin section (× 4 400)

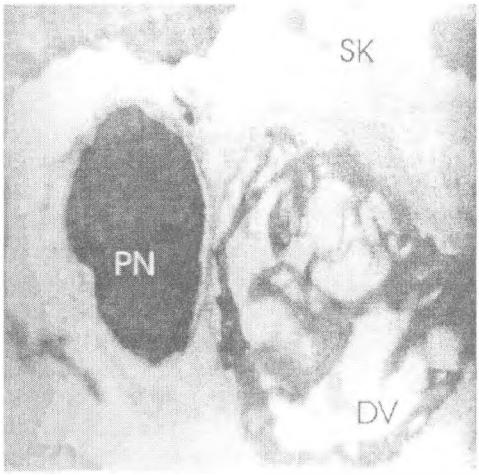

Fig. 13. A part of cytoplasm of Sertoli cell (SK) in degeneration with a pycnotic nucleus (PN) and a digestive vacuole (DV). Ultrathin section (× 12 000)

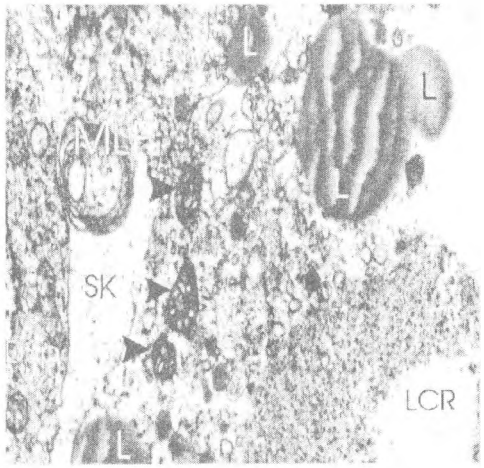

Fig. 14. A part of cytoplasm of Sertoli cell (SK) in degeneration, with lysosomes with "myeline-like" figures (MLF), lysed cytoplasmic regions (LCR), mitohondria in degeneration (black arrows), lipid droplets (L) with different size. A part of one spermatogonium in degeneration (DSp) is shown. Ultrathin section (× 8000)

sence of transversal cut fragments of flagellumes of sperm residues in the cytoplasm of Sertoli cells (Fig. 9) or phagolysosomes with already digested material of sperm origin (Fig. 10) supported this fact. In the later phase of the life cycle of Sertoli cells a more distinct vacuolisation of their cytoplasm could be observed, which caused a degeneration of these somatic cells, characterised by karyopycnosis. The final phases of Sertoli cells' life cycle were followed by exfoliation from the wall of the seminiferous lobules, disintegration and complete destruction of the cells, presence of detritus, i.e. residues in the lumen of the lobules, as well as lysis. Disintegration and destruction of some Sertoli cells which are manifested with torn cell borders, presence of vesicular nucleus or nucleus in pycnosis with emphasized hyperchromatic characteristics, undifferentiated nucleolus were evident on ultrathin sections (Fig. 11). The degeneration of the Sertoli cells was followed by detachment of the nuclear membrane, a process which was well distinguished at an ultrastructural level (Fig. 12). In the cytoplasm of Sertoli cells in degeneration, excluding the presence of pycnotic nucleus, digestive vacuoles, i.e. autophagosomes were noticed, indicative for autophagia occurring on the level of these cells (Fig. 13). On ultrathin sections the degeneration of Sertoli cells was demonstrated by a presence of lysosomes with "myeline-like" figures in their cytoplasm, endoplasmic reticulum in disorganisation, mitochondria with initial signs of degeneration, with widened crysts and thickened matrix, chyaloplasm with granular structure and lysed cytoplasmic regions (Fig. 14). All these changes occurring on the level of Sertoli cells showed their degeneration in the period after the spawning.

Discussion

The ultrastructural analysis of testes of Salmonidae from Ohrid Lake during the spermatogenetic cycle showed certain features which provided a characteristic histological picture of testes in this period. In the period after the spawning visible changes on the level of seminiferous lobules, especially in the Sertoli cells were observed. All these changes occurred successively. In the initial phase of the period after the spawning sperm residues were still present in the lumen of seminiferous lobules. As changes progressed, degeneration of Sertoli cells took place. The mentioned changes, especially those which happened in the final phase of the period after the spawning, at a sufficient extent, changed the histoarchitectonic of the testes, in comparison with the period before spawning. On the basis of consequent characteristic changes which happened on the level of the testes in the period after the spawning in Salmonidae from Ohrid Lake, we can concluded that this was a period of regeneration of the testes. The seminiferous lobules underwent important transformations in the period after the spawning. As a somatic component of the seminiferous lobules Sertoli cells suffered significant degenerative changes which caused their involution, i.e. involution of seminiferous lobules themselves. This process in Salmonidae repeats every year. The seminiferous lobules and the Sertoli cells themselves, in Salmonidae, are not constant elements of testes, but temporary formations which are formed every year after the spawning. The findings of this study confirmed our preliminary investigations (Tavciovska-Vasileva; Tavciovska-Vasileva & Dimovska; Tavciovska-Vasileva & Rebok) [13, 14, 15, 16, 17, 18, 19] on changes which happen on the level of testes of Salmonidae from Ohrid Lake, i.e. collapsing and disintegration of the lobules, degeneration, i.e. involution of the Sertoli cells, ect. This process was also noted in other Teleostei (Turner; Tavciovska-Vasileva) [20, 12]. Therefore, our results support the difference between mentioned species and mammals, where seminiferous lobules or tubules are constant elements of the testes. There are literature data for different Teleostei species which point out the presence of degenerative changes of Sertoli cells during the period after the spawning. After phagocytosis of the residual bodies by Sertoli cells, they later suffer lipid degeneration, i.e. involution. So, in *Perca Flavescens* Mitch. an involution of the seminiferous tubules in the period after the spawning was described, which in an idirect way points to involution of Sertoli cells, as a unique somatic component of the tubules in this period (Turner) [20], while *Perca fluviatilis* L., (Kulaev) [7] concretely points to some degenerative changes which happen with Sertoli cells in the period after the spawning. Also, similar statements were given about the fate of the Sertoli cells after the finished sexual cycle with *Perca fluviatilis macedonica* Kar. by Dimovska et al. [2] and Tavciovska-Vasileva [12]. After the expulsion of sperm cells in the lumen of tubules, in several species of Teleostei, Sertoli cells suffer lipid degeneration, and probably, finaly are resorbed (Nagahama et al.) [9]. Similarly, it was pointed out that in *Cymatogaster aggregata*, many Sertoli cells suffer degeneration (Gardiner) [3]. The degeneration of Sertoli cells in some species of Atheriniformes, as *Poecilia reticulata* was also described (Billard) [1]. According to Turner [20], the genesis of seminiferous tubules in Teleostei during their embryonic development is similar to that in mammals, but it happens only once in their life, while in Teleostei it repeats every year with the new reproductive cycle. Recently the phenomenon of the life cycle of Sertoli cell has been noted by other authors, not only with Teleostei, but in other low Vertebrata as well (Lofts) [8]. However, the fact is that a small number of authors have dealt with this problem. Relatively few authors have treated the changes which happen immediately after the spawning, and later (Billard, 1970; Tavciovska-Vasileva, 1992;

Tavciovska-Vasileva & Dimovska, 1997) [1, 12, 16]. Our investigation in Salmonidae from Ohrid Lake pointed out that directly after the spawning, similarly to other examined Teleostei, an intensive phagocytosis of sperm residues by Sertoli cells took place. The phagocytic activity of these somatic elements of seminiferous lobules was accompanied at the same time by numerous changes which reflected upon their cytomorphological appearance. Namely, in the prespawning period Sertoli cells are characterised with squamous appearance, whereas in the postspawning period they gradually lost the squamous form and increased their dimensions. The presence of increased number of vacuoles of different size was evident in their cytoplasm. Close to or in contact with these Sertoli cells, as in their cytoplasm numerous sperm residues were edident. In favour of this fact was the presence of transversally and longitudinally cut fragments of flagelumes of sperm residues in the cytoplasm of these cells, later its lysis, which indicated the phagocytotic role of these somatic elements of the seminiferous lobules during this period of the year. In other species of Salmonidae the phagocytotic activity of Sertoli cells in the period after the spawning was reported in *Salmo gairdneri* by Hurk et al. [6]. The phagocytotic activity of Sertoli cells was demonstrated also by the ultrastructural findings of Grier [5]. Gresek et al. [4] noticed presence of philopodia and residual bodies on the level of Sertoli cells in the period after the spawning in *Oryzias latipes*. The presence of philopodia and residual bodies of Sertoli cells has been also pointed out in Poecilidae, *Poecilia latipina* (Pudney & Callard) [11]. The presence of philopodia in Sertoli cells of different species of Teleostei in the period after the spawning was reported in *Cyclostoma nigrofasciatum* (Nicholls & Graham) [10], in *Cymatogaster aggregata* (Gardiner) [3]. In Salmonidae as *Oncorhynchus kisutch* and *Oncorhynchus gorbuscha* the presence of philopodia on a level of Sertoli cells was determined by Nagahama et al. [9].The phagocytotic activity of Sertoli cells in Salmonidae from Ohrid Lake is characterised by subsequent considerable cytological changes, manifested by intensive vacuolisation of the cytoplasm, lipid gegeneration, karyopycnosis, total destruction and delamination, presence of their residues in the lumen of the seminiferous lobules, as well as its lysis, mitochondria with disintegrated crysts, autophagosomes, "myeline-like" structures. All these structural changes point out the degeneration of these somatic cells, i.e. these changes cause their involution and with that the involution of the seminiferous lobules themselves. In other species of Salmonidae similar statement concerning the definitive fate of Sertoli cells in the period after the spawning was given by Hurk et al. [6]. In their study which concerns the testes of *Salmo gairdneri*, Hurk et al. [6] pointed out that in the period of intensive phagocytic activity some Sertoli cells which separate from the wall of the seminiferous lobules could be observed, which cause their degeneration.

Conclusions

The successive cytological changes based on ultrastructural findings in some regions of testes of Salmonidae from Ohrid Lake during the spermatogenetic cycle, with a special emphasis of Sertoli cells, can be defined like this:

1. Sertoli cells as an integral part of seminiferous lobules suffered considerable changes, changing their cytomorphological aspect. Namely, out of cells with squamous appearance characteristic for the period before the spawning, they gradually increased their dimensions. Lipid vacuoles of different size can be noticed in their cytoplasm while the nuclei acquired a polymorphic form.

2. The close contact of Sertoli cells with the sperm residues, as well as the presence of fragments of their flagellumes in the cytoplasm of Sertoli cells, showed their phagocytic activity.

3. The degenerative changes of Sertoli cells were manifested by extreme vacuolisation, mitochondria in degeneration with widened crysts and thickned matrix, disorganised ER, digestive vacuoles (autophagosomes), "myeline-like" structures and lysed cytoplasmic regions. The above-mentioned changes were followed by karyopycnosis, complete degeneration and delamination of the cells from the wall of the seminiferous lobules, their detritus (Sertoli necrotic material) in the lumen of the lobules and its lysis.

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Enzyme histochemical expression of lipoprotein lipase and localization of mast cells in the paranal sinus in sexually mature and immature dogs

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In this histochemical study, we established for the first time the expression of lipoprotein lipase, lipids and their relationship with mast cells localization in the canine paranal sinus. Intensive enzyme reaction for LPL was present in the cytoplasm of some cells in all layers of the stratified squamous cornified epithelium, in some cells of apocrine and sebaceous glands of PS wall in both sexually mature and immature dogs. LPL localization was also observed in the stroma.

Mast cells were observed in vicinity of the stratified squamous cornified epithelium, the apocrine and sebaceous glands of the studied organ. These structures exhibited positive reaction for both lipids in the Sudan III staining and for LPL activity. The expression of LPL, single lipid deposits of a various size and in some instances mast cells were observed in the three layers of blood vessels, supplying with blood the paranal sinus. In the microcirculation bed, LPL activity and mast cells were also shown.

In this study, there were individual particularities in LPL expression on the paranal sinus, but not such related to age or gender (sexual dimorphism).
An attempt to explain the relationship between LPL expression, lipids and mast cells localization in the PS wall was performed. This was probably related not only to the organ function, but also with the development of pathological processes within.

Key words: lipoprotein lipase, paranal sinus, dog.

Introduction

The role of mast cells in binding both lipoprotein lipase (LPL) (van Tilbeurgh et al., 1994, Kokkonen and Kovanen, 1987) and low-density lipoproteins (LDL) (Kokkonen and Kovanen, 1990) is acknowledged. This allowed some investigators to assume the involvement of these cells in the development of atherosclerosis (Ma and Kovanen, 1997). The relatively high amount of mast cells observed in canine PS

(Stefanov and Vodenicharov, 2007) is probably important not only for the normal function of the organ, but also for the development of various pathological states. Lipoprotein lipase (LPL) is the primary lipolytic enzyme, involved in the intravascular metabolism of lipoproteins (Goldberg I J, 1996). The major quantity of LPL in the body is localized in the capillary endothelium. A small amount is detected in the arterial endothelium and that part is believed to participate in atherogenesis (Zilversmit, 1973). A subendothelial localization of the enzyme in the arterial intima is reported. Theoretically, LPL of arterial intima could originate from circulating LPL or from the local synthesis of various cells of the intima. According to some authors, LPL is produced by macrophages and smooth muscle cells (Yla-Herttuala et al., 1991). This enzyme is synthesized and secreted in a catalytically active form by adipocytes and myocytes. Then, it is transported to the capillary endothelial surface. Its physiological role to hydrolyze triglycerides from chylomicrons, very low density lipoprotein (VLDL) and intermediate density lipoprotein (LDL) particles 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 (Merkel et al., 1998). LPL activity was investigated in adipose tissue in birds (Sato and Akiba, 2002) and cats (Backus et al., 2001), the heart muscle in mice (Liu et al., 2008) and sheep (Boonet et al., 2000), mammary gland in mice (Jensen, et al., 1991), canine skeletal muscle (Budohoski, 1985), rat skin (Ma and Kovanen, 1997). Montagna and Parks (1948) found out only traces of lipase in the apocrine cells and the sebum. The granules of mast cells have reacted positively. The authors did not provide detailed information about the type and mode of action of enzyme.

In the available literature, there are no data with regard to LPL expression and distribution in the paranal sinus.

The purpose of the present study was to establish the relationship between LPL activity and mast cells in canine PS.

Material and Methods

In this study, the paranal sinuses of 7 male and 7 female healthy mongrel dogs at the age of 2 months to 6 years were used.

Immediately after the euthanasia of the dogs, specimens from PS 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 is based upon the Tween method consisting in the deposition of insoluble calcium soaps at the sites of enzyme activity. They 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 and mast cells were detected on cryostat cross-sections by means of histochemical reaction with Sudan III (Feinchemie KG. Sebnitz, Germany) according to Daddy technique, replacing haematoxylin with toluidine blue with pH 3. Semi-thin cross-sections of 1 μm were stained with toluidine blue (pH 3) and Azur II.

Results

An intensive enzyme histochemical expression for LPL was observed in the cytoplasm of most cells of all layers of the stratified squamous cornified epithelium of the sinus both in adult and sexually immature dogs (Fig. 1). The highest number

of reacted cells was observed in the basal layer and their amount decreased in the direction of stratum corneum. Clusters of granules with a various size were mainly observed in the apical part of LPL-positive apocrine glandular cells (Fig. 2). 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 (Fig. 3). 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. In some dogs from both genders, LPL expression was present only in single cells of apocrine and sebaceous glands whereas others exhibited reaction in a considerable part of cells of these structures.

Mast cells were localized in the stroma, adjacently to lipid droplets, mainly near the stratified squamous cornified epithelium of the sinus, around the tubules of apocrine glands and around the acini of sebaceous glands. Less frequently, mast cells were detected among the acini in glandular complex. Single intraepithelially located mast cells (1-2 mastocytes) were observed only in some glandular tubules (Fig. 3), as well as in some acini of sebaceous glands (1-2 mastocytes).

Various-sized lipid droplets were found out at some sites in the stroma as well as in a major part of apocrine glands cells (Fig. 3). These glands exhibited a weak to moderate reaction for lipids. In some individuals, only single cells of apocrine glands were positive whereas in others, many secretory cells have reacted. In the cornified epithelium, the reaction for lipids was weak to moderate at some sites. A strong reaction was exhibited in sebaceous glands and their excretory duct, as well as in the lumen of the duct and the sinus lumen.

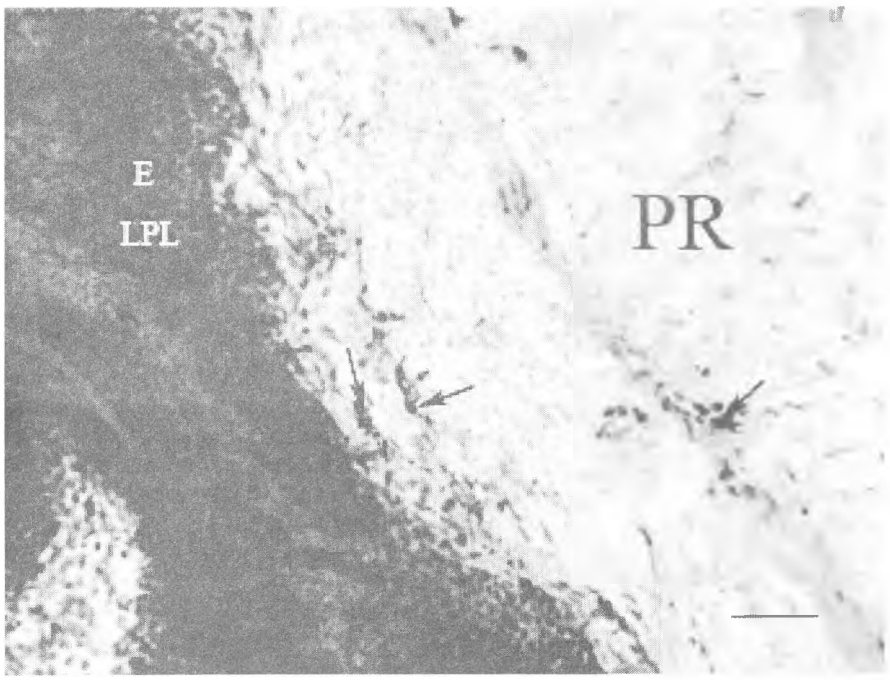

Fig. 1. Localization of (LPL) in all layers of the cornified epithelium (E), including the cornified layer. Some of cells in the propria (PR) showed a positive LPL reaction. Magnification × 100 (bar = 50 μm)

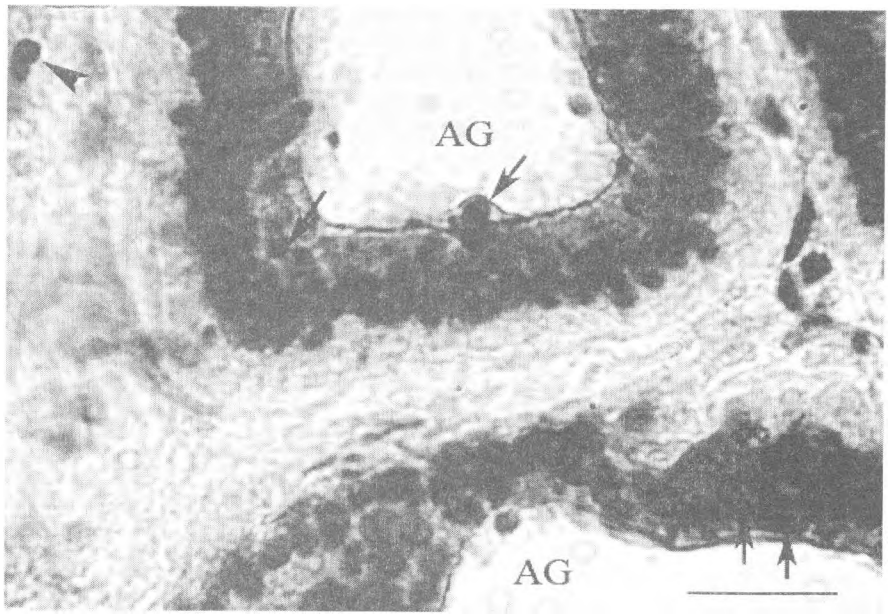

Fig. 2. Localization of LPL activity in the apical part of some secretory cells (arrows) of apocrine glands (AG), as well as in the interstitium (arrowheads). Magnification × 400 (bar = 20 μm)

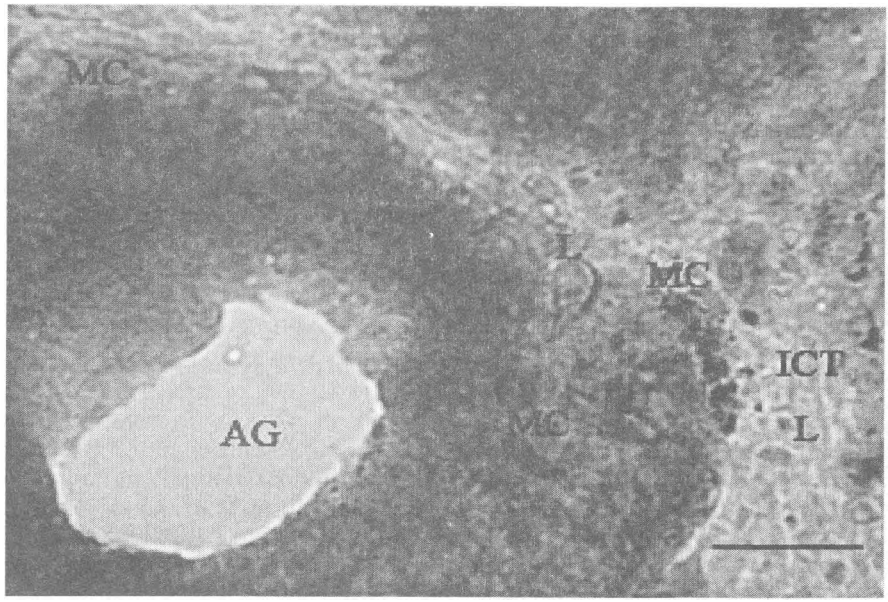

Fig. 3. Localization of lipid droplets (L) in the cytoplasm of some cells of the apocrine glands (AG) and in interstitial connective tissue (ICT). Mast cells (MC) are situated in ICT, near the apocrine glands, and single cells — intraepithelially as well. Sudan III and toluidine blue (pH 3) staining. Magnification × 400 (Bar = 20 μm)

In the blood vessels supplying PS with blood, the pattern of LPL localization in the three vascular wall layers was irregular and appeared as single brown granules with a round irregular 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. Mast cells were also detected in the three layers of some blood vessels. In the microcirculation bed, mastocytes were localized in the vicinity of capillaries, arterioles and venules. LPL activity was observed in apical part capillary endothelium. Single reaction deposits were present in the endothelium, the cells of tunica media and in tunica externa of arterioles. There were individual but neither age- nor gender-related particularities in the expression of LPL in the paranal sinus in dogs.

Discussion

The localization of LPL in PS stroma under the form of various-sized reaction deposits observed by us, could be explained by the ability of this enzyme to bind to proteoglycans of the extracellular matrix, similarly to arterial intima. This could result in LDL retention and consequently, its modification in these structures (Jonasson, 1987, Yla-Herttuala et al., 1991, Pentikainen et al., 2000). There are data that collagen-bound proteoglycan decorin could bind LPL and collagen (Pentikainen et al., 2000). The dimeric structure of LPL provokes an increased affinity to heparin, because both monomers could participate in binding one molecule heparin (van Tilbeurgh et al., 1994). Both components of mast cell granules, the proteases and the heparin proteoglycan act simultaneously to promote LDL uptake by macrophages in vitro (Kokkonen and Kovanen, 1989). The binding of LDL to mast cell granules is performed through the interaction of the apolipoprotein B component of LDL and VLDL with the heparin proteoglycan component of the granules. Secretory granules exocytosed from rat serosal mast cells bind LDL, and on being phagocytosed by macrophages, carry the bound LDL into these cells. LDL is bound to the heparin proteoglycan component of the exocytosed granules whether they are expelled into the free extracellular space or remain associated with the mast cells. The proteolytic degradation of the granule-bound LDL results in its modification such that large fused LDL particles are formed on the granule surface. phagocytosis, by macrophages, of the granules containing fused LDL particles leads to lysosomal degradation of LDL and cholesterol accumulation in macrophages as nonmembrane-bound cholesteryl ester droplets, typical of foam cells. Cholesterol is accumulated in mast cells under the form of large, partially degradated and modified LDL particles, bound to granules (Kokkonen and Kovanen, 1990). Ma and Kovanen (1997) found out that degranulation of mastocytes induced transendothelial transport and the local accumulation of LDL in rat skin. Therefore, mast cells, being an important component of canine PS stroma (Stefanov and Vodenicharov, 2008), could possess a similar activity and this could help to elucidate their role not only in lipid metabolism, but in the pathogenesis of illnesses of this organ too. The cellular and extracellular localization of lipid droplets and LPL in vicinity of mast cells, observed by us in the various structures of the paranal sinus, confirm this view of ours. The intraepithelial localization of mast cells in PS, evidenced also by using semi-thin sections, could be probably related to lipid metabolism and determined their role for physiological and pathological events in this organ. These data are corresponding to the intraepithelial localization of mast cells in the epithelium of porcine ureter, reported by Vodenicharov et al. (2005).

On the basis of data obtained in this study, we presumed a role of LPL through LPL in the pathogenesis of the frequently encountered diseases of the paranal sinus. LPL also bridges native and modified lipoproteins to heparan sulfate at the cell surface and thus facilitates the uptake of lipoproteins by intimal cells (Ory, 2007). LPL provokes a selective uptake of cholesterol from LDL — a process that requires cell surface proteoglycans but that is not dependent on lipoprotein receptors and LPL activity (Seo T et al., 2000; Pentikainen et al., 2001). On the basis of observed LPL expression in the PS wall, we hypothesize that in this organ too, the utilization of cholesterol from LDL is also possible taking into consideration of LPL ability to bind cell surface proteoglycans. The hydrolysis of VLDL by LPL results in formation of free fatty acids that increase the permeability of arteries to LDL, and consequently, causing LDL entry and retention (Rutledge et al., 1997).

In this study, the localization of LPL activity in the three layers of the vascular wall — the subepithelial connective tissue and the intimal endothelium, in tunica media and tunica externa of arteries was found out to be irregular. Single lipid deposits of a various size, and in some instances mast cells were also present in the three layers of blood vessels. In the microcirculation bed, mast cells were localized adjacently to capillaries, arterioles and venules. LPL activity was also observed in the luminal surface of the endothelium of capillaries and arterioles. Single reaction deposits were detected in the cells of tunica media and in tunica externa of arterioles. The data of the present study show that there was a relationship between mast cells, lipids and LPL (van Tilbeurgh et al., 1994; Kokkonen and Kovanen, 1990), all of them exerting their effect upon the various PS structures. LPL plays an important role in the metabolism of chylomicrons, involved in the transport of dietary lipids, as well as in the metabolism of VLDL and LDL, involved in the transport and metabolism of endogenously synthesized lipids (Smithe et al., 1998, Ory, 2007). It could be therefore assumed that exogenous lipids could influence the lipid content of sebum in canine sinus, similarly to events reported in human skin sebaceous glands (Smithe et al., 1998).

Montagna and Parks (1948) established expression of fatty acids in the apical part of tall columnar apocrine cells, in the cells and the secretion of sebaceous glands and in stratum corneum of the lining epithelium of the duct. According to them, no reaction for lipids was present in Sudan III staining as well as no cholesterol in apocrine gland cells, whereas the main amount of lipids was synthesized in the cells of sebaceous glands. Unlike these authors, we observed a positive reaction for lipids in the cells of the stratified squamous cornified epithelium, sebaceous glands, and to a lesser extent, in apocrine glands cells and the stroma in Sudan III staining. It is proved that the skin and skin glands were important sites for de novo lipid synthesis in primates and rats (Feingold et al., 1982, 1983). It is known that apocrine and sebaceous glands in human skin dermis secrete lipids, mainly triglycerides, fatty acids, cholesterol and its esters. Low density lipoprotein receptor (LDL R) and LPL were also detected in skin apocrine and sebaceous glands. These data demonstrate that these glands could release endogenous cholesterol and fatty acids and that this could be important for understanding both acne and axillary odour (Smithe et al., 1998). By means of the observed LPL localization in the cells of apocrine, sebaceous glands and the lining epithelium of PS, we support the opinion of some investigators, affirming that in most cells, cholesterol is probably synthesized de novo or endogenous cholesterol under the form of LDL is uptaken by LPL and glycosaminoglycans (Smithe et al., 1998; Ory, 2007). Via regulating the metabolism of lipoproteins, LPL enhances the extracellular accumulation of lipids (Pentikainen et al., 2001). We share a similar opinion, assuming that this pathway is important for

lipid accumulation in LPL-synthesizing cells. Our data allowed believing that the presence of lipids in the secretion of PS apocrine glands was probably related to the control of its strong odour.

It could be speculated that the observed localization of LPL in canine PS could be a result from synthesis in the studied organ. Furthermore, LPL is able to bind to glycosaminoglycans both on cell surface and of the extracellular matrix. This way, LPL could penetrate through the vascular wall (Jonasson, 1987; Yla-Herttuala et al., 1991; Pentikainen et al., 2000) and to occur in PS structures.

The present investigation provided evidence that in the paranal sinus, there was an intensive enzyme histochemical expression of LPL and lipids i both sexually mature and immature dogs that could be attributed to the main function of the organ. Taking into consideration the results of our study, a possible relationship between LPL and lipids expression, on one hand, and the localization of mast cells in PS, on the other, could be assumed. This was probably important not only for the course of physiological, but for the pathological events in this organ.

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Gougerot—Carteaud confluent and reticulated papillomatosis

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Gougerot—Carteaud confluent and reticulated papillomatosis is a dermatosis due to a genetically determined keratinization defect. It is a rare disease with typical histopathologic findings of hyperkeratosis, papillomatosis and modest epidermic acanthosis. We report two patients suffering from confluent and reticulated papillomatosis, presented with extensive lesions of symmetrically disposed plaques formed by the confluence of grayish-brown rough papules, distributed on the skin of the trunk and upper extremities. In both cases the histological examination was very important for establishing the proper diagnosis. We began treatment with topical calcipotriol and obtained remission that persisted during the control visits.

Key words: confluent and reticulated papillomatosis, Gougerot—Carteaud syndrome, histopathology, hyperkeratosis, therapy.

Introduction

Gougerot—Carteaud confluent and reticulated papillomatosis (CRP) is a rare disease due to a genetically determined keratinization disorder. There are reports of familial cases where two or more members of the same family were affected [7], but sporadic cases are more frequently described. Female are more frequently affected with a peak incidence during the pubertal period.

CRP lesions are usually persistent, asymptomatic, verrucous papules with a tendency to coalesce. Previously they were interpreted as pseudo acanthosis nigricans, but now it is a separate nosological entity with not well defined etiopathogenesis and treatment [4]. Histopathologic examination of the skin efflorescence is characteristic and shows hyperkeratosis, papillomatosis and modest epidermic acanthosis. Therapeutic opportunities include systemic antibiotics, retinoids, topical tazarotene, tacalcitol, or calcipotriol. Dermoabrasion, UVA phototherapy and laser treatment are also proposed [2, 5, 9].

We describe two cases of this rare disease CRP where the microscopic findings played a very important role for establishing the diagnosis.

Materials and Methods

A 16-year-old white female presented with a ten months history of a skin eruption of papules with a rough verrucous surface. They were localized on the nuchal region and forearms. They had no enlarged in size but had a tendency to coalesce. A slight scratch with fingernails produced a fine powdery material.

The other case was an 11-year-old white female presented plaques symmetrically disposed on the skin of the abdomen and upper extremities. The lesions consisted of grayish-brown rough papules, measuring some millimeters in diameter and had first appeared one year before. She had neither pruritus nor ache. The patient had no other compliances except the bad aesthetic view. She was treated with emollients and cold cream but only improvement of the skin mildness was achieved.

Laboratory investigations including complete blood counts, erythrocyte sedimentation rate (ESR), blood sugar, hepatic and renal function tests, serum electrolyte levels, lipid analysis and urinalysis, were performed. Skin biopsies of the papules were obtained. They were fixed in 10% neutral-buffered formaldehyde solution, processed routinely, and stained with hematoxylin-eosin.

Results

We formulated the diagnoses of CRP in both cases on the basis of the clinical picture, the histological findings, evolution of the disease and the age of the patients. The physiologic and pathologic anamnesis gave negative results and laboratory investigations did not reveal any alterations worthy of note. Wood's lamp observation showed no fluorescence. The histological examination evidenced the following alterations of the epidermis: hyperkeratosis, papillomatosis, mild acanthosis and the absence of melanocyte hyperplasia. Periodic acid- Schiff reaction did not evidence the presence of hyphal cells or mycetes (Fig. 1). The second case showed mild hyperkeratosis, mild acanthosis and almost an absence of papillomatosis, no spongio-

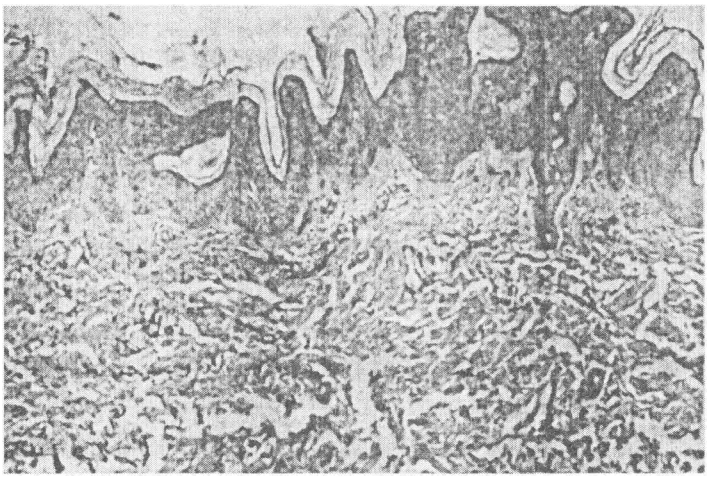

Fig. 1. Hyperkeratosis, papillomatosis, modest acantosis and absence of melanocyte hyperplasia. Hematoxylin-eosin stain × 100

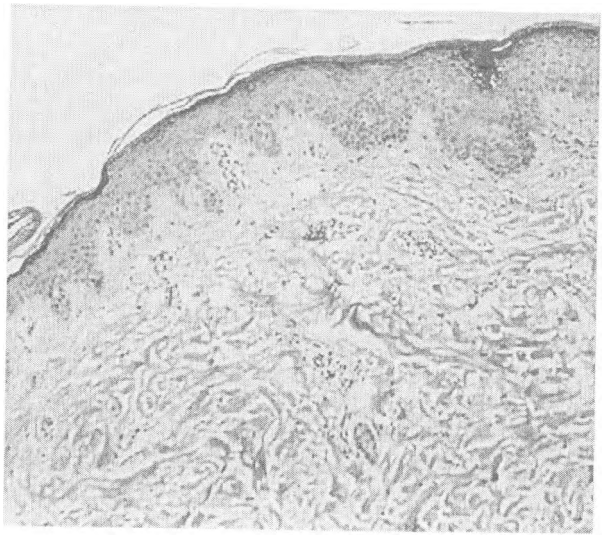

Fig. 2. Mild orthohyperkeratosis, acanthosis and almost absence of papillomatosis. Hematoxylin-eosin stain × 100

sis or significant inflammatory infiltrate (Fig. 2). These findings were very important to make the proper diagnosis as there were some differential diagnoses including acanthosis nigricans, Darier's disease, follicular hyperkeratosis, pityriasis versicolor and epidermodysplasia verruciformis. We began treatment with topical calcipotriol 0,005% applied twice daily for a two months period. On control visits performed at 1 and at 2 months no relapses were evident and a complete resolution was reached.

Discussion

Gougerot—Carteaud CRP is a rare dermatosis, which has a unique clinical feature and shows hyperkeratosis and papillomatosis histologically. This disease was first described by Gougerot and Carteaud in 1927 [6]. It is characterized by persistent, asymptomatic, verrucous papules that have a tendency to coalesce. The intermammary region is ussualy affected first with subsequent spread to the breast and abdomen. The interscapular area, neck and axilla also may be involved. The eruption often begins during puberty and more commonly affects women. Endocrine-metabolic alterations, an anomalous reaction of the host to colonization by the Pityrosporum orbiculare, a keratinization disorder either genetically determined or induced by toxic substances produced by unidentified agents, an early form of cutaneous amyloidosis or an anomalous epidermic response to UV rays with consequent aberrant keratinization, have been reported in an attempt to explain the pathogenesis of CRP [1].

Both evidence of papillomatosis clinical and histological has been pointed as important to achieve the diagnosis of CRP. These findings are presented in our first patient, the 16-year-old one. Almost the lack of papillomatosis in biopsy specimen from the lesions of the skin of our 11-year-old patient is interesting and unexpected.

We think that the reason for this is that this girl clinically had not verrucous surface of the eruption.

In our cases, the distribution of the lesions and histopathologic findings are consistent with those of CRP. One of the most important sign for the disease in histological examination is hyperkeratosis. In a recent publication an electronic microscopic study showed a marked increase in the number of lamellar granules in the granular layer [8]. This finding is interesting to speculate the pathophysiology of hyperkeratosis of CRP. Lamellar granules can mediate cell cohesion of stratum corneum by realizing contents and lipids. The hydrolytic contents of the granules are, in part, responsible for their reorganization and subsequent assembly in the intercorneocyte spaces to form the intercellular lamellae in the stratum corneum.

A treatment of choice for CRP does not exist. We treated our patients with calcipotriol, which is an analogous synthetic of 1,25 dihydroxyvitamin D3, a potent regulator of the cellular differentiation and an inhibitor of keratinocytes proliferation. Its mechanism is based on the regulation of an anomalous keratin expression. In CRP it leads the keratinocytes towards a more normal maturation and differentiation, reducing the expression of keratins of high molecular weight that are normally anomalous [3].

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Medullary *HLA-DR* immunopositive cells of human fetal thymus are involved in negative T-lymphocytes selection

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Accumulating evidence shows that the T-cell precursors move from the bone marrow to the thymus where they are selected for self-tolerance by exposure to MHC antigens on stromal cells. Those developing thymocytes that bind too strongly to self MHC molecules will be induced to undergo apoptosis (negative selection) because these cells would have the potential to cause autoimmune diseases. We applied monoclonal antibodies, immunocytochemistry, electron microscopy and flow cytometry to investigate the HLA-DR immunoreactivity and apoptosis of thymus medullary cells in human fetuses. The results presented provide new proofs about the role of fetal HLA-DR immunopositive cells in thymus medulla as eventual sites of negative prenatal T-cell selection processes.

Key words: fetal thymus, negative T-lymphocytes selection.

Introduction

It is well established that the mature T cells expressing αβT cell receptors (TCRs) are generated in the thymus via a complex process of positive and negative selection. Those developing thymocytes that bind too strongly to self major histocompatibility complex (MHC) molecules will be induced to undergo apoptosis (negative selection) because these cells would have the potential to cause autoimmune diseases [1, 2]. Evidence is presented that negative selection occurs at a relatively late stage of thymocyte differentiation and affects a population of CD4+CD8-/αβTCR+ and CD4-CD8+/αβTCR+ cells found in the medulla. Several important questions about positive and negative selection in the thymus still remain to be answered though [3, 4]. Some kinds of thymic cells such as epithelial cells, dendritic cells, macrophages, B lymphocytes, activated T lymphocytes and thymic "nurse" cells (TNC) express MHC class II antigens, including human leukocyte-associated antigen-DR (HLA-DR). However, relatively little is known of the details of HLA expression and

T-lymphocyte selection in human thymus [1, 5, 6]. In view of the above, the present study was focused on the detection and analysis of HLA-DR immunoreactivity and apoptosis of medullary cells in fetal human thymuses as possible sites of prenatal negative T-cell selection, using monoclonal antibodies, immunocytochemistry, electron microscopy and flow cytometry.

Material and Methods

Our study covered human fetuses (6th-7th month of gestation; n=12) during interruption of normal pregnancy and thymuses of old (aged 60-70 years; n=6) individuals, obtained from thoracic surgery cases. They had no pathological disorders. Annexin V (FL), sc-4252, (Santa Cruz Biotechnology) and three kinds of monoclonal antibodies (Ab), namely Anti-Pan cytokeratin Ab (C 1801, Sigma Chemical Co.), Anti-CD 14 Ab (UCH-M1, sc-1182, Santa Cruz Biotechnology) and Anti-HLA-DR Ab /HK 14, IgG2a, Sigma Chemical Co.) were tested. Indirect immunoperoxidase (IIP), immunofluorescence and transmission electron microscopy (TEM) were performed according to the protocols that we described previously [5, 6]. To define the nature of the thymic cell types we stained serial tissue sections with Anti-cytokeratin Ab, Anti-CD14 Ab and Annexin V which reacted with epithelial cells, monocyte/macrophages and apoptotic cells, respectively (according to the manufacturer's instructions). Control experiments were carried out in parallel. Labomikroscop Axioskop 20 (Fb Carl Zeiss Opton) and electron microscope Hitachi H500 were used. Two-colored flow cytometry was performed using FACSalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA). Histogram and dot plots of HLA-DR positive cells were presented [5].

Results

Normal fetal thymus showed a lobulated structure and prominent Hassall's corpuscles in the medulla (Fig. 1). HLA-DR immunopositive epithelial cells, macrophages and lymphocytes, as well as CD14 immunopositive macrophages were scattered throughout the thymus medulla. Strong HLA-DR staining of Hassall's corpuscles and adjacent thymic cells was seen (Fig. 2). Some lymphocytes, macrophages and epithelial cells showed co-localization of Annexin V and HLA-DR immunopositivity. Double staining demonstrated that the green Annexin V signal co-localized with the red HLA-DR signals, leading to yellow-staining cells and clusters of cells (data not shown). The ultrastructural characteristics of lymphocyte apoptosis, i.e., cell shrinkage, rearrangement of the chromatin structures (chromatin margination, aggregation and condensation), nuclear fragmentation, cytoplasm condensation and/or vacuolization, cellular disintegration and formation of apoptotic bodies which are phagocytosed and digested by adjacent macrophages were observed. The apoptotic bodies were degraded within the lysosomes. Clusters of apoptotic cells were organized by thymus macrophages (Fig. 3). We quantified the presentation of MHC molecules by flow cytometry. The two-color flow cytometric analysis revealed the presence of HLA-DR positive and HLA-DR/CD3 double positive lymphocytes (Fig. 4).

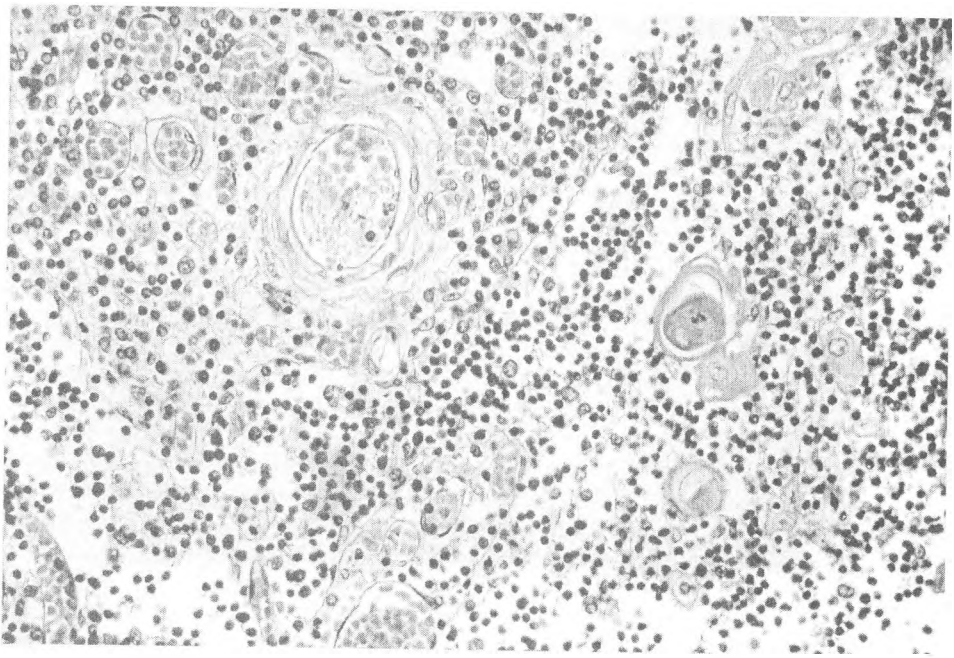


Fig. 1. Medulla of fetal thymus with Hassall's corpuscles, containing keratinized epithelial layers, intact epithelial cells, macrophages and thymocytes; HE staining; Magnification × 100

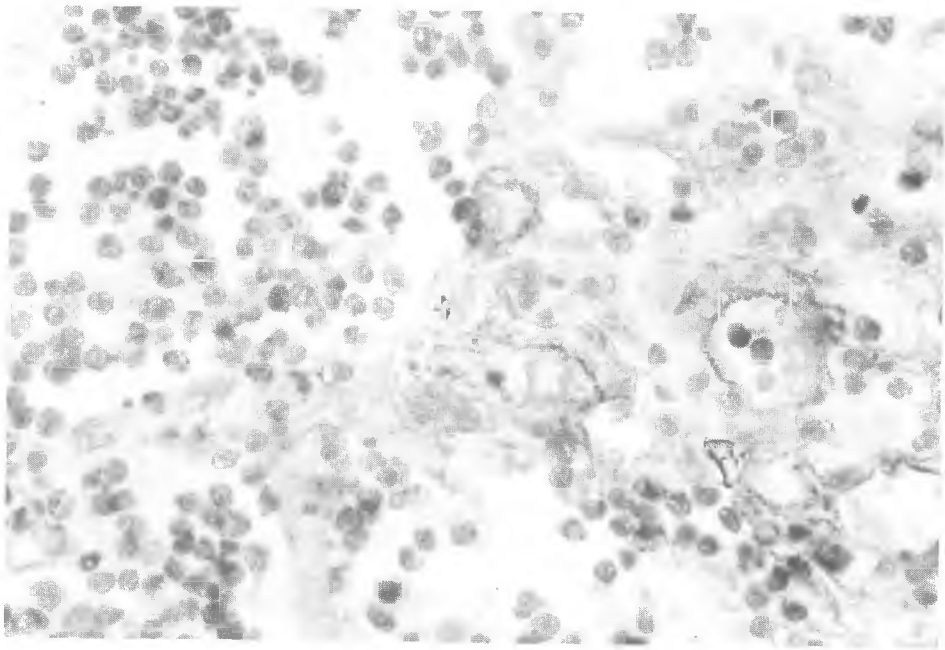


Fig. 2. HLA-DR immunopositive medullary epithelial cells, macrophages and Hassll's corpuscles; IIP staining; Magnification × 200

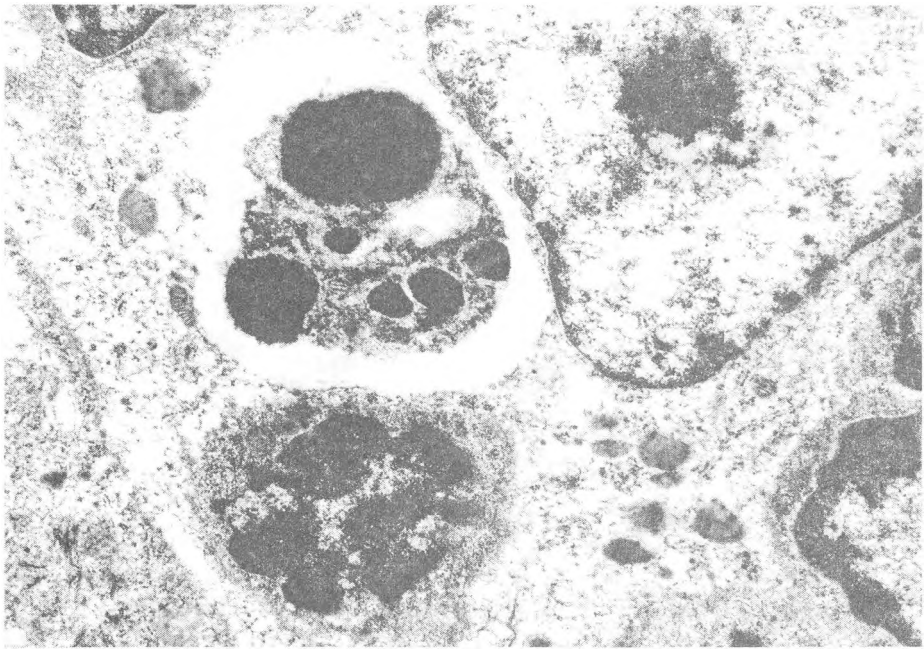
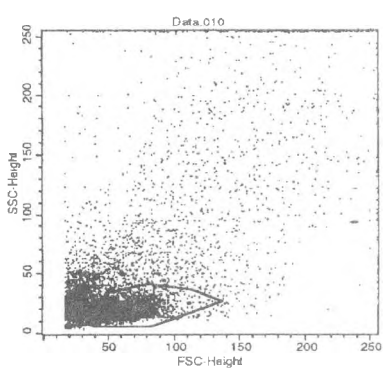
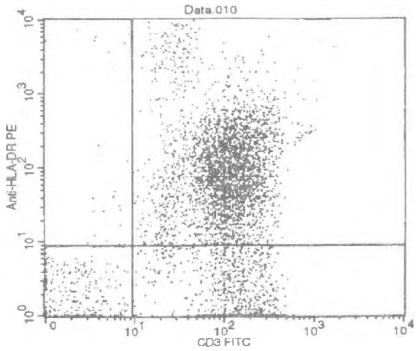
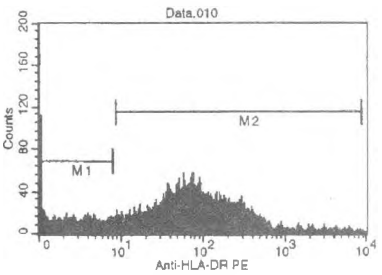


Fig. 3. Medullary macrophage containing a phagolysosome with nuclear fragments of an apoptotic thymocyte; TEM; Magnification × 10 000







Quad	Events	% Gated	% Total
UL	23	0.48	0.26
UR	3539	73.33	40.07
LL	262	5.43	2.97
LR	1002	20.76	11.34

Marker	Left, Right	Events	% Gated	% Total	CV	Median	Peak Ch
All	1, 9647	4826	100.00	54.64	333.85	48.70	1
M1	1, 8	1224	25.36	13.86	77.82	1.43	1
M2	8, 8354	3584	74.26	40.58	277.72	80.58	74

Fig 4. Representative flow cytometric analysis of lymphocytes from fetal human thymus: Status of HLA-DR and CD3 expression; Histogram and dot plot analysis

Discussion

Accumulating evidence shows that the T-cell precursors move from the bone marrow to the thymus where they are selected for self-tolerance by exposure to MHC antigens (class I and/or class II) on stromal cells. Self-tolerance induction is largely a reflection of negative selection (deletion) of autoreactive T cells in the thymus by apoptosis. [1, 4]. Whether thymocytes undergo negative selection in the cortex or medulla or in both sites has long been controversial [4, 7]. It is still controversial if a specialized class of thymic antigen-presenting cells (APCs) are responsible for the negative selection [3, 4]. We found an elimination of thymocytes in human fetal thymus by apoptosis and subsequent phagocytosis. Under physiological conditions, thymocytes selection probably occurs largely in the thymus medulla with the participation of HLA-DR immunopositive macrophages, epithelial cells and Hassll's corpuscles as sites of negative T-lymphocytes selection.

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Histomorphological changes in testicular tissue on patients with hydrocele and infertility

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Spermatological and pathohistological examination is carried out in 116 male patients with hydrocele (mean age 37.85 years). Scrotal thermographs have also been conducted in these patients.

The pathohistological examination of tunica vaginalis testis sample shows moderate fibrosis with collagen deposition and hyalinization as well as desquamation of the mesothelial cells layer. Mild focal interstitial fibrosis is present in the testicular specimen. Insignificant reduction of spermatozoa number in the seminal ducts was observed, together with disorganization of the germinative epithelium in certain zones, while in others spermatogenesis has taken place at the level of spermatids. Changes in the interstitial Leydig cells were not observed.

The authors substantiate the hypothesis that hydrocele can act as a endogenous factor for deterioration of gonads function with disturbance in spermatogenesis and subsequent infertility.

Key words: hydrocele, scrotal thermography, hydrocele fluid, testis, *tunica vaginalis testis*, infertility.

Introduction

Wide range of endogenous and exogenous factors can have a substantial deleterious effect on male fertility — temperature, light, environmental toxins, physical and endocrine factors and many others. Some local pathophysiological changes in testicles, as well as accompanying pathological conditions, have a significant negative role on the reproductive function in the adult male. Hydrocele is one of this local pathological conditions of the testis.

Etiological factors that lead to the emergence of scrotal hydrocele are multiple and diverse by nature, with wide range of negative influence on the spermatogenesis, and more specifically on the spermatological parameters with subsequent infertility [2].

Dating back to 1873, Cannelogul [3] emphasizes that hydrocele even if doesn't affect the spermatogenesis, suppress spermatozoids' maturation. At present days, Politoff et al. [11] and Tzvetkov et al. [4] state that there is a marked depression of spermatogenesis with aberrations in the maturation of the primary spermatocyte (degeneration of first degree — general terminology for thermal injury on the spermatogenesis).

In this article, we present data from spematological and pathohistological examinations of tunica vaginalis testis and testicular tissue in patients with hydrocele, aiming to establish the degree of deterioration of the reproductive function in males with this condition.

Materials and Methods

The authors studied 116 postoperative unilateral hydrocele patients. The patients' ages varied between 5 and 72 years (mean age 37.85 ± 12.11), 30 of them (25.86% of the studied cases) were married without children.

We have conducted contact thermography according to Clark's method in 23 patients (mean age 27.8 years) with scrotal hydrocele and concomitant infertility [7]. Parallel we conducted thermography in control group of 13 healthy males with preserved fertility.

We use correlative analysis, based on the hydrocele's fluid volume compared to spermatological parameters (according WHO criteria, 1996) [14].

An opened testicular biopsy and biopsy of tunica vaginalis testis by Vilar's method were performed in 11 patients [12]. Two specimens of tunica vaginalis testis, fixed in a 10% solution of neutral formalin, and a piece of testicular tissue in Bouin's fixative, were taken. Both specimens underwent a routine histological treatment technique, and the prepared samples were stained with hematoxylin and eosin, and after van Giesson, for collagen presence.

Results and Discussion

From the research on the 116 patients, operated due to scrotal hydrocele, we establish:

In active reproductive age are

n = 59 → 50.85 % [Tabl. 1]

Most common age of appearance of hydrocele

age → over 26 years of age
n = 96 → 81.35 %

Table 1. Age distribution of the patients with hydrocele (n = 116)

Patients	under 14 years of age	15 – 25 y.	26 – 35 y.	36 – 46 y.	over 46 y. of age
n	9	11	35	24	37
%	7.78	9.48	30.17	20.68	31.89

Hydrocele and concomitant diseases of male reproductive system

On table 2 are illustrated several concomitant pathological conditions in patients with testicular biopsy and unfavorable fertility prognosis.

Table 2. Concomitant pathological conditions in patients with testicular biopsy and unfavorable fertility prognosis

Condition	Number of patients
Incarcerated hernia, ipsilateral	1
Kryptorchism	3
Motor and mental retardation	2
Testicular torsion	1
Varicocele Dissociation testis/epididymis	1 1

The most common concomitant condition is Kryptorchism.

Spermatological examinations, volume of scrotal fluid and hydrocele

Table 3. Sperm analysis, before and after sclerotherapy in hydrocele, compared to control group of healthy fertile males

Parameter	Hydrocele fluid volume 2.73 ml	Hydrocele fluid volume ≥ 10.00 ml	Hydrocele fluid volume ≥ 30.00 ml	Control group	PNS
Ejaculate volume (ml)	2.53 ± 1.46	2.19 ±1.22	2.45 ± 0.97	2.84 ±1.27	< 0.001
Spermatozoids count (mln/ml)	23.11 ±15.44	25.40 ± 11.01	28.86 ± 1.22	63.45 ± 15.32	< 0.001
Spermatozoids motility (%)	41.56 ± 15.84	33.20 ± 11.45	10.23 ± 23.00	74.81 ± 19.62	< 0.01

The motility of spermatozoa with 60 % forward motion is found only in 26.06% of hydrocele cases. With volume of the hydrocele fluid over 30 ml, the motility of the gametes drop out with 15%, compared to the control group of healthy subjects with normospermia. Therefore we found direct parallel inversed connection between the volume of the hydrocele fluid and the spermatozoa motility (Table 3).

Other parameters, such as ejaculate volume, pH and spermatozoa count, did not show significant differences, when compared to hydrocele fluid volume.

Thermography in scrotal hydrocele and infertility

We observed the following results, divided by groups according to the etiological factor for the hydrocele and its localization:

In the ***first group*** of patients (n=7) there is bilateral hydrocele following inflammatory process — orchiepididymitis, we found elevation of scrotal temperature with mean value of 1.5°C. In all of these patients azoospermia is found.

In the ***second group*** of patients (n=11) with unilateral hydrocele, following trauma, we found elevation of the temperature with mean value of 1.2°C and different degree of oligospermia I to III degree.

In the ***third group*** — 4 patients with unilateral hydrocele, the temperature is elevated with 0.8°C with normal scrotal parameters.

In the ***fourth group*** — healthy males with normospermia and preserved reproductive function mean the scrotal temperature is 0.46°C.

Histomorphological changes in testicular tissue on patients with hydrocele

In evaluation of the results one must take into consideration the fact that the patients were with unilateral hydrocele, duration of the disease less than 3 years, and quantity of the hydrocele fluid less than 50 ml.

The pathohistological examination of tunica vaginalis testis sample shows moderate fibrosis with collagen deposition and hyalinization as well as desquamation of the mesothelial cells layer. The small blood vessels and capillaries in the tunica wall are dilated and engorged with blood. Extensive round-cell inflammatory infiltrate is observed mostly around those vessels, but also diffusively through the entire thickness of the tunica. Lymphocytes, plasmatic cells, and fibrocytes predominate in the infiltrate (Fig. 1).

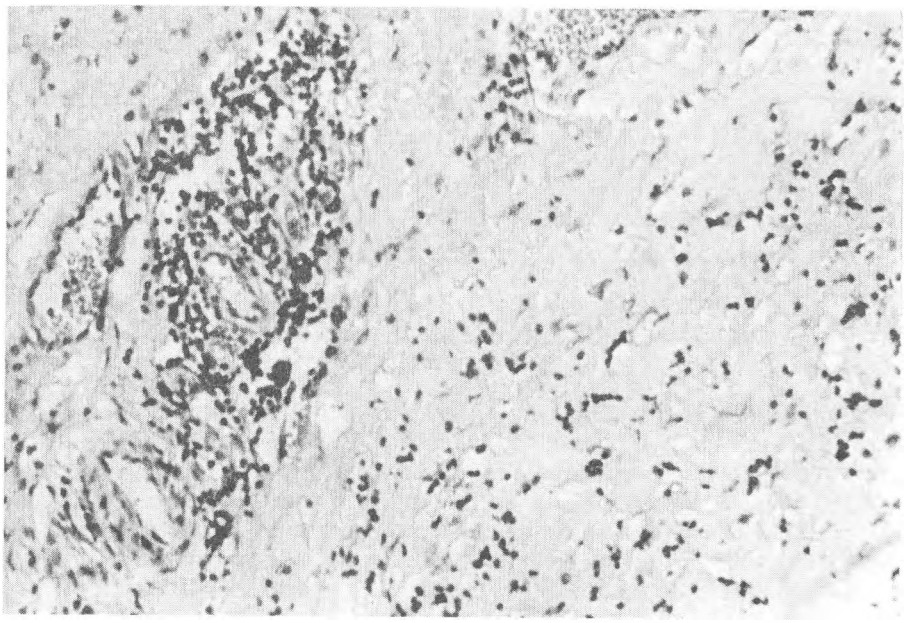

Fig. 1 Chronic nonspecific inflammatory process with collagenization of tunica vaginalis testis. The round-cell inflammatory infiltrate has a predominantly perivascular localization. HE, × 100

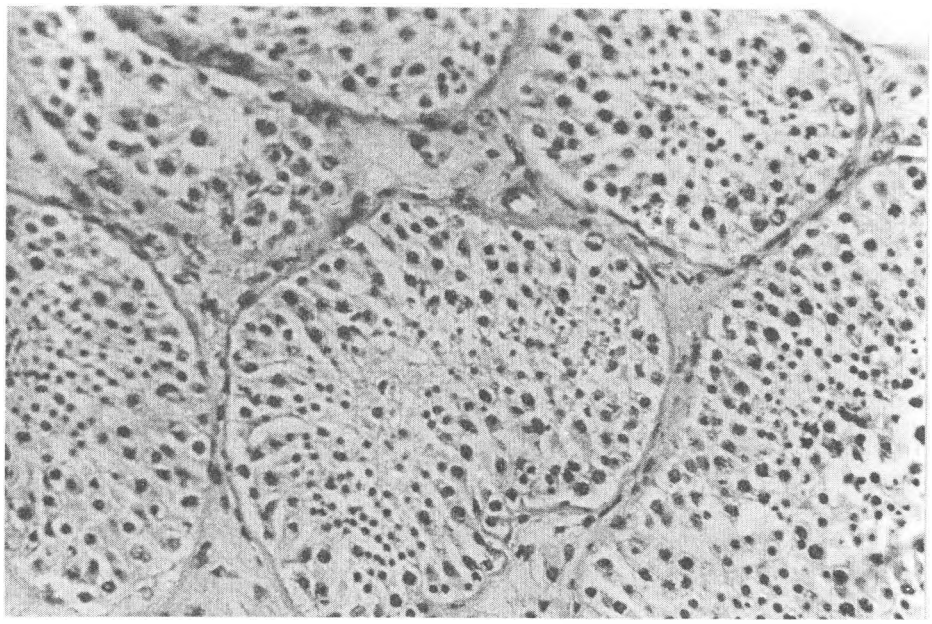

Fig. 2. Mild interstitial fibrosis of the testicular tissue, reduction of spermatozoa number, and disorganization of germinative epithelium in certain zones of the seminal ducts. HE, × 125

Mild focal interstitial fibrosis with scattered thickening of lamina propria of the seminal ducts is present in the testicular specimen. Insignificant reduction of spermatozoa number in the seminal ducts was observed, together with disorganization of the germinative epithelium in certain zones, while in others spermatogenesis has taken place at the level of spermatids (Fig. 2). Changes in the interstitial Leydig cells were not observed. No data of inflammatory process in testicle were found.

Our results, as well as those of Osegbe [9], show that the hydrocele can be a endogenous factor for injury of the gonad function and disturbances in spermatogenesis. Data point towards diminished fertilization capacity of these patients, especially if we compare sperm parameters with hydrocele fluid volume and scrotal thermography. Gannizzaro reaches similar constelations [5].

The morphological changes in tunica vaginalis testis that we have observed in patients with hydrocele have nonspecific character. An insignificant hypospermatogenesis is observed in testicular tissue, similar to that in patients with infertility due to orchitis, orchiepididymitis, testicular and paratesticular tumors. As shown by other authors [6], the testicular tissue atrophy is rare in hydrocele patients.

Different levels of inflammatory changes in tunica vaginalis testis are found in hydrocele patients. Mostly, they are chronic and nonspecific. In some cases, granulations, total or partial preservation of mesothelial layer (where the cells turn to low-cubic ones), or marked mesothelial proliferation with formation of several layers, occur on the inside wall of hydrocele sac [1, 13]. None of these changes was observed in our cases. The acquired forms of hydrocele are often due to neoplasms, inflammatory processes, and injuries of the testicle, epididymis, or other paratesticular structures [10, 8]. However, in our cases there were no data of inflammatory

process in the testicle, while there were marked chronic nonspecific inflammatory changes in the testicular tunics. Those changes appear to be the milestone of the clinically diagnosed hydrocele and its determination as symptomatic.

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Morphological changes in the neonatal murine gut induced by SCF and EGF in organ culture — electron microscopy study

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The aim of our study was to determine the developmental effect on the murine small intestine in organ culture in presence of EGF and SCF. Morphological changes in connection with the effect of both growth factors were observed under transmission and scanning electron microscopes. Organ culture preparation was done by the methods of Playford et al. Specimens were subject to autoradiography and electron microscopy observation.

Using autoradiography approach we found that the number of dividing nuclei was increased both for the samples treated with EGF and SCF. Transmission electron microscopy and scanning electron microscopy showed well shaped enterocytes with typical microvilli for the organ explants treated with EGF and SCF. We found correlation in results obtained from different investigation methods-autoradiography and electron microscopy. Both growth factors had beneficial effect and affected maturation and development of the neonatal murine small intestine.

Key words: SCF, EGF, colostrum, gut development, morphology.

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 epidermal growth factor (EGF) has major role in gut maturation and development along with stem cell factor (SCF). Many studies prove that EGF directly or indirectly regulates growth, function and maintenance in epithelial tissues and postnatal somatic and bone growth. It was proven that EGF can suppress adipocyte differentiation and maturation. Systemic treatment with EGF in rats reduced amounts of adipose tissue, decreasing muscle and fat mass [1]. Stem cell factor and its interaction with c-kit are considered to be important for the homeostasis of epithelial barrier function in the intestinal tract [2].

The aim of our study was to determine the effect of the latter growth factors on the development of murine small intestine in organ culture.

Morphological changes in connection with the effect of both growth factors were observed under transmission and scanning electron microscopes. Quantitative analysis of the number of dividing nuclei, stimulated or non stimulated with both factors was done.

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 Playford et al. [3]. They were opened so that the medium could wash the villi inside. The explants were incubated in culture medium RPMI 1640 containing 10 % fetal calf serum with 50 ng/ml rm EGF or 20 ng/ml rm SCF /Immunotools /Germany/ for 24, 48 and 72 hours at 37° C, 5% CO_2 at 100 % humidity.

Autoradiography:

For the purpose of autoradiography investigation we cultured bowel specimens as described above and we added 5 µl of ^{3}H-Thymidine/Amersham, UK/ to both SCF and EGF treated cultures. Gut explants were put into Tissue Tek culture medium and frozen at — 25°C. Later they were sectioned and applied to glass slides. Autoradiographic emulsion/Hypercoat EM-1, Amersham, UK/ was used to incorporate at a dark room. When dry the slides were placed in a slide box over silicagel, wrapped in foil and placed in a refrigerator for two weeks. Autoradiographic emulsion was developed at dark room and finally slides were washed with distilled water. Counterstaining with hematoxylin showed the histological details of the tissue.

Electron microscopy observation:

Incubated explants were removed from the culture and washed three times in PBS buffer and fixed in 2.5% glutaraldehyde, containing 0.4 M Na-caccodilate. Post fixation was performed with 1% osmium tetra oxide and then specimens were dehydrated in graded ethanol solutions (50-100%) and embedded in resin. Ultra thin sections were contrasted with uranyl acetate and Reynold's lead citrate and examined with transmission electron microscope.

Preparation for scanning electron microscopy was performed as follows: specimens were fixed in 2.5% gluaraldehyde, washed twice in 0.1M caccodilate buffer and then dehydrated in graded ethanol solutions (50-100%). Critical point drying was performed and then specimens were coated with gold in a sputter coater and observed under scanning electron microscope.

Results

Using autoradiography approach we measured number of dividing nuclei at the small gut wall. For that purpose we used StatMost for Windows. One-Way ANOVA Results were used to prepare graphs. For all days of incubation the number of dividing nuclei was increased for the samples treated with EGF (Fig. 1). The incorpora-

tion of ^{3}H-thymidine was also stimulated at the samples treated with SCF (Fig. 2). Data are presented as the mean ± SEM. *P* value < 0.05 was considered statistically significant.

Transmission electron microscopy of the three parts of the small intestine stimulated with EGF showed well shaped enterocytes with typical microvilli, characteristic

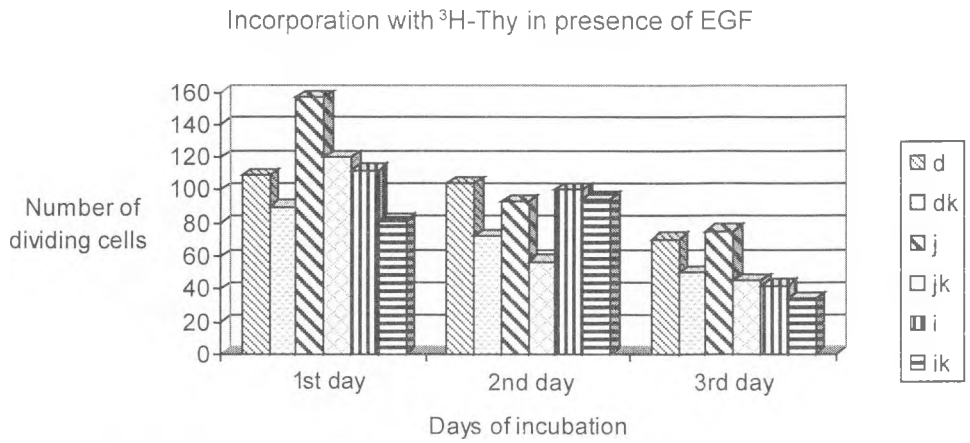

Fig. 1. Explants treated with EGF from all days of incubation. The increased number of dividing cells in presence of EGF is visible. Obvious is the increased incorporation with ^{3}H-Thymidine (^{3}H-Thy) at the duodenum. P — value < 0.05; d — duodenum, dk — duodenum control, j — jejunum, jk — jejunum control, i — ileum, ik — ileum control

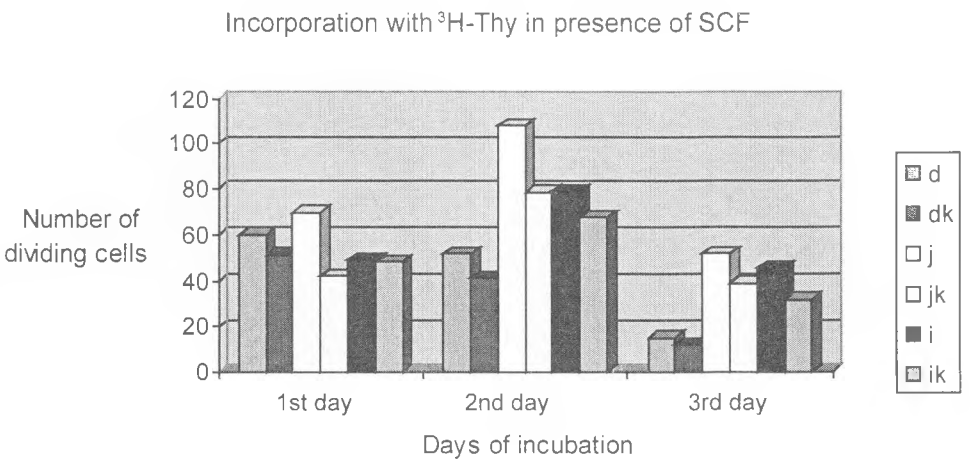

Fig. 2. Incorporation with ^{3}H-Thymidine in presence of SCF. Increased number of dividing cells for all specimens treated with SCF. P — value < 0.05. d — duodenum, j — jejunuk, i — ileum; dk — duodenum control, jk — jejunum control, ik — ileum control

for the brush border (Fig. 3a). Tight junctions were also observed between the enterocytes. A great number of nuclei were activated. Untreated specimens showed less activated nuclei along with fewer amounts of microvilli covering the brush border region. Some regions showed lack of microvilli or if present they were thinner and irregular. Loose junctions between different enterocytes were observed, which we suppose were in connection with the absence of EGF in the culture medium (Fig. 3b).

Organ explants stimulated with SCF showed well shaped microvilli covering the brush border in comparison to the control specimens (Fig. 4a, b), where microvilli

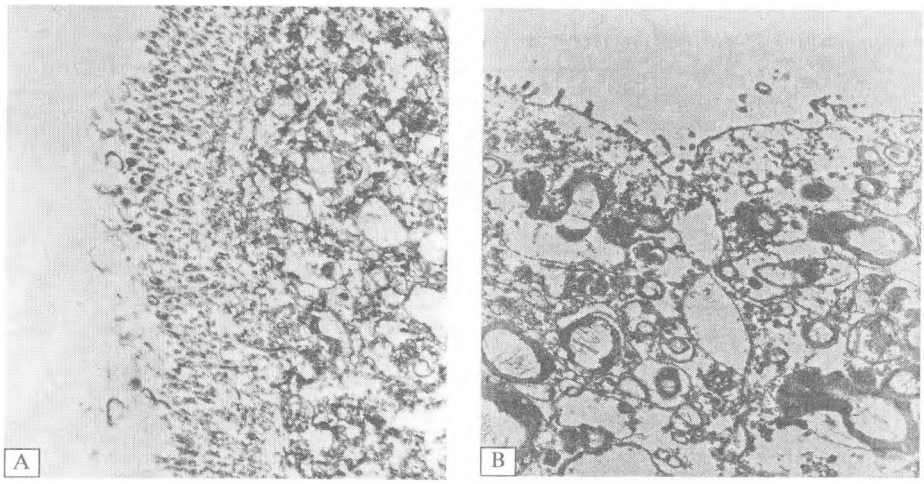

Fig. 3. Transmission electron micrograph of duodenum of 5-day-old mouse, treated with EGF-second day of incubation. In presence of EGF (A) microvilli are thicker. When cultured without EGF microvilli are fewer and loose junctions are seen between cells. Originally × 12000 for both micrographs

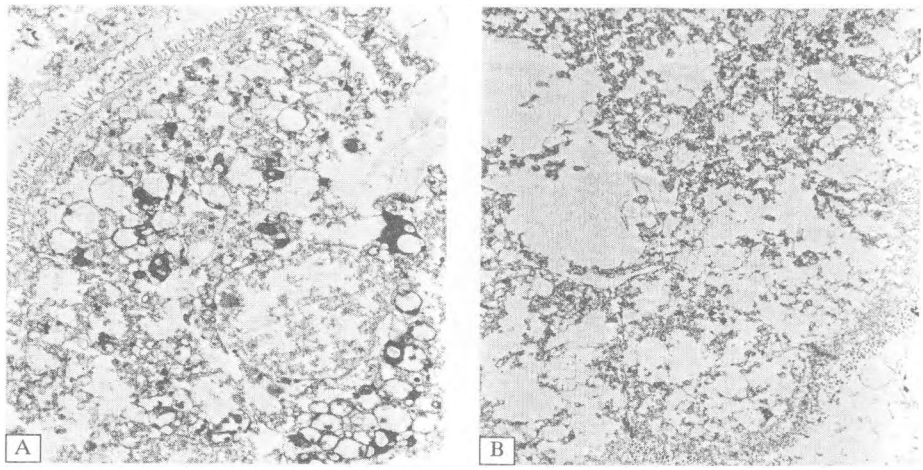

Fig. 4. Transmission electron micrograph of jejunum of 5-day-old mouse. Specimen treated with SCF (A) showed well shaped enterocytes with typical microvilli and activated nuclei. Originally × 4400. Cultured in absence (B) of the growth factor microvilli were fewer. Originally × 3000

were shorter and had more gaps in between. Both cases showed tightly situated cells and activated nuclei.

Scanning electron microscopy (SEM) provides vivid three-dimensional images which are easy to understand in comparison to TEM. For this point of view SEM is advantageous in morphological research and it can serve as adequate method to reveal intracellular structures [4].

Scanning electron microscopy of duodenum, jejunum and ileum cultured with EGF showed straight villi covering viscera (Fig. 5a). Controls observed were thicker and shorter (Fig. 5b). In most ileums not treated with EGF we found curved villi with loosely situated enterocytes.

Scanning electron microscopy of murine small gut stimulated with SCF revealed longer villi with more contrasting and tightly situated enterocytes (Fig. 6a). Controls had shorter and thicker villi and more space between them (Fig. 6b) Using

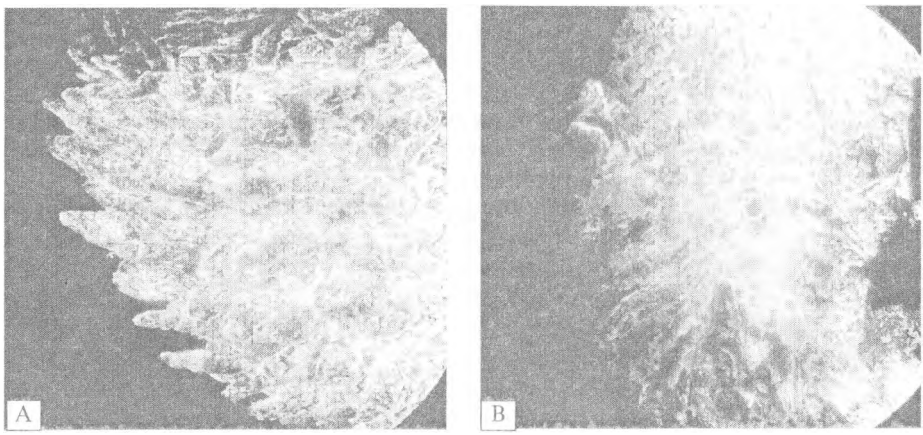

Fig. 5. Scanning electron micrograph of duodenum of 5-day-old mouse, second day of incubation. Treatment with EGF increases the number of villi covering viscera (A). In absence of EGF villi are fewer and curved (B). Originally × 180

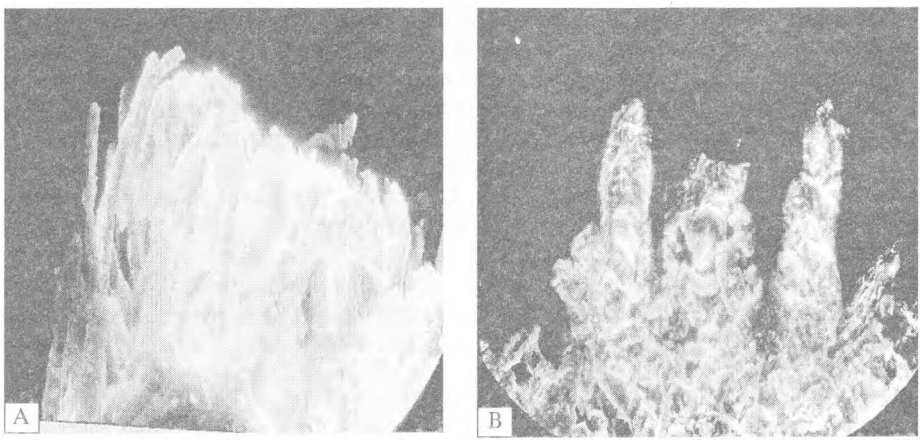

Fig. 6. Scanning electron micrograph of ileum, second day of incubation with SCF. Long finger-like villi are observed over treated viscera (A). Fewer irregular villi are observed in controls (B). Originally × 180

higher magnifications discrete details of villous morphology were revealed. Corrugations of the surface of the villi were obvious in all investigated samples. Those treated with SCF showed tight cell to cell location. Specimens that weren't treated with SCF had more gaps between enterocytes and cells were unequal.

Discussion

EGF and SCF are present in colostrum and milk but data suggests different results about their influence on gastro-intestinal tract.

Epidermal growth factor (EGF) is a 53 amino acid peptide that is produced by the salivary glands, mammary glands, Brunner's glands of the duodenum and kidneys [5]. It is a potent stimulant of proliferation and healing of the gastrointestinal tract, acting as a cytoprotective agent and is also stabilizing cells against noxious agents such as indomethacin [6]. It was shown that EGF can prevent hepatic injury and multiorgan injury [7, 8] Clinical trials of EGF are presently under way for treatment of ulcerative conditions of the bowel. A rapid intervention with EGF may maintain organ viability [9]. Addition of EGF to organ culture had beneficial effect on gut mucosa, accelerating the maturation rate and proliferation of intestinal cells in early embryonic murine gut [10]. It was shown that enterocyte maturation was more sensitive to EGF than cell proliferation [11].

The autoradiography investigation gave us quantitative results abut those nuclei that were in the S phase of division during the exposure to ^{3}H-Thymidine. The quantities of dividing nuclei at the gut wall were in correlation to our electron microscopy results. Enlarged number of dividing cells for the explants treated with EGF and for those treated with SCF showed the stimulating and maturating effects on enterocytes of both growth factors.

Stem cell factor (SCF) has the ability to stimulate growth of early hematopoetic progenitors capable of maturing to erythroid, megacaryocyte, granulocyte, lymphocyte, and macrophage cells. Mammals treated with SCF increase hematopoetic cells of myeloid and lymphoid lineages. [1] It also promotes the development of mast cells from CD 34+stem cells in vitro and in vivo [12]. The interaction of SCF and c-kit is considered to be an important signalling event for the homeostasis of the epithelial barrier function in the intestinal tract [13].

Scanning electron microscopy of the small bowel revealed the morphology of the stimulated explants. At birth the villi are finger-like and regular. Simultaneously the villi shape changes from finger- to leaf- or tongue-like and the number of dividing villi and villi with indentations increase remarkably in time [14-15]. Observations based on SEM analysis indicate very dynamic growth-related changes that occur in association with the growth factors added. It was shown that in the unsuckling piglets the villi were short and dense [16]. In our case in presence of EGF we found straight and thick situation of the villi. On the other hand, unstimmulated explants had shorter and denser villi. As visualized, small intestinal histological features were similar to those described in other species. [17]. Specimens treated with SCF were again dense and a finger-like shape was obvious. Explants that were not treated with SCF had shorter villi with finger-like form. The villi looked twitched. It was due to a reduction in basal vascular resistance simultaneous with dramatic increase in local intestinal blood flow and lymph formation [16]. Higher magnification graphs showed many corrugations between cells and weak cell to cell contacts.

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Case of variable drainage of the superior branch of the left pulmonary vein with patent foramen ovale

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During routine dissection course we have found abnormal drainage of the superior branch of pulmonary vein. The vein collecting the blood from the superior lobe of the left lung was draining into the left brachiocephalic vein. After that the venous drainage was following its usual way. The inferior lobe of the left lung was drained normally into the left atrium. The two pulmonary veins of the right lung had no variations. During classical dissection of the heart we have found foramen ovale enormous in size. Variation of that type has significant value in performing manipulations and operations of the heart.

Key words: pulmonary venous drainage, lungs, foramen ovale.

Introduction

With the advancing of medicine and performing more complex and invasive methods of examinatoin and treatment of the human heart, like heart catheterization and on-beating heart surgery, detailed knowledge of the veins and arteries is required. Interestingly, Winslow [10] first described anomalous connection of the pulmonary veins of a lung more than 200 years ago in 1739. In the second half of 20th century, numerous reports of different type of pulmonary vein anomalies with or without clinical manifestation have been published. Arthurton [1] published a case report of total anomalous pulmonary drainage into the coronary sinus, a rare case of total pulmonary drainage into the portal vein has been described by Butler [4], Winter et al. [11] described an interesting case about 10-week-old infant with total pulmonary drainage into the right atrium. Choo Yung Suh [9] published two cases of pulmonary venous anomaly, clinically manifested in two young soldiers. Recently, with helical CT Shinozaki et al. [8] have found total pulmonary vein drainage into the superior vena cave in a 41-year-old man without any significant complains in his daily life (except palpitation by atrial flutter) due to patent foramen ovale and absence of pulmonary artery stenosis. In the last few years the MRI made the diagnosis of

those anomalies in infants, children and adults a lot easier. In 2003 Haramati [6] published retrospective CT examination of 29 patients. Seventy-nine per cent (23 of 29 patients) had an anomalous left upper lobe vein connecting to a persistent left vertical vein, draining into the left brachiocephalic vein.

Embryologically the lungs are derived from the foregut with which they share a common blood supply. In early stages the pulmonary veins are derived from the splanchnic plexus and have multiple communications with 2 systems, the cardinal system of veins and the umbilicovitelline system. From the cardinal system the superior vena cava, innominate veins, and coronary sinus are ultimately derived. In the final stages of development the umbilicovitelline system is represented principally by the portal venous system. In this early stage the primordia of the lungs have no direct connection with the heart. Subsequently a direct connection with the heart occurs as a result of the union of these primary lung veins with an outgrowth from the dorsal wall of the sinoatrial region known as the common pulmonary vein. After the lungs acquire a route of drainage directly into the heart, the connections between the pulmonary portion of the splanchnic plexus and the cardinal and umbilicovitelline veins are lost [2].

Case report

During routine dissection course, we dissected a 75-year-old male cadaver at the dissection halls in the Department of Anatomy, Histology and Cytology in Medical University, Pleven and found variation in the drainage of the left pulmonary veins. While we were dissecting the veins of the left side of the neck and the left shoulder region we found a huge venous vessel arising from the upper lobe of the left lung (Fig. 1). That

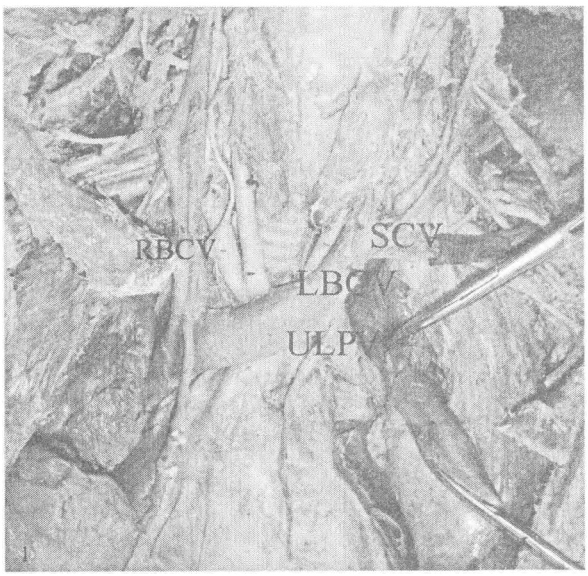

Fig. 1. In situ dissection of the lungs showing the anomalous upper left pulmonary vein (ULPV). Right brachiocephalic vein (RBCV); Left brachiocephalic vein (LBCV); Subclavian vein (SCV)

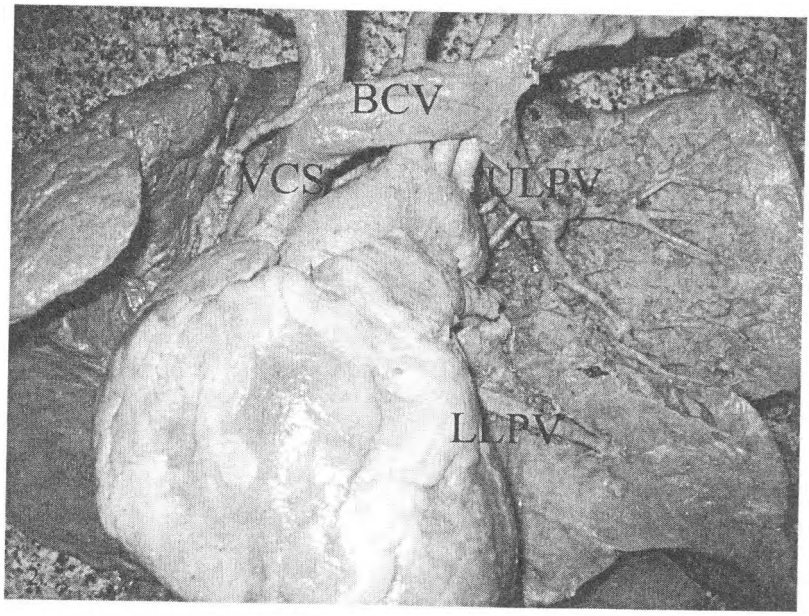

Fig. 2. Eviscerated heart and lungs showing the anomalous upper left pulmonary vein (ULPV) and partially dissected left lung. Left brachiocephalic vein (BCV); Vena cava superior (VCS); Lower left pulmonary vein (LLPV)

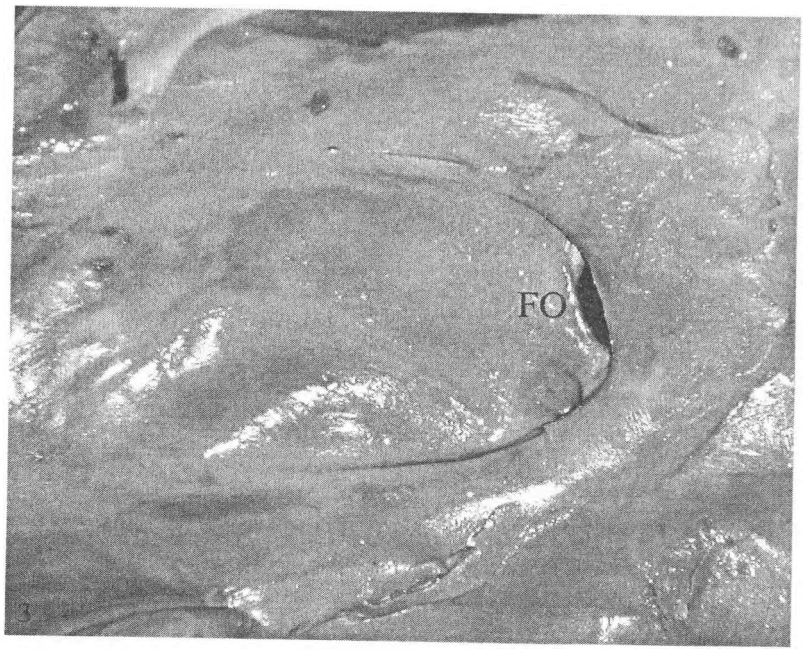

Fig. 3. Classic dissection of the cadaver heart revealing foramen ovale apertum (FO)

vessel had entered the left innominant vein under the clavicle near to the top of the left lung. While dissecting in situ the hilus of the left lung we found normal drainage of the left lower pulmonary vein. After precise dissection of the vessels in the thoracic cavity, neck and the shoulder region we eviscerated the heart and the lungs (Fig. 2). At first sight we did not find any other external anomalies of the drainage of the vessels except the pulmonary vein draining blood from the upper lobe of the left lung into the left brachiocephalic vein by vertical vein. The other three pulmonary veins drained into the left atrium. We made classical anatomical dissection of the heart and we found large fossa ovalis and patent foramen ovale (Fig. 3). There were no pathological aberrations of the thickness of the myocardium.

Discussion

We found PAPVD (Partial anomalous pulmonary venous drainage) with patent foramen ovale in 75-year-old male cadaver. In the literature it is described as an anomally without any clear clinical symptoms. Until the end of the dissection course we did not find any macroscopic pathology in the heart besides patent foramen ovale. The lungs and the other internal organs were intact which showed us that this particular type of anomaly (PAPVD with patent foramen ovale) was not clinically manifested and it was not the direct reason for the death of the man.

According to Edwards [5] the cause in most examples of anomalous pulmonary venous connection is either 1) failure of connection of the atrial portion of the heart with the pulmonary portion of the splanchnic plexus or 2) secondary obliteration of normally developed comnmnunications between the atrial portion of the heart and the pulmonary portion of the splanchnic plexus. In either event that portion of the pulmonary tissue that fails to make direct connection with the heart has no route for drainage other than the primitive connection between the splanchnic plexus or umbilicovitelline system of veins.

In a study of cases Brody [3] stated that, there are two major types of anomalous drainage, i.e. total and partial anomalous drainage. The case that we have described belongs to the second group which usually survives to adult life. At present it is recognized that anomalous drainage of a portion of one or both lungs is relatively common and frequently accompanies atrial septal defect.

In 1953 Muir [7] published detailed summary of the different types of anomalous pulmonary drainage, total and partial, found under different circumstances. Most of the patients with total anomalous pulmonary drainage had patent foramen ovale or other cardiac anomaly and have survived several months, rarely years after birth. The cases with partial anomalous pulmonary drainage were found mostly after necropsy or accidently during different examinations [7], like the case we represent — found during routine dissection course.

Conclusion

The described cases of anomalous pulmonary drainage after necropsy, angiography, heart catheterizations, CT and MRI are of great importance for the medical practice. Some of them are found accidentlally by doctors, performing routine heart examination, while others have been found because of different complains like dyspnoea, palpitation, pulmonary edema and various types of rhythm disorders. Also another type of this anomaly exists, the one without any clinical manifestation because

of the patent foramen ovale which compensates the venous pressure and prevents the pulmonary hypertension.

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Anthropology

Underweight in Bulgarian Boys and Girls from 3 till 17 Years of Age Living on the Borderline between 20th and 21st Century

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The aim is to characterize and classify the underweight in children and adolescents from Sofia, and to assess the gender differences in the frequency of separate categories underweight.

Totally 2931 children and adolescents aged 3-17 years are studied. Underweight are defined according to the BMI cut-offs elaborated by Cole et al. In both genders till 13 years of age the frequency of children with underweight reaches to 15.0 % in boys aged 3 and girls aged 9. Between 14 and 17 years of age the frequency of underweight children is higher, more emphasized in girls — 27% in 17 years old girls. The established underweight among present generation of Bulgarian children and adolescents could serve as a base for analyses, evaluations and prognoses for their health status. The frequency rise of thinness with ages among adolescents represents a special interest, as well as necessity of profound investigations and wide discussion in parallel with the problem of obesity.

Key words: BMI cut-offs, underweight, growing up period.

Introduction

One of the most important indicators for predisposition to some serious, socially important diseases in adulthood, or the presence of such ones during growing up period, is the Body Nutritional Status (BNS). And if the international accepted criteria for BNS for adults have been elaborated far ago, this methodological problem concerning BNS criteria during growth period of the young generations started its unification for international use hardly before several years [11]. Different indicators as weight-for-stature, weight-for-age have been used for the BNS to be categorized, as well as dif-

ferent cut-offs concerning BMI and elaborated by various percentiles, z-score or SD approaches, based mainly on national data, have been applied [15, 16, 19, 20, 21]. In many countries were taken data and published results mainly for the epidemiology of obesity [1, 2, 4, 8, 12, 18]. The underweight compared to obesity is also reliable health problem but before 2-3 years the reports in the literature about population investigations concerning undernutrition were very few and mainly for the countries in which hunger and undernourishment were officially recognized [7, 9].

As a result of constant searching and more precise definition, a team of international scientists (International Obesity Task Force — IOTF) originated criteria about fixing age and sex specific cut-offs for BMI during growth [12]. On the basis of these criteria T. Cole et al. [5, 6] elaborated and published in the British Medical Journal internationally accepted BMI cut-offs for annual determination of the underweight and overweight BNS categories in children and adolescents from 2 till 17 years concerning both genders separately. This afforded an opportunity to the scientists for assessing the epidemiology of both categories unhealthy nutritional status concerning these ages, as for the separate countries, so internationally. Furthermore according to the criteria of IOTF, the cut-offs for every age group between 2 and 17 years are mathematically equalized by the LMS method to the WHO cut-offs for BMI in adults: 16, 17, 18.5, 25, and 30 kg/m^2. This fact gives possibility for the frequency of the different categories Body Nutritional Status in children, adolescents and adults to be compared.

The **aim** is to characterize and classify the underweight in 3-17 years old children and adolescents from Sofia living at the end of 20th and the beginning of the 21st century and to assess the gender differences in the frequency of separate categories underweight.

Material and Methods

Object of the study were: 640 pre-school children — 3-6 years (2004-2005) and 2291 children and adolescents between 7 and 17 years of age (1995-2002); or totally 2931 boys and girls uniformly distributed into 16 age groups for both genders separately.

The mean age of the investigated boys and girls in all 16 age groups is 3.5, 4.5, 5.5, 6.5, 7.5, and so on. To facilitate the presentation of the data, they are marked in whole numbers (in years) on our tables, for example in the group of the 3 years old children are enlisted those ones aged from 2 years 11 months and 30 days till 3 years 11 months and 29 days and so on for concerning all age groups.

The study was carried out in Sofia. The nests in which the investigation had to be carried out have been defined by the lottery method. Seven kindergartens and seven schools were included, situated in the center of the city; in the near areas to the city and in the outskirts.

The anthropological investigation were carried out by a team consisting of specialists in medical anthropology, who were trained in advance and tested for individual and inter-researcher's errors. All requirements for the medico-biological studies were followed, as well: the investigations were carried out in the morning, in specially equipped rooms and the boys from 3 till 17 years were in shorts and the girls in bikini. The data taken are elaborated and analyzed statistically (carried out by means of SPSS version 13.0). The statistical significance of gender differences was established by the T-criterion of Student at $P < 0.05$.

The evaluation concerning epidemiology of the different categories body nutritional status being subject of the present paper is made on the basis of the data about

Table 1. BMI cut-offs adapted according to this elaborated by Cole et al. about underweight (2007)

Age (years)	BOYS			GIRLS		
	Thinness grade III	Thinness grade II	Thinness grade I	Thinness grade III	Thinness grade II	Thinness grade I
2.5	x-13.2	13.3-13.9	14.0-14.9	x-13.1	13.2-13.7	13.8-14.6
3.5	x-13.0	13.1-13.6	13.7-14.6	x-12.9	13.0-13.5	13.6-14.3
4.5	x-12.8	12.9-13.4	13.5-14.3	x-12.6	12.7-13.2	13.3-14.1
5.5	x-12.6	12.7-13.2	13.3-14.1	x-12.4	12.5-13.0	13.1-13.9
6.5	x-12.4	12.5-13.1	13.2-14.0	x-12.3	12.4-12.9	13.0-13.8
7.5	x-12.4	12.5-13.1	13.2-14.1	x-12.3	12.4-13.0	13.1-13.9
8.5	x-12.4	12.5-13.2	13.3-14.2	x-12.4	12.5-13.1	13.2-14.1
9.5	x-12.6	12.7-13.3	13.4-14.5	x-12.5	12.6-13.3	13.4-14.4
10.5	x-12.8	12.9-13.6	13.7-14.8	x-12.8	12.9-13.6	13.7-14.8
11.5	x-13.0	13.1-13.9	14.0-15.2	x-13.2	13.3-14.0	14.1-15.3
12.5	x-13.4	13.5-14.2	14.3-15.6	x-13.6	13.7-14.6	14.7-15.9
13.5	x-13.8	13.9-14.7	14.8-16.1	x-14.2	14.3-15.1	15.2-16.6
14.5	x-14.4	14.5-15.3	15.4-16.7	x-14.6	14.7-15.7	15.8-17.2
15.5	x-14.9	15.0-15.8	15.9-17.3	x-15.2	15.3-16.2	16.3-17.7
16.5	x-15.4	15.5-16.3	16.4-17.8	x-15.6	15.7-16.6	16.7-18.1
17.5	x-15.8	15.9-16.8	16.9-18.3	x-15.9	16.0-16.9	17.0-18.4

BMI. The categorization is made according to the body mass index cut-offs elaborated by Cole et al. [5, 6] and recommended by the WHO when interage, intergroup and international comparisons in boys and girls aged 2-17 years had to be assessed (Table 1). According to these BMI cut-offs, underweight children are divided into three grades: thinness grade I, thinness grade II and thinness grade III.

Results

The means of BMI for every age-gender group are presented on Table 2 and in Figure 1.

Between 3 and 8 years, i.e. during childhood, the means of BMI didn't change tangible staying into the range 15.38-16.35 kg/m^2. For these ages, however, the BMI values are higher in boys, even if not much, and significant gender differences are found only in the 5 years old children.

From 9 years henceforth, when the beginning of puberty could be marked, and the annually changes of BMI means in both genders become greater with ages, the BMI increment is from 17.0 kg/m^2 at 9 till 21.0 kg/m^2 at 17. The year increasing is statistically significant between 8 and 9 years for both genders, i.e. on the borderline between childhood and puberty. Significant is also the increment between 11 and 12 in girls and one year later (between 12 and 13) in boys, which could be explained by the boys' later puberty. The last significant annual increasing of BMI values is

Table 2. Biostatistical data of BMI in children and adolescents between 3 and 17 years of age

Age (years)	Boys						Girls						Differences			
	n	mean kg/m²	SD	SEM	min	max	*n*	mean kg/m²	SD	SEM	min	max	Gender	Interage		
														Age period	Boys	Girls
3	80	15.91	1.43	0.16	13.2	21.6	80	15.76	1.26	0.14	12.9	20.3	0.15	**3-4**	-0.17	-0.01
4	80	15.75	1.83	0.20	12.5	25.9	80	15.76	1.48	0.17	13.2	19.1	-0.01	**4-5**	0.17	-0.38
5	80	15.92	1.87	0.21	13.3	26.6	80	15.38	1.42	0.16	13.2	20.7	0.53*	**5-6**	0.22	0.33
6	80	16.14	2.04	0.23	13.4	26.3	80	15.72	2.04	0.23	12.1	23.9	0.42	**6-7**	0.11	0.34
7	110	16.24	1.95	0.19	12.9	26.0	110	16.06	2.04	0.19	12.4	22.6	0.19	**7-8**	0.11	-0.07
8	100	16.35	1.97	0.20	13.1	24.2	101	15.99	1.96	0.20	12.3	21.3	0.36	**8-9**	0.99*	1.20*
9	100	17.27	2.54	0.25	12.8	26.1	101	17.23	2.84	0.28	12.0	27.8	0.04	**9-10**	0.20	0.41
10	100	17.79	2.92	0.29	13.8	27.7	98	17.57	2.86	0.29	12.6	25.1	0.22	**10-11**	0.65	0.78
11	99	18.28	2.76	0.28	13.6	25.0	100	18.32	3.19	0.32	13.4	30.1	-0.04	**11-12**	0.21	0.67*
12	97	18.47	2.65	0.27	14.2	26.9	100	19.44	3.44	0.34	13.2	29.1	-0.97*	**12-13**	1.15*	0.63
13	101	19.57	3.33	0.33	13.9	28.7	99	19.87	4.01	0.40	11.9	36.1	-0.30	**13-14**	-0.04	-0.35
14	99	19.45	3.14	0.32	14.1	28.9	101	19.13	2.72	0.27	12.8	31.2	0.32	**14-15**	0.54	0.57*
15	100	19.92	3.33	0.33	15.4	35.9	100	19.98	3.03	0.30	14.7	36.0	-0.06	**15-16**	0.36	0.36
16	119	20.40	3.23	0.30	14.7	31.0	120	20.26	3.13	0.29	15.4	32.4	0.15	**16-17**	0.58	0.00
17	118	20.99	3.21	0.30	14.9	31.4	118	20.25	2.97	0.27	15.5	31.8	0.73			

* Statistical significance at $P<0.05$

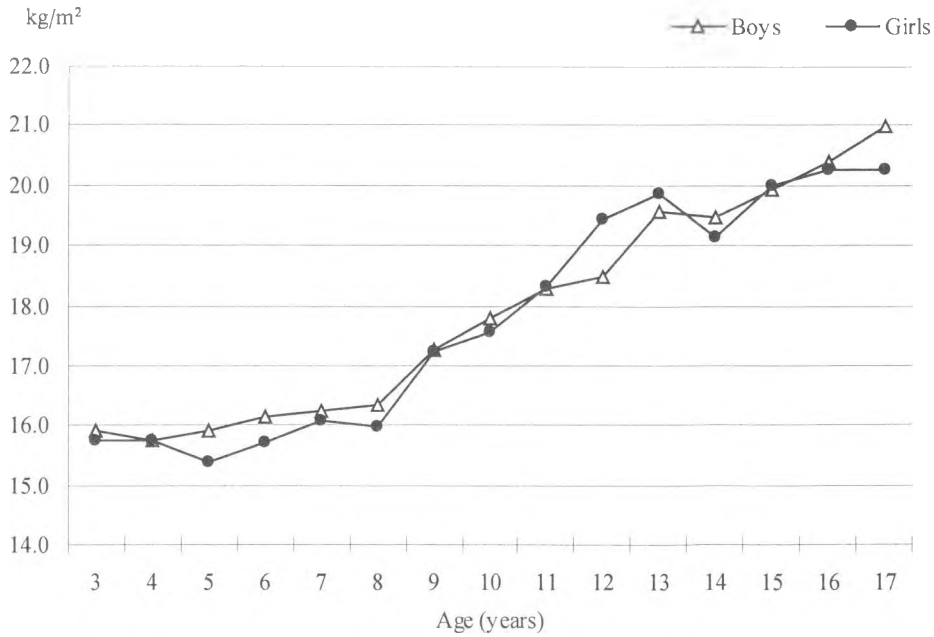

Fig. 1. Means of BMI between 3 and 17 years

established only for girls between 14 and 15, i.e. at the beginning of the postpubertal period by them.

After 9 the gender differences are already more distinctly expressed. During active puberty (11-13 years) the values of BMI become higher in girls, the gender difference being statistically significant in the 12 years old boys and girls. The BMI again is higher in boys aged 16 and 17, when postpubertal period comes.

The frequency of children and adolescents with underweight is presented on Tables 3 and 4, and in Figures 2 and 3.

Till 13 years the frequency of underweight children is not so low — from 3 till 13 concerning underweight girls it is between 5.0% and 15.84%, and for boys it is between 6.0% and 15.0%. From 14 till 17 when the puberty nearly ends, frequency of the underweight adolescents increases — in boys it comes till 22.0% and in girls it reaches even 26.67%.

The children with thinness grade I are comparatively frequent till the end of puberty. During postpuberty the underweight frequency for both genders increases considerably, more markedly in girls. The ages in which more boys with thinness grade I were found are: 3 years (11.25%), 10 years (12.0%) and between 12 and 15 years, reaching at 15-18.0%. In girls are found also variations with ages concerning frequency of thinness grade I — it is high at 4 years (11.25%), at 6 years (10.0%) and during puberty between 9 and 12, when its frequency varies among 10.0% and 12.87%. Considerable gender differences are found after 14 years when the frequency of girls with thinness grade I is constantly high (among 13.0% and 17.5%).

The frequency of children with thinness grade II more often is higher in school age girls. Most underlined are the gender differences in 17 years age boys and girls — the thinness grade II for girls comes till 9.17% and in boys it reaches hardly 2.54%.

Table 3. Frequency of the individuals with underweight

Age (years)	Underweight			
	boys		girls	
	n	%	*n*	%
3	12	15.00	7	8.75
4	9	11.25	9	11.25
5	7	8.75	4	5.00
6	6	7.50	11	13.75
7	7	6.36	10	9.09
8	7	7.00	13	12.87
9	8	8.00	16	15.84
10	16	12.00	14	14.28
11	6	6.00	15	15.00
12	11	11.61	12	12.00
13	13	12.87	15	15.00
14	19	19.00	22	21.78
15	22	22.00	16	16.00
16	20	16.67	29	24.16
17	18	15.25	32	26.67

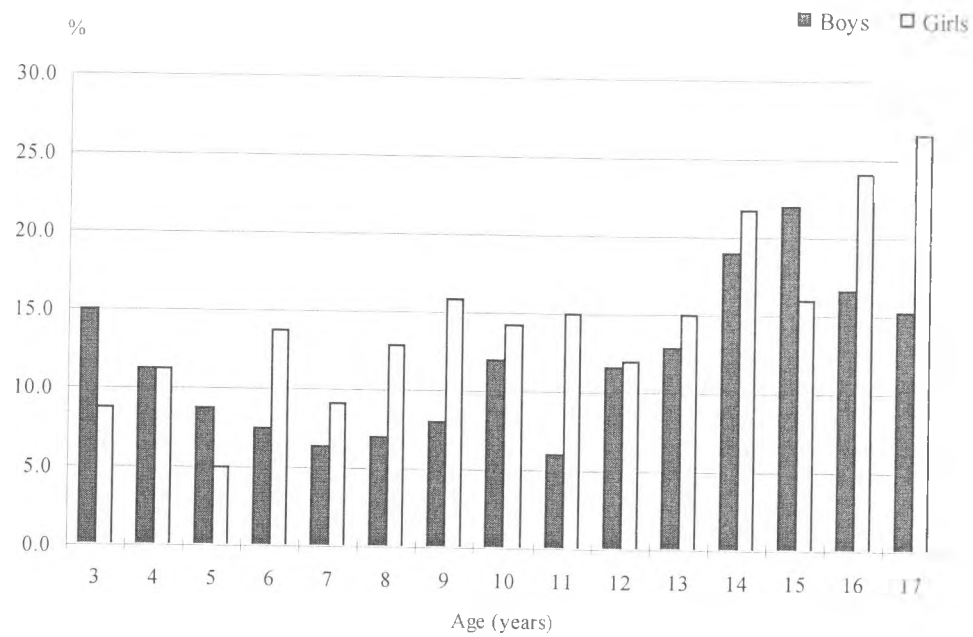

Fig. 2. Frequency of boys and girls with underweight

Table 4. Frequency of the separate underweight categories

Age (years)	BOYS							GIRLS						
	n	Thinness grade III		Thinness grade II		Thinness grade I		*n*	Thinness grade III		Thinness grade II		Thinness grade I	
		n	%	*n*	%	*n*	%		*n*	%	*n*	%	*n*	%
3	80	-	-	3	3.75	9	11.25	80	-	-	1	1.25	6	7.50
4	80	1	1.25	2	2.50	6	7.50	80	-	-	-	-	9	11.25
5	80	-	-	-	-	7	8.75	80	-	-	-	-	4	5.00
6	80	-	-	-	-	6	7.50	80	1	1.25	2	2.50	8	10.00
7	110	-	-	1	0.91	6	5.45	110	-	-	3	2.73	7	6.36
8	100	-	-	1	1.00	6	6.00	101	1	0.99	4	3.96	8	7.92
9	100	-	-	3	3.00	5	5.00	101	2	1.98	1	0.99	13	12.87
10	100	-	-	-	-	12	12.00	98	1	1.02	2	2.04	11	11.22
11	100	-	-	1	1.00	5	5.00	100	-	-	3	3.00	12	12.00
12	97	-	-	1	1.30	10	10.31	100	1	1.00	1	1.00	10	10.00
13	101	-	-	2	1.98	11	10.89	99	2	2.00	5	5.00	8	8.00
14	99	1	1.00	5	5.00	13	13.00	101	2	1.98	4	3.96	16	15.84
15	100	-	-	4	4.00	18	18.00	100	1	1.00	2	2.00	13	13.00
16	120	3	2.50	6	5.00	11	9.17	120	1	0.83	7	5.83	21	17.50
17	118	4	3.39	3	2.54	11	9.32	118	1	0.83	11	9.17	20	16.67

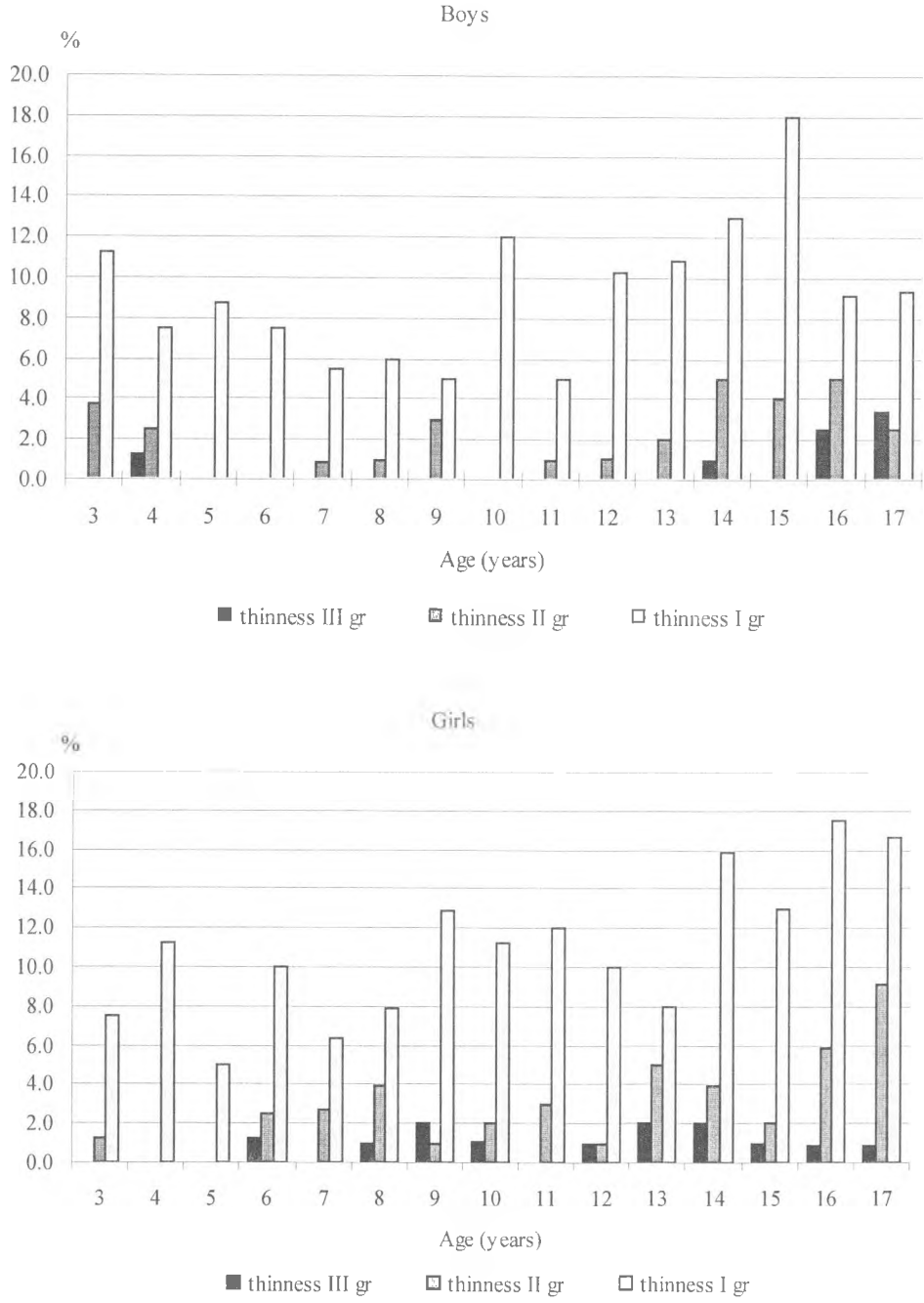

Fig. 3. Frequency of the separate underweight categories

Almost in all ages between 6 and 17 years there are underweight girls — thinness grade III, i.e. skinny ones, as their frequency at 9, 13 and 14 years reaches 2.0%. Boys with such nutritional status (thinness grade III) are found only at the end of the studied period (14, 16 and 17 years), the frequency of those ones being 3.4% at 17

Discussion

The analysis and evaluation of our results are conformed to the decision of international scientists concerning application of the age and gender specific criteria for BNS categorisation during growth [19]. It is the period from birth till body maturity during which in specific manner for both genders, the two basic components of body weight — Fat Tissue (FT) and Lean Body Mass (LBM) developed. Something more, the morphological body maturity, which during pre-puberty begins to developed more quickly and its basic realisation happens in puberty, naturally goes along with the accumulation of more FT in girls while in boys it is accompanied with the increment of LBM too. By these processes, the formation of typical for adult man and women body structure and body composition come true [3, 10].

The followed age changes of BMI means in our study confirm the underlined dependence of these values from children's age and gender. Concerning Bulgarian children and adolescents the interage and the respectively BMI gender differences follow as a whole the stage decrease or increase of BMI during growth described by many authors and summarized by Cole et al. [5, 6]. The established age BMI curves in our study reflect the dependences between specific life conditions in Bulgaria and the specificity of growth and body maturity in Bulgarian children. The borderline of childish Body Nutritional Status and the coming formation of youth Body Nutritional Status could be found between 8 and 9 years. Reliable changes in Body Nutritional Status during active puberty in the Bulgarian girls come between 11 and 12, and in boys they come one year later reflecting later puberty in them.

Concerning gender differences of interest is the permanently higher BMI in boys till puberty. During active puberty (11-13 years) the BMI values are higher in girls reflecting the specificity of body maturity in them. The quicker accumulation of subcutaneous fat tissue in female individuals during this period is an initial period that later drive to the formation of Body Nutritional Status type pear in them.

From medico-biological point of view most interesting are the results about frequency of both unhealthy categories Body Nutritional Status. Both underweight and overweight dissemble enough high risk for health, as during growth, so being predisposition for some illnesses in adult ages. Unfortunately, in Bulgaria and in other European countries, the only direction in which are going on discussions concerning Body Nutritional Status in growing up boys and girls is obesity. On the other hand, the results published in international journals very often are generalized affecting large age periods that doesn't correspond to the accepted and natural age stages of growth [14, 20]. From medico-biological point of view, however, such age generalization is not correct because it is not clear to which age group are belonged the corresponding data. Such generalisation periods couldn't be able to give possibility for a connection between specificity of growth process and body nutritional status to be sought.

Of special interest in national aspect is the established frequency of underweight among present growing up Bulgarian population. Our results have also international meaning because they are made according to the unified criteria of IOTF and WHO and could be compared to similar data elaborated in other countries.

It is established that if till 13 years a big part of the Bulgarian boys and girls are predisposed to obesity, not very few of them are predisposed to illnesses as a result from underweight. More serious health problem is the one concerning high frequency of underweight in post puberty period, mainly for girls.

Important result from medical point of view is the established presence of thinness grade III in girls (i.e. skinny girls) for nearly all ages between 6 and 17. In our opinion this result is an alarm at necessity of profound investigations elaborating respective national strategy concerning prevention from forthcoming health complications.

Similar results for increment of the underweight frequency in children were already published in other European countries. The cited team of Czech scientists [17] established that during last decade increases considerably the frequency of underweight children in the lowest age categories (till 14.0%) and among older adolescents (till 13.0%). Martinez et al. [13] reported about increment of the underweight frequency in Spanish children from both genders — from ≈ 3.0% during 1992 till ≈ 10.0% during 2004.

Conclusion

The established frequency of underweight and overweight among present generation of Bulgarian children and adolescents could serve as a base for analyses, evaluations and prognoses for their health status. The frequency rise of thinness with ages among adolescents represents a special interest, as well as necessity of profound investigations and wide discussion in parallel with the problem of obesity.

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Relation between the Nutritional Status Type and the Arterial Blood Pressure in 9-15-year-old Schoolchildren from Sofia

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The aim of the present work is to investigate the age-dependent changes of Nutritional Status (NS) and Arterial Blood Pressure (ABP) in 9-15-year-old schoolchildren from Sofia, the generation living at the beginning of the 21st century and to evaluate the relationships between ABP and the anthropologically determined NS. The analyzed data represent a part of complex cross-sectional anthropological study including 1142 schoolchildren, attending three schools in Sofia. The study was carried out during the years 2001 and 2002. It was established that the "hypertension" ABP was found to be four times more often among the overweighed schoolchildren than among the ones with normal NS. The frequency of girls with "hypertension" Systolic Blood Pressure (SAP) between 9 and 14 years was consistently higher than it was in the boys between these ages. Among obese schoolchildren the frequency of those with "hypertension" SBP has increased markedly, better expressed in girls.

Key words: adolescents, anthropometric, anthropometrical nutritional status, arterial blood pressure, relationships

Introduction

The morphological and functional entity of organism determines the scientific interest in the revelation and the impartial assessment of relationship between basic and important for the human health characterizations. Purposeful investigations into this direction are important especially when covering the period of growth, within which the childish and/or adolescent organism transforms itself into a mature one [3, 5, 10, 12, 14, 21]. Recently more and more specialists report on the established trend about the predisposition towards considerable and socially important diseases for adulthood to be detected since the period of growth [8, 9, 16, 20]. Among clinical specialists is accepted the existence of such a trend about two important characteristics in the human physical development. Cardiologists established the so called

"trace phenomenon" concerning Arterial Blood Pressure (ABP), i.e. if a borderline or raised ABP have been revealed for a person during his childhood and adolescent ages, the expectancy for him to suffer from hypertension in his adulthood increases [1, 6, 8, 13, 18, 26]. The same trend is also established about overweight in young ages, which more often goes deeper into obesity in adulthood [4, 5, 8, 17, 26]. The close relation between ABP and the type of Nutritional Status (NS), which exists regularly, gave reason for numerous investigators to apply different approaches for an objective evaluation of this connection [7, 8, 11, 17, 18]. The detailed literature review made by us pointed out that articles dealing with purposive evaluation of the relationship between the NS type expressed by Body Mass Index (BMI) ("normal" or healthy NS, "overweight" NS and "obesity" NS) and the ABP categories ("hypotension", "normal tension", "heightened tension" or "pre-hypertonic" and "hypertension") could be found rarely, and in the Bulgarian scientific literature such articles weren't detected at all.

The aim of the present work is to evaluate the relationship between nutritional status type and arterial blood pressure level in 9-15-year-old in boys and girls from Sofia city who are representatives of the young Bulgarian generation living at the beginning of the 21st century.

Subjects and Methods

The data analyzed are part of a complex cross-sectional anthropological study (Mitova, 24) including 1142 schoolchildren aged 9-15 years from three schools in Sofia city, carried out during the years 2001 and 2002. The boys and girls under investigation were separated uniformly each sex in seven age groups — mean ages of 9.5, 10.5, 11.5, 12.5, 13.5, 14.5, and 15.5 years. The groups of the 9-year-old children comprise 81 boys and 81 girls, aged from 9.00 years to 9.99 years. The rest investigated boys and girls were ranged according to the same age affiliation.

The data concerning each schoolchild comprise: stature and body weight utilized to compute the BMI (body weight/height^2 in kg/m^2), Systolic Blood Pressure (SBP) and Diastolic Blood Pressure (DBP). The stature was measured using a standard anthropometer (allowance of 0.1 cm), and the body weight, using a Body Fat Monitor "Tanita TBF 612" (allowance of 0.1kg), respectively. The Arterial Blood Pressure was measured on the right hand in a seating position, after 10 minutes rest, using mercury sphygmomanometer according to the method of Korotkov (reading I and V phase with exactness of 0.1mmHg). When a value outside the norm for the physiological ABP was registered, two additional measurements were performed, at intervals of 5 minutes each, and the average value of the three trials were used.

The differences in ABP categorization of the students who belong to the three types NS — using respectively χ^2 (at $p<0.05$). To project the interrelations of the three types NS and the ABP was used the z-score procedure ($SDS=X_i-X/SD$, by norm for $SDS=0$).

The NS type was defined in three categories according to the data about BMI (normal, with overweight and with obesity) on the ground of the recommended by WHO and published by Cole et al. [2] cut off points for BMI about each age-gender group.

To classify the examined schoolchildren into different ABP-categories were used the borderline values for SBP and DBP on the generally accepted normative percentile values (P_5, P_{90}, P_{95}) for each age-gender group and computed according to the data in the present study (Table 1). The values for ABP<P_5 showed presence of

"hypotension"; the ABP values between P_5 and P_{90} showed "normal tension"; those between P_{90} and P_{95} showed normal-but-higher tension that is not pathological for the respective age, defined also as "heightened tension" (a risky tension, pre-hypertonic, normal but higher); and values for ABP>P_{95}, which marked a "hypertension" for the concerned age.

Results

Distribution of the investigated schoolchildren into types concerning NS

The data analysis concerning distribution of the examined schoolchildren into NS types (Tabl. 1 and Fig. 1) showed that in all the investigated age-gender groups the schoolchildren with normal nutritional status prevail, their frequency being established to increase from the beginning to the end of the examined period. For the NS type "overweight" the frequency was rarer in the 15-year-old boys (6.3%) and girls

Table 1. Distribution of schoolchildren into NS types according to the data of BMI

BOYS							
Age (yrs)	Total	Normal NS		Overweight		Obesity	
	N	n	%	n	%	n	%
9	81	66	81.5	13	16.0	2	2.5
10	80	68	86.1	10	11.4	2	2.5
11	82	62	75.6	17	20.7	3	3.7
12	83	69	83.1	13	15.7	1	1.2
13	75	64	84.2	11	14.7	1	1.3
14	83	68	81.9	13	15.7	2	2.4
15	80	72	90.0	5	6.3	3	3.8
GIRLS							
Age (yrs)	Total	Normal NS		Overweight		Obesity	
	N	n	%	n	%	n	%
9	78	66	81.5	10	12.3	5	6.2
10	80	62	77.5	15	18.8	3	3.8
11	80	68	85.0	12	15.0	0	0.0
12	85	77	90.6	8	9.4	0	0.0
13	83	72	86.8	11	13.3	0	0.0
14	82	72	87.8	9	11.0	1	1.2
15	82	81	98.8	1	1.2	0	0.0

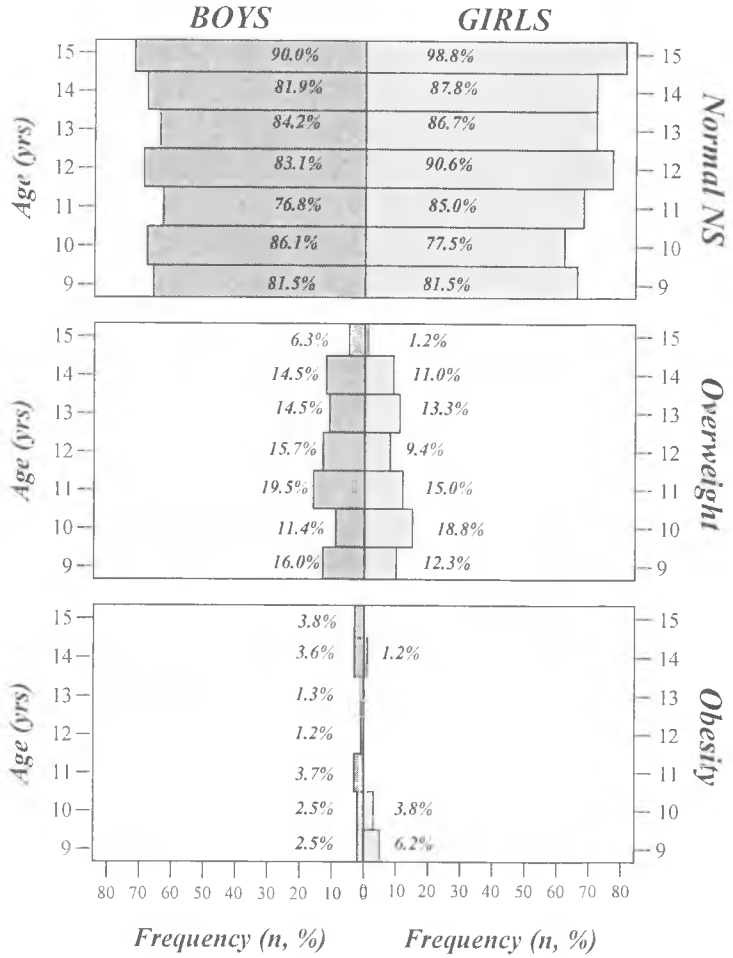

Fig. 1. Distribution of schoolchildren into NS types according to the data of BMI

(1.2%). Most often this NS category was found in the boys at 11 years (20.7%) and in the girls at 10 years of age (18.8%). Boys who got into the NS type "obesity" was revealed rarely concerning all age groups (between 1.2% at 12 years and 3.8% at 15 years of age), while opposite to them the girls with NS type "obesity" was revealed only for three age groups (at 9, 10 and 14 years of age), their frequency being higher — 6.2%, 3.8% and 1.2% respectively.

Distribution of the schoolchildren into categories of ABP

The schoolchildren with "normal" SBP and "normal" DBP were met most frequently in all the investigated age groups (Tabl. 2). The frequency of individuals having normal SBP varies between 82.3% in the 10-year-old and 92.8% in the 14-year-old boys, and between 81.7% in the 14-year-old and 97.5% in the 11-year-old girls.

Table 2. Distribution of the schoolchildren into ABP categories

Systolic Blood Pressure																			
	BOYS									GIRLS									
Age (yrs)	N	Hypotension		Normotension		Heightened tension		Hypertension		N	Hypotension		Normotension		Heightened tension		Hypertension	
		n	%	n	%	n	%	n	%		n	%	n	%	n	%	n	%
9	81	4	4.9	73	90.1			4	4.9	81	1	1.2	74	91.4	1	1.2	5	6.2
10	79	3	3.8	65	82.3	10	12.7	1	1.3	80	4	5.0	70	87.5			6	7.5
11	82	6	7.3	71	86.6			5	6.1	80	1	1.3	78	97.5			1	1.3
12	83	2	2.4	75	90.4			6	7.2	85	8	9.4	71	83.5	3	3.5	3	3.5
13	80	3	3.8	68	85.0	8	10.0	1	1.3	83	1	1.2	75	90.4			7	8.4
14	83	2	2.4	77	92.8			4	4.8	82	8	9.8	67	81.7			7	8.5
15	80	3	3.8	69	86.3	1	1.3	7	8.8	82	4	4.9	69	84.1	4	4.9	5	6.1
Diastolic Blood Pressure																			
	BOYS									GIRLS									
Age (yrs)	N	Hypotension		Normotension		Heightened tension		Hypertension		N	Hypotension		Normotension		Heightened tension		Hypertension	
		n	%	n	%	n	%	n	%		n	%	n	%	n	%	n	%
9	81	5	6.2	69	85.2			7	8.6	81	3	3.7	70	86.4	4	4.9	4	4.9
10	79	1	1.3	77	97.5			1	1.3	80	3	3.8	65	81.3	11	13.8	1	1.3
11	82	3	3.7	78	95.1			1	1.2	80	8	10.0	59	73.8	11	13.8	2	2.5
12	83	5	6.0	75	90.4			3	3.6	85	5	5.9	77	90.6			3	3.5
13	80	4	5.0	72	90.0			4	5.0	83	4	4.8	75	90.4			4	4.8
14	83	4	4.8	71	85.5	6	7.2	2	2.4	82	4	4.9	74	90.2			4	4.9
15	80	4	5.0	71	88.8	1	1.3	4	5.0	82	4	4.9	68	82.9	6	7.3	4	4.9

The frequency of boys having SBP and DBP hypotension varies between 2.4% at the age of 12 and 14 years and 7.3% at the age of 11 years, while the frequency of corresponding girls varies between 1.2% at the age of 9, 11 and 13 years and 9.8% at the age of 14 years.

Schoolchildren of only three age groups have fallen into the category "heightened tension" SBP and their frequency decreased in the ages — in boys at the ages of 10 (12.7%), 13 (10.0%) and 15 (1.3%). The frequency of girls going to this SBP category was three times lower but was registered as early as at the age of 9 (1.2%), followed by the 12 (3.5%) and 15 (4.9%) years old girls and their frequency increased in the ages. The frequency of boys with hypertonic values of SBP varies between 1.3% at 10 years and 8.8% at 15 years, and the frequency of girls — between 1.3% at 11 years and 8.5% at 14 years respectively.

The distribution of schoolchildren into the DBP categories was analogous to this one concerning the categories of SBP. Hypotonic DBP was registered in both genders, as the frequency varies for boys from 1.3% in the 10-year-old ones and 5.0-6.0% in boys from the rest age groups. The lowest frequency of girls with hypotension DBP was revealed at the ages of 9 and 10 years (3.8%), while the maximal frequency about this characteristic was observed — at the age of 11 years (10.0%). Within the age interval 12-15 years, like it is in boys, the frequency of girls with hypotension DBP is about 5.0%. The DBP of the category "heightened tension" were found only in boys at 14 years (7.2%), while in the 15 years old ones it was 1.3%. Unlike boys, the girls fall 2.5 times more frequently in this category — 4.9% at the age of 9 years, 13.8% at the age of 10 and 11 years, and 7.3% at the age of 15 years. Into the category "hypertension" DBP were met nearly 1.0% less schoolchildren than the ones with "hypotension" DBP. The frequency of individuals with "hypertension" DBP varied between 8.6% at 9 years and 1.2% at 11 years in boys, and between 1.3% at 10 years and 4.9% at 9, 14, and 15 years in girls.

Relation of NS type and ABP in the investigated schoolchildren

In schoolchildren having normal NS, the normal ABP tension was established again most frequently (Figs. 2 and 3). In this NS category, the frequency of boys with hypotension SBP was about 5.0% and the frequency of those with hypertension SBP and DBP was 3.0% (lower by 2.0%). The frequency of boys with "pre-hypertonic" SBP was 3.0%, while only 1.0% of them displayed a "heightened tension" DBP.

The distribution of SBP and DBP among girls who have normal nutritional status is similar to that in boys. Pathologically high SBP values were found in 4.0% of the girls (1.0% more than in boys), while the "heightened tension" DBP were more frequent (3.6%) in girls than the "heightened tension" SBP (1.4%).

About NS type "overweight" the impression was made that in both sexes the frequency of schoolchildren with "hypotension" SBP was lower than the frequency of those ones who fall into the "normal" type of NS (by nearly 3.0% in boys and 2.0% in girls), while the schoolchildren with "hypotension" SBP were several times more frequent (12.2% in boys and 16.5% in girls). The frequency of schoolchildren with "hypotension" DBP, however, decreased only in boys, and in girls it increased if only by 1.0%. Concerning the NS-type "overweight", boys with "hypertension" DBP were twice more frequent (6.1%) than girls (3.0%).

Interesting was also the distribution of schoolchildren after different ABP categories towards NS-type "obesity". In both gender groups were not found obese schoolchildren who have hypotension ABP. On the other hand, relatively lower was

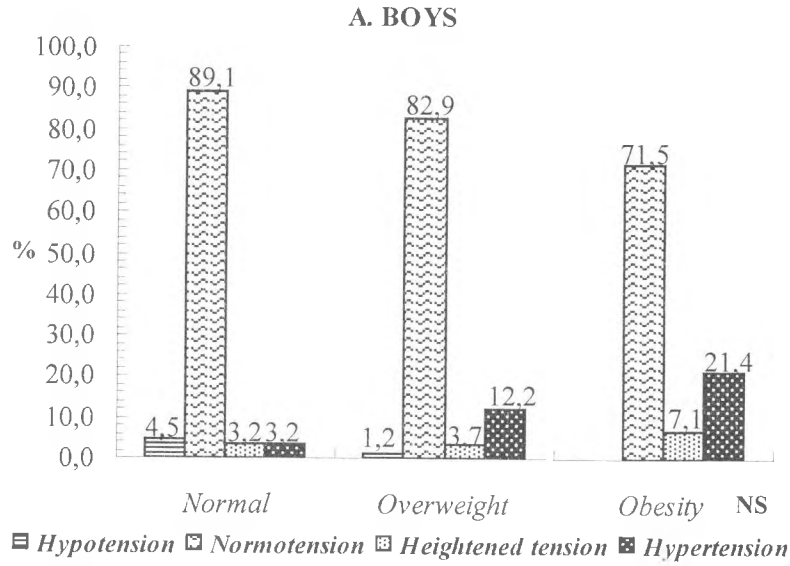

Fig. 2. A) Relation of NS type and SBP categories in boys

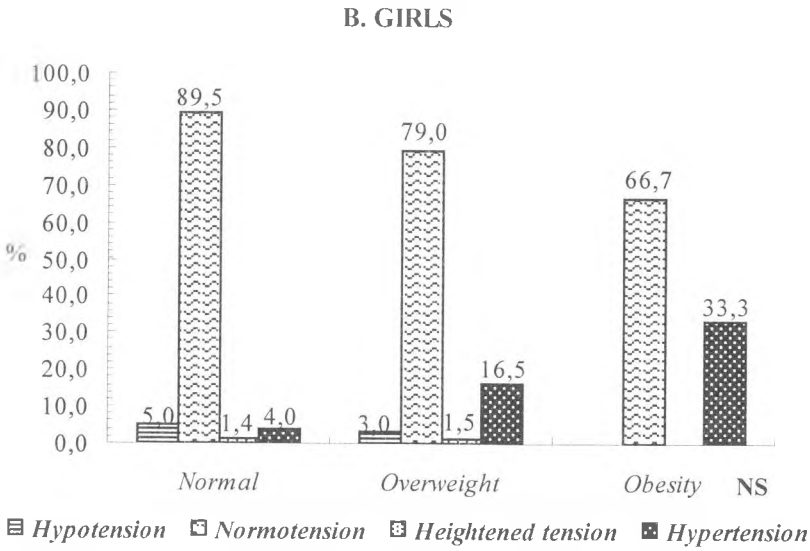

Fig. 2. B) Relation of NS type and SBP categories in girls

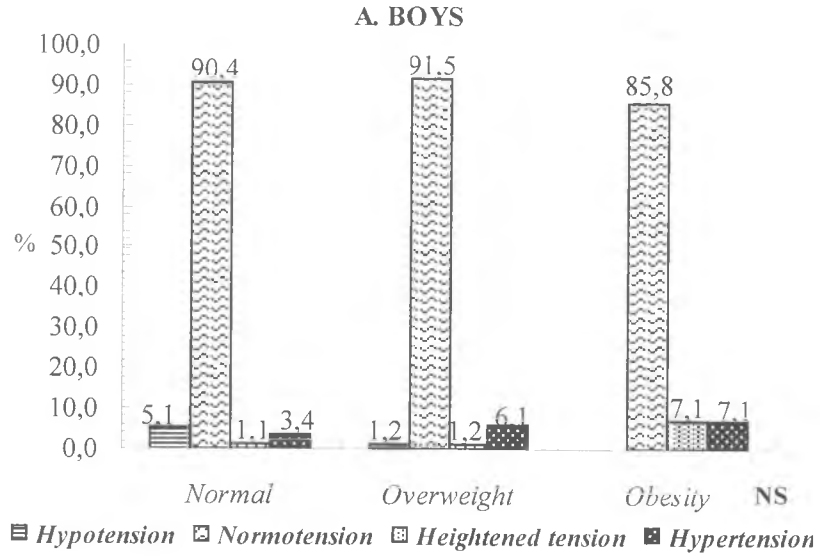

Fig. 3. A) Relation of NS type and DBP categories in boys

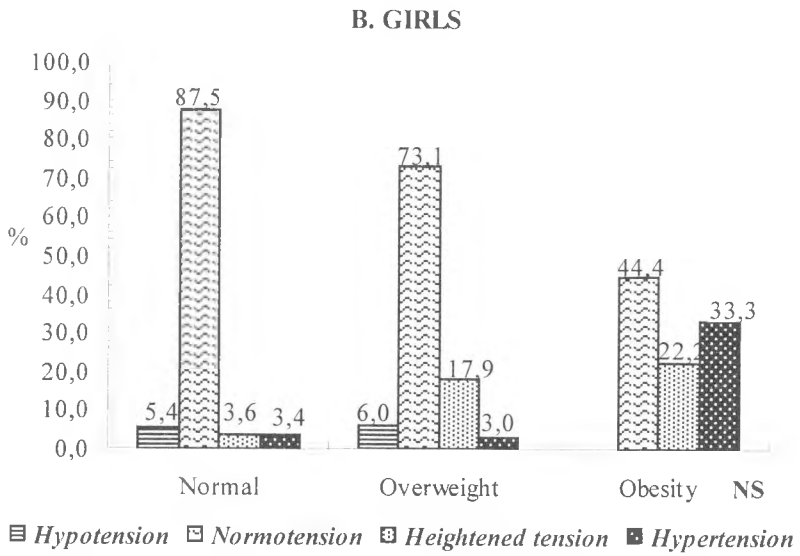

Fig. 3. B) Relation of NS type and DBP categories in girls

the frequency of schoolchildren with "normal" ABP concerning SBP — 71.5% for boys and 66.7% for girls, as with "normal" ABP concerning DBP the frequency was 85.8% for boys and 44.4% for girls. Into this NS type the boys with "hypertension" SBP were at three times more frequent (21.4%) compared to the ones with "hypertension" DBP (7.1%). Concerning obese boys, the frequency of those who have "heightened tension" SBP and the ones with "heightened tension" DBP was identical (7.1%). In obese girls, the frequency of those with "hypertension" SBP and "hypertension" DBP was again identical (33.3%). Obese girls who have "hypertension" SBP were established 1.6 times more frequently, while obese girls who have respectively "hypertension" DBP were 4.6 times more frequently. Among obese girls such with "heightened tension" SBP were not found, while 22.2% of them had "heightened tension" DBP.

The results obtained, about interconnection of the NS type evaluated by BMI and the SBP and DBP values in 9-15 years old schoolchildren, could be shown clearly by the z-score procedure (Fig. 4). In comparison with schoolchildren having normal NS, in the adolescents being overweighed the Standard Deviation Score (SDS) increased progressively (up to SDS~0.5 for SBP and up to SDS~0.3 for DBP), as in those having "obesity" NS — the SDS values came to~0.9 for SBP and SDS>1.0 for DBP (in the investigated by us schoolchildren having normal NS the SDS<0, as in the same category were incorporated the individuals with "undernutrition" NS). By the χ^2 procedure about reliability of the differences in the distribution of ABP categories concerning the three types NS were established that the obesity boys and girls had higher values (statistically significant) for SBP compared to the children with normal NS (boys at $p<0.01$; girls at $p<0.001$). Reliably higher DBP for the schoolchildren with normal NS were found only in the obesity girls ($p<0.001$). According to the χ^2 procedure concerning ABP, the differences between overweighed and obesity adolescents, as the difference between adolescents with normal and overweighed NS were not statistically significant.

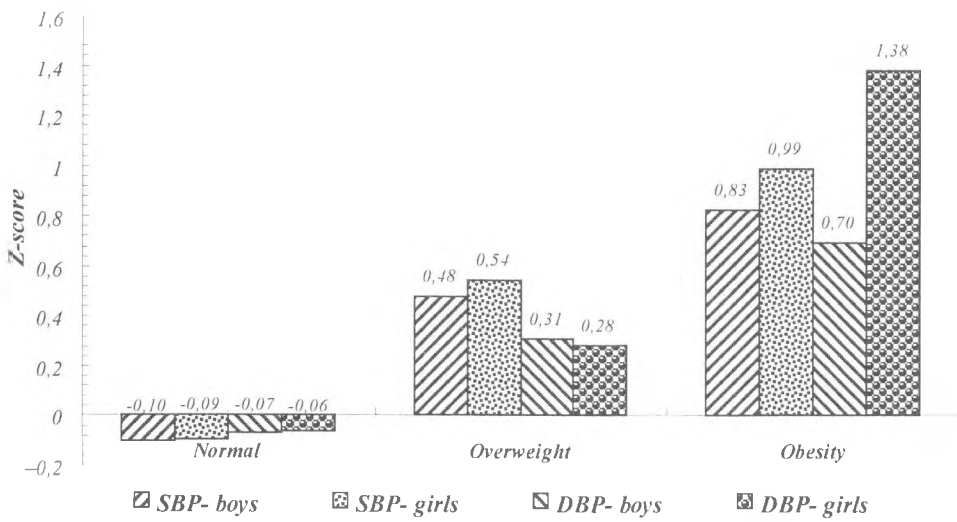

Fig. 4. Z-score values of nutritional status type and arterial blood pressure

Discussion

Our results that concerned the frequency of schoolchildren into different NS categories were similar to those found by Stanimirova [27]. She studied the changes of obesity's frequency in children and adolescents (from 0 till 20 years) using norms elaborated by her, which concern three populations from the region of Pleven town during a period of 20 years (1973, 1988 and 1993). According to the author mentioned above, the frequency of obese children was found to be different for all ages throughout childhood and youth, during which three maximums were established. The first maximum was found to be between the ages 3 and 6 years, when the obesity's frequency reached 10.0%, the second had coincide with the beginning of puberty and the pubertal growth acceleration (frequency of 15.0-16.0%), and the third maximum was established to lie respectively in the period 15-16 years of age — the end of puberty. In the investigated by us 9-15-year-old schoolchildren from Sofia were established also a maximal frequency of overweighed and obese children at the beginning of children's puberty and pubertal growth acceleration. The next frequency's maximum was established only in boys at 15 years of age.

According to the international data collected by Cole et al. [2], about the age period from 2 till 18 years, the frequency of overweight NS vary between 5.0% and 18.0%, and that of obesity NS — between 0.1% and 4.0%. The established by us frequency of "overweight" and "obesity" NS types has followed the same framework of the quoted authors. In our study, the maximal frequency of overweighed boys was higher by nearly 3.0% only at the age of 11, and the maximal frequency of obese girls was higher by nearly 2.0% at the age of 9 years.

Compared with the scarce data from Bulgaria obtained on the basis of the same cut-off values by Cole et al. [2], the overweight and obesity in 9-15-year-old schoolchildren from Sofia appear to have low frequency than in children of the same age in Plovdiv (frequency of 18.0% for overweight and 5.0% for obesity) [15] and some of the smaller towns (frequency of 20.5% for overweight and 8.2% for obesity) as Haskovo, Lyubimets, and Svilengrad [28].

Growth dynamics of arterial blood pressure in investigated 9-15-year-old schoolchildren corresponds to the data published in literature [8, 9, 10, 12, 13, 21, 22, 23, 26]. The comparison of our results that concerned the frequency of SBP and DBP hypertensions in schoolchildren studied during 2001-2002 with the results found by Rahneva [26], correspondingly for 6-15-year-old schoolchildren from Sofia city investigated in the years 1972/3 and 1982/3, showed that the frequency of hypertension ABP was equal in all three generations (about 6.0%). The average frequency of boys and girls with ABP hypertension at 10-14 years found in the present study was lower (from 2.0 till 4.0%) compared to the average frequency found in their coevals studied at the beginning of the 70s and 80s of the 20th century. At the age of 15 years, however, the schoolchildren with hypertension ABP represented 6%, i.e. they were about two times less than the schoolchildren with hypertension ABP established in the years 1973 and 1983 (12.2% and 11.2%, respectively). This could be explained by the fact that among the investigated by us boys and girls at 15 years during 2001-2002, the overweighed ones were 6.3% and 2.4% respectively and obesity were established only in 3.8% of the boys.

The results obtained by us, concerning relationship between the type of NS determined through BMI data, and the SBP and DBP categories, confirmed the fact that "heightened tension" and "hypertension" ABP have been found considerably more frequently in schoolchildren with overweight and obesity NS, the same being established by many authors who used other methodological approaches, as well [6, 7, 16, 17, 19, 20, 21, 22, 25].

Conclusions

The results in the anthropological investigation of 9-15-year-old schoolchildren from Sofia city show that:

The frequency of schoolchildren with overweight and obese NS was higher at the beginning of the growing up period (9-11 years of age) for both sexes, while at the age of 15 the frequency of boys and girls with overweight NS was found to be considerably smaller, and the obese NS was established only in boys.

It was established that the frequency of girls with "hypertension" SBP between 9 and 14 years was consistently higher than it was in the boys between these ages.

The "hypertension" ABP was found to be in times more often among the overweighed schoolchildren than among the ones with normal NS.

Among obese schoolchildren the frequency of those with "hypertension" SBP has increased markedly, better expressed in girls.

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Comparative Anthropometric Characteristics of Bulgarian Students (1986-2002)

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It is made a comparison of basic anthropometric data of total 1019 university students in Sofia. Of them 297 male and 580 female students, were investigated in 1986 and 72 male and 70 female students were investigated in 2002. Twenty-one directly measured parameters and eight relative ones were analyzed. The study demonstrated that the height has increased in both sexes, more significantly in males. Unlike height, weight decreases sharply, especially in female students. This affects the massiveness of the body /BMI decreases/ and is due primarily to the reduction of subcutaneous fat tissue. Most body diameters and circumferences also decrease, especially in female students. These changes in some degree coincide with the worldwide tendency of gracilization and acceleration, but seem influenced also by the socioeconomic and cultural changes during this period in our country.

Key words: university students, physical development, anthropometric characteristics, secular changes, gracilization.

Introduction

Human growth and development are genetically determined, but they are under influence of a series of environmental factors. Among them the socio-demographic factors hold a special position [2, 13, 14]. Therefore the alteration in the anthropometric characteristics are used for evaluation of the living conditions in the society [1, 4, 10].

The late 20th and the early 21st century is a time of great socioeconomic changes in Bulgaria, sudden changes in living standards (mostly in the negative direction) and increasing social stratification. Although university students (especially these in the capital) are usually children of more educated and with better material status strata; they are not isolated of the society. Therefore the anthropometric characteristics of the students and their eventual alterations are of special interest. Moreover, research on physical development of young adults (about 20 years) in Bulgaria are sparse - the last reliable data for the whole Bulgarian young population, aged 18-25, are from measurements in 1970s [15].

Material and Methods

In the present paper are used anthropometric data of 297 male and 580 female students, investigated in the spring of 1986 in Sofia by R. Stoev and of 72 male and 70 female students, investigated in the spring of 2002 in Sofia by N. Atanassova-Timeva and Y. Zhecheva. The mean age of students investigated in 1986 was 23.9 years for male individuals and 22.0 years for female ones while those ones investigated in 2002 were younger — a mean of 19,5 and 19.6 years. In both cases it refers to young adults which growth is almost closed [3], thus the comparison between the both sample data is justified. Twenty-one absolute anthropometric features were analyzed, taken by standard anthropometric methods [6, 7]: height, body weight, sitting height, lower extremity length (height to *illiospinale*), circumferences of: chest in pause, waist, hip, thigh, calf, upper arm and forearm; biacromial diameter (breadth of shoulders), transversal and sagital chest diameters, bicristal diameter (bicristal breadth), skinfolds of: thigh, X-th rib, subscapular, calf, suprailiac and triceps. On the basis of these absolute features, 8 relative parameters are calculated: Body Mass Index (BMI), relative (relation to the height) sitting height, relative lower extremity length, relative chest circumference, relative biacromial and bicristal diameters, chest index (relation of sagital and transversal chest diameters) and pelvic index (relation of bicristal to biacromial diameters).

Data processing is used a standard variation-statistical analysis including Student's *t* criterion to compare the accuracy of the identified differences and standard deviation method of accounting for their relative size (variation statistic, t-test of Student and z-score).

Results and Discussion

Height of the investigated students of both samples (Tables 1 and 2, Figs 1 and 2) is greater than the national average, and this for Sofia, even compared with investigation data from 1980 [12]. This is easily explained — as noted, students are usually children of families with higher social status than the average. Such excess of students' height in comparison to the relevant age group of the whole population is well described in the literature in general and in particular in Bulgaria [5, 11]. It is more interesting that since 1986 the increase continues, which is more pronounced in male students. Obviously the socioeconomic difficulties in the 90s have not affected the height of male individuals of this social group. One possible explanation is that the early childhood of the students, investigated in 2002, falls in early 1980s, which is the period of maximal living standard in Bulgaria. The investigated students in 2002 have been around 8,5 and 14,5 years during the great economic difficulties in 1991 and 1997, i.e. before and after puberty leap in growth. However, socioeconomic crisis has probably left its mark — in male students, whose growing period lasts longer, the increase in growth is greater than that of the female ones, whose growth is almost completed by 16-17 years [3]. The difference in height between the two reference groups for males is 3,2 cm, while for females is twice less — 1,5 cm.

Unlike height, weight decreases sharply, especially for female students (by 6,7 kg = –0,75 SD). This affects the massiveness of the body (BMI decreases) and is due primarily to the reduction of subcutaneous fat tissue (skinfolds thickness). It conld not be said yet to what extent this is a consequence of changes in nutrition, associated with major socioeconomic and cultural changes in the late 20th century, but the general tendency of gracilization coincides with the observed secular

Table 1. Basic anthropological features — male students

	1986				2002				$p\leq$	Z
	N	M	SD	SE	N	M	SD	SE		
Body weight	297	75,01	10,4	0,60	72	70,4	8,63	1,02	0,001	–0,44
Height	297	175,61	5,78	0,34	72	178,78	6,63	0,78	0,001	0,55
Sitting height	297	90,88	3,93	0,37	72	93,17	3,76	0,44	0,001	0,58
Lower extremity length	297	101,34	4,7	0,27	72	99,09	5,18	0,61	0,001	–0,48
Chest circumference — pause	296	92,4	6,38	0,37	72	87,37	5,79	0,68	0,001	–0,79
Waist circumference	297	80,54	7,82	0,45	72	77,53	6,11	0,72	0,001	–0,38
Hip circumference	296	96,06	5,92	0,34	72	91,06	5,54	0,65	0,001	–0,84
Thigh circumference	296	55,4	4,37	0,25	72	56,05	4,11	0,48		0,15
Calf circumference	297	37,42	2,57	0,15	72	35,65	2,49	0,29	0,001	–0,69
Upper arm circumference	297	29,68	3,05	0,18	72	29,73	2,84	0,33		0,02
Forearm circumference	297	27,85	2,04	0,12	72	27,11	1,62	0,19	0,001	–0,36
Biacromial diameter	296	40,01	1,97	0,11	72	41,19	2,05	0,24	0,001	0,60
Transversal chest diameter	296	29,3	1,84	0,11	72	29,51	2	0,24		0,11
Sagital chest diameter	296	21,1	1,94	0,11	72	20,91	1,71	0,20		–0,10
Bicristal diameter	296	28,14	2,01	0,12	72	27,23	1,65	0,19	0,001	–0,45
Thigh skinfold	293	18,28	6,45	0,38	72	14,74	6,12	0,72	0,001	–0,55
Xth rib skinfold	293	11,49	5,11	0,30	72	7,08	2,87	0,34	0,001	–0,86
Subscapular skinfold	293	12,46	4,74	0,28	72	9,66	3,56	0,42	0,001	–0,59
Calf skinfold	292	10,69	5,08	0,30	72	10,76	3,77	0,44		0,01
Suprailliac skinfold	293	11,04	5,91	0,35	72	6,99	3,04	0,36	0,001	–0,69
Triceps skinfold	293	11,28	4,14	0,24	72	9,39	3,48	0,41	0,001	–0,46
BMI	297	24,29	2,93	0,17	72	22,04	2,56	0,30	0,001	–0,77
Relative sitting height	297	51,58	3,38	0,20	72	52,12	1,31	0,15	0,05	0,16
Relative lower extremity length	297	57,70	1,49	0,09	72	55,41	1,67	0,20	0,001	–1,54
Relative chest circumference	296	52,66	3,72	0,22	72	48,92	3,49	0,41	0,001	–1,01
Relative biacromial diameter	296	22,80	1,10	0,06	72	23,06	1,21	0,14	(0,15)	0,24
Relative bicristal diameter	296	16,03	1,05	0,06	72	15,24	0,91	0,11	0,001	–0,75
Chest index	296	72,09	5,94	0,35	72	71,05	6,43	0,76		–0,18
Pelvic index	296	70,38	4,73	0,27	72	66,22	4,43	0,52	0,001	–0,88

Table 2. Basic anthropological features — female students

	1986				2002				$p \leq$	Z
	N	M	SD	SE	N	M	SD	SE		
Body weight	580	59,13	9,03	0,37	70	52,39	6,99	0,84	0,001	–0,75
Height	580	162,55	5,59	0,23	70	164,05	5,06	0,60	0,05	0,27
Sitting height	580	86,44	3,45	0,14	70	87,22	3,12	0,37	(0,10)	0,23
Lower extremity length	579	93,36	4,25	0,18	70	89,97	3,91	0,47	0,001	–0,80
Chest circumference — pause	578	85,33	6,58	0,27	70	73,05	4,45	0,53	0,001	–1,87
Waist circumference	579	67,43	6,22	0,26	70	65,92	5,18	0,62	0,05	–0,24
Hip circumference	580	95,54	7,07	0,29	70	87,21	5,43	0,65	0,001	–1,18
Thigh circumference	580	55,38	5,2	0,22	70	54,13	4,42	0,53	0,05	–0,24
Calf circumference	579	34,9	2,65	0,11	70	33,1	2,65	0,32	0,001	–0,68
Upper arm circumference	578	24,98	2,85	0,12	70	24,72	2,29	0,27		–0,09
Forearm circumference	579	23,19	1,82	0,08	70	22,73	1,39	0,17	0,05	–0,25
Biacromial diameter	577	35,77	1,89	0,08	70	34,84	1,87	0,22	0,001	–0,49
Transversal chest diameter	576	25,46	1,77	0,07	70	24,91	1,56	0,19	0,01	–0,31
Sagital chest diameter	577	18,17	1,66	0,07	70	17,72	1,48	0,18	0,05	–0,27
Bicristal diameter	576	27,25	2,71	0,11	70	25,45	1,46	0,17	0,001	–0,66
Thigh skinfold	562	25,49	6,14	0,26	70	19,29	4,5	0,54	0,001	–1,01
Xth rib skinfold	563	13,45	6,19	0,26	70	8,79	3,34	0,40	0,001	–0,75
Subscapular skinfold	563	14,63	6,18	0,26	70	10,53	3,75	0,45	0,001	–0,66
Calf skinfold	560	21,61	6,24	0,26	70	14,61	3,37	0,40	0,001	–1,12
Suprailliac skinfold	563	14,94	7,00	0,30	70	8,35	2,77	0,33	0,001	–0,94
Triceps skinfold	562	15,93	5,46	0,23	70	13,62	3,53	0,42	0,001	–0,42
BMI	580	22,36	3,12	0,13	70	19,44	2,21	0,26	0,001	–0,94
Relative sitting height	580	53,19	1,56	0,06	70	53,17	1,26	0,15		–0,01
Relative lower extremity length	579	57,43	1,41	0,06	70	54,83	1,32	0,16	0,001	–1,84
Relative chest circumference	578	52,53	4,04	0,17	70	44,55	2,70	0,32	0,001	–1,98
Relative biacromial diameter	577	22,02	1,11	0,05	70	21,24	1,04	0,12	0,001	–0,70
Relative bicristal diameter	576	16,77	1,63	0,07	70	15,52	0,84	0,10	0,001	–0,77
Chest index	577	71,49	5,87	0,24	70	71,32	6,26	0,75		–0,03
Pelvic index	576	76,19	6,63	0,28	70	73,16	4,68	0,56	0,001	–0,46

Fig. 1. Z-score in male students

Fig. 2. Z-score in female students

Table 3. Comparable anthropometric features for students in Sofia (1986, 2002) and in Plovdiv (1993-1996)

	Males			Females		
Year	1986	1993-1996	2002	1986	1993-1996	2002
Height	175,6	176,9	178,8	162,6	163,3	164,0
Body weight	75,0	72,7	70,4	59,1	54,9	52,4
BMI	24,3	23,2	22,0	22,4	*20,6	19,4
Sitting height	90,9	94,4	93,2	86,4	87,8	87,3
Lower extremity length	101,3	100,0	99,1	93,4	91,4	90,0
Biacromial diameter	40,0	39,3	41,2	35,8	34,5	34,8
Bicristal diameter	28,1	27,8	27,2	27,2	25,7	25,4

*The index is calculated by us from the means.

changes in the late 20th century and in other countries (after [8]). The gracilization is manifested in the fact that most body diameters and circumferences, also decrease, especially in female students. It is especially pronounced against the background of increasing growth (decrease of the relative indicators). A larger reduction of body fat in females could be associated with some cultural changes in diet, which particularly affects young women from educated families (distribution of different diets).

An exception of the gracilization tendency is biacromial diameter of male students, which increases. Back to male individuals the upper arm circumference, thigh circumference and chest diameters remained stable, whereas in female students these measures follow the general decreasing tendency. Thus in male students a slight tendency to more athletic constitution is presented, which tendency is missing in female students. One possible explanation is that this phenomenon may be related to increasing sporting activity in male youths.

In both sexes is observed a reduction of absolute and relative lower extremity length ($p<0,001$), which is accompanied by increase of the sitting height. In male students, these changes are more pronounced in comparison with the changes in female students.

During the period between our two investigations, namely in 1993-1996, it has been conducted a research of the physical development of students in Plovdiv university [9, 14]. The published data do not include all anthropological features studied by us. The comparison of the results of our two studies and the aforementioned study are listed on Table 3. They confirm the established tendency in Sofia students to increase of height and decrease of weight, of the lower extremity length and of bicristal diameter. The results in Plovdiv well fit between the results of two other investigations. Discrepancy with the tendencies established in Sofia observed in biacromial diameter (which in Plovdiv students is less) and in sitting height (which in Plovdiv students is bigger). This may be due to small differences in measurement technique.

Conclusion

The anthropometric characteristic of Bulgarian students in the late 20th and the early 21st century is changing. These changes partly coincide with the worldwide tendency of gracilization, but seem influenced by the socioeconomic and cultural changes during this period in our country.

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Anthropometrical indices and pubertal maturation of boys in Bulgaria

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Puberty is a maturational process that leads to ability for reproduction. There should be reference ranges for every population for pubertal progress and therefore we describe the pubertal development of Bulgarian males. 131 boys from two primary schools in Sofia were included in this longitudinal study. They were monitored from the first to the seventh class of the primary school. The testicular volumes, pubic hair stage, penile length, and penile circumference as well as height, weight, waist and hip circumferences were registered. The anthropometric and genital characteristics according to the chronological age were presented to serve as a basis for further comparisons. There is some evidence for earlier or more rapid pubertal development in Bulgaria now in comparison to 1970s. Probably, the taller and heavier boys enter the puberty earlier, however, larger studies are necessary to confirm or reject this hypothesis.

Key words: puberty, boys, height, testicular volume, weight.

Introduction

Puberty is a time of immense developmental change. From the early work of Marshall and Tanner is known that the process occurs in a predictable sequence of events in both girls and boys. The first sign of puberty in boys is testicular enlargement, which is followed by pubic hair development, changing of the scrotum, penile growth, linear growth spurt [9]. The differences in the age of sexual maturation between populations are largely due to the different socio-economic conditions, as well as genetic factors. Consequently, each ethnic group has to construct its own normative data [4].

Therefore, we present the results of a longitudinal study on the pubertal development of Bulgarian boys. They show the pubertal progression and the corresponding anthropometrical indices.

Material and Methods

In the study were included 131 boys from two schools in Sofia chosen at random. The children were examined every consecutive year from 1993 to 2000. The signs of pubertal development (testicular volumes, pubic hair, penile length, penile circumference) and anthropometric indices /height, weight, waist circumference, hip circumference and body mass index (BMI)/ were determined. For technical reasons, in 1996 only the anthropometric values were described. At the beginning of the study 9 of the children were 6 years old, 112 were 7 years old, 9 were 8 years old and one was 9 years old. Some of the boys were monitored for a period shorter than 7 years, because of transfer to another school, disease or refusal of examination. The pubertal development of all boys was estimated by only one experienced investigator (Philip Kumanov) in order to avoid the inter-observational error. The anthropometrical indices were measured at the same time by well-trained team from the Institute of Experimental Morphology and Anthropology by the Bulgarian Academy of Sciences, Sofia. The study was approved by the Bulgarian Ministry of Education and Science. Statistical analysis was conducted with SPSS v. 11.0 (Chicago, IL, USA). Descriptive statistics and frequency analysis were used. Differences in the anthropometric indices were established through independent sample t-test or Mann—Whitney test after a Kolmogorov—Smirnov test for the normality of distribution. The data were shown as means and standard deviations.

Results and Discussion

Our data describing the pubertal development and the corresponding changes in the anthropometrical indices are shown on the Table 1. Rapid growth of the testes occurred between 11 and 14 years of age. The increase in the penile length and circumference was more gradually. These results supported completely the conclusions of our previous cross-sectional study of 6200 Bulgarian males between 0 and 19 years of age [17].

One of the most important questions discussed in the literature is whether secular trends in male pubertal development still exist. Therefore, we tried to compare our data to those of previous studies. An investigation of 3254 boys from the region of Blagoevgrad was conducted in the 1970s [16]. The results showed more rapid progress in the testicular volumes and a greater increase in the penile length in the current study in comparison to previous study (Fig. 1). However, data from another transversal study of 6207 boys from the region of Pleven, published at the same time (1980), leads to the conclusion that pubertal events start nowadays at similar age as before [15]. According to our study, volume of the right testis exceeded 2 ml at 11.50 $\pm$ 1.03 years, while the corresponding age for the left testis was 11.63 $\pm$ 1.05 years. Pubic hair reached stage 2 soon after the testicular enlargement at the mean age of 11.76 years $\pm$ 1.24. According to the study of Stanchev and Stanimirova [15], the puberty began at 11.50$\pm$1.44 years with the development of the genital stage 2, while the pubic hair stage 2 occurred at 11,61$\pm$1,55 years which is very close to our results.

The comparison between these three studies could not lead to definitive conclusions, because of some serious limitations. First of all, the two previous studies were cross-sectional, while we examined the boys longitudinally. The three studies were conducted in Pleven, Blagoevgrad and Sofia respectively, and thus the interregional differences as well as inter-observer errors could not be excluded. The other bias

Table 1. The anthropometrical and pubertal characteristics of the investigated boys.

	7 years	8 years	9 years	10 years
VRT	1.26±0.49	1.25±0.48	1.77±1.05	
VLT	1.26±0.47	1.22±0.46	1.67±0.98	
PH	1.01±0.10	1.02±0.20	1.11±0.44	
PL	4.89±0.65	5.12±0.67	5.19±0.67	
PC	5.66±0.50	5.87±0.49	5.87±0.51	
Height	125.90±5.35	131.55±5.68	136.60±6.14	141.75±6.51
Weight	25.82±4.25	28.41±4.98	32.62±6.15	35.41±7.34
BMI	16.22±1.90	16.36±2.03	17.39±2.45	17.51±2.70
WC	54.55±4.72	56.29±5.05	58.33±5.61	60.06±6.22
HC	57.49±5.10	59.73±5.36	62.86±6.51	64.86±7.59
	11 years	12 years	13 years	14 years
VRT	3.09±2.07	5.70±3.73	10.24±5.19	11.20±4.89
VLT	2.97±2.09	5.13±3.47	9.60±5.34	10.14±4.20
PH	1.45±0.70	2.08±1.02	3.40±1.32	3.39±1.33
PL	5.41±0.88	5.54±1.41	6.72±1.77	7.79±1.58
PC	6.29±0.85	6.80±1.30	8.32±1.50	8.60±1.35
Height	147.00±6.68	152.40±7.32	158.57±8.29	163.60±8.88
Weight	39.36±7.69	43.66±8.80	48.00±10.10	52.82±10.64
BMI	18.10±2.71	18.68±2.84	18.97±3.11	19.63±3.13
WC	62.07±6.64	63.70±6.44	66.28±6.67	67.97±6.52
HC	66.55±7.42	69.63±7.94	73.26±7.61	74.69±7.43

VRT — right testis volume; VLT — left testis volume;
PH — pubic hair stage according to Tanner;
PL — penile length; PC — penile circumference;
WC — waist circumference; HC — hip circumference.

could result from the different age of the boys. We examined children mostly aged between 7 and 14 years, while in the 2 previous studies were included boys from 7 to 18-20 years and late pubertal events were also registered. Consequently, some data suggests earlier or more rapid pubertal development in Bulgaria now in comparison to the 1970s but solid ground for this definitive conclusion is lacking. Our results about the pubertal onset in Bulgaria were close to those in other Balkan countries like Greece and Turkey, where earlier pubertal maturation was not demonstrated [3, 10, 11].

Stanchev and Stanimirova [15] mentioned in their paper that almost every table that represented mean anthropometric indices was based on the chronological age. However, the mean anthropometrical characteristics varied significantly in boys at the same chronological age, but in different pubertal stages. Among 12 years old boys the authors described children from pubertal stages I to IV and the difference in their mean height was 16,63 cm. With the progression of puberty this difference

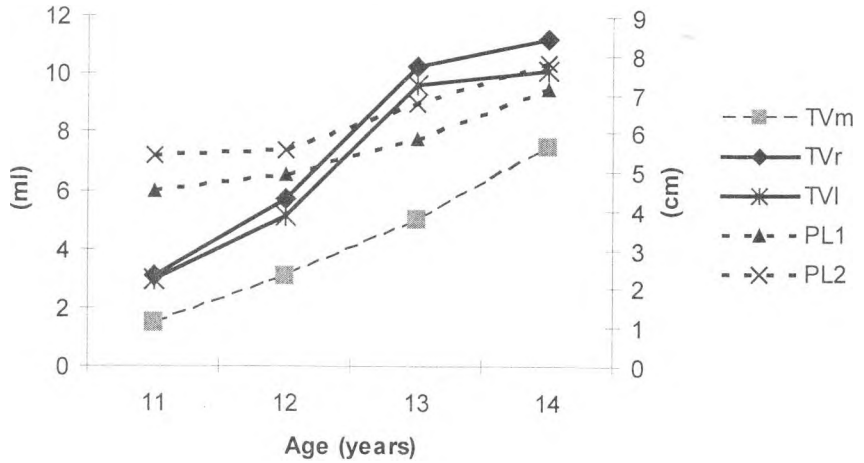

Fig. 1. Comparison between the data from our longitudinal study and from the previous study [16]. TVm — Mean testicular volume, 1970s; TVr — right testicular volume and TVl — left testicular volume in the present study; PL1 — penile length,1970s; PL2 — penile length in the present study

decreased and in the 17 years old boys it was only 1,03 cm [15]. Similarly in our study, pubic hair stages from I to IV were observed in 12 years old boys and the difference in the mean height between them was 12.73 cm. Consequently, the pubertal progression should be considered by the evaluation of the physical development in adolescent boys.

One of the most interesting unsolved problems in the literature is if the height, weight and BMI have any influence on the pubertal development in males. The recent secular trends in pubertal maturation especially in girls seem to coincide with the increasing prevalence of overweight and obesity and have raised considerable discussion as to whether the early maturation is due to the obesity epidemic [2]. According to He and Karlberg, a higher BMI gain in childhood is related to an earlier onset of puberty in both sexes [5]. However, according to Lee et al. [8], boys in the highest BMI trajectory (mean BMI *z* score at age of 11.5 years, +1.84) had a greater relative risk of being prepubertal compared with boys in the lowest BMI trajectory (mean BMI *z* score at age 11.5 years, −0.76). The authors suggest that the relationship between body fat and timing of pubertal onset is not the same in boys as it is in girls [8]. Besides the contradictory results it should be mentioned also that some longitudinal studies used only anthropometrical data like the onset of pubertal growth spurt and the peak height velocity to establish the onset and the progress of puberty [2, 5]. Obviously, it would be better to make conclusions considering not only the height and weight of the children, but also testicular volumes or pubic hair stages.

Therefore, we decided to compare the boys that had testicular volume 3 ml or more at the age of 12 years (earlier maturing boys, group A) to those that did not enter puberty and respectively did not show any testicular enlargement at the same (12 years) age (later maturing boys, group B). As expected, the children in puberty were higher and heavier than those which did not enter puberty at the age of 12 (Table 2).

Table 2. Differences in the anthropometrical indices at the ages of 7 and 12 years in earlier maturing boys (right or left testis 3 ml or more at the age of 12) and later maturing boys (no testicular enlargement at the age of 12 years)

7 years old boys	**Height***	**Weight***	**BMI**
Earlier maturing	126.39±5.07 cm	26.04±3.73 kg	16.25±1.70 (kg/m^2)
Later maturing	123.02±6.32 cm	23.72±3.08 kg	15.63±1.18 (kg/m^2)
12 years old boys	**Height****	**Weight****	**BMI***
Earlier maturing	154.27±6.74 cm	45.51±8.42 kg	19.05±2.86 (kg/m^2)
Later maturing	146.58±7.33 cm	37.39±7.06 kg	17.32±2.25 (kg/m^2)

* — $p<0.05$; ** — $p<0.001$.

However, it was more important to find if the earlier maturing boys were more obese in their childhood in comparison to the others. Therefore, we compared the height, weight and BMI of the earlier maturing boys (group A) at the age of 7 years to the same indices in the later maturing children (group B) (Table 2). The results showed that in their childhood the earlier maturing boys were at the same time taller and heavier than the later maturing boys. Probably, because of the unidirectional distinctions in the height and weight of the children, the difference in the BMI did not reach statistical significance. Thus, we could speculate that the taller and heavier but not more obese boys enter the puberty earlier. However, we think that the number of investigated children is small for making definitive conclusions.

Laron [7] investigated also the relationships between the obesity and the sexual maturation in males. He found that at all ages, the obese boys were taller and their bone age was more advanced than the controls up to age 14. However, there was no difference between the two male groups in the time of appearance and development of axillary or pubic hair, moustache, beard, acne or breaking of the voice, testicular volume, and penile size. The mean overall pubertal score and age of first ejaculation were also similar [7]. On the other hand, a study in Spain established a significant positive correlation between the age of the pubertal onset and BMI in boys, indicating a tendency towards higher BMI at pubertal onset when onset is later [12]. Wang analyzed data for 1520 American boys and established that comparing to their counterparts, early maturing boys were thinner [13]. On the contrary, a recently published study of 21 612 Danish boys born between 1940 and 1969 suggested that during the study period the heaviest category of prepubertal children entered puberty significantly earlier than the lightest category of children. However, the secular change was found in all BMI categories suggesting that the obesity is not solely responsible for the earlier maturation [2]. It should be mentioned, that conclusions from data collected between 1940s and 1970s should not be automatically transferred to 1990s, because of dramatically changed life conditions. For instance, Juul et al. found that the Danish boys in 1991-1993 were significantly taller compared with data from 1964, but timing of pubertal maturation seemed unaltered [6].

Clear associations between childhood adiposity, as reflected in BMI, and early pubertal development in girls have been established [1]. Increased adiposity in prepubertal children could lead to higher conversion rate of androgens to estrogens, thereby overexposing tissues to estrogens during prepubertal years. Insulin

resistance in obese subjects is associated with compensatory hyperinsulinaemia and lower levels of sex-hormone-binding globulin (SHBG), resulting in increased sex steroid bioavailability. Thus, there are several mechanisms whereby the increased prevalence of overweight and obesity could trigger early pubertal development by increased exposure to sex steroids in girls.

However, as we have shown, the influence of the fat tissue on the sexual maturation in boys remained unexplained. Thus, the information about the anthropometrical and pubertal indices in investigated by us children could serve for further comparison and conclusions for the pubertal development and acceleration in Bulgarian boys.

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Third Head of Biceps Brachii

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The anterior compartment — biceps brachii muscle is very variable one. In about 12% of arms, a humeral head is found in addition to those usually found arising from the coracoid process (medial or short head) and the glenoid lip of the scapula (lateral or long head). A biceps with more than two heads is found in 10% of white Europeans. In our case we reveal an additional third head of this muscle. The origin of this head is not unusual and it is one of the most common cases of this accessory slip arising from the humerus at the insertion of coracobrachialis, extending between it and the brachialis muscle. Some other varieties are discussed from the available literature. Finally the knowledge of the existence of the third head of the biceps brachii may become significant in preoperative diagnosis and during surgery of the upper limbs.

Key words: anatomical variants, biceps brachii, progressive muscular varieties.

Introduction

The biceps brachii is one of the muscles of the anterior compartment of the upper arm. It is characteristically described as a two-headed muscle that originates proximally by a long head and a short head (Williams, [10]). Distally, these two heads join to form a common tendon which inserts into the radial tuberosity, and some aponeurotic fibres form the bicipital aponeurosis which merges with the deep fascia of the forearm. This muscle mainly contributes to flexion and supination of the forearm (Williams, 1995). Testut has described the biceps brachii muscle as one of the muscles with most frequent anatomical variations [9]. These variations may present as a group of accessory fascicles arising from the coracoid process, the pectoralis major tendon, head of the humerus, articular capsule of the humerus or from the shaft of the humerus itself (Sargon, [8]). This last variation is also known as the humeral head of the biceps brachii muscle. Several authors have reported the presence of this anomaly with a varying frequency (Asvat, 1993; Bergman, 1988; Kopuz, 1999). A biceps with more than two heads is found in about 8% of Chinese, 10% of white Europeans, 12% of black Africans, and about 18% of Japanese (Bergman, 1988). A biceps may be composed of 1 to 5 heads. The most common accessory slip is one arising from the humerus at the insertion of coracobrachialis, extending between it and the brachialis muscle. It joins the short head, but most of its fibers

join the bicipital or semilunar fascia. It may be isolated and terminate entirely in the fascia. Mori described various origins of the third or accessory head as follows: In 50 arms there were 10 (20%) arms with a third head of the biceps. The origins of these additional heads were:

1. The distal portion of the deltoid tuberosity, 4 arms, 8%.
2. Near the point of the humeral insertion of coracobrachialis, 3 arms, 6% and
3. The terminal tendon of pectoralis major, 2 arms, 4% (Mori, 1964).

Description

During routine student dissection of a left upper limb from an adult male cadaver a third head of biceps brachii muscle was found. This additional head was observed deeper to the other two standard heads. The origin of the variable muscle is from the anterior surface of the humerus between the insertion of coracobrachialis and the origin of brachialis muscles. The third head is inserted medially on the most distal part of the common muscle body and ends on the usual place for biceps brachi with its tendon and apponeurosis. The length of the third head, which is strip-like, is 157 mm and its width is 19 mm almost along its whole course. The nerve supply for this structure is from the musculocutaneous nerve and the brachial artery is sending several arterial branches to it. The other two heads has its usual course, size and nerve and blood supply (Figs 1, 2).

Discussion and Conclusion

The muscles are usually variable structures. A reduction and even a total agenesis of muscles are observed as well as appearance of additional heads, attachment to adjacent muscles and expansion or reduction of their origin or insertion. These

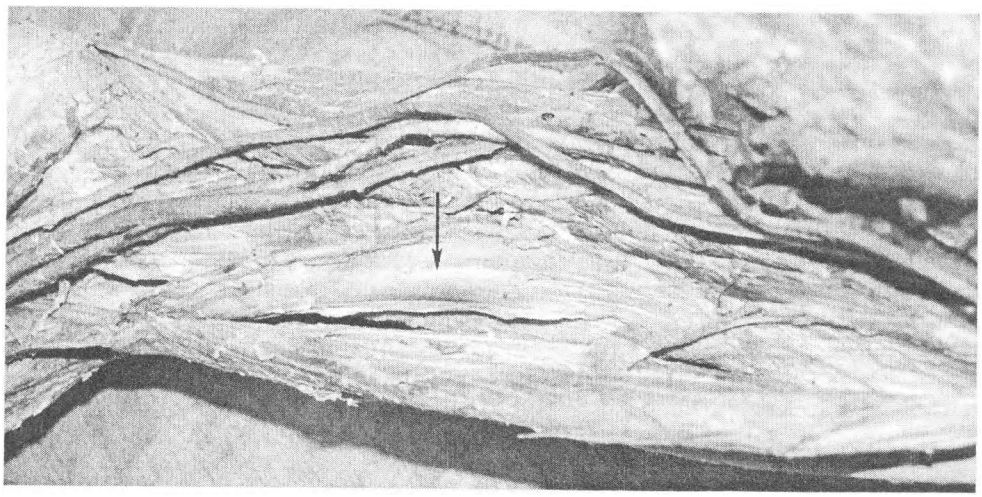

Fig. 1

Fig. 2

varieties are usually congenital and could be regressive and progressive type. The former are closer to more primitive forms like sternalis m., the third head of biceps brachii m., functional auricular mm., peroneus quartus muscle, etc. (Койчев,1995). The incidence of the third head of the biceps brachii muscle has been reported in several articles. Gray's Anatomy reported the incidence of this variation to be as much as 10% (Williams, 1995), which concurs with the observations of Bergman et al. in white Europeans (Bergman, 1988). Asvat et al. reported an incidence of 21.5% in their study group consisting of blacks (Asvat, 1993). It appears that the incidence varies among ethnic groups. Kopuz et al. attributed the appearance of these variants to evolutionary or racial trends (Kopuz, 1999). Santo Neto et al reported an incidence of 9% among blacks, which was significantly lower than the reported incidence for whites in his series (Santo Neto, 1998). Khaledpour contradicted Santo Neto et al.'s results by comparing his series to the results from other authors. He reported that the third head of biceps brachii was rare in whites and relatively high among blacks (Khaledpour, 1985). Given its innervation and relationships, the third head of biceps brachii in humans is probably derived from the muscles of the anterior compartment of the arm. Notably, humans, in contrast to other primates, lack the long head of coracobrachialis [1]. In those cases in which the third head arises from the insertion area of coracobrachialis, it is possible that it represents a remnant of the long head of coracobrachialis, the ancestral hominoid condition [1]. As Dobson (1881) found in Cercopithecus, the long head of coracobrachialis may find an insertion onto the radial tuberosity in common with biceps brachii. Embryological observations by Testut described this variation of the third head of biceps brachii as a portion of the brachialis muscle supplied by the musculocutaneous nerve, in which its distal insertion has been translocated from the ulna to the radius (Testut, 1902). Knowledge of the existence of the third head of the biceps brachii may become significant in preoperative diagnosis and during surgery of the upper limbs. Because of the association of the third head with unusual bone displacement subsequent to fracture, such variation has relevance in surgical procedures. Therefore, surgeons, in particular orthopedic surgeons, should be aware of this anatomical variation when dealing with some of the clinical syndromes.

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A Medico-Anthropological Study of the Skeleton and a Plastic Reconstruction of the Skull of Tsar Samuil

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In the investigation of the "St. Achilles" basilica ruins on the St. Achilles Island in The Small Prespa Lake (1965-1975) carried out by prof. N. Mutsopulos in the third sarcophagus (Grave B.1) an intact skeleton of an adult man was found. The burial is primary. The skeleton belongs to a male individual (about 70 years of age) identified by prof. Mutsopulos as that of Tsar Samuil.

Bone wounds were established on the bones of the left fore-arm and the skull. The sizes of the broken fore-arm bones and the skull characterize a male individual of short stature and a small head. The latter is confirmed by the performed reconstruction of the head after the skull was completed at the beginning of 2008.

Taking into account all the anthropological and historical data the thesis of prof. Mutsopulos that the buried man is the Bulgarian Tsar Samuil is most convincingly confirmed.

Key words: Tsar Samuil, skull, head reconstruction.

Prof. Mutsopulos investigated the ruins of the "St. Achilles" church on the island in the Small Prespa Lake bearing the same name for the period 1965-1975 [9]. Four sarcophagi were discovered in the middle of the southern nave of the wooden-roofed basilica. A skeleton of an adult male was found untouched in the third sarcophagus (Grave B.1).

The cranium was lying on an erected head-prop and on the left arm were found remains of a chain-mail woven with very fine gold threads. A valuable silk fabric decorated with parrots placed in circles was located on the pelvis and the silk threads were also interwoven with gold lines. The entire skeleton and cranium were of an intense red colour.

In the inspection procedure it was established that there were traces of a poorly healed wound on the fore-arm bones of the left arm. Prof. Mutsopulos concluded that skeleton belonged to a male wounded in battle who was later hampered in the correct caring of his wound. Based on the above-mentioned data in 1965-1966 he identified the skeleton from the grave B.1 with the one of Tsar Samuil. This hypothesis of his gave rise to a significant amount of interest. The fracturing of the left fore-arm bones is attributed to the wounding of Tsar Samuil in the battle at the

river of Sperchei and their poor and incomplete healing to the difficult and lengthy relocation to Prespa.

The age of the buried one determined after the skeleton by Aris Pylyanos and Peter Boev is about 70 years [1, 2, 3, 13].

In 2007 Bozhidar Dimitrov, director of the National History Museum, presented to me a plaster copy of Tsar Samuil's cranium and plastic copies of the left fore-arm bones — ulna and radius of the skeleton from sarcophagus B.1. The idea was for me to carry out a plastic anthropological reconstruction of the head after the plaster cast copy of the skull (a copy of Tsar Samuil's skull sent by prof. Mutsopulos in Bulgaria in 1987 via the consul general of Bulgaria in Thessaloniki Mr. Ilia Petrov.

The reconstruction of the head after the skull of Tsar Samuil was completed at the beginning of 2008 and on March 20th it was presented to the scientific community in the National Anthropological Museum at BAS [5, 4, 8]. During the period of work on the image of Tsar Samuil amounting to half a year I had the opportunity to get acquainted in detail with the skull and the two left fore-arm bone copies, which were given to me.

I have to note that I have not seen (except in photograph) the bone remains from grave B.1 and never had the opportunity to conduct medico-biological and anthropological investigations on them.

In his monograph "The St. Achilles basilica in Prespa — a historical monument — a sanctuary", published VION, Plovdiv, 2007) prof. N. Mutsopulos noted that the study on the hurt bones (ulna and radius) was performed in the Laboratory of Descriptive Anatomy at the Medical Faculty at the "Aristotle's University" in Thessaloniki by prof. Marius Polizoni (report 10.12.1983) [9]. He wrote in his report and was quoted by prof. Mutsopulos that "In the Laboratory of Descriptive Anatomy a left radius and a left ulna from a human skeleton have been delivered displaying the following typical features (fig. 1). Radius: The radial bone is with an unhealed fracture. The ulna: Almost right in the middle of the bone there is a fracture coalesced at an angle of 135°. Taking into account the form of coalescence of the bone the fracture seems to have occurred at least a year prior to the death of the person".

The cranium bearing the initials St.Ach.Grave B M65 (fig. 2) belongs to a male of approximately 70 years of age. It has been dated by the archaeologist who has found it the 10^{th}-11^{th} century. The diameter of the skull indicates an individual shorter than the average height*.

The zygomatic width as well as the small forehead reveals a person with a small face. The height of the nose root is rather big and the nose itself is strongly protruding. The relief of the whole cranium is very even. The orbital cavities are rather big. These features would suffice for the classification of the skull as an Europoid one belonging to the Aegean or the Aegeocaucasian type in the broadest sense of this notion. The photographs of the bone objects are from prof. Mutsopulos's monograph.

The left ulna is with a formed bone callus located in the middle of the bone. The amassment of bone tissue is predominantly found on the front surface of the broad angle open to the front. The hind surface of the callus displays a narrow (2-3 mm) rough portion with discernible traces of granulation. The length of both coalesced fragments calculated by us is 20.5 cm.

* This is corroborated also by the juxtaposition of the fore-arm bones of the left arm from the grave B.1 with an analogous portion on the fore-arm of a normal skeleton (fig. 135 from the monograph of prof. N. Mutsopulos).

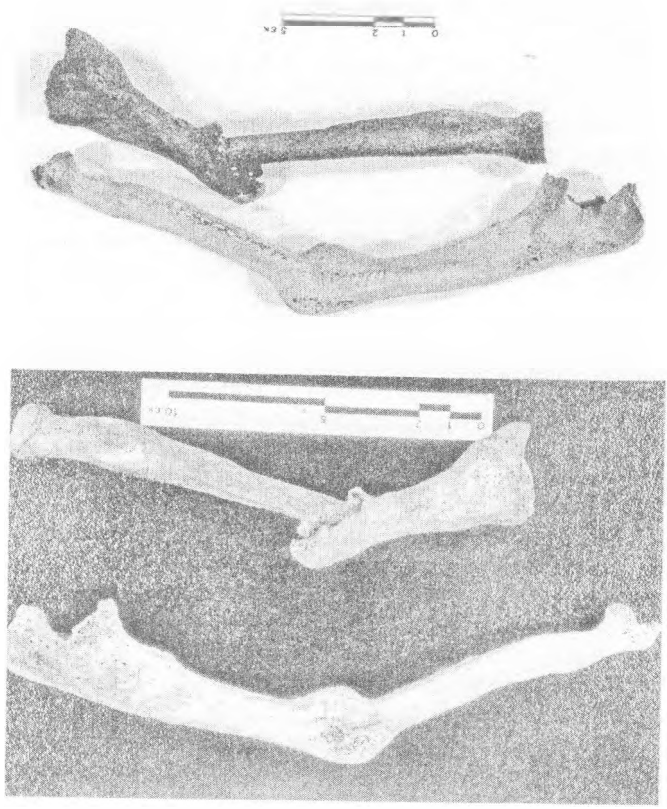


Fig. 1. Left ulna and left radius, Grave B.1, "St. Achilles" basilica

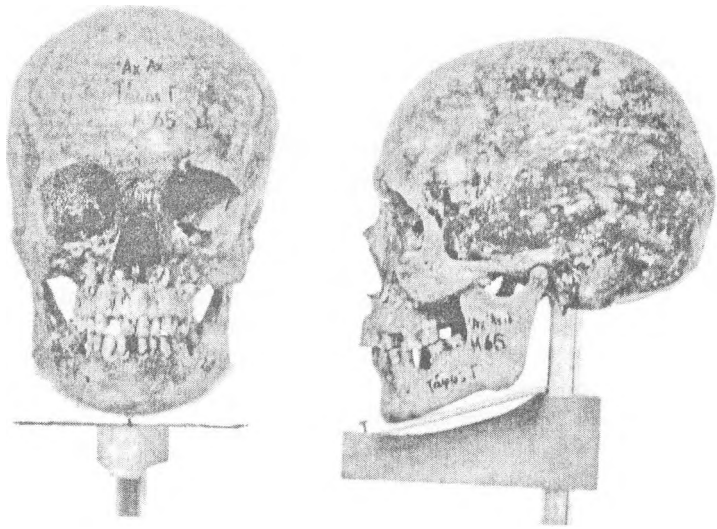


Fig. 2. The skull from Grave B.1, "St. Achilles" basilica

The left radius is with an uncoalesced fracture (fig. 3). The break is between the medium and lower third of the bone. The fronto-lateral surface of the shorter distal fragment is with a bony cavity of sizes of roughly 20/14 cm which is confined from below and medially by a bony callus. Its medical surface from the end of the cavity to the medial edge displays a furrow of 18 mm with a width from 2 to 5 mm, of an uneven bottom as well as short (2-3 mm) bone protrusions. According to the length of both fragments measured by us the probable length of the bone is 17.8 cm.

The height calculated after the Trotter-Gleser formula is 150.61 cm [6, 7, 10].

The comparison of the bone wounds on the radius and ulna indicates to the direction of the stroke from the front to the back, from above to below and from the outside to the inside. The more strongly damaged bone — the radius (of an outer location) has suffered the greater damage of the stroke. This is made possible in a probable state of the fore-arm with a palm towards the sagittal line (to the face) and the stroke has been dealt from the front to the back [11, 12, 14].

The most probable situation of the left arm (weapon free) is raised above, stretched in front of the attacker standing before the victim.

The assumption for such a position is also supported by the lesions on the left upper half of the skull: the left temporal area and the left zygomatical arch and

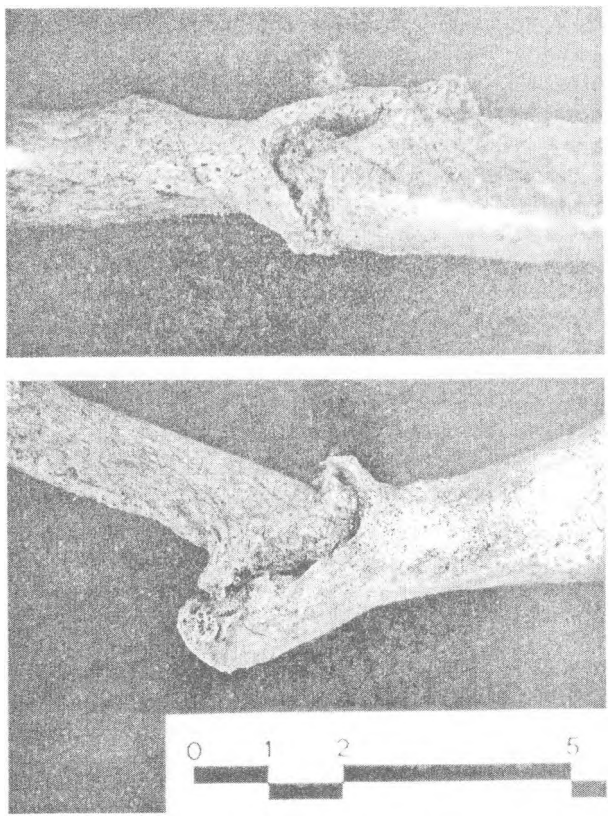


Fig. 3. The left radius — uncoalesced fracture

bone, the outer edge of the orbita. The left temporal pit in its upper third to 2 cm behind the orbital edge is of a rough surface of concave and convex (2-3 mm) character with indentations and protrusions (2-3 mm).

The left cheek-bone arch is indented (a healed fracture without displacement) at the point of binding the two excrescences forming it. The left cheek-bone is jutting out and compared with the right one it is of a more convex surface and a marked furrow-like indenture of the bone suture which connects it to the left maxilla. As a result the canine pit (fossa canina) on the left is more shallow [4, 5, 8].

The reconstruction of the action in the process of the traumatic injuries on the bones of Tsar Samuil would ontline the following picture:

The attacker is in front and a little to the right from the Tsar. He is right-handed — in his right hand is the weapon that has caused the injury. It is of the type of "a hard object with a non-cutting edge". As he lifts it for the head of the Tsar, Tsar Samuil, as anybody would do, stretches his left arm to the front at the height of the lower forehead with a palm turned towards the face. The direction of the blow is oblique — from the front to the back which is confirmed by the localization of the fractures of the two bones. Also, this time with a lesser force the weapon (picket, spear, etc) inflicts damage with its front end to the cheek bone area, the zygomatic arch and cheek-bone itself which has led to lesions in the soft tissues in these areas and the development of osteomyelitis in the unhealed radius indicates to an open wound. Explicit proofs in the identification of Tsar Samuil such as a grave inscription, location of the burial site, description of his looks are missing. The proofs provided for the identification of the bone remains from the sarcophagus (grave B.1) as ones belonging to Tsar Samuil are as follows:

— The "St. Achilles" basilica had been built by Tsar Samuil after his conquest of Larissa and his return from his march to the Southern Hellenic lands (984-986). There are no data suggesting that another nobleman could be buried there (after the Byzantine author M. Ataliates). The find of remains from a gold-woven chain-mail on the left hand and the valuable silk fabric woven with golden threads and decorated with parrots being placed on the pelvis.

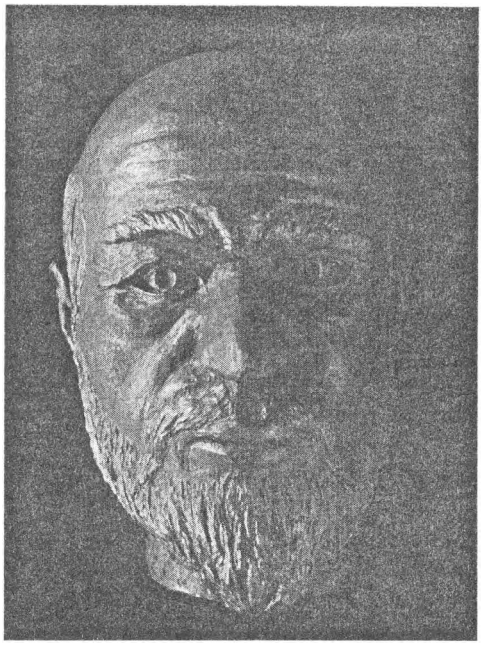
— The traumatic injuries on the left fore-arm bones and the skull show that the buried man (at a mature age) is wounded in the battle and was deprived of the chance for a proper medical treatment [6, 10].

In the battle at the river Sperchei Tsar Samuil and his son Gavrail-Radomir (the Byzantine authors Scylitzes and Kebrin state that Gavrail-Radomir has been superior in strength and body-build to his father Samuil but not intellectually) have been seriously wounded and have marched for a long time to Prespa (a distance roughly amounting to 350-400 km) over an undulating cross-country terrain. This fact most possibly is the reason for the lack of therapy which has led to the described condition of both left fore-arm bones. With his left hand Tsar Samuil has been half crippled [6, 10, 11]. The fracture of the left zygomatic arch having squeezed the temporal muscle (most probably injured) has eventually accounted for the limited movement of the mandible (the opening of the mouth) with all consequences arising from that fact.

What is the summing up that can be drawn from this short presentation?

The burial in the third sarcophagus (B.1) is primary, i.e. after the laying of the dead body the latter has not been moved, shifted, and no action has ever been undertaken with regard to it.

The skeleton belongs to an individual of the male sex of about 70 years of age with a height of about 155 cm (after the data of prof. Mutsopulos). There are remains of a gold-woven fabric on it. Skeletal wounds are found on the bones of the



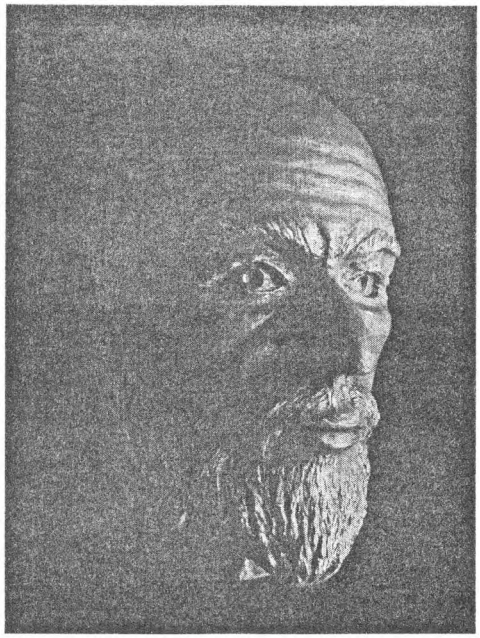


Fig. 4 and Fig. 5. The plastic reconstruction of the head after the skull of Tsar Samuil — full-face and semi-profile view

left fore-arm and the skull. The radius is with an unhealed fracture resulting from traumatic osteomyelitis.

The formation of a callus on the ulna implies a period of a year following its fracture. The small sizes of the broken fore-arm bones (probably also the ones of the rest of the bones of the skeleton) and those of the skull indicate to a male individual of a small stature and small head [6, 7, 10]. The latter is confirmed by the reconstruction of the head made after the skull [5]. Taking into account all this, the wounding of Tsar Samuil in the battle at the river Sperchei, his long and difficult journey to Prespa and the ensuring ailment as well as the anthropological study of the skull and the reconstruction of the soft tissues of the head, support the thesis of prof. Mutsopulos that the buried one in the third sarcophagus (grave B.1) in the "St. Achilles" basilica on the island by the same name in the Little Prespa lake is the Bulgarian Tsar Samuil (figs 4, 5).

If made possible, an additional study on the remains from grave B.1 kept by prof. Mutsopulos would elucidate certain details about the skeleton such as age, height, body-build, strength and anthropological type.

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Influence of cobalt on male fertility

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Cobalt is an essential oligoelement for mammals in the form of cobalamin (vitamin B_{12}). Cobalt does not accumulate in the organism but high doses of cobalt could exert adverse effects.
The present article is focused on the negative influence of cobalt on male fertility. Significant reduction in epididymis and testis weight was found in cobalt treated animals. Genotoxic effects of cobalt involved significant increase in the frequency of chromosomal aberrations in male gametes. Impaired fertility was manifested by low sperm count, decreased motility, accompanied by morphological abnormalities. Structural alterations include enlargement of interstitium, desorganisation of peritubular area and degeneration of seminiferous epithelium (vacuolation of Sertoli cells, multinuclear germ cells, containing degenerative spermatocytes and spermatids). Cobalt interferes with the hormonal balance as well.

In conclusion, cobalt exposure could be considered as a risk factor for male reproductive development and function and hence for male reproductive health.

Key words: cobalt, male fertility, reproductive organs, spermatogenesis.

1. General role of cobalt

Cobalt is a naturally occurring, relatively rare element of the earth's crust [8, 14]. It circulates in surface environment through many natural processes and anthropogenic activities. Cobalt is an essential oligoelement for mammals involved as a constituent of vitamin B_{12} (cobalamin), mainly. Congenital disturbances related to absorption and function of vitamin B_{12} give rise to pathological alterations such as megaloblastic anemia, retardations, neurological and ocular defects and other syndromes and diseases [10]. Cobalt is found in very small amounts in food although fish and sea foods, meat, eggs, liver and other animal products are relatively rich in cobalt [27]. The adult human body contains approximately 1 mg of cobalt, 85% of which is in the form of vitamin B_{12}. Human dietary intake of cobalt varies between 5 and 50 mg/day [14].

There are three ways of cobalt intake — by food and drinks, by inhalation and by skin absorption. Ingestion of cobalt by food and beverages represents the main source of cobalt for human general population. Absorption of vitamin B_{12}

from food under physiological conditions involves no less than five separate vitamin B_{12}-binding molecules, receptors and transporters and each molecule has separate affinity and specificity for vitamin B_{12} and a separate cell receptor, as well [21]. Initially in the stomach vitamin B_{12} is bounded by heptacorrin. After that in ileum (the only place of vitamin B_{12} absorption) vitamin B_{12} bounds to intrinsic factor before being absorbed by the intestinal epithelial cells. Transportation into all other cells is possible only after preliminary proteolitical release of vitamin B_{12} and its subsequent binding to another transport protein — transcobalamin II [10, 21]. By blood circulation cobalt could be delivered and subsequently accumulated in different organs — most significant amount is accumulated in liver and kidneys, but higher doses of cobalt are detected in hematopoietic organs, brain, reproductive organs etc. Increased uptake of cobalt by inhalation is typical for workers in specific occupational settings such as alloys and metals manufacturing, diamond polishing, dental laboratory materials production etc [13, 14]. These workers are exposed to dust full of cobalt and other metals and hence combined effect of these elements couldn't be rule out. Main target of cobalt is respiratory system. Chronic exposure to high cobalt concentrations in the working environment leads to impaired lung function - asthma, hard metal lung disease and predisposition to lung cancer. Skin absorption rarely occurs, for example by jewelry. Cobalt has relatively high allergic potential being one of the five top global allergens [16]. Exposure to cobalt could give rise to allergic reactions and contact dermatitis [25].

Prolonged exposure to cobalt leads to different pathological alterations such as cardiomyopathy, impaired function of thyroid gland and liver. Cobalt has been shown to exert genotoxic and carcinogenic effect. Embryotoxic activity was also revealed due to transplacental route. Experimental treatment of cobalt results in increased incidence of total growth retardation, embryolethality and severe congenital abnormalities [24]. Apart its potential toxic effect, cobalt is not cumulative toxin and it is rapidly excreted in urine and to a lesser extent via faeces. Concentration of cobalt in blood and/or urine is proposed as a biomarker for cobalt exposure as elevated concentrations in body fluids mainly reflects recent contamination [12, 14]. Moreover ingestion of controlled amounts of soluble cobalt compound resulted in significantly higher concentrations of cobalt in urine and blood from females compared with that from males [4]. Cobalt toxicity could be treated with Dimercaprol, $CaNa_2$-EDTA, D-Penicillamin [27].

Some cobalt-compounds were shown to possess therapeutic potential. In the past cobalt was used for treatment of anemia, due to stimulation of erythropoietin synthesis [6]. Some cobalt-based compounds possess high antiproliferative and cytotoxic activity against human lung, ovarian, colon, uterine carcinomas and against leukemia and lymphoma cells, as well [1]. Cobalt significantly reduces plasma glucose levels and body weight in streptozotocin-diabetic rats and these data opens new perspectives for diabetic treatment strategies in the future [26].

2. Role of cobalt in male reproductive function and fertility

Cobalt-treated experimental animals show different pattern of response depending on duration of exposure (acute or chronic), applied doses and the particular species' characteristics as well. It was proven that ruminants need much higher doses of dietary cobalt for conducting of normal life than the non-ruminant animals. Cobalt toxicity in ruminants is relatively rare phenomenon in comparison with the non-

ruminants due to some physiological features of vitamin B_{12} acquirement. Duration of cobalt exposure is very important for the subsequently induced abnormalities and for the following period of recovery [1]. It is also of a great importance the period of life during which the cobalt treatment was take place.

The crucial negative effects of cobalt on testis were rendered to its ability to induce conditions characterized with more or less decreased level of oxygen. Cobalt chloride is widely used pharmacological agent for inducing hypoxia. Cobalt displaces ferrous ion from haeme, resulting in reduced oxygen-binding capacity of the molecule and hence chemically simulating hypoxia [20]. There are two main hypotheses explaining the effect of cobalt-induced hypoxia and its influence on testicular vasculature. One hypothesis is that the veins and arteries become blocked due to erythrocyte packing associated with cobalt-induced disturbance of vascular permeability. The other hypothesis is that cobalt induces polycythemia (increased erythrocyte concentration), which precipitates hypoxia due to increased blood viscosity. The testis is much more susceptible to hypoxic state than the other organs and it is under constant hypoxic environment probably due to specific organization of the testis and its vasculature.

The unique coiled testicular artery and the closely applied pampiniform plexus of veins assist in achieving the lower temperature required for spermatogenesis. The coiled testicular artery also reduces the pulse height of arterial blood flow. On the other hand, testicular blood and lymphatic vessels are restricted to capsular and interstitial tissue which commonly comprises 10 to 20% of testis volume and that result in morphological restriction of the testicular vasculature [22]. In addition human testis is more sensitive to hypoxic state in comparison with other mammals due to higher level of convolutions of seminiferous tubules which lead to increased irregularity of the blood vasculature within the testes. Moreover, human testes have lower density of intertubular and peritubular capillaries as compared to other mammals. It can be suggested that any impediment to testicular blood flow will rapidly precipitate hypoxic state. Therefore these structural specificities of testicular vasculature make testis much more sensitive to induced hypoxic states. Ischemia of testis due to torsion has been shown to result in a permanent loss of spermatogenesis [20]. Smaller degree of testicular torsion did not reduce testicular secretion of testosterone whereas prolonged torsion diminished testicular steroidogenesis in man although the role of hypoxia in modulating of testicular steroidogenesis is not well studied [20].

Experimental treatment with cobalt induced a lot of abnormalities in reproductive organs that seriously affect male fertility. Experimental animals showed decreased weight of testes and epididymis while weight of seminal vesicles and preputial glands was significantly increased. Structural changes in the testis involved necrosis and degeneration of seminiferous epithelium and interstitium [3, 7]. Corrier et al. [5] found that damaged tubules often presented side by side with normal tubules. Degeneration of seminiferous epithelium was initially manifested by vacuolation of Sertoli cells, formation of abnormal spermatid nuclei and multinucleated cells that often contained degenerative spermatocytes and/or spermatids. Spermatogonia, primary spermatocytes and round spermatids were markedly affected, while elongated spermatids, and spermatozoa were more resistant to cobalt treatment and Sertoli cells were the last surviving cells [3, 5]. Cobalt chloride-induced oxidative stress leads to alteration in behavior of tesmin — a testis specific protein with stage-specific distribution and to induction of apoptotic signals in spermatocytes, as well [23]. Sloughing of germ and Sertoli cells was also found as well as formation of empty spaces within the seminiferous epithelium [15]. Bitner et al. [3] reported shrinkage of

the tubules with accumulation of "calcified" necrotic debris accompanied by disorganization of peritubular cells and folding of basal lamina.

Cobalt increased the number of abnormal spermatozoa that consequently reduced fertility in human and animals [9]. Depletion of live sperm and reduced motility of the spermatozoa was observed [7, 19]. Testicular/epididymal sperm counts and daily sperm productions were significantly decreased. The negative effect of cobalt on sperm involved head and tail abnormalities [9, 17]. The head shape abnormalities reflect changes in the DNA content while tail alterations include loss of filaments and degeneration of mitochondria.

Morphometric analysis revealed significant decrease in relative volume of seminiferous epithelium in cobalt treated animals, whereas the relative volume of interstitium was significantly increased. Diameter of seminiferous tubules was increased probably due to higher value of luminal diameter. The number of cell nuclei per defined area was also elevated [15] although heigh of seminiferous epithelium remained relatively constant. Enlargement of interstitial space was accompanied by hypertrophy of Leydig cells and thickening of testicular vessels [7]. Blood capillaries were dilated and transmission of blood elements into the interstitium was detected, indicating oedematization [15].

Experimental treatment with cobalt influenced Leydig cells steroidogenesis - serum testosterone levels were dramatically increased, while FSH and LH serum levels remained normal. Data suggests that cobalt interfere with local regulatory mechanisms in testosterone synthesis [19]. It is well known that regulation of steroidogenesis by luteinizing hormone is mediated by cAMP and calcium [11]. Cobalt (Co^{2+}) is a calcium channel blocker, and hence it could interfere with the signal transduction pathways involved in steroidogenesis. The increase in size of interstitial Leydig cells and possibly their activity could be responsible for elevated testosterone levels that in turn could explain higher weight of seminal vessels in cobalt-treated mice [19].

Impairment of male fertility as a result of cobalt treatment was demonstrated by experimental model in which untreated females were mated with cobalt-treated males, subjected to chronic cobalt treatment before mating. Impaired male fertility results in lower number of pregnant females and number of implantation sites. Moreover, the total number of resorptions and the number of females with resorptions were significantly increased [7]. The number of viable fetuses as well as the number of total and live births was decreased. The authors suggested that these effects may be attributed to poor development of fertilized ova due to alterations in sperm quality resulted from cobalt-treatment [7].

It was recognized that cobalt possesses mutagenic and carcinogenic activity. The genotoxic effect of cobalt concerning male reproduction was poorly investigated. Hassan et al. [9] established that $CoCl_2$ exerted genotoxic effect on mice somatic and germ cells, e.g. significant increase in the frequency of chromosomal aberrations in mouse spermatocytes. The mutagenic potential of cobalt and its compounds was evaluated by International Agency for Research on Cancer. Cobalt (II) compounds were reported to induce DNA damage, DNA protein cross links, gene mutation, sister chromatid exchanges, and aneuploidy in in vitro studies with animal and human cells [9].

Regarding to the direct genotoxic mechanisms, cobalt (II) induces formation of reactive oxygen species (ROS) when combined with hydrogen peroxide in cell free system and the ROS were suggested to give different kinds of site-specific DNA damage. Cobalt ions were shown to substitute for zinc in protein-zinc finger domains which control gene expression. Such substitution is suggested to generate free

radicals close to DNA which in turn caused DNA damage [9]. Some of the genotoxic effects of cobalt (II) are attributed to its activity as poison of topoisomerase II demonstrated in cultured cells [2]. It was also shown that cobalt interferes in the DNA repair processes causing their inhibition [6]. Cobalt competes with the essential Mg (II) ions [6] suggesting possible interference with the processes/biochemical reactions required Mg (II) ions.

It is important to note that acute cobalt exposure did not give rise to any significant or irreversible alterations concerning male sexual function and reproductive system whereas the consequences of the chronic exposure are much more powerful. It was reported that following acute occupational cobalt inhalation the urinary elimination is rapid for 24 h followed by a slower excretion phase lasting several weeks. The repeated dose treatment with food may result in its accumulation in the tissue beyond the capacity to be discharged through the natural physiological mechanisms [9].

In conclusion, cobalt could be considered as a risk factor for male reproductive health and therefore men exposed to higher cobalt concentrations on their working places should be carefully monitored. Moreover, it would be beneficial if they are additionally subjected to treatment with drugs such as complex of selenium and vitamins A, C, E which are able to reduce the negative effect of cobalt [9].

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Review articles

Importance of Androgens and Estrogens for Mammalian Spermatogenesis

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Normal proceeding of spermatogenesis required gonadotrophic (FSH and LH) and steroid (testosterone and estradiol) hormones during different developmental stages. The importance and mechanism of action of each hormone is demonstrated by the data from experimental models and transgenic animals lacking androgen or estrogen receptors, gonadotrophins and their receptors. Endocrine disrupters are estrogenic and/or anti-androgenic chemicals widely spread in the environment. Acting as agonist or antagonists of steroid receptors they interfere in hormonal balance having potentially hazardous effects on male reproductive function. Most of the studies in the literature concerning the fine steroid balance in regulation of spermatogenesis have investigated Sertoli and total germ cell population. The mechanism of estrogen action on different stages of male germ cell development is poorly investigated. The absence of information about this problem requires implementation of profound study on the mechanisms via which estrogens regulate particular phases of spermatogenesis (mitotic, meiotic and postmeiotic stages).

Key words: androgens, estrogens, spermatogenesis, endocrine disrupters.

Normal male fertility relies on normal spermatogenesis, process by which immature germ cells undergo division, differentiation and meiosis to give rise to haploid elongated spermatides and finally spermatozoa. These events occur in close association with somatic cells, namely Sertoli cells in the seminiferous epithelium that communicate with germ cells directly via Ligand /Receptor mediated interactions or via paracrine signallization. Germ cell development requires as expression and secretion of many Sertoli cell proteins in stage specific manner as well as regulation by steroid hormones (androgens and estrogens) [18]. The predominant sources of testosterone (T) are the Leydig cells dispersed in intesrtistium of the testis (together with fibroblasts, macrophages and leucocytes). Besides control within

the testis the full fertilizing potential of the released spermatozoa is also dependent on the progression and maturation of sperm trough the excurrent duct system and epididymis.

The hormones secreted in the testis are required for various functions in the body including maintenance of secondary sexual functions and feedback on the hypothalamus and the pituitary to control the secretion of the gonadotropins LH and FSH. It is well known that gonadotropins are major endocrine regulators of spermatogenesis [16, 19, 20, 29]. In response to GnRH front part of pituitary secrets LH and FSH that act on different target cells in the testis realizing particular functions in its endocrine regulation [20]. FSH targets the Sertoli cells to regulate spermatogenesis by stimulating the production of numerous growth factors. Leydig cells are main target of LH and they are primary involved in the secretion of androgens, notably T, as well other steroids including estrogen as end products obtained from the irreversible transformation of androgens by aromatase. The role of FSH and T is greatly investigated but there are still lots of confusions about the mechanisms of action of these hormones on their target cells in supporting of germ cell development. Quantitative studies on FSH- and FSH receptor knock-out mice demonstrated lower sperm count although mice are fertile [12]. Consequently while FSH is not essential for qualitative aspects of spermatogenesis the hormone is clearly essential for quantitative normal spermatogenesis. During postnatal life Sertoli cells and spermatogenesis are differently sensitive to FSH and T, connected with switch from mainly FSH dependent to day 20 to more T dependent after day 40 [20]. It has been shown that androgens alone stimulated all phases of germ cell development in hypogonadal mouse (*hpg*), which is congenitally deficient in GnRH and therefore LH and FSH [26] enhancing the necessity of androgens and its determination as survival factor for spermatogenesis. In adulthood T supported qualitative normal spermatogenesis in hypophisectomised rats. Moreover quantitative parameters were reached by T application after treatment with GnRHa or ethane dimethanesulphonate (EDS) in spite of lack or reduced FSH levels [20]. FSH or T alone increased Sertoli cell number in *hpg* mice and there is observed pronounced synergetic effect. FSH alone is able to maintain proliferation of spermatogonia whereas meiotic and postmeiotic differentiation is dependent on T and required synergy of both hormones [10]. Both FSH and T are considered as major survival factors for germ cells in the male as experimental deprivation of these hormones induced profound germ cell apoptosis [27]. To some extent preferential importance of T is suggested although the role of FSH should not be underestimated [2]. Androgens act on androgen receptors (AR) to control spermatogenesis. In the pubertal and adult testis AR is localized in interstitium - in Leydig and peritubular cells and in seminiferous tubules only in Sertoli cells but not in germ cells. In adult testis stage-specific expression is found in Sertoli cells with lowest level in late stages of spermatogenic cycle and highest in stage VII-VIII when spermatozoa are released into the lumen. This stage is considered as androgen-dependent stage at which androgens preferentially acts on spermatogenesis [3]. Importance of androgen signaling for male reproductive development and function is demonstrated by transgenic mice lacking AR. DeGendt and colleagues [6] have generated two types of AR knock-out animals — total knock-out in all target cells (ARKO) and selective knock-out in Sertoli cells in the testis (SCARKO). ARKO males have very small testes in abdominal position. Spermatogenesis is arrested at very early stage of germ cell differentiation and reproductive tract and external genitalia are not developed, so they are phenotypic female. SCARKO animals have testes with normal scrotal position but with reduced testis weight. Reproductive tract and external genitalia

are developed and spermatogenesis proceeds completion of meiosis and postmeiotic differentiation. Comparative analysis of two models demonstrated the autonomous role of Sertoli cells in classical genomic mechanism of androgen action in the male [28]. High level of intratesticular T is required for spermatogenesis normal in quantitative and qualitative manner. The plasma levels of T are also adequate to normal male sexual and reproductive function [20]. Circulating plasma T and its derivates dihydro-testosteron (DHT) and estradiol (E_2) realize feedback regulation of LH and FSH secretion. Normal proceeding of spermatogenesis required exact hormones during different developmental stages: 1) initiation and proceeding of first spermatogenic wave during puberty, 2) support of spermatogenesis in adulthood, 3) re-initiation of spermatogenesis after temporary disturbance or lost of germ cells [2].

Whereas the role of androgens, FSH and LH is incontestable for spermatogenesis the recent investigations in endocrine regulation of spermatogenesis show that estrogens (E) should be added to the group of hormones involved in this regulation. A new role of estrogens for male reproductive function was suggested. Discovery of the expression of estrogen receptors (ER) in testis, entire reproductive tract, several hypothalamic nuclei and pituitary supports the suggestion that E regulate the hypothalamus-pituitary-testis axis [23]. In order to exert their biological role estrogens interact with ER which in turn modulate the transcription of specific genes. Until 1996 only ER-α was discovered and than the novel ER-β was identified. It was shown that the ER-α and the ER-β are not always present in the same cells (or are present in different amounts) within the male genital tract [4]. ER like AR are members of the steroid/ thyroid hormone super-family of nuclear receptors, which share common structural architecture, and consist of six independent but interacting functional domains [1]. In addition to the classic genomic pathway (mediated by ER) estrogens can also induce extremely rapid response via nongenomic mechanism of action involving membrane associated ER (particular important in cardiovascular and neuronal tissues) [18]. Immunohistochemical studies for ER-α show that this protein is present in mouse undifferentiated gonad at day 10. In prenatal Leydig cells ER-α is expressed before existence of AR. These findings support the suggestion that estrogens may have a significant role very early in the gonadal differentiation process. Expression of ER-β in gonocytes, Sertoli and Leydig cells until birth was also observed. Around the time of birth the testis continues to express both ER subtypes and aromatase. In adult testis ER-α is restricted to Leydig cells whereas ER-β is widely distributed- confined to Leydig cells, peritubular cells, Sertoli cells and some populations of germ cells- spermatogonia, late primary spermatocites (pachytene) and round spermatides. This data support the hypothesis of direct estrogen action as on somatic cells (Sertoli cells, peritubular and Leydig cells) as well as on germ cells in the testis. The first definitive demonstration that estrogens were required for male fertility was the use of knock-out models for ER. Mice lacking functional ER-α (ERαKO) were infertile due to defect in efferent ductile development and function [11, 14] in this way spermatozoa can not reach their full fertilizing capacity. Conversely, in the ER-β knock-out mice (ERβKO) no abnormal development of germ cells has been observed and the male are fertile but it has been noted hyperplasia of the epithelium of seminal vesicles, bladder and prostatic gland [13]. Impaired fluid reabsorbtion in efferent ducts leads to accumulation of the fluid in the tubular lumen that in tern exerts pressure on seminiferous epithelium affecting spermatogenesis [8]. Dealing with the double knock-out mice (ERα/β) the phenotype is identical to that of ERαKO and the males are sterile [5]. Mice lacking a functional aromatase gene (aromatase knock-out, ArKO) are also

infertile. Evidence from several studies indicates that ER-α, ER-β, and aromatase are encoded by separate genes but are co-expressed with AR in the male reproductive tract [1]. Data of Ebling et al. [7] show that treatment for 70 days with estradiol induced full qualitatively normal spermatogenesis in *hpg* mice where T production is lacking and FSH levels were 1/3 from control value. These results clearly indicate that estrogens may play a role in spermatogenesis, via some stimulatory effects on FSH secretion in addition to direct effect via ER in the testis [19]. Quantitative studies of spermatogenesis by Atanassova et al. [2] showed that high doses of estrogens induced pronounced germ cell apoptosis and affect spermatogenesis by suppressing FSH and T whereas low doses of estrogens have mild stimulatory effect and suppressed apoptotic index [2]. All data provide strong evidence for an important role of estrogen in the regulation of the testis and male reproductive tract and hence for male fertility.

Several environmental contaminants are known to interfere at various stages of germ cell development interfering in the normal hormonal balance and thereby causing reduced sperm count. Endocrine disrupters are estrogenic and/or antiandrogenic chemicals widely spread in the environment that have potentially hazardous effects on male reproductive function resulting in infertility and erectile dysfunction. Endocrine disrupters are able to mimic natural hormones. They can inhibit the production and/or action of hormones and /or alter the normal regulatory function of the endocrine systems [25]. In this way this compounds disrupt the hormonal balance in particular estrogen / androgen balance by binding to hormone receptors during fetal and postnatal development and give rise to reproductive abnormalities persisting to adulthood. Besides reduced fertility and erectile disfunction endocrine disrupters can induce testicular and prostate cancers, abnormal sexual development, alterations in pituitary and thyroid gland functions, embryo/ fetal loss, bird defects, immune suppression, neurobehavioral disruption [25]. Rats exposed in utero to certain phthalates also exhibit disorders of sperm production (even in normal descended testes) and reduced fertility. These changes are probably related to the occurrence of dysgenetic areas and germ-cell-depleted (Sertoli cell-only) testes [9] and epididymal lesions. The disorders induced by phthalates are remarkably similar to testis dysgenesis syndrome (TDS) disorders in the human [9, 15]. These changes are therefore reflection of endocrine disruption, but the latter occurred secondary to the disgenesis [21]. Many environmental xenobiotic chemicals, such as polychlorinated biphenyls (PCBs), dichlordiphenyltricloroethane (DDT), dioxin, and some pesticides have estrogenic effects [24]. A large part of agricultural products (phytoestrogens), industrial chemicals and heavy metals impair normal reproductive function because of their widespread presence in the environment and their ability to accumulate and resist biodegradation. In addition many pharmacological and biological agents including radiation therapy affect male fertility disrupting hormonal balance. One of compounds, diethylstilbestrol (DES) was greatly investigated throughout the years because of its identifying as a transplacental carcinogen and its proven negative effect in both male and female offspring exposed prenatally to DES. It is a potent synthetic estrogen that for many years was thought to prevent complications of pregnancy and between the late 1940s and the early 1970s DES was prescribed for million women in USA [30] and Europe. The male offspring exposed prenatally to DES has an excess prevalence of reproductive abnormalities (criptorhydism, hypospadia, low sperm count, epididymal cysts) and infertility [30]. It was found that DES is associated also with many reproductive difficulties in young women whose mothers had been given this drug during pregnancy, like clear-cell adenocarcinoma of the vagina and cervix, inferti-

lity, miscarriage, preterm delivery and fetal/infant death. Much of our understanding of the fetal/ neonatal effects of DES has come from studies of animal models demonstrating that DES caused retardation of testis development and suppressed spermatogenesis acting on differentiation of germ cells via direct and indirect mechanisms [2]. McKinnell and colleagues [17] demonstrated that in rats treated neonatally with DES androgen receptor immunoexpression was virtually absent from all affected tissues including the testis and entire reproductive tract. Suppression of androgen production and action (expression of AR) is an integral part of the mechanism via which estrogen effect male reproduction [2]. Comparison of the effects in animal studies of administrating either an anti-androgen or a potent estrogen such as DES reveal remarkable similarities in the changes that are induced at birth (cryptorchidism, hypospadias, epididimal and/or prostate abnormalities) and in adulthood (small testes, low sperm count, testicular germ cell cancer) [22]. The similarity in phenotypic changes suggests that common pathways of action may be involved in at lest some of this changes. One possible explanation is that administration of anti-A may elevate endogenous E levels and that this might contribute to some of the adverse effects in addition to blockage of androgen action. Conversely, E administration might interfere with androgen production or action in addition to activating ER-mediated pathways. This both possibilities would fundamentally alter the androgen/estrogen balance by lowering androgen action and elevating estrogen action. All studies in the literature concerning the fine steroid balance in regulation of spermatogenesis have investigated Sertoli and total germ cell population. The mechanism of estrogen action on different stages of male germ cell development is poorly investigated. The absence of information about this problem requires implementation of profound study that would elucidate our understanding about the mechanisms via which estrogens regulate particular phases of spermatogenesis (mitotic, meiotic and postmeiotic stages). Such studies will contribute to evaluation of the importance of estrogen/androgen balance in functional maturation of germ and somatic cells in the testis and to discern direct and indirect mechanisms of estrogen action on different testicular cell populations.

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Differentiation of stem and progenitor cells in activated gene-engineered dendritic cells with anti-malignant properties

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Studies on the biology of dendritic cells (DCs) are mainly focused on their role as immune activators and modulators. In their appropriate cultivation and/or modifications, they have shown abilities for an enhanced expression of specific effective molecules. These properties have characterized them as promising candidates for construction of novel safe vaccines and gene-engineering products on their basis. In this aspect, in the last years the attention is directed to development of new safe therapeutic methods and techniques with DCs.

Key words: dendritic cells, stem/progenitor cells, recombinant viral vectors.

Introduction

Dendritic cells (DCs) have been found to play a pivotal role in the processess of immune response initiation and modulation, mainly as powerful antigen-presenting cells (APCs) [5, 9, 10, 21, 26—31, 33—36, 38, 42]. On the other hand, they have been found to participate in the maintenance of peripheral tolerance, and their capacity to induce anti-nuclear auto-immune response has been proven in experimental models [12].

Biological properties of dendritic cells and their role in generation of adequate immune response

In the light of the unique properties of DCs, they have been proposed as powerful immunomodulation agents, including in the composition of novel vaccines and gene-engineering products for treatment of malignant disorders [1, 2, 6, 8, 9, 12, 15—17, 19, 23, 24, 26, 28, 29, 33, 37—40, 44, 45, 48]. Complex mechanisms, which include molecular, genetic and cellular components, such as *Wnt-*, *BMP-* and *Notch/*

Delta-signalling pathways, have been found to underlie differentiation and functions of stem and progenitor cells [5, 7—10, 13, 21, 26, 29, 31, 34, 35, 42]. By use of polymerase chain reaction in real time (RT-PCR), an ability for initiation of erythroid (β-globin) and/or myeloid (myeloperoxidase) gene expression programs by the same cell prior to exclusive commitment to the erythroid and/or, respectively, myeloid lineages for it, has been shown [21, 29]. On the other hand, protein BCL-6 has also been detectable in inter- and intra-follicular CD4+ T-lymphocytes, but not in other follicular components, including B-lymphoid cells, plasma cells, monocytes/macrophages and DCs [5, 10, 13, 17, 21, 26, 29, 31, 34, 35, 43].

Origin of dendritic cells by differentiation of myeloid precursors in the presence of specific antigens

According to many literature data, granulocyte-macrophage colony-stimulating factor (GM-CSF) mobilizes CD34+ bone-marrow progenitor cells both *in vitro* and *in vivo* with an increased frequency and generation of DCs with anti-malignant properties [8, 9, 13, 35, 37, 39]. Similarly, in the addition of GM-CSF plus tumor necrosis factor-α (TNF-α), induced development of DCs from purified CD34+ cells of bone marrow, cord blood and peripheral blood, has been observed. The critical role of TNF-α for the differentiation of DCs has been supported by the demonstration that this cytokine induces the expression of molecule CD40 in CD34+ cells. Besides that, CD34+/CD40+ cells have been found to express only myeloid markers, significantly increase allo-antigen presenting function, compared with total CD34+ cells, and have also given rise to DCs' number. Capable of modulating differentiation of DCs from these bipotent CD34+/CD40+ cells during the later stages of their cultivation, has also been shown to be cytokine interleukin-4 (IL-4) [38].

The possibility of generating or expanding tolerant DCs *ex vivo* has been found to open novel therapeutic perspectives. Their *in vitro* and/or *ex vivo*-maturation has been characterized as a critical step in the induction of T-cell responses and it has been proven to depend on the activation of transcription factors from the family of Nuclear Factor-kappaB (NF-κB) [29]. It has also been suggested that kinetic and the quality of DCs' activation is controlled by cytokine IL-10, which has been characterized as alternative promising pathway of their differentiation [25]. On the other hand, DCs, differentiated in the presence of vaso-active intestinal peptide (VIP), have shown impaired allogeneic haplotype-specific responses to donor CD4+ T-lymphocytes in mice, and have been found to induce generation of regulatory T-cells in the graft [10, 21]. As a critical component for optimal function of DCs, has been characterized the TNF supper-family member lymphotoxin-αβ (LTαβ), independently of its described role in maintaining of the lymphoid tissue organization [30]. In the absence of LTαβ on antigen-specific T-cells, DCs' dysfunction *in vivo* could be rescued via CD40 or LTβ receptor stimulation, respectively, which has suggested a possibility for eventual cooperation of these pathways. It has also been indicated that DCs, induced by ligand Flt3, are well positioned to regulate the qualitative nature of intestinal immune responsiveness, depending on the presence or absence of appropriate inflammatory signals [37]. In this way, a potential use of ligand Flt3 as a mucosal vaccine adjuvant in conjunction with the inflammatory mediator IL-1 has been suggested.

Development of novel therapeutic strategies with dendritic cells

In the last years, the development of novel therapeutic strategies with DCs has become extensively investigated [1—7, 9—11, 14—26, 28, 30, 36 - 48]. So genetically modified DCs have been widely tested in pre-clinical studies, included as anti-malig-

nant agents. After such application of DCs, peptide-specific responses by cytotoxic T-lymphocytes (CTLs), improvement in performance status, decrease in malignancy markers levels, regression of malignancies, and, at the same time, no toxic side effects have been accounted [33]. Because isolated DCs, loaded with malignant antigens *ex vivo* and administered as effective cellular vaccines, have induced protective and therapeutic anti-malignant immunity in experimental animals with induced malignant disorders, adjuvant treatment of malignancies at high risk for recurrence after operation, as well as methods for targeting malignant antigens to DCs *in vivo*, have been explored [38]. On the other hand, appropriate modifications of DCs to express malignancy-specific antigens by *in vitro* and/or *ex vivo*-transfer of genes, coding respective antibodies, has been suggested [1, 2, 6, 9, 11, 13—15, 17, 18, 22, 23, 25, 27, 28, 32, 36, 37, 40, 42, 44 - 48]. Therefore, exploitation of the antigen-presenting properties of DCs offers promise for the development of effective anti-malignant immunotherapy. For this aim, different therapeutic strategies of DCs, have been developed.

Development of novel therapeutic strategies with hybrid vaccine constructs, received by fusion of dendritic cells with malignant cells

As alternative method for delivery into DCs, their fusion with malignant cells has been utilized, as well as the hybrid cells-based vaccines have shown high therapeutic activity, even in patients with malignant diseases and disorders [3, 17, 19, 20, 22, 26, 27, 40—42, 44—47]. The immunization with such hybrid conjugates, derived by fusion between DCs and malignant cells, has significantly increased the production of Th1 cytokine-producing cells, the number of antigen-specific CD8+ T-cells, as well as the anti-malignant immunity. The observed anti-malignant immunity, induced by vaccination with DCs/malignant cells hybrid fusion products has reacted differently to injected malignant cells and autochthonous malignancies [46]. It has also been shown that immunization with such fusion cells induces rejection of metastases. Hybrid cells, obtained by fusion between DCs and malignant cells, have been found to express major histo-compatibility complex (MHC) molecules, both class I and class II-restricted malignancy-associated epitopes and might, therefore, be useful for the induction of specific malignancy-reactive CD8+ and CD4+ T-lymphocytes both *in vitro* and *in vivo*, by human vaccination trials [30]. The observed greatly reduced number of established pulmonary metastases, both with and without *in vivo*-administration of *IL-2*-adoptive transfer of T-lymphocytes, derived from *B16/DC* vaccine-primed lymph nodes into *B16* tumor-bearing mice, has suggested a role of malignant cells/DCs hybrids as effective cellular vaccines for eliciting T-cell-mediated anti-malignant immunity [19]. It has also been demonstrated an enhanced immunization with received by fusion of DCs with mouse 4TOO plasmacytoma cells *FC/4TOO* hybrid cells, plus anti-malignant immunity of IL-12 [20]. Findings, according which fusions of ovarian cancer cells to autogenic or allogeneic DCs induce cytolytic T-cell activity and lysis of autologous malignant cells, mediated by MHC molecules class I-restricted mechanism, have suggested that the fusions are probably functional, when are generated by autolgenic and/or allogeneic transplantation of DCs [30]. It has also been demonstrated that sequential stimulation with DCs/breast carcinoma cells fusion hybrids results in a marked expansion of activated malignancy-specific T-lymphocytes, which has suggested these fusion cells are probably effective APCs, which stimulate inhibitory T-cells that limit vaccine efficacy [39, 44]. Similarly, hybrids, derived by fusion of spleen DCs from C57BL/6 mice with *B16* melanoma cells, have expressed MHC-

molecule B7, as well as *B16* tumor marker M562, and have been characterized as an attractive strategy for immunotherapy of malignancies [9]. On the other hand, the results, according to which the *ex vivo*-exposure of DCs on the presence of cytokine transforming growth factor-beta (TGF-β) hasn't appeared to lessen the efficacy of DCs vaccines, have suggested that this cytokine, derived from malignant cells, has probably reduced the their efficacy via *in vivo*-mechanism, and the neutralization of produced by the fusion cells TGF-β might enhance it [24, 27, 40]. An increase in the immunogenic potential of DCs/malignant cells fusion cell-based vaccines has been observed in heat-treated malignant cells [26].

Development of novel therapeutic strategies with dendritic cells, transduced by recombinant viral vectors, coding malignant antigens

DCs have shown a possibility to be genetically engineered to express constitutively respective genes of interest, coding immune-modulating cytokines, antibodies and/or antigens, derived from transformed cells or other pathogens [1, 2, 6, 11, 13—15, 18, 22, 25, 28, 32, 37, 48]. In laboratory conditions, human DCs, transduced with recombinant *adenoviral* vectors, have shown inhibition of a mixed leukocyte culture, reduced cell surface expression of co-stimulatory molecules CD80/CD86, as well as inability for production of the potent allo-stimulatory cytokine IL-12 [11, 14, 15, 17, 25, 32, 37, 48]. In investigation on the *in vivo*-properties of the so modified DCs, skin transplantation of experimental mice with non-obese diabetes, combined with severe immunodeficiency (NOD/SCID), reconstituted via intraperitoneal injection with allogeneic mononuclear cells (MNCs) mixed with autologous to the skin donor DCs, transduced with either recombinant *adenoviral* gene construct *AdV/IL-10* or *AdV/MX-17*, a reduced skin graft rejection, characterized by reduced mononuclear cell infiltration and less destruction of derma—epidermis junctions, in comparison with the animals with inoculation of DCs, has been observed [10]. *Adenovirus*-transduced immature DCs have shown ability to differentiate in the presence of lipopolysaccharide (LPS) or a monocyte-/macrophage-conditioned medium to express the surface markers of mature DCs, such as CD25, CD83, high levels of molecules CD86 and HLA-DR, as well as to secrete IL-12. Their ability to induce T-lymphocytes' growth has also been enhanced. It has also been suggested that *adenoviruses* probably have mediated minor effects on the phenotype of DCs, which, however, could be seen only when a sufficient number of particles enter in each cell [37]. Recombinant *adenoviral* vectors have also been found to transduce effectively DCs and direct the generation of specific CTLs, which would be a potent strategy in the immunotherapy of Hodgkin's lymphoma [14]. According to the results from other study, transduction of DCs with recombinant vectors with insertion of gene *mTRP-2* (encoding tyrosinase-related protein-2, respectively), provides a potential therapeutic strategy for the management of melanoma, especially in the early stage of that disease [25]. So modified DCs have also shown high stimulatory activity in both allogenic and autogenic mixed lymphocyte reaction. Similarly, mouse DCs, infected with recombinant *fowlpox virus* (*rFWPV*) vector, have stimulated a powerful, MHC class I-restricted immune response against the recombinant antigen [5]. These data have also supported the efficiency of the recombinant viral vectors in studies on the biologic properties of DCs, including the expression of specific antigens for active immune therapy.

Development of combined therapeutic strategies with dendritic cells

The fact that an increased Th1 cytokine production and stronger anti-malignant effect haven't been observed in mice, depleted of gamma-interferon (IFN-γ), has also supported the maintenance of DCs/malignant cells conjugates as potent anti-malignant vaccines, as well as the cytokine *IL-18* [42, 44]. These data could be additionally administrated by gene transfection of cells for enhancement of the immunity, which is probably mediated mainly by IFN-γ.

Development of combined therapeutic strategies with gene-engineered dendritic cells

For further increase of the potency of the vaccine, a combined variation of both technologies has been applied, in which *IL-18*-transfected DCs have been used to prepare DCs/malignant cells conjugates [27, 36, 40, 42, 44—48]. It has also been indicated that *GM-CSF* gene-modified DCs might lead to the generation of hybrid vaccines with potentially increased therapeutic efficacy [9]. Although the observed elicited anti-malignant effect with participation of both CD4+ and CD8+ T-lymphocytes by the hybrid vaccine *IL-18DC-E.G7*, derived by fusion between gene-engineered DCs, transduced with recombinant *adenoviral* vectors, carrying genes for enzyme β-galactosidase (*AdlacZ*) and/or for cytokine IL-18 (*AdIL18*), respectively, and *E.G7* malignant cells, derived from *EL4* cells, transfected with *cDNA*, carrying gene for chicken egg albumin, it has been largely blocked by anti-IFN-γ antibodies [23].

Development of combined therapeutic strategies with gene-engineered malignant cells

Results from experiments for immunization with fusion hybrids, derived by fusion of DCs with *IL-12* gene-transferred malignant cells, have shown an ability to elicit a previously enhanced anti-malignant effect in experimental therapeutic models [27, 40—44]. Such novel *IL-12*-producing fusion cell vaccine has been characterized as a promising intervention for future immune therapy of malignant diseases [36].

Development of combined therapeutic strategies with gene-engineered both malignant and dendritic cells

In immunization of mice with gene-engineered *DCRMAT/J558-IL-4* fusion hybrids, an elicited stronger *J558* tumor-specific CTLs immune response has been induced, in comparison of hybrid vaccine *DCRMAT/J558 in vivo* [27]. Similar results have been observed in immunization of C57BL/6 mice with gene-engineered *DC/J558-IL-4* hybrids, and gene-engineered fusion hybrid vaccine constructs have been characterized as an attractive strategy for immunotherapy of malignancies [27, 44, 45, 47].

Conclusion

Dendritic cells (DCs) have been characterized as hopeful vehicles for appropriate modulation of the immune response, including in composition of vaccine constructs and gene-engineered products with anti-malignant activity. They have also shown

abilities for enhanced expression of specific molecules in appropriate conditions of cultivation and/or by appropriate modifications. These properties characterize them as promising candidates for construction of novel and safe therapeutic products on their basis, by use of new technologies, as their fusion with malignant cells; transduction with recombinant viral vectors, as well as a combined variation, in which malignant cell, DC or both components of the hybrid fusion vaccine might be genetically transduced.

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Acromegaly

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Acromegaly is a hormonal disorder that results from too much growth hormone (GH) in the body. The excess GH comes from noncancerous tumors on the pituitary. The most common symptoms are abnormal growth of the hands and feet, brow and lower jaw protrude, the nasal bone enlarges, and the teeth space out. Surgery is the first option recommended for most people with acromegaly, as it is often a rapid and effective treatment. If the surgery is successful, facial appearance and soft tissue swelling improve within a few days. Medical therapy is most often used if surgery does not result in a cure. From anthropological point of view contemporaries studies on acromegaly are small number. Acromegaly is often difficult to diagnose. Scientists have based their diagnoses almost entirely upon phenotypic characteristics. So further medico-anthropological studies of patients with acromegaly are needed to determine level of abnormal changes.

Key words: acromegaly; pituitary; growth hormone; hypertrophy of the hands, feet and the face.

Introduction

Acromegaly (from Greek 'acros-end' and 'megalos-big') is a hormonal disorder that results from too much growth hormone (GH) in the body. Due to its low frequency and "hidden" onset it is hard to diagnose. In Bulgaria there are 400 registered cases of acromegaly and no full anthropological characterization of specific anomalies (enlargement) that affect the hands and feet as a consequence of the disease.

In 1886 Pierre Marie (1853-1940 Paris, France) used the term "acromegaly" for the first time andgave a full description of the characteristic clinical picture: "A condition characterized by hypertrophy of the hands, feet and the face exists which we propose to be called "acromegaly" which means hypertrophy of the extremities. In reality the extremities are swollen during the disease course and their increase in volume is the most characteristic feature of this disease. Acromegaly is different from myxedema, Paget's disease or leontiasis ossea of Virchow." [8]. Marie, however, was not the first physician to give a clear description of the clinical picture of acromegaly. Others had done this years before him, like (possibly) the Dutch surgeon and active

opponent of superstition and witch-burning, Johannes Wier (1515-1588) already in 1567, or Saucerotte in 1772. Other physicians had also given the disease different names including Alibert in 1822 calling it "Ge'ant scrofuleux", Verga in 1864 calling it "Prosopo-ectasia"and Lombroso in 1869 calling it "Macrosomia". A total of more than 20 physicians had already published ondisorders, which later could be reclassified as cases of acromegaly [11]. In 1886, Marie was not yet aware of any pituitary pathology in patients with acromegaly. In the following years he and his co-workers J. D. Souza-Leite and G. Marinesco significantly contributed to further knowledge on the clinical features and pathology of acromegaly by publishing many important papers in this field: 1. Souza-Leite J. D. (1890) De l'acrome'galie maladie de Marie, Paris; 2. Marie P., Marinesco G. (1891) Sur l'anatomie pathologique de l'acrome'galie.; 3. Marie P (1888) L'acrome'galie.; 4. Marie P. (1888) L'acrome'galie. etc. (see ref. Wouter, W. [13])

Discussion

The pituitary, a small gland in the brain, makes GH. In acromegaly, the pituitary produces excessive amounts of GH. Usually the excess GH comes from benign, or noncancerous, tumors on the pituitary. These benign tumors are called adenomas.

Acromegaly is most often diagnosed in middle-aged adults, although symptoms can appear at any age. If not treated, acromegaly can result in serious illness and premature death. Acromegaly is treatable in most patients, but because of its slow and often "sneaky" onset, it often is not diagnosed early or correctly. The most serious health consequences of acromegaly are type 2 diabetes, high blood pressure, increased risk of cardiovascular disease, and arthritis. Patients with acromegaly are also at increased risk for colon polyps, which may develop into colon cancer if not removed [5].

When GH-producing tumors occur in childhood, the disease that results is called gigantism rather than acromegaly. A child's height is determined by the length of the so-called long bones in the legs. In response to GH, these bones grow in length at the growth plates–areas near either end of the bone. Growth plates fuse after puberty, so the excessive GH production in adults does not result in increased height. However, prolonged exposure to excess GH before the growth plates fuse causes increased growth of the long bones and thus increased height. Pediatricians may become concerned about this possibility if a child's growth rate suddenly and markedly increases beyond what would be predicted by previous growth and how tall the child's parents are.

Hormones never seem to act simply and directly. They usually "cascade" or flow in a series, affecting each other's production or release into the bloodstream.

GH is part of a cascade of hormones that, as the name implies, regulates the physical growth of the body. This cascade begins in a part of the brain called the hypothalamus. The hypothalamus makes hormones that regulate the pituitary. One of the hormones in the GH series, or "axis," is growth hormone-releasing hormone (GHRH), which stimulates the pituitary gland to produce GH.

Secretion of GH by the pituitary into the bloodstream stimulates the liver to produce another hormone called insulin-like growth factor I (IGF-I). IGF-I is what actually causes tissue growth in the body. High levels of IGF-I, in turn, signal the pituitary to reduce GH production [1].

The hypothalamus makes another hormone called somatostatin, which inhibits GH production and release. Normally, GHRH, somatostatin, GH, and IGF-I levels

in the body are tightly regulated by each other and by sleep, exercise, stress, food intake, and blood sugar levels.

In more than 95 percent of people with acromegaly, a benign tumor of the pituitary gland, called an adenoma, produces excess GH. Pituitary tumors are labelled either micro- or macro-adenomas, depending on their size. Most GH-secreting tumors are macro-adenomas, meaning they are larger than 1 centimeter. Depending on their location, these larger tumors may compress surrounding brain structures. For example, a tumor growing upward may affect the optic chiasm–where the optic nerves cross–leading to visual problems and vision loss. If the tumor grows to the side, it may enter an area of the brain called the cavernous sinus where there are many nerves, potentially damaging them [9].

Compression of the surrounding normal pituitary tissue can alter production of other hormones. These hormonal shifts can lead to changes in menstruation and breast discharge in women and erectile dysfunction in men. If the tumor affects the part of the pituitary that controls the thyroid, another hormone-producing gland, then thyroid hormones may decrease. Too little thyroid hormone can cause weight gain, fatigue, and hair and skin changes. If the tumor affects the part of the pituitary that controls the adrenal gland, the hormone cortisol may decrease. Too little cortisol can cause weight loss, dizziness, fatigue, low blood pressure, and nausea.

Some GH-secreting tumors may also secrete too much of other pituitary hormones. For example, they may produce prolactin, the hormone that stimulates the mammary glands to produce milk. Rarely, adenomas may produce thyroid-stimulating hormone. Doctors should assess all pituitary hormones in people with acromegaly.

Rates of GH production and the aggressiveness of the tumor vary greatly among people with adenomas. Some adenomas grow slowly and symptoms of GH excess are often not noticed for many years. Other adenomas grow more rapidly and invade surrounding brain areas or the venous sinuses, which are located near the pituitary gland. Younger patients tend to have more aggressive tumors. Regardless of size, these tumors are always benign.

Most pituitary tumors develop spontaneously and are not genetically inherited. They are the result of a genetic alteration in a single pituitary cell, which leads to increased cell division and tumor formation. This genetic change, or mutation, is not present at birth, but happens later in life. The mutation occurs in a gene that regulates the transmission of chemical signals within pituitary cells. It permanently switches on the signal that tells the cell to divide and secrete GH. The events within the cell that cause disordered pituitary cell growth and GH oversecretion currently are the subject of intensive research [7].

Rarely, acromegaly is caused not by pituitary tumors but by tumors of the pancreas, lungs, and other parts of the brain. These tumors also lead to excess GH, either because they produce GH themselves or, more frequently, because they produce GHRH, the hormone that stimulates the pituitary to make GH. When these non-pituitary tumors are surgically removed, GH levels fall and the symptoms of acromegaly improve [12].

In patients with GHRH-producing, non-pituitary tumors, the pituitary still may be enlarged and may be mistaken for a tumor. Physicians should carefully analyze all "pituitary tumors" removed from patients with acromegaly so they do not overlook the rare possibility that a tumor elsewhere in the body is causing the disorder.

What are the symptoms of acromegaly?

The name acromegaly comes from the Greek words for "extremities" and "enlargement," reflecting one of its most common symptoms–the abnormal growth of the hands and feet. Swelling of the hands and feet is often an early feature, with patients noticing a change in ring or shoe size, particularly shoe width. Gradually, bone changes alter the patient's facial features: The brow and lower jaw protrude, the nasal bone enlarges, and the teeth space out.

Overgrowth of bone and cartilage often leads to arthritis. When tissue thickens, it may trap nerves, causing carpal tunnel syndrome, which results in numbness and weakness of the hands. Body organs, including the heart, may enlarge [14].

Other symptoms of acromegaly include:

- joint aches
- thick, coarse, oily skin
- skin tags
- enlarged lips, nose, and tongue
- deepening of the voice due to enlarged sinuses and vocal cords
- sleep apnea–breaks in breathing during sleep due to obstruction of the airway
- excessive sweating and skin odor
- fatigue and weakness
- headaches
- impaired vision
- abnormalities of the menstrual cycle and sometimes breast discharge in women
- erectile dysfunction in men
- decreased libido

How common is acromegaly?

Small pituitary adenomas are common, affecting about 17 percent of the population. However, research suggests most of these tumors do not cause symptoms and rarely produce excess GH. Scientists estimate that three to four out of every million people develop acromegaly each year and about 60 out of every million people suffer from the disease at any time. Because the clinical diagnosis of acromegaly is often missed, these numbers probably underestimate the frequency of the disease [7].

How is acromegaly diagnosed?

If acromegaly is suspected, a doctor must measure the GH level in a person's blood to determine if it is elevated. However, a single measurement of an elevated blood GH level is not enough to diagnose acromegaly: Because GH is secreted by the pituitary in impulses, or spurts, its concentration in the blood can vary widely from minute to minute. At a given moment, a person with acromegaly may have a normal GH level, whereas a GH level in a healthy person may even be five times higher [1, 6].

More accurate information is obtained when GH is measured under conditions that normally suppress GH secretion. Health care professionals often use the oral glucose tolerance test to diagnose acromegaly because drinking 75 to 100 grams of glucose solution lowers blood GH levels to less than 1 nanogram per milliliter (ng/ml) in healthy people. In people with GH overproduction, this suppression does not occur. The oral glucose tolerance test is a highly reliable method for confirming a diagnosis of acromegaly [3].

Physicians also can measure IGF-I levels, which increase as GH levels go up, in people with suspected acromegaly. Because IGF-I levels are much more stable than GH levels over the course of the day, they are often a more practical and reliable screening measure. Elevated IGF-I levels almost always indicate acromegaly. However, a pregnant woman's IGF-I levels are two to three times higher than normal. In addition, physicians must be aware that IGF-I levels decline with age and may also be abnormally low in people with poorly controlled diabetes or liver or kidney disease [6].

After acromegaly has been diagnosed by measuring GH or IGF-I levels, a magnetic resonance imaging (MRI) scan of the pituitary is used to locate and detect the size of the tumor causing GH overproduction. MRI is the most sensitive imaging technique, but computerized tomography (CT) scans can be used if the patient should not have MRI. For example, people who have pacemakers or other types of implants containing metal should not have an MRI scan because MRI machines contain powerful magnets.

If a head scan fails to detect a pituitary tumor, the physician should look for non-pituitary "ectopic" tumors in the chest, abdomen, or pelvis as the cause of excess GH. The presence of such tumors usually can be diagnosed by measuring GHRH in the blood and by a CT scan of possible tumor sites.

Rarely, a pituitary tumor secreting GH may be too tiny to detect even with a sensitive MRI scan [12].

How is acromegaly treated?

Currently, treatment options include surgical removal of the tumor, medical therapy, and radiation therapy of the pituitary [14].

Goals of treatment are to:

- reduce excess hormone production to normal levels
- relieve the pressure that the growing pituitary tumor may be exerting on the surrounding brain areas
- preserve normal pituitary function or treat hormone deficiencies
- improve the symptoms of acromegaly

Surgery

Surgery is the first option recommended for most people with acromegaly, as it is often a rapid and effective treatment.

Medical Therapy

Medical therapy is most often used if surgery does not result in a cure and sometimes to shrink large tumors before surgery. Three medication groups are used to treat acromegaly.

Somatostatin analogs (SSAs) are the first medication group used to treat acromegaly. They shut off GH production and are effective in lowering GH and IGF-I levels in 50 to 70 percent of patients.

The second medication group is the GH receptor antagonists (GHRAs), which interfere with the action of GH. [3].

Dopamine agonists make up the third medication group. These drugs are not as effective as the other medications at lowering GH or IGF-I levels, and they normalize IGF-I levels in only a minority of patients [3, 10].

Although first researches on acromegaly concern phenotypical onset of the disease and are mainly descriptive (scopic) and includes some basic measurements (metric) so they can be qualified as anthropological studies. From anthropological point of view contemporaries studies on acromegaly are small number. Such a study is published by Dostalova S. et al. [4] and includes 38 patients (12 women and 36 men) passed trough cephalometric examination and 86 persons of control group (36 women and 50 men). The results are showing notables anomalies in patients with acromegaly from both sexes: increased facial high, longer ramus of the mandible and greater distance *basion-supramentale.* In conclusion significant anomalies affecting all orofacial bones except maxilla are found.

Another study concerning body composition of patients with acromegaly regarding quantity of water and fats in the body is presented by Brummer, R. et al. [2]. The design includes 10 patients submitted examination of cellular weight, extra cellular water and fats-free extra cellular solids. The measurement techniques consisted of anthropometry, bioelectrical impedance analysis (BIA)-applying various established regression equations-tritiated water dilution, whole body 40K-counting, and whole body computed tomography. The results are: CT-calibrated anthropometric predictions significantly overestimated body fat. It is concluded that in patients with active acromegaly, the determination of body composition using either certain two-compartment models based on measurement of total body water or bioelectrical impedance, or a four-compartment model based on total body water and total body potassium measurements show good agreement with CT-determined body composition. But this study is mainly comparison of most exact way to determinate the body composition.

Conclusion

Since acromegaly is often difficult to diagnose until later in life, recent studies are focusing on the best and most efficient way to determine a problem before major irreversible damage occurs. Unfortunately, since the disease is so rare, major symptoms generally have to occur before the afflicted is even tested for the disease. The problem is until recently, scientists have based their diagnoses almost entirely upon phenotypic characteristics and what is known about pituitary adenomata. So further medico — anthropological studies on metric and scopic characteristics of patients with acromegaly and comparison with control groups to determine level of abnormal changes occurring orofacial and somatic structures, and may be discover some anthropological signs evoking eventual on set of the disease.

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