ISSN 0861-0509

Acta morphologica et anthropologica (8)

8 • Sofia • 2003 • Institute of Experimental Morphology and Anthropology

Contents

Institute of Experimental Morphology and Anthropology

Acta morphologica et anthropologica, 8
Sofia • 2003

Morphology

Serum GD1a Ganglioside in Patients with Multiple Sclerosis

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In order to obtain more information concerning the neuronal damage in early multiple sclerosis(MS), the relative distribution of GD1a, one of the major human brain neuronal ganglioside fraction, was determined in the serum of patients with MS and of healthy subjects. The MS patients were with primary progressive MS (PPMS) and with remitting-relapsing MS during the first attack of the disease (FARRMS). An increase of GD1a portion in the serum of patients with PPMS and FARRMS was observed. The difference in relative proportion of GD1a between healthy subjects, patients with PPMS and FARRMS was statistically significant . It could be suggested that these findings are connected with the neuronal injury in the early phases of MS pathogenesis.They further support the concept of MS as a neuronal disease.

Key words: multiple sclerosis; ganglioside GD1a; serum.

Introduction

Multiple sclerosis (MS) is considered to be prototype of primary demyelinating disease in the central nervous system (CNS) in every textbook of neurology. However, in the past few years a considerable body of evidence indicates that neurons are also targets of the disease process [3, 4, 7, 9, 10, 15, 16]. Furthermore, in 2000 the Editorial paper of W a x m a n [17] in Archives of Neurology was entitled "Multiple Sclerosis as a Neuronal Disease". Although there is an increasing agreement that axonal loss is a major factor contributing to disability in the later stages of MS [2, 11, 13] the relation of neuronal damage to the pathogenesis of MS in the early stages of the disease remain to be elucidated.

Recent studies have shown axonal damage early in the evolution of MS [5, 13, 21]. The role of axonal degeneration in MS suggests that neuronal or axonal markers could be used to monitor disease progression.

Ganglioside GD1a is one of the major gangliosides in human brain neurons [19]. It was reported that GD1a was also the predominant ganglioside fraction in the bovine brain axons (without axolemma) [6]. The ganglioside spectra of normal blood plasma are remarkably stable and show only minor variations in samples from healthy donors of different age and sex [12]. However, in pathological conditions the plasma ganglioside spectrum may undergo pronounce changes [1, 14].

Recently, we first reported evidence that the serum ganglioside pattern undergoes significant changes in remitting-relapsing multiple sclerosis (RRMS) [20]. In order to obtain more information concerning the neuronal damage in early MS in this study the relative distribution of GD1a was determined in the serum of patients with primary progressive MS (PPMS) and of RRMS patients during their first MS attack (FARRMS).

Materials and Methods

Serum Samples

Sera were obtained from 20 patients with clinically definite MS and from 20 healthy subjects. Eight patients were evaluated during their first attack of the dis-

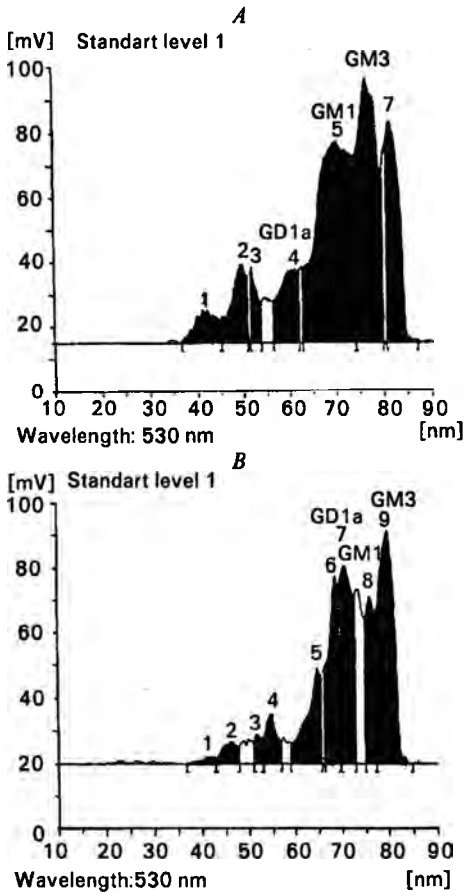

Fig. 1. Densitograms of Serum Gangliosides of MS Patients and Healthy Subjects
A — Healthy Subjects; *B* — Patients with first RRMS attack.(FARRMS)

ease of what later was definitely diagnosed as RRMS and 12 patients were with primary progressive MS (PPMS).

Isolation of serum gangliosides was performed by the method of I l i n o v et al. [8]. It includes the following stages:

a) dehydration of the sample by azeotropic distillation of the mixture of serum water/n-propanol = 1:10 (v/v);

b) total lipid triple extraction with cyclohexane (I), chloroform : methanol =1:1 (v/v) (II), and chloroform : methanol = 1:2 (v/v) (III);

c) non-polar lipids removal by preparative TLC with a mobile phase: chloroform : methanol: 0,3 % $CaCl_2$= 30:18:4 (v/v/v);

d) elimination of the blood sugar by Sep Pak technique according to W i l l i a m s and M c C l u e r [18].

e) HPTLC of the ganglioside fractions with a mobile phase: chloroform : methanol : 0,1 M sodium lactate = 55:40:10 (v/v/v).

The spots were visualized by spraying with orcinol reagent followed by local heating at 110°C and the gangliosides were quantified densitometrically. Bovine brain gangliosides (Calbiochem) were used as a test mixture for identification. Four independent analyses and quantifications were conducted for each MS group and healthy subjects.

The relative distribution of three serum gangliosides (GM3, GM1 and GD1a) in the serum of patients with RRMS during their first MS attack (FARRMS), of patients with primary progressive MS (PPMS) and of healthy subjects (HS) was recalculated on the basis of densitograms (Fig. 1 — *A* and *B*).

The Student's test was used to determine statistical differences between the groups using *P* value of less then 0,05 as the level of confidence. The data are presented as a mean value (M) ± standard error of mean (SEM) (Table 2).

Results

An increase of GD1a portion in the serum of PPMS and FARRMS was observed (Fig. 2 and Table 1).

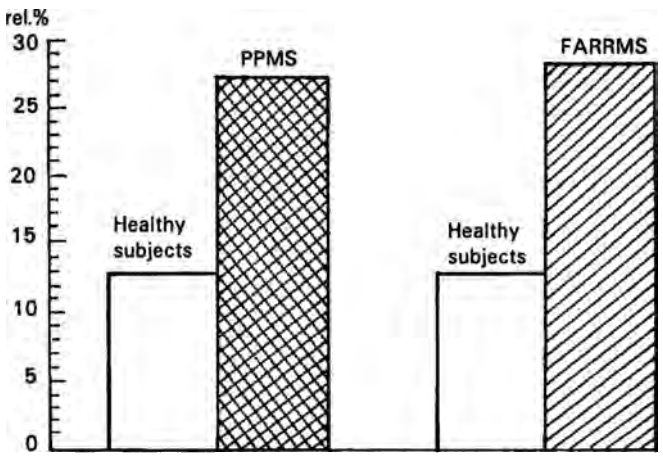

Fig. 2. Serum GD1a Gangliosides (in rel.%) in patients with PPMS, FARRMS and of Healthy Subjects

The difference in relative proportion of GD1a between healthy subjects patients with PPMS and FARRMS was statistically significant ($p<0.05$) (Table 2).

T a b l e 1. Relative Percentage of Serum GD1a Ganglioside Fractions in Healthy Subjects and in MS Patients

Group*	M ± SEM
HS (n = 20)	12.9 ± 0.40
FARRMS (n = 8)	28.3 ± 0.30
PPMS (n = 12)	27.3 ± 0.18

*HS — healthy subjects; FARRMS — patients with first RRMS attack; PPMS — patients with primary progressive MS; SEM — standard error of mean; n — number of evaluated subjects

T a b l e 2. Results of Student's Test Indicating Significance of Differences between HS, FARRMS and PPMS Patients with P Value of less than 0.05 Considered Significant

Group*	P value
HS vs FARRMS	0.02
HS vs PPMS	0.009

*HS — healthy subjects; FARRMS — patients with first RRMS attack; PPMS — patients with primary progressive MS

Discussion

This study has revealed a considerable increase of GD1a portion in the serum of PPMS and FARRMS patients. A statistically significant difference of GD1a portion was found between the healthy subjects and the patients with PPMS and FARRMS. These findings are in full concordance with our previous data concerning serum ganglioside spectrum in patients with RRMS [20]. The group of RRMS patients with their first MS attack had significantly rise of GD1a portion in the serum. The increase of serum GD1a in patients with PPMS was demonstrated for the first time in this study.

Early neuronal damage in patients with MS has been observed in vivo by magnetic resonance spectroscopy (MRS) which shows decreased levels of the neuronal specific marker N-acetylaspartate (NAA) in early stages of MS [5]. By assessing central brain NAA in MS patients with a wide range of disability and disease duration D e S t e f a n o et al. [5] showed that diffuse cerebral axonal damage begins in the early stage of RRMS and develops more rapidly in the earlier clinical stage of the disease.

Pathological studies of F e r g u s o n et al. [7] and Trapp et al. [15] applying modern morphological techniques, have provided evidence of axonal injury throughout active MS lesions. F e r g u s o n et al. [7] used amyloid precursor protein as a histopathological marker of damage axons, while T r a p p et al . [15] used confocal microscopy and immunohistochemistry applying an antibody to non-phosporylated neurofilament epitopes, which are increased in demyelinating axons.

Our recent electronmicroscopic investigations revealed early axonal damage in chronic relapsing experimental allergic encephalomyelitis which shares histopathological and immunological parameters with MS [21].

Considering the data mentioned above, and the fact that GD1a is one of the major ganglioside in human brain neurons, we could suggest that the increase of GD1a portion in the serum of FARRMS and PPMS is connected with the neuronal injury in the early phases of MS pathogenesis. Therefore, serum GD1a gangliosides could be used as neuronal markers to monitor disease activity. Our findings further

support the concept of MS as a neuronal disease. The neuronal pathology in the early MS argue for the early treatment of MS with agents directed toward neuronal protection.

References

1. Ayabe, M., S. Shichijo, M. Yokoyama. Diagnostic value of ganglioside patterns in plasma of human diseases. — J. Clin.Lab. Analysis, 3, 1989, 301-306.
2. Barnes, D., P. M. G. Munro, B. D. Youl, J. W. Prineas, W. I. McDonald. The longstanding MS lesion: a quantitative MRI and electron microscopic study. — Brain, **114**, 1991, 1271-1280.
3. Bjartmar, C., X. Yin, B. D. Trapp. Axonal pathology in myelin disorders. — J. Neurocytol., **28**, 1999, 383-395.
4. Compston, A., A. Coles. Multiple sclerosis. — Lancet, **359**, 2002, 1221-1231.
5. De Stefano, N., S. Narayanan, G. S. Francis, R. Arnaoutelis, M. C. Tartaglia, J. P. Antel, P. M. Matthews, D. L. Arnold. Evidence of axonal damage in the early stages of multiple scerosis and its relevance to disability. — Arch. Neurol., **58**, 2001, 65-70.
6. De Vries, G., W. Norton. The lipid composition of axons from bovine brain. — J. Neurochem., **22**, 1974, 259-264.
7. Ferguson, B., M. K. Matyszak, M. M. Esiri, V. H. Perry. Axonal damage in acute multiple sclerosis lesions. — Brain, **120**, 1997, 393-399.
8. Ilinov, P., D. Deleva, S. Dimov, E. Zaprianova. A variant for isolation of serum gangliosides. — J. Liquid Chrom. Rel. Technol., **20**, 1997, (8), 1149-1157.
9. Kornek, B., H. Lassman. Axonal pathology in multiple sclerosis: a historical note. — Brain Pathol., **9**, 1999, 651-656.
10. Lovas, G., N. Szilagyi, K. Majtenyi, M. Palcovits, S. Komoly. Axonal changes in chronic demyelinated cervical spinal cord plaques. — Brain, **123**, 2000, 308-317.
11. Scoldihg, N., R. Franklin. Axon loss in multiple sclerosis. — Lancet, **352**, 1998, 340-341.
12. Senn,H. J., M. Orth, E. Fitzke, H. Wieland, W. Gerok. Gangliosides in normal human serum. Concentration, pattern and transport by lipoproteins. — Eur. J. Biochem., **181**, 1989, 657-662.
13. Silber, E., M. K. Sharief. Axonal degeneration in the pathogenesis of multiple sclerosis. — J. Neurol. Sci., **170**, 1999, 11-18.
14. Suzuki, K. Gangliosides and disease: a review. — Adv. Exper. Med. Biol., **174**, 1985,407-418.
15. Trapp, B. D., J. Peterson, R. M. Ransohoff, R. Rudick, S. Mork, L. Bo. Axonal transection in the lesions of multiple sclerosis. — N. Engl. J. Med., **338**, 1998, 278-285.
16. Trapp, B. D., R. Ransohoff, R. Rudick. Axonal pathology in multiple sclerosis:relationship to neurologic disability. — Curr. Opin. Neurol., **12**, 1999, 295-302.
17. Waxman, S. G. Multiple sclerosis as a neuronal disease. — Arch. Neurol., **57**, 2000, 22-24.
18. Williams, A., R. McCluer. The use of Sep-Pak C18 cartidges during the isolation of gangliosides. — J. Neurochem., **35**, 1980, 266-270.
19. Yu, R., K. Iqbal. Sialosylgalactosyl ceramide as a specific marker for human myelin and oligodendroglial perikarya: gangliosides of human myelin, oligodendroglia and neurons. — J. Neurochem., **32**, 1979, 293-300.
20. Zaprianova, E., D. Deleva, P. Ilinov, E. Sultanov, A. Filchev, L. Christova, B. Sultanov. Serum ganglioside patterns in multiple sclerosis. — Neurochem. Research, **26**, 2001, (2), 95-100.
21. Zaprianova, E., O. Sotnikov, S. Sergeyeva, D. Deleva, A. Filchev, B. Sultanov. Axonal reaction precedes demyelination in experimental models of multiple sclerosis. — Morphology (in Russian), **5**, 2002,54-59.

Institute of Experimental Morphology and Anthropology

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Leucocytes in Ovarian Corpus Luteum During its Lifespan

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The appearance of different leucocytes and macrophages in the guinea pig corpus luteum of pregnancy and of the oestrous cycle was investigated electronmicroscopically. During the neovascularization in the early corpus luteum, eosinophills , neutrophills and lymphocytes were seen between the luteinizing cells, while during the corpus luteum involution autophagocytosis and heterophagocytosis (with participation of macrophages) were available. The role of the leucocytes (granulocytes, lymphocytes) and the macrophages for the development and function of the corpus luteum was discussed.

Key words: ovary, corpus luteum, leucocytes (granulocytes, lymphocytes), macrophages, ultrastructure.

Recently appeared some studies on the presence of different blood cells (eosinophils, neutrophils, lymphocytes, macrophages) in the ovary during the preovulatory period and the formation, functioning and regression of the corpus luteum [2, 11]. It is well known that the processes of the luteinization of the ovulated follicle is accompanied by the neovascularization, but the understanding of its cellular and molecular mechanisms is still uncomplete [12].There is some evidence that eosinophils play a role in the degradation of the follicle wall at the time of ovulation and exert a major influence on the neovascularization of the corpus luteum [10]. On the other hand, recent evidence indicates that the cells of the immune system (T- and B-lymphocytes, macrophages, granulocytes) and their large network of secretory products or cytokines, play an active role in the ovary throughout the oestrous cycle [9].

The aim of this study is to describe the appearance of the leucocytes and macrophages during the formation and involution of the guinea pig cyclic corpus luteum and the corpus luteum of pregnancy.

Material and Methods

Guinea pigs corpora lutea of pregnancy (at the 9^{th},15^{th},35^{th},45^{th}, 63^{th} day and two days after parturation) as well as during the oestrous cycle (3, 9, 14 and 20 days after ovulation) were prepared for electronmicroscopical study (fixation in 6% glutaral-

dehyde in phosphate buffer, postfixation in 1% osmium acid with 3,4% saccharose, dehydration and embedding in Epon) .The semithin sections were stained with Toluidin blue-pironine. The thin sections were stained with Uranyl acetat and Lead citrat.

Results and discussion

At early pregnancy of guinea pig (9 th and 15th day) and on third and 9 th days after ovulation, various leucocytes subsets (lymphocytes, eosinophils, neutrophils)

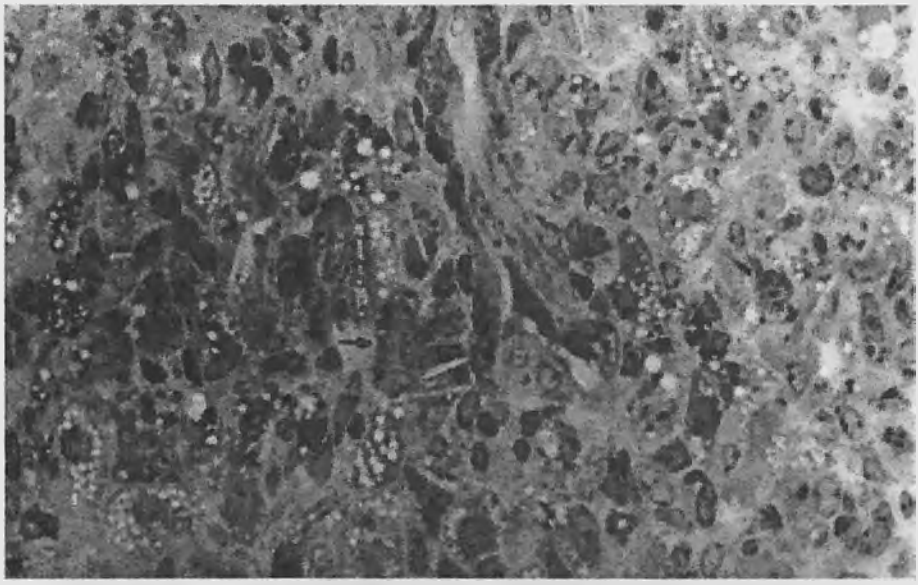

Fig. 1. Corpus luteum at early pregnancy (9th day) — around the capillaries many leucocytes are seen (arrow) (× 100)

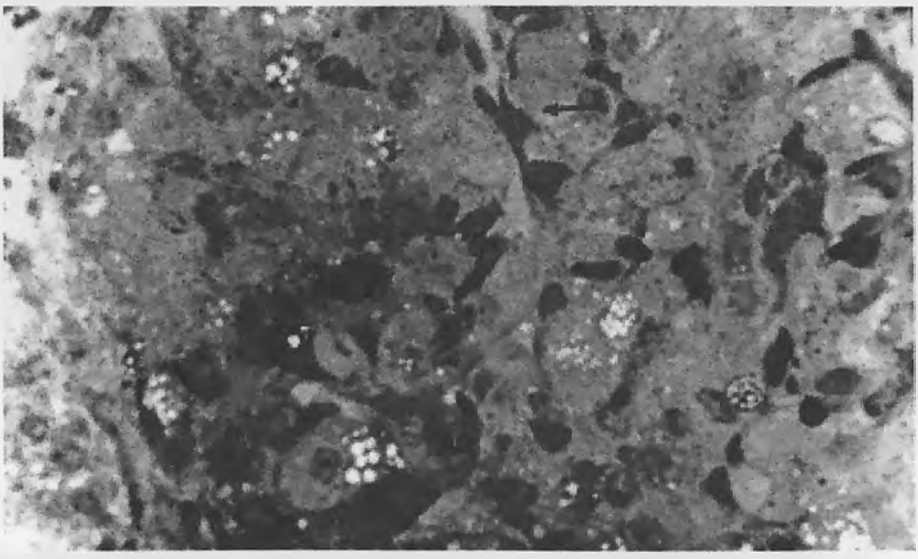

Fig. 2. Corpus luteum at midpregnancy (35th day) — around the capillaries single leucocytes are seen (arrow) (× 100)

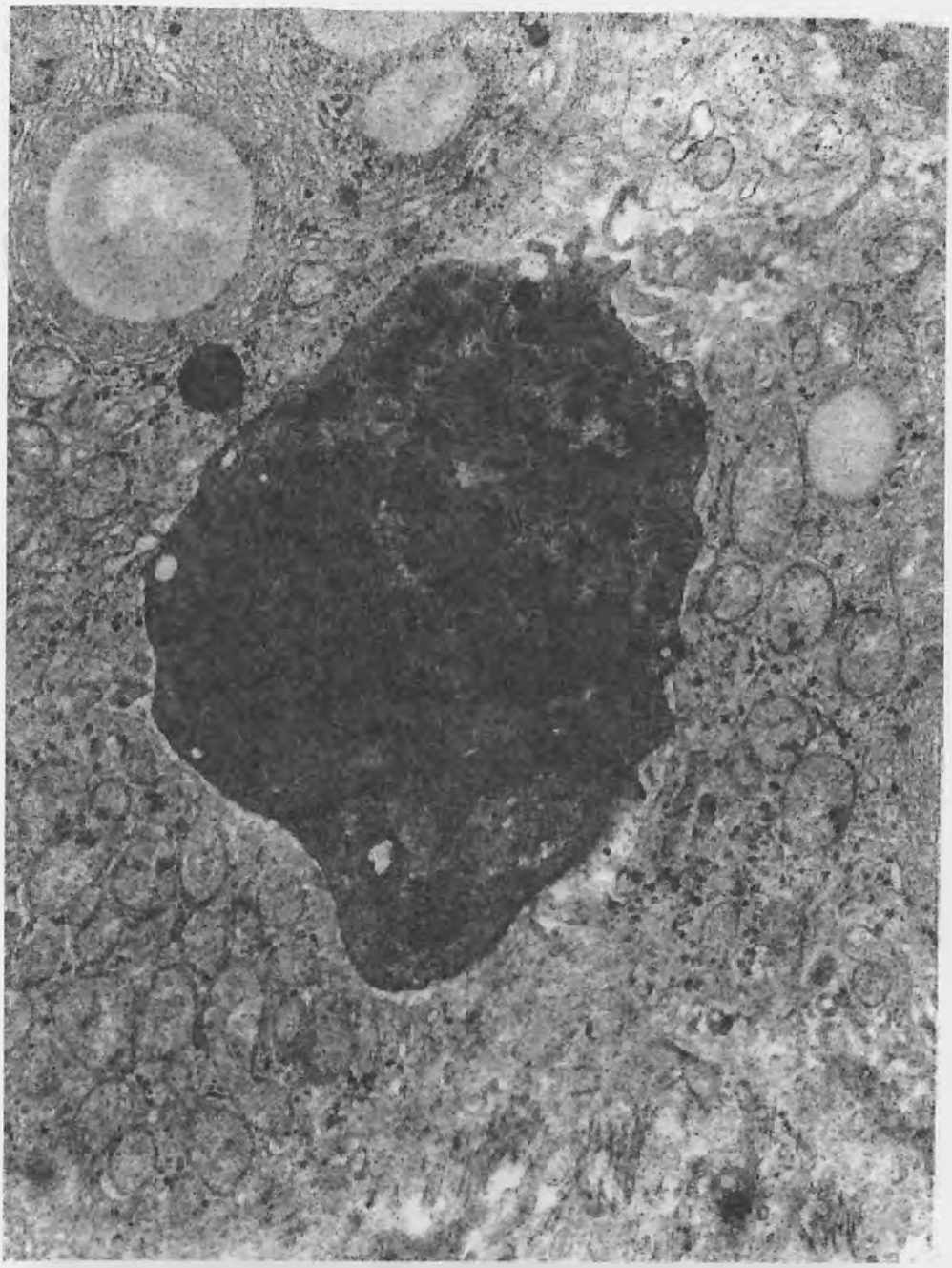

Fig. 3. Corpus luteum at early pregnancy (9th day) eosinophil (Eo) between the luteal cells (L) (× 16 000)

were seen in the vessels and around its when the neovascularization was available (Fig.1, 3, 4).

The luteal cells possess the well developed agranular endoplasmic reticulum (whorls), mitochodria with tubular cristae and lipid droplets, which are the ultra-

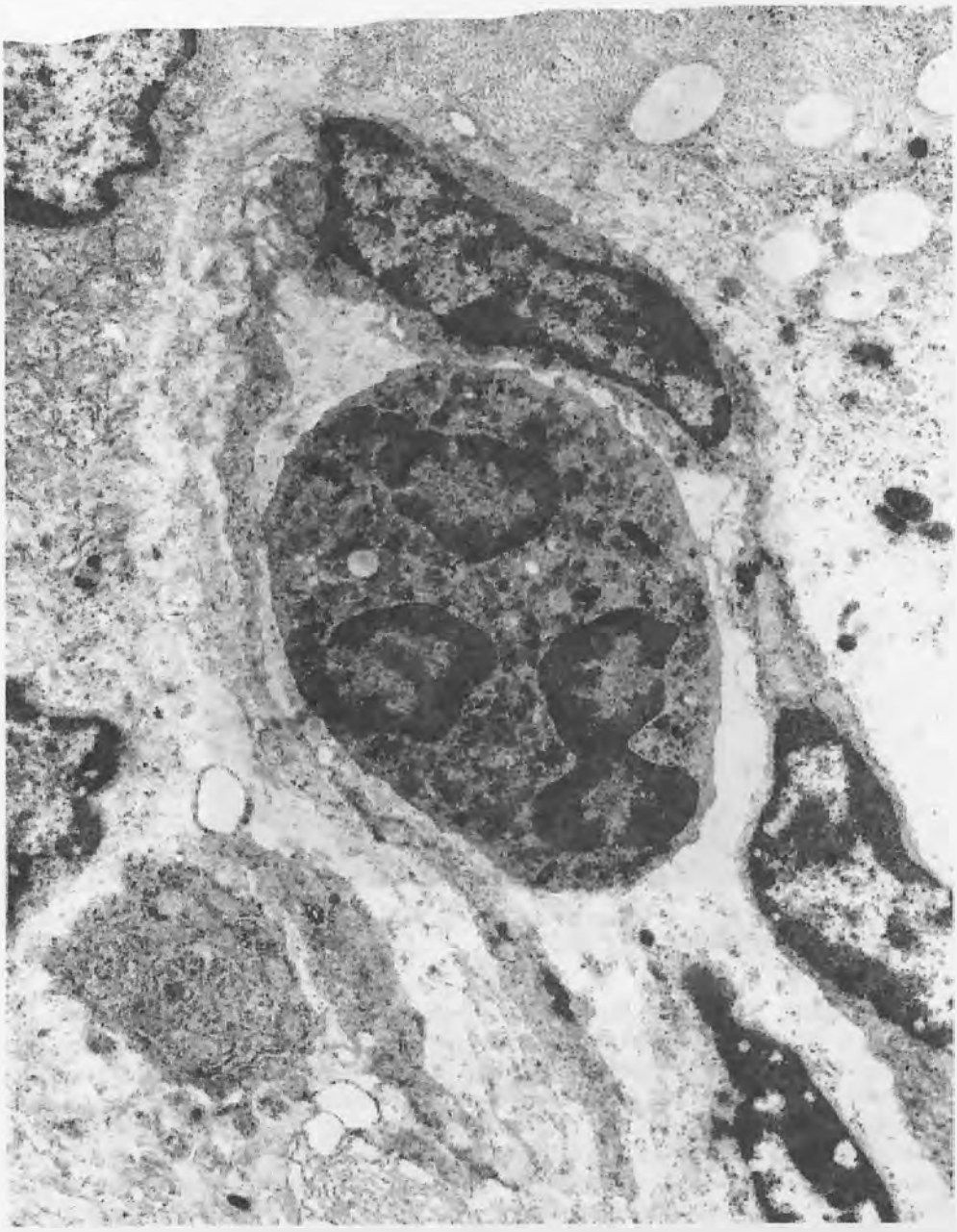

Fig. 4. Corpus luteum at early pregnancy (9th day) — polymorphonuclear leucocyte (PMN) in the capillary (× 14 000)

structural features of steroid producing cells (Fig. 3, 4).

Corpus luteum is a model of transicient physiological formation of the new blood vessels in the adult [12, 5]. In this early stage of guinea pigs corpus luteum formation eosinophils and polymorphnuclear leucocytes could be seen (Fig.3, 4).

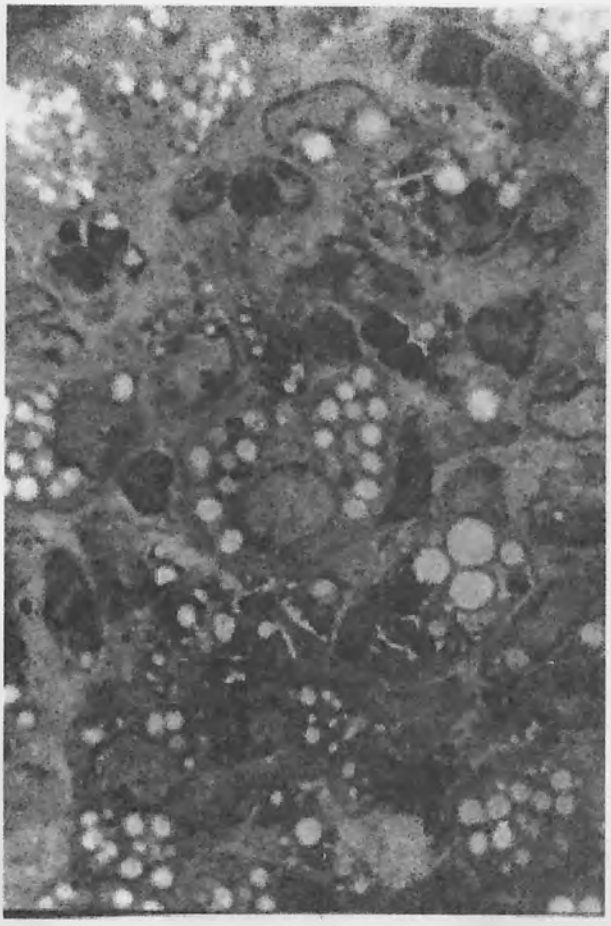

Fig. 5. Corpus luteum 20th day after the ovulation — many macrophages are seen (× 100)

Different trafficking mechanisms involved in the appearance of these leucocytes during the luteinization have been suggested in the literature. V i g a n o et al. [13] pointed out the cyclic expression of adhesion mollecules in the mammalian ovary. After R o h m et al. [11] the selective migration of various leucocytes subpopulations into ovary during the preovulatory period and follicular regression is accompanied by changes of the distribution pattern of adhesion molecules on the leucocytes and endothelial cells. On the other hand, the morphological signs of diapedesis and emperiopolesis can be asked. The emperiopolesis /term given by H u m b l e et al. [7] for engulfment of viable cells within other viable cells without reciprocal damage/, has never been reported for internalization of leucocytes by arterial muscular cells [6]. In our electronograms we have also not seen the emperiopolesis of leucocytes in corpus luteum endothelial cells. After R o h m et al. [11] eosinophil accumulation at the time of ovulation and early corpus luteum development is due to their diapedesis from the circulation.

During the development and the bloom stage of the guinea pig corpus luteum of pregnancy (mid pregnancy) and of the oestrous cycle (on the 15th day of the

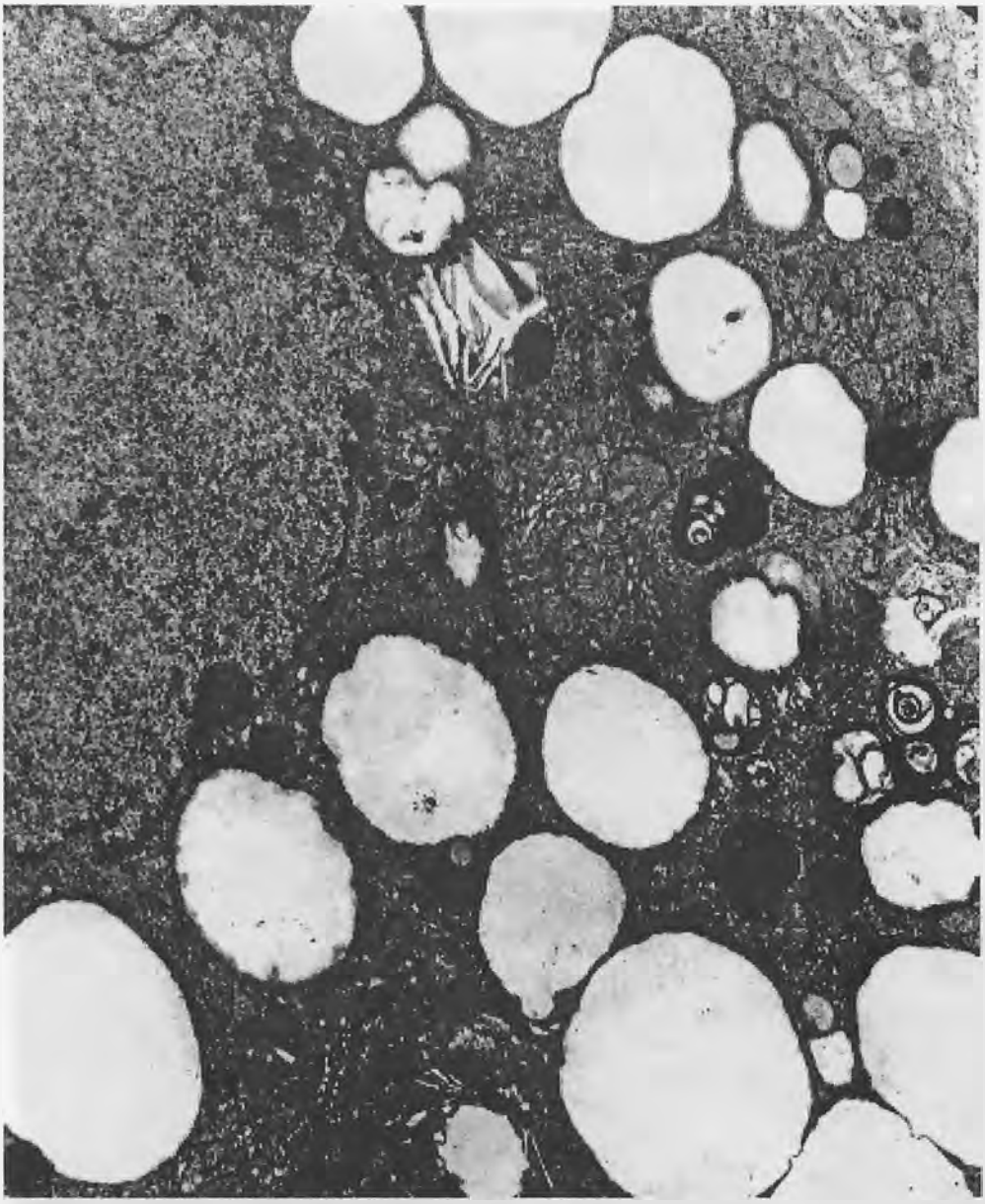

Fig. 6. Luteal cells two days after the parturation — autophagic structures in the cytoplasm (× 15 000)

cycle), the eosinophils and neutrophils desappeared (Fig. 2) and appeared cells with phagocytic cell debris.

The results presented in this study show some differences in ultrastructural changes durind involution of corpus luteum of pregnancy and corpus luteum cyclicum. At the end of guinea pig pregnancy autophagic processes (degenerative changes in the cytoplasm, enlarged cysternae of agranular endoplasmic reticulum, lipid droplets or the formation of autophagic vacuoles — i.e. secondary lysosomes) predominate in the luteal cells (Fig. 6). During the regression of the corpus luteum

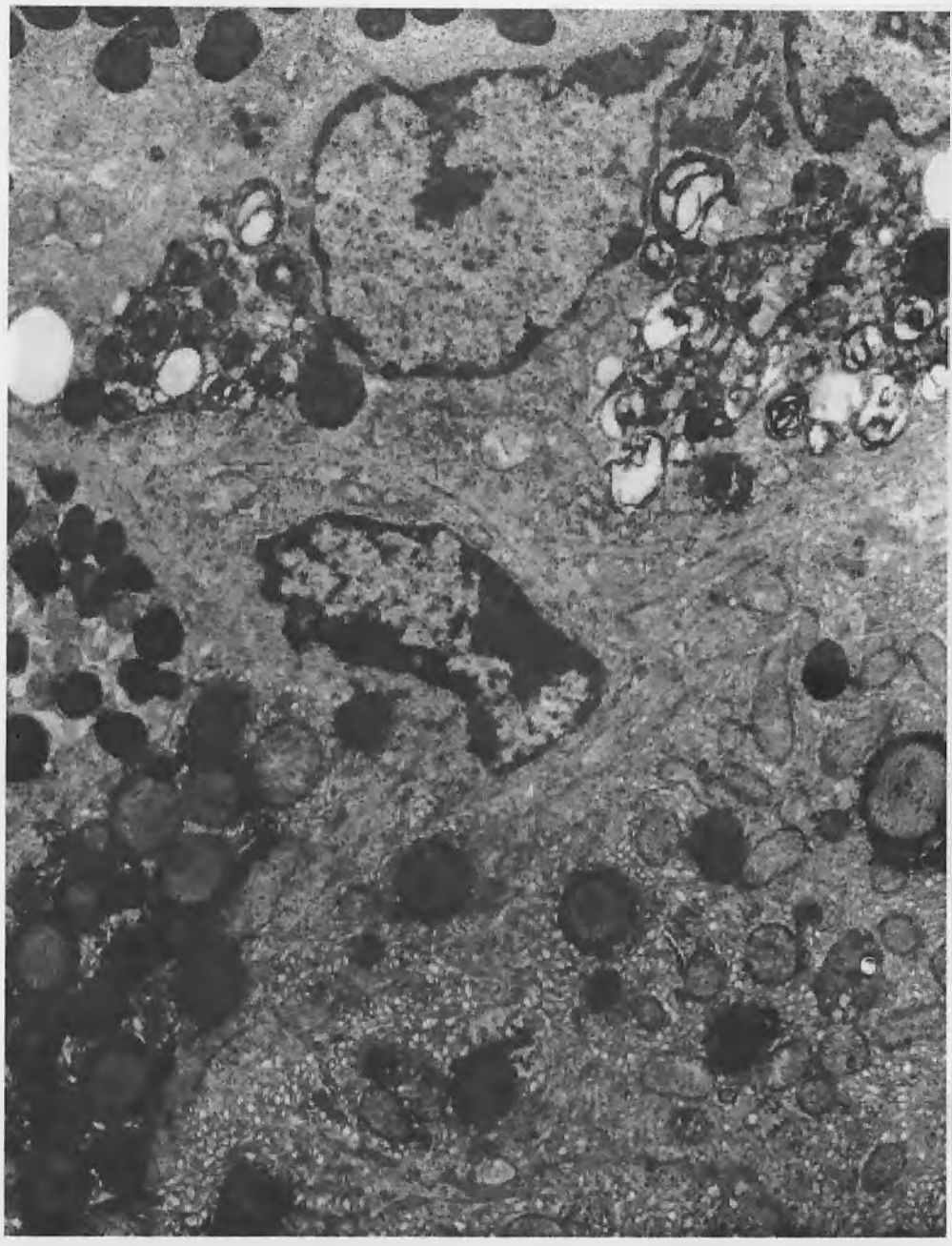

Fig. 7. Macrophages in the cyclic corpus luteum 20 days after ovulation (× 10 000)

cyclicum the heterophagocytosis is prominent and round large cells are seen among the luteal cells. Its nuclei are excentric situated and in the cytoplasms the large vacuoles and abundant large and small lysosomes could be visualized (Fig. 5, 7). K a s u y a and K a w a b u c h i [8] have been reported for the first time macrophages within the growing follicles of the guinea pig. There are different points of view

about the genesis of macrophages in corpus luteum: from theca interna [1], from capillar litoral cells and pericytes [4], by monocyte migration from the blood vessels [3].

Further investigations on the mechanisms involved in the selective increase of eosinophils, neutrophils and lymphocytes during the neoformation of the corpus luteum and of the increase of macrophages during its involution could be directed to the processes of blood cell migration and its physiological role in the corpus luteum functions.

References

1. Adams, E. C., A. T. Hertig. Studies on the human corpus luteum. I. Observations on the ultrastructure of development and regression of the luteal cells during the menstrual cycle. — Cell. Biol., **4**, 1969, 696-715.
2. Aust, G., C. Simchen, U. Heider, F. A. Hmeiden, V. Blumenauer, K. Spanelborowski. Eosinophils in the human corpus luteum: the role of RANTES and eotaxin in eosinophil attraction into preovulatory structures. — Molec.Hum.Reprod., **6**, 2000, No 12, 1085-1091.
3. Bauer, M., I. Reibiger, K. Spanel-Borowski. Leucocyte proliferation in the bovine corpus luteum. — Reproduction, **121**, 2001, 297-305.
4. Bourneva, V. G., L. Gitsov. Electron microscopical studies on corpus luteum during involution. — Folia Anat. Iugosl., **4**, 1975, 77-81.
5. Bourneva, V. G., L. Gitsov. Die Feinstruktur der Kapillaren und des perikapilaren Gewebes im Gelbkoerper von Meerschweinchen. — Verh. Anat. Ges., **70**, 1976, No 8, 165-169.
6. Donetti, E., R. Batta, C. Comparato, C. Altana, S. Sartore, R. Paoletti, P. Castano, G. Gabbiani, A. Corsini. — Polymorphonuclear leucocyte-myocyte interaction. An early event in collar-induced rabbit carotid intimal thickening. — Exper.Cell Res., **274**, 2002, 197-206.
7. Humble, I. G., W. H.W. Jayne, J. V. Pulvertaft. Biological interaction between lymphocytes and other cells. — Br. J. Haematol., **2**, 1956, 283-294.
8. Kasuya, K., M. Kawabuchi. Macrophages are present not only in atretic mature follicles but also in the growing follicles of the guinea pig ovary. — New Trends in Microanat.of Reprod., **6**, 1998, 183-189.
9. Lawler, D. F., J. Hopkins, E. D. Watson. Immune cell populations in the equine corpus luteum throughout the oestrous cycle and early pregnancy: an immunohistochemical and flow cytometric study. — J. Reprod. and Fertil., **117**, 1999, No 2, 281-290.
10. Murdoch, W. J., L. E. Staedman. Investigations concerning the relationship of ovarian eosinophils to ovulation and luteal function in the sheep. — Amer.J.Reprod.Immunol., **25**, 1991, 81-87.
11. Rohm, F., K. Spanelborowski, W. Eichler, G. Aust. Correlation between expression of selectins and migration of eosinophils into the bovine ovary during the preovulatory period. — Cell Tiss. Res., **309**, 2002, 313-322.
12. Vailhe, B., D. Vittet, J. J. Feige. In vitro models of vasculogenesis and angiogenesis. — Lab. Invest., **81**, 2001, No 4, 439-452.
13. Vigano, P., B. Gaffuri, A. M. Di Blasio, M. Vignati. Intercellular adhesion molecule-1 is expressed on human granulosa cells and mediates their binding to lymphoid cells. —J. Clin. Endocrinol. Metab., **82**, 1997, 101-105.

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Morphofunctional Aspects of Rat Leydig Cell Development

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In the present study the rat testes on postnatal days 5, 10, 15, 20, 24 , 27 and of mature Wistar rats, received single i.p. injection of ethane dimethane sulphonate (EDS) were used. The ultrastructural characteristics of the LC, 3β hydroxysteroid dehydrogenase (3βHSD), and glucose-6-phosphate dehydrogenase enzyme activities were studied. The dynamics of appearance and intensity of enzymes investigated corresponded with postnatal structural differentiation of the LC. The restoration of new LC population after EDS administration repeats the process of normal LC development with a similar time range. The results obtained strengthen the view for the differentiation processes of the LC during the postnatal development.

Key words: Leydig cells, ultrastructure, enzymohistochemistry, EDS

Introduction

The postnatal development of the Leydig cells (LC) in mature, differentiated cell forms with low proliferation ability involves the processes of proliferation, differentiation and testosterone production. It has been reported that during rat development two LC populations existed: fetal type LC, producing androgens required for the fetal masculinization and postnatal LC [9]. The fetal LC originate prenatally from mesenchymal-like precursor cells situated in the testicular interstitium, one part of which transform into rounder, testosterone-producing fetal LC, while others remain spindle shaped and differentiate after birth into the mature LC [9]. The fetal type LC rapidly regreses during the first three weeks after birth [13]. It should be noted that the adult LC derive not from the fetal type, but from the undifferentiated mesenchymal-like precursor cells or stem cells. The embryonic origin of Leydig stem cells is still disputable. There are data in this direction providing evidence that the mesonephric mesenchymal cells in the interstitium are a potential source of LC stem cells [3]. During the ontogenesis the interstitial mesenchymal cells form mixed population of stem cells from which derived also peritubular myofibroblasts, testicular macrophages and interstitial fibroblasts [2]. The difficulty to identify the Leydig stem cells is caused by lack of specific markers.

Based on data reported by H a r d y et al. [7], by 14 postnatal day Leydig stem cells proliferate and transform into progenitor cells, which are an intermediate stage in the LC development and could be found in rat testis between days 14 and 28 after birth. The LC progenitors differentiate morphologically into immature LC between days 28 and 56 postpartum which divide once and then transform into adult type LC.

Ethane-1, 2-dimethanesulphonate (EDS) is a unique toxin with cytotoxic action confined almost exclusively to adult, but not immature, rat LC [16, 12]. EDS selectively decreases or eliminates both basal and LH-stimulated testosterone production *in vivo* and *in vitro*, thus providing a model in which there is a complete loss of LC within the testis [12, 14]. It has been shown that a single dose of EDS injected into adult rats caused a temporary impairment of fertility, reduced levels or loss of serum and intratesticular testosterone and elevated pituitary secretion of LH and FSH as a result of the destruction of existing LC [11, 20, 16]. During the first two days after EDS application, the LC population in the rat may be eliminated by macrophages [5] and subsequently two to three weeks new LC appeared and regenerated completely eight to ten weeks after treatment, apparently from proliferating interstitial mesenchymal-like precursor cells [11, 18].

The purpose of our study was to establish the morphological and enzymehistochemical features of rat LC during the postnatal development and of newly formed LC population after treatment with EDS.

Material and Methods

Testes of Wistar rats (n=16) on days 5, 10, 15, 20, 24 and 27 after birth and of adult rats (n=7), received single i.p. injection of EDS (75mg/kg body weight) were used as material. The experimental animals were killed 14, 21 and 30 days after initial EDS administration.

Electron microscopy — testicular fragments were fixed in phosphate-buffered 2.5% glutaraldehyde and postfixed in 1% osmium tetraoxide. Tissue was dehydrated in ethanol and embedded in Durcupan (Fluka). The ultrathin sections were contrasted with lead citrate and uranyl acetate.

Electronograms were made on a Philips-CM 12.

Enzymohistochemistry — on fresh cryostat sections (5 μm thick) the enzyme activities of 3β hydroxysteroid dehydrogenase with substrate dehydroepiandrosterone [15] and glucose-6-phosphate dehydrogenase with substrate glucose-6-phosphat [8] were studied. *Controls.* Sections were incubated in medium without the specific substrate for the corresponding enzyme.

Results

The electron microscopic observation showed that in rat testicular interstitium on the 5th and 10th postnatal day abundant fetal type Leydig cells with large size and high density of the cytoplasm could be seen. The fetal LCs were recognizable by their spherical nucleus with dense chromatin, lipid droplets, well-developed smooth endoplasmic reticulum (SER) and mitochondria with mainly tubular cristae. Strong 3βHSD and glucose-6-phosphate dehydrogenase activities were found in the LC at these stages of postnatal development. Between 15 and 24 postnatal days the number of fetal type LC progressively decreased and structural features of their

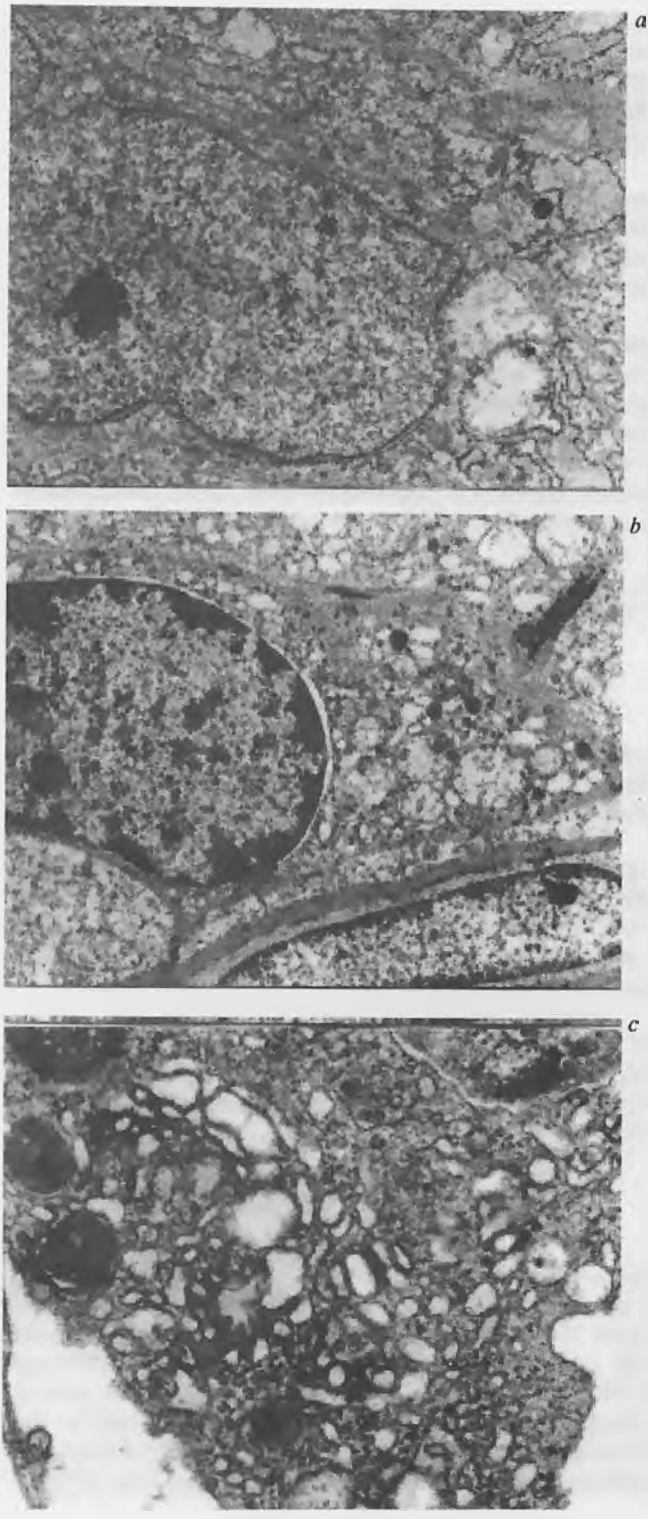

Fig.1. Electron micrographs
a — ultasructure of progenitor type LC (postnatal day 15) without lipids and well- developed SER (× 9600)
b — ultrastructure of immature LC (postnatal day 27). Numerous lipid droplets, steroidogenic type mitochondria and well-developed SER are evident in the cytoplasm (× 12 400)
c — ultrastructure of adult type LC (30 day after EDS). An abundance of smooth endoplasmic reticulum, Goldgi complex and tubular mitochondria was observed (× 19 000)

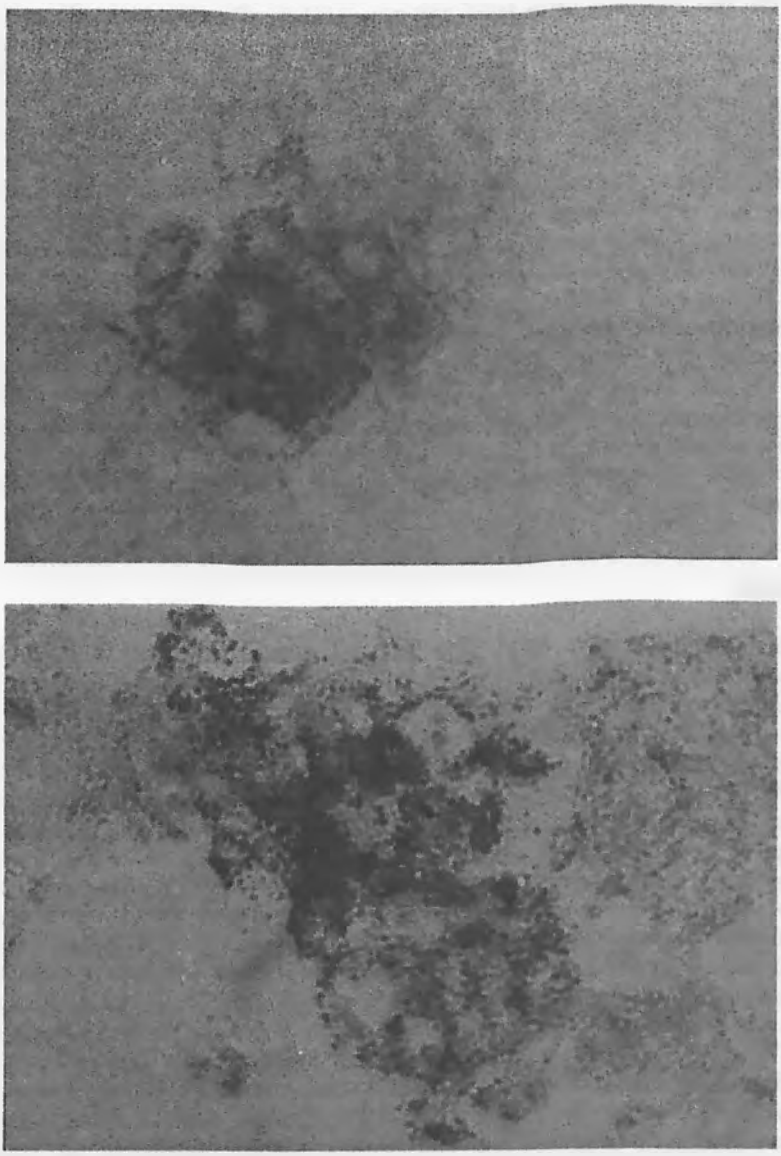

Fig. 2. Histochemical visualization of 3βHSD enzyme activity
a — in the immature Leydig cells (postnatal day 20) (× 400)
b — in the newly formed LCs 21 days after EDS treatment (× 400)

involution could be observed — volume reduction, nuclear condensation and vesicular form of smooth endoplasmic reticulum. In the intertubular space a few elongated or spindle shaped LC occurred whose ultrasructure corresponded to that of progenitor type LC — small cytoplasmic volume, no lipids and absence of well-developed SER (Fig. 1 — *a*). In comparison with the fetal type LC, 3βHSD and glucose-6-phosphate dehydrogenase enzyme activities in the LC progenitors were

reduced. On the 20th day after birth the appearance of interstitial cells with similar electron microscopic pattern to those of immature type LC were observed. In contrast to LC progenitors, immature LC were larger and rounder, with lipid droplets. Their cytoplasm was tightly packed with tubular cisterns of SER, pleomophic steroidogenic type mitochondria and rough endoplasmic reticulum (RER), (Fig. 1 — *b*). They were intensively stained for 3βHSD (Fig. 2 — *a*) and glucose-6-phosphate dehydrogenase activities (Fig. 3 — *a*). Two weeks after EDS administration the electron microscopical observation revealed spindle shaped progenitor type LC with small cytoplasm and well developed RER. The progenitors showed 3βHSD and glucose-6-phosphate dehydrogenase activities with low staining intensity. Between 21 and 30 days after treatment with EDS electron microscopic analysis demonstrated that immature type LC predominate in the interstistial space. A strong activity for 3βHSD (Fig. 2 — *b*) and glucose-6-phosphate dehydrogenase (Fig. 3 — *b*) was found in the cytoplasm of immature LCs. On day 30 after EDS adult type LCs can be observed between the seminiferous tubules. They were recognized by their abundant SER, steroidogenic type mitochondria, decline in lipid droplets (Fig. 1 — *c*). and intensively staining for 3βHSD and glucose-6-phosphate dehydrogenase activities.

Discussion

It has been reported that fetal type LC secreting androgens during the fetal period, progressively reduce in number and involute postnatally [9]. Our findings confirmed this fact, as well as other publication, in which the fetal LC were described as cells with numerous lipid droplets, well-developed SER and strong 3βHSD enzyme activity [4, 6, 13]. We found involution markers in the cytoplasm of some of the fetal LC such as vesiculation of SER and membrane whorls. Our results showed that between 15 and 24 postnatal days, besides the reduced number of fetal LC, elongated or spindle shaped progenitors were observed, which corresponds to previously data for the presence of LC progenitors in rat testis between days 14 and 28 postpartum [7]. In the present study moderate to low 3βHSD and glucose-6-phosphatdehydrgenase enzyme activities were detected in the LC during this period. Because of using of NAD as a cofactor, the activity of 3βHSD is dependent of glucose-6-phosphate dehydrogenase. In this regard, the developmental changes in glucose-6-phosphate dehydrogenase enzyme activity are similar to 3βHSD intensity pattern. The mechanism by which LC progenitors produce androgen when they lack well developed SER and have weak 3βHSD enzyme activity is still obscure. An explanation in this direction is that the LC progenitors maybe have mitochondrial form of steroidogenic enzymes [1]. The present results confirmed the established transformation of progenitors into immature type LC with abundant SER and lipid droplets and increase in steroidogenic enzyme activities in them between postnatal days 28 and 56 [4, 6, 17]. Following EDS administration, we found the first 3βHSD enzyme activity on the day 14 after treatment and strong increasing labeling intensity for this enzyme in the newly formed LC on days 21 and 30. Our data confirm the results by T e e r d s et al. [19] indicating 3βHSD enzyme activity as a marker for LC differentiation after EDS treatment and provide additional evidence for the similarity between steroidogenic enzyme pattern in rat LC during the postnatal development and after EDS. Therefore, the increase in steroidogenic enzyme activity occurs in tandem with the structural features of steroidogenic differentiation.

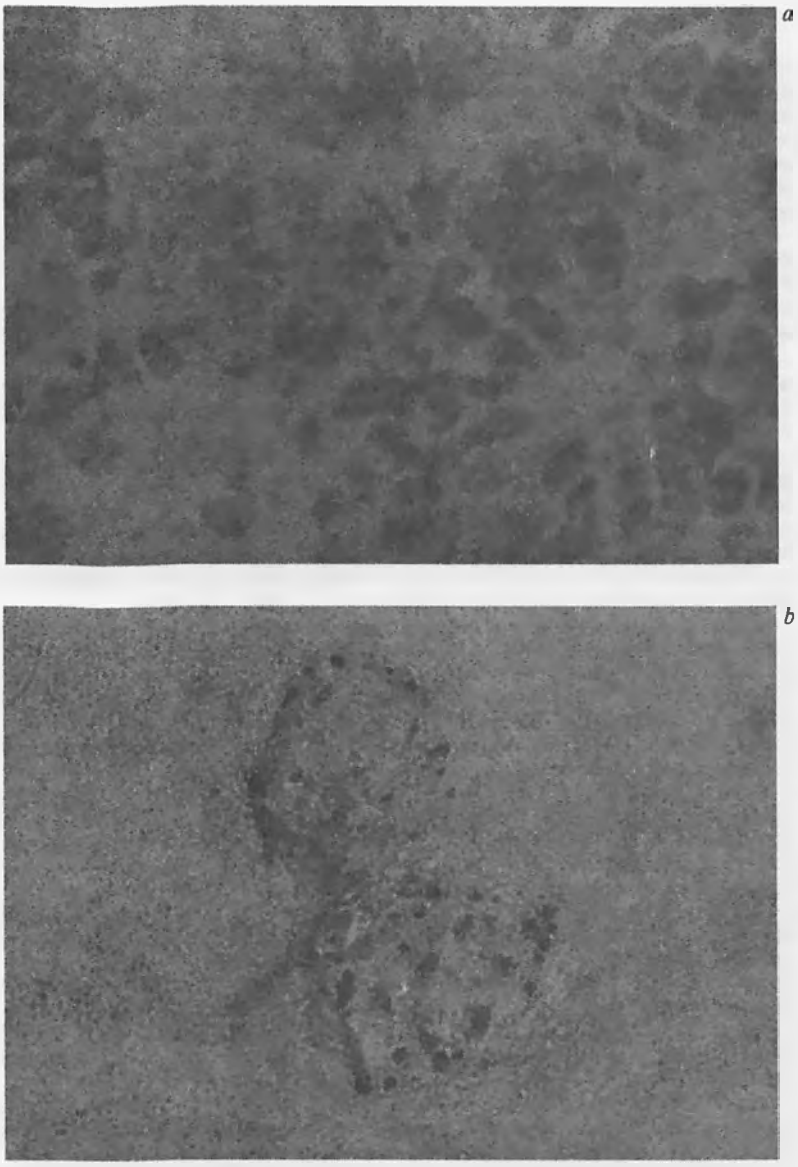

Fig. 3. Histochemical visualization of glucose-6-phosphate dehydrgenase enzyme activity
a — in the clusters of interstitial LCs on day 24 postpartum (× 400)
b — in the newly formed LCs 21 days after EDS treatment (× 400)

In conclusion, as far as the regeneration of LC repopulation in adult testis after EDS treatment repeats the prepubertal LC development, the results obtained strengthen the view for the differentiation processes of LC in rat testis.

References

1. Cherradi, N., G. Defaye, E. M. Chambaz. Dual subcellular localization of the 3-beta-hydroxysteroid dehydrogenase isomerase: Characterization of the mitochondrial enzyme in the bovine adrenal cortex. — J. Steroid Biochem. Mol. Biol., **46**, 1993, 773-779.
2. Dechelotte, P., J. Chassagne, A. Labbe, M. Afane, T. Scheye, B. de Laguillaumie, D. Boucher. Ultrastructure and immunohistochemical evidence of in situ differentiation of mononuclear phagocyte system cells in the interstitium of the human fetal testis. — Early Hum., **20**, 1989, 25-26.
3. de Kretser, D. M., J. B. Kerr. The cytology of the testis. -In: The Physiology of Reproduction (Ed. E. Knobil, J. D. Neill, G. S. Greenwald, C. L. Markert, D. W. Pfaff). New York, Raven Press, 1994, 1177-1290.
4. Dupont, E., F. Labrie, V. Luu-The, G. Pelletier. Ontogeny of 3β-hydroxysteroid dehydrogenase/ Δ^5- Δ^4 isomerase (3β- HDS) in rat testis as studied by immunocytochemistry. — Anat. Embryol, **187**, 1993, 583-589.
5. Gaytan, F, J. L. Romero, C. Morales, C. Reymundo, C. Bellido, E. Aguilar. Response of testicular macrophages to EDS-induced Leydig cell death. — Andrologia **27**, 1995, 259-265.
6. Haider, S. G., D. Passia, G. Overmeyer. Studies on the fetal and postnatal development of rat Leydig cells employing 3β- hydroxy steroid dehydrogenase activity. — Acta Histochem., **32**, 1986, 197-202.
7. Hardy, M. P., W. R. Kelce, G. R Klinefelter, L. L. Ewing. Differentiation of Leydig cell precursors in vitro: A role for androgen. — Endocrinology, **127**, 1990, 488-490.
8. Hess, R., D. G. Scarpelli, A. G. E. Pearse. — Nature, **181**, 1958, 1531-1532 (In: Гистохимия, Э.Пирс, Москва, Изд. Иностранной Литературы, 1962, 513—515).
9. Huhtaniemi, I., L. J. Pelliniemi. Fetal Leydig cells: Cellular origin, morphology, life span, and special functional features. — Proc. Soc. Exp. Biol. Med., **201**, 1992, 125-140.
10. Jackson, N. C., H. Jackson, J. Shanks, J. S. Dixon. Study using in vivo binding of 125 I-labeled hCG, light and electron microscopy of the repopulation of rat Leydig cell after destruction due to administration of EDS. — J. Reprod. Fertil., **76**, 1986, 1-10.
11. Jackson, C. M., I. D. Morris. Gonadotrophin levels in male following impairment of Leydig cell function by ethane dimethanesulphonate. — Andrologia, **9**, 1997, 29-35.
12. Kerr, J. B., K. Donachie, F. F. G. Rommerts. Selective destruction and regeneration of rat Leydig cells in vivo. A new method for the study of seminiferous tubular-interstitial tissue interaction. — Cell Tissue Res., **242**, 1985, 145-156.
13. Kuopio, T., J. Tapanainen, L. J. Pelliniemi, I. Huhtaniemi. Developmental stages of fetal-type Leydig cells in prepubertal rats. — Development, **107**, 1989 (a), 213-220.
14. Laskey, J. W., G. R. Kleinefelter, W. R. Kelce, L. L. Ewing. Effects of ethane dimethanesulfonate (EDS) on adult and immature rabbit Leydig cells: comparison with EDS-treated rat Leydig cells. — Biol. Reprod., **50**, 1994, 1151-1160.
15. Levy, H., H. W. Deane, B. L. Rubin. Visualization of steroid 3β ol dehydrogenase activity in tissues of intact and hypophysectomized rats. — Endocrinology, **65**, 1959, 932-943.
16. Molenaar, R., D. G. Rooij, F. F. G. Rommerts, P. J. Reuvers, H. J. van der Molen. Specific destruction of Leydig cell in mature rats after *in vivo* administration of ethane dimethyl sulphonate. — Biol. Reprod., **33**, 1985, 1213-1222.
17. Shan, L. X., L. J. Zhu, C. W. Bardin, M. P. Hardy. Differential regulation of steroidogenic enzymes during differentiation optimizes testosterone production by adult rat Leydig cells. — Endocrinology, **133**, 1993, 2277-2283.
18. Teerds, K. J. Regeneration of Leydig cells after depletion by EDS: a model for postnatal Leydig cell renewal. — In: The Leydig Cell (Ed. A. H. Payne, M. P. Hardy, L. D. Russell). Vienna Cache River Press, IL, 1996, 203-219.
19. Teerds, K., M. de Boer-Brouwer, J. Dorrington, M. Balvers, R. Ivell. Identification of markers for precursor and Leydig cell differentiation in the adult rats testis following ethane dimethanesulfonate administarion. — Biol. Reprod., **60**, 1999, 1437-1445.
20. Tena-Sempiere, M., I. Pinilla, E. Aguilar. Follicle-stimulating hormone and luteinizing hormone secretion in male rats orchidectomized or injected with ethylene dimethane sulphonate. — Endocrinology, **133**, 1993, 1173-1181.

Institute of Experimental Morphology and Anthropology

Acta morphologica et anthropologica, 8
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Direct and Indirect (Gonagotropin-mediated) Effects of Estrogens on Developing Rat Testis

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This study sought to establish the relative importance of direct and gonadotropin-mediated effects on the testis at age 18 days after neonatal treatment with a high (10 μg) or a low (0.1 μg) dose of diethylstilbestrol (DES). Rats were treated on alternative days from day 2 — 12 with DES or GnRH antagonist (GnRHa), alone or combined. Treatment with 10 μg DES or GnRHa, alone or combined, had similar negative effects on Sertoli cell nuclear volume/number per testis, germ cell apoptotic index, Leydig cell volume per testis and spermatocyte volume per unit Sertoli cell. Injection of DES 10 μg ± GnRHa, but not GnRHa or 0.1μg DES alone, induced major overgrowth/distension of rete testis. However, co-administration of 0.1μg DES + GnRHa caused a similar enlargement of the rete. Treatment with 0.1μg DES alone failed to produce adverse changes in spermatogenesis. However, when rats were treated with 0.1μg DES + GnRHA, the germ cell apoptotic index, spermatocyte volume/Sertoli cell and Leydig cell volume were all impaired to a lesser extent than in GnRHa treated rats. It is concluded that gonadotropin suppression is a likely explanation for the negative effects of neonatal treatment with 10 μg DES on Sertoli, Leydig and germ cells, but this plays no role in induction of rete testis overgrowth, which probably results form altered androgen:estrogen balance. A significant "stimulatory" effect of 0.1 μg DES, compared to GnRHa alone, on germ cell survival and Leydig cell volume, despite the co-administration of GnRHa, is suggestive of direct (estrogen receptor-mediated) effects of DES on one or more testis cell types.

Key words: estrogens, spermatogenesis, apoptosis, Leydig cells and testis.

Introduction

Numerous studies in rodents have demonstrated that neonatal exposure of the male to high doses of potent estrogens, such as DES, results in major adverse changes in the testis and reproductive tract [10, 3]. In earlier studies, the negative effects of estrogens were explained as a result of suppression of gonadotropin secretion during the treatment [5]. More recent studies of our own, involving comparison of the effects of neonatal treatment with DES or GnRHa, have raised the possibility of direct effects of the DES on testicular cells [13, 1]. This possibility has been reinforced by the demonstration that estrogen receptor-β (ERβ) is expressed in Sertoli and Leydig cells as well as in most germ cells [12] and Leydig cells also expressed

ERα [7]. However, because the Sertoli and Leydig cells are also target cells for gonadotropins, it is difficult to distinguish unequivocally between direct (ER-mediated) and indirect (gonadotropin-mediated) effects of DES on the testis.

Recently it was demonstrated a stimulatory effect of very low estrogen levels on spermatogenesis in adult hypogonadal mouse [6] and in the immature at [2] associated with elevated plasma levels of FSH. Therefore, for the effects of both low and high doses of estrogens on the rodent testis, it remains uncertain whether the major changes observed reflect modulation of gonadotropin secretion or direct activation of ERs in testis cell types or a combination of both effects. The present study was designed to address this issue by comparing the effects on the developing rat testis of treatment with a high or a low dose of DES alone or in combination with a GnRHA antagonist. We have evaluated endpoints that we have previously shown to reflect most clearly inappropriate estrogen exposure (germ cell volume per Sertoli cell, germ cell apoptotic index, Sertoli and Leydig cell volume/number per testis, rete testis size).

Materials and Methods

Beginning on postnatal day 2, rats were subjected to one of the following treatments administered by s.c. injection: a) DES at a dose of 10 or 0.1μg in 20 μl corn oil on days 2,4,6,8,10 and 12; b) 10 mg/kg of long acting GnRHa (Antarelix) in 20 μl 5% mannitol on days 2 and 6; c) 10 μg DES as in (a) + GnRHa as in (b); d) 0.1μg DES + GnRHa; e) 20 μl corn oil (vehicle) as control. Rats from all treatment groups were subsequently sampled on day 18. Paraffin Bouin's fixed 5-μm testicular sections were used for cell quantification studies and visualization of apoptotic germ cells identified by TUNEL method as described previously [13]. Different testicular cell types were counted using 121-point eyepiece graticule and the data were used to determine the following: a) nuclear volume of viable (non-apoptotic) spermatocytes per Sertoli cell nuclear volume as an index of spermatogenic efficiency; b) the germ cell apoptotic index as a ratio of the nuclear volume of apoptotic/normal germ cells per testis; c) seminiferous tubule lumen % volume as an indicator of the onset of spermatogenesis at puberty. Optical dissector method was applied for quantification of Sertoli cell number per testis [1] and to confirm data for Sertoli cell nuclear volume obtained by point counting. Leydig cell volume per testis was determined on sections immunostained for 3β-hydroxysteroid dexydrogenase (3β-HSD) [15]. The 3β-HSD positive nuclei and cytoplasm were scored separately by point counting and the data for absolute volumes (nuclear and cytoplasm) were collected. Comparison of the different parameters for the various treatment groups was made using ANOVA.

Results

Testicular weight and gross morphology, seminiferous tubule lumen formation
Testicular weight on day 18 was reduced to 70% by treatment with GnRHa, DES 10μg or DES 10μg + GnRHa, as compared to the control. Treatment with 0.1μg DES induced a small (18%) but not significant reduction in that parameter whereas combined treatment with DES 0.1μg + GnRHa caused a comparable reduction to that induced by treatment with GnRHa (Fig.1). Injection of DES 10μg ± GnRHa, but not GnRHa or 0.1μg DES alone induced major overgrowth/distension

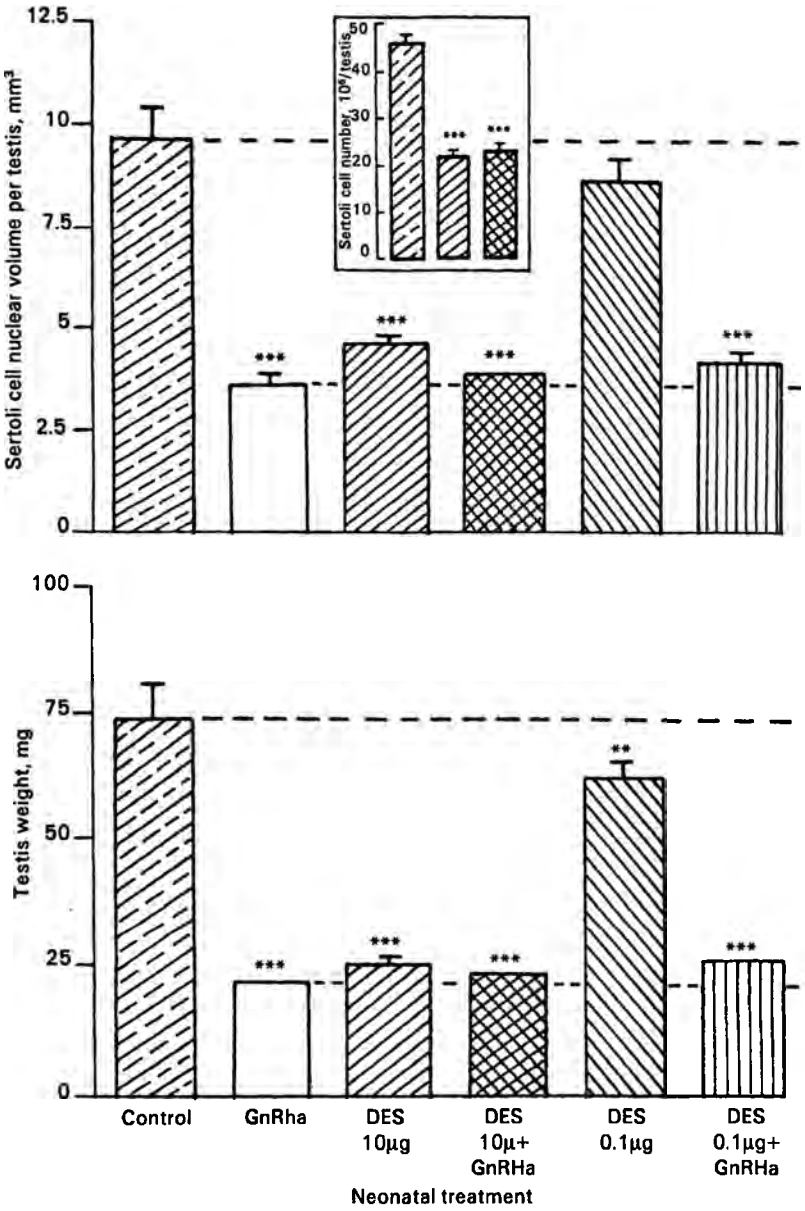

Fig. 1. Effect of neonatal treatment with DES (10 or 0.1 μg) or GnRHa, alone or in combination, on Sertoli cell nuclear volume per testis (top) and testis weight (bottom) on day 18th. Insert in the top panel shows the effect of treatment with DES 10μg alone or DES 10 μg + GnRHa on Sertoli cell number determined by optical disector method. Dashed lines show mean values for control and GnRHa-treated groups to aid comparison. Data represent the mean ± SEM (n=5 per group; ** p<0.01, *** p<0.001, in comparison with control)

of rete testis (data not shown). However co-administration of 0.1μg DES + GnRHa caused a similar enlargement of the rete. The formation of seminiferous tubule (% lumen volume per testis) was 7.0±0.4% in control and was reduced by 66, 80, 84, 6 and 61% respectively in groups treated with GnRHa, DES 10μg, DES 10μg + GnRHa, DES 0.1μg or DES 0.1μg + GnRHa. With an exception of the DES 0.1μg treatment group, all of these values were significantly reduced ($p<0.01$) compared with controls.

Changes in Sertoli and germ cell nuclear volume per testis

Based on point counting, Sertoli cell nuclear volume per testis showed changes after treatment that paralleled the reduction in testicular weight (Fig. 1). With an exception of the DES 0.1μg, all treatments resulted in a marked decrease in Sertoli cell nuclear volume. For three of the groups Sertoli cell number was determined by optical dissector method and the data confirmed those obtained for Sertoli cell nuclear volume using point counting (see insertion of the chart). Treatment with either GnRHa or DES 10 μg alone both significantly reduced spermatocyte nuclear volume per unit Sertoli cell volume, though the degree of suppression was slightly greater ($p<0.05$) with DES 10μg than with GnRHa (Fig. 2). Combined treatment with DES 10μg + GnRHa reduced germ cell volume to a comparable extent as did treatment with DES 10 μg alone. Treatment with DES 0.1μg alone had no effect on spermatocyte volume per unit Sertoli cell volume but combined treatment with DES 0.1 μg + GnRHa significantly suppressed this cellular ratio and the magnitude of this suppression was less than that observed in animals treated with GnRHa.

The germ cell apoptotic index was expressed as a ration of apoptotic/normal germ cells and showed the mirror image of changes to spermatocyte volume per Sertoli cell. Thus treatment with GnRHa or DES 10 μg alone, or these two treatments combined, increased the germ cell apoptotic index by comparable amounts (6-fold increase, Fig 2). In contrast, treatment with DES 0.1μg alone had no significant effect on the germ cell apoptosis. Combined treatment with DES 0.1 μg + GnRHa significantly increased ($p<0.01$) the germ cell apoptotic index when compared with control or DES 0.1μg alone. However, this increase was significantly less ($p<0.05$) than that induced by treatment with GnRHa alone.

Changes in Leydig cell volume per testis

Leydig cell volume per testis was reduced by 98% in rats treated with DES 10 μg or GnRHa alone or in combination (Fig. 3). Treatment with DES 0.1 μg alone reduced Leydig cell volume, compared with control, but this was not statistically significant. Combined treatment with DES 0.1μg + GnRHa reduced Leydig cell volume by 93%, but this reduction was significantly less ($p< 0.01$) than in rats treated with GnRHa alone.

Discussion

To assess the relative importance of direct and indirect effects of estrogens on the testis, we chose two doses of DES (10 μg and 0.1 μg) that our previous studies have shown to exert radically different effects on spermatogenesis and on FSH secretion [2]. Thus, the high dose (10 μg) of DES induced several major adverse effects on the testis and suppressed FSH secretion whereas the lower dose (0.1 μg) of DES

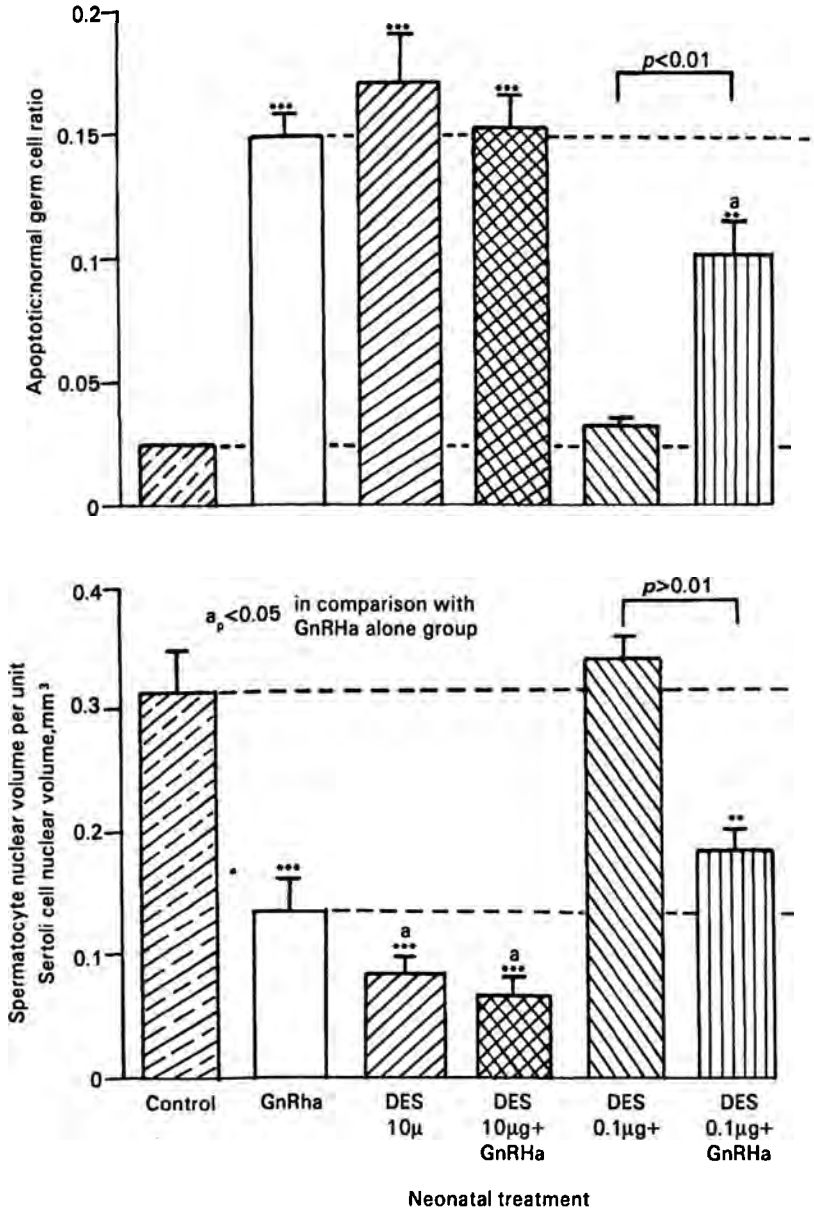

Fig. 2. Effect of neonatal treatment with DES (10 or 0.1 μg) or GnRHa, alone or in combination, on the germ cell apoptotic index expressed as a ratio of apoptotic/normal germ cells (top) and spermatocyte nuclear volume per unit Sertoli cell nuclear volume (bottom) on day 18th. Dashed lines show mean values for control and GnRHa-treated groups to aid comparison. Data represent the mean ± SEM (n=5 per group; ** p<0.01, *** p<0.001, in comparison with control)

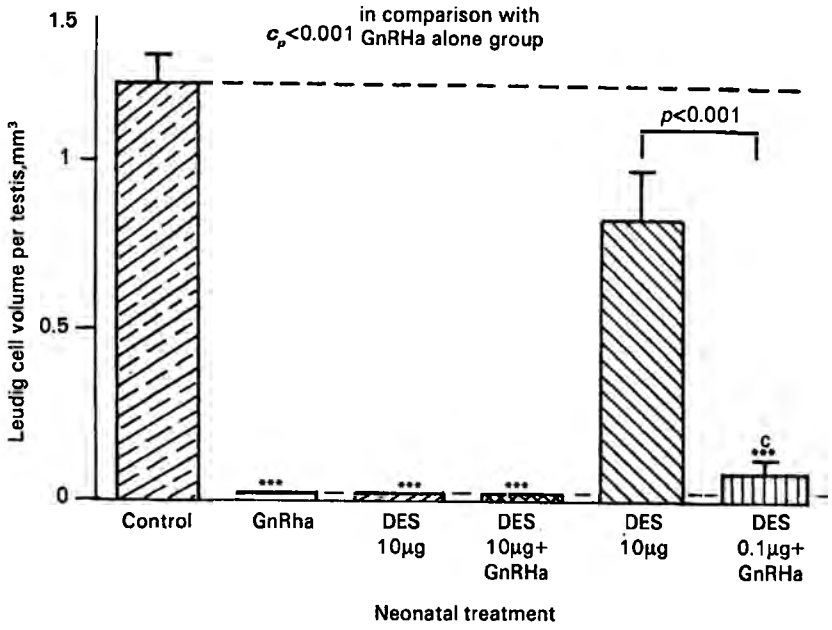

Fig. 3. Effect of neonatal treatment with DES (10 or 0.1 μg) or GnRHa, alone or in combination, on Leydig (3β-HSD positive) cell volume per testis on day 18th. Dashed lines show mean values for control and GnRHa-treated groups to aid comparison. Data represent the mean ± SEM (n=4 per group; *** p<0.001, in comparison with control)

failed to produce adverse effect on spermatogenic process. It was reasoned that demonstration of a testicular change in the DES + GnRHa-treated groups, but the absence of a comparable change in animals treated with GnRHa alone, would provide persuasive evidence for a direct effect of DES on the testis. Induction of rete testis overgrowth/distension is a change that fall into this category. We had earlier concluded that gross distortion of the normal androgen:estrogen balance (suppressed androgen production + supranormal estrogen action) underlay the gross morphological changes in the testis and reproductive tract of rats treated neonatally with high dose (eg 10 μg) of DES [3, 10] This interpretation is reinforced by some of the present findings. Thus, treatment with DES 0.1 μg alone will have raised estrogen levels but has no effect on testosterone levels [see 16] and did not affect the rete testis. Treatment with GnRHa alone will not have raised estrogen levels but does significantly reduce testosterone levels and also had no effect on the rete. However, when these two treatments were combined, presumably leading to raised estrogen and lowered testosterone levels concomitantly, it resulted in a marked distension of rete testis, comparable to that caused by high dose (10 μg) of DES. Similarly, the attenuation of germ cell apoptotic index in rats treated with DES 0.1μg + GnRHa when compared with that treated with GnRHa alone, is interpreted as possible evidence for a direct (stimulatory) effect of DES. The significantly lower suppression of Leydig cell volume in rats treated with DES 0.1 μg + GnRHa, compared with rats treated with GnRHa alone, may also fall into this category.

On the other hand, where similar testicular changes were induced by treatment with DES 10μg or GnRHa alone, and combined treatment had no additive effect,

we can conclude that gonadotropin suppression is a likely explanation for the observed adverse effects. Suppression of Sertoli cell nuclear volume/number and testis weight, increase in the germ cell apoptotic index and decrease in spermatocyte volume per unit Sertoli cell are endpoints which changes support this suggestion. We cannot exclude other possibilities, for example that treatment with DES 10 μg reduced Sertoli cell proliferation by a direct effect on Sertoli cells mediated via ERβ which then resulted in inhibition of the FSH-stimulated intracellular signaling pathway that normally up-regulated Sertoli cell proliferation. It is established that one of the major driving forces for Sertoli cell proliferation in the neonatal period of the rat is FSH [11, 14] and the present demonstration of an approximate halving of Sertoli cell number/volume after neonatal GnRHa treatments (alone or combined) reinforces this conclusion.

There is also a further, untested possibility, namely that the low dose of DES elevates prolactin secretion [5] which then positively acts on the function of Sertoli cell [8], germ [9] or Leydig [4, 9] cells. These various possibilities are difficult to dissect apart because of the complexity both of the endocrine system involved and of the testicular cell-cell interactions. It seems apparent from the points discussed above that future studies of direct, positive effect of estrogens on testicular germ cell development should use animals of a younger age or in vitro approaches such as germ cell-Sertoli cell co-cultures or short-term cultures of isolated seminiferous tubules from prepubertal rats.

In conclusion, the present study provides strong evidence in support of a role for gonadotropin suppression in many, but not all, of the adverse testicular changes induced by neonatal treatment with high dose of DES. We have not excluded the possibility that estrogen effects within the testis may negatively impact on the same signalling pathways as those activated by gonadotropins. The present findings also demonstrate that DES-induced changes in the testis results also from a disturbance of the normal androgen:estrogen balance and therefore a close interrelationship between actions of androgens and estrogens is involved in regulating normal development of the testis.

References

1. Atanassova, N., C. McKinnell, M. Walker, K. J. Turner, J. S. Fisher, M. Morley, M. R. Millar, N. P. Groome, R. M. Sharpe. Permanent effect of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number and the efficiency of spermatogenesis in adulthood. — Endocrinology, **140**, 1999, 5364-5373.
2. Atanassova, N., C. McKinnell, K. J. Turner, M. Walker, J. S. Fisher, M. Morley, M. R. Millar, N. P. Groome, R. M. Sharpe. Comparative effects of neonatal exposure of male rats to potent and weak (environmental) estrogens on spermatogenesis at puberty and relationship to adult testis size and fertility: Evidence for stimulatory effects of low estrogen levels. — Endocrinology, **141**, 2000, 3898-3907.
3. Atanassova, N., C. McKinnell, K. Williams, K. J. Turner, J. S. Fisher, P. T. K. Saunders, R. M. Sharpe. Age-, cell-, and region-specific immunoexpression of ERα (but not ERβ) during postnatal development of the epididymis and vas deferens of the rat and disruption of this pattern by neonatal treatment with diethylstilbestrol. — Endocrinology, **142**, 2001, 874-886.
4. Barkey, R. J., E. Weiss-Messer, H. Hacahm, S. Herscovitch, R. Ber, T. Amit. Prolactin and testicular Leydig cell function: characterization of prolactin receptors in the murine MA-10 testicular Leydig cell line. — Proceedings of the Society for Experimental Biology and Medicine, **206**, 1994, 243-248.
5. Bellido, C., L. Pinilla, R. Aguilar, F. Gaytan, E. Aguilar. Possible role of changes in postnatal gonadotropin concentrations in permanent impairment of the reproductive system in neonatally oestrogenized male rats. — J. Reprod. Fertil., **90**, 1990, 369-374.

6. Ebling, F. J. P., N. Brooks, A. S. Cronin, H. Ford, J. B. Kerr. Estrogenic induction of spermatogenesis in the hypogonadal mouse. — Endocrinology, **141**, 2000, 2861-2869.
7. Fisher, J. S., M. R. Millar, G. Majdic, P. T. K. Saunders, H. M. Fraser, R. M. Sharpe. Immunolocalisation of estrogen receptor-α (ERα) within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. — J. Endocrinol., **153**, 1997, 485-495.
8. Guillaumot, P., M. Benahmed. Prolactin receptors are expressed and hormonally regulated in rat Sertoli cells. — Mol. Cell. Endocrinol., **149**, 1999, 163-168.
9. Hondo, E., M. Kurohmaru, S. Sakai, K. Okawa, Y. Hyashi. Prolactin receptor expression in rat spermatogenic cells. — Biol. Reprod., **52**, 1995, 1284-1290.
10. McKinnell, C., N. Atanassova, K. Williams, J. S. Fisher, M. Walker, K. J. Turner, P. T. K. Saunders, R. M. Sharpe. Suppression of androgen action in the induction of gross abnormalities of the reproductive tract in male rats treated neonatally with diethylstilbestrol. — J. Androl., **22**, 2001, 323-338.
11. Meachem, S. J., R. I. McLachlan, D. M. deKretser, D. M. Robertson, N. G. Wreford. Neonatal exposure of rats to recombinant follicle stimulating hormone increases adult Sertoli and spermatogenic cell numbers. — Biol.Reprod., **54**, 1996, 36-44.
12. Saunders, P. T. K., J. S. Fisher, R. M. Sharpe, M. R. Millar. Expression of estrogen receptor beta (ERβ) occurs in multiple cell types, including some germ cells, in the rat testis. — J. Endocrinol., **156**, 1998, R13-R17.
13. Sharpe, R. M., N. Atanassova, C. McKinnell, P. Parte, K. J. Turner, J. S. Fisher, J. B. Kerr, N. P. Groome, S. Mcpherson, M. R. Millar, P. T. K. Saunders. Abnormalities in functional development of the Sertoli cells with diethylstilbestrol: a role of estrogens in Sertoli cell development? — Biol. Reprod., **59**, 1998, 1084-1094.
14. Sharpe, R. M., K. J. Turner, C. McKinnell, N. P. Groome, N. Atanassova, M. R. Millar, D. L. Buchanan, P. S. Cooke. Inhibin-B levels in plasma of the male rat from birth to adulthood: effect of experimental manipulation of Sertoli cell number. — J. Androl., **20**, 1999, 94-101.
15. Sharpe, R. M., M. Walker, M. R. Millar, N. Atanassova, K. Morris, C. McKinnel, P. T. K. Saunders, H. M. Fraser. Effect of neonatal gonadotropin-releasing hormone antagonist administration on Sertoli cell number and testicular development in the marmoset: Comparison with the rat. — Biol.Reprod., **62**, 2000, 1685-1693.
16. Williams, K., C. McKinnell, M. Walker, J. S. Fisher, K. J. Turner, N. Atanassova, R. M. Sharpe. Perinatal oestrogen exposure and abnormalities of the male reproductive system: a role for the androgen: oestrogen balance. — Human Reprod. Update, **7**, 2001, 236-247.

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The Role of α-Smooth Muscle Actin (α-SMA) and Desmin in Human Testicular Peritubular Matrix

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The peritubular matrix has a great significance in endothelial organization during the process of testicular angiogenesis. As a response endotelial cells migrate into interstitium and secrete new matrix components for basal membrane of the seminiferous tubules. The aim of the study was to examine the changes in peritubular matrix under pathological conditions and to investigate localization and distribution of α-SMA and desmin, both are cytoskeletal proteins characteristic for smooth muscle cells. Materials were obtained from testicular biopsies of 9 infertile men and immunohistochemical techniques were applied using monoclonal antibodies against α-SMA and desmin. The results showed positive immunostaining for α-SMA and desmin in peritubular myoid cells and around small blood vessels in interstitium. Our data brings additional support to the view that testicular peritubular matrix proteins play key role in endothelial organization especially in process of angiogenesis and testicular microvasculature is important for normal spermatogenesis.

Key words: peritubular matrix, immunohistochemistry, α-SMA, desmin.

Introduction

The peritubular matrix has a great significance in endothelial organization during the process of testicular angiogenesis. As a response endothelial cells migrate into testicular interstitium and secrete new matrix components for basal membrane of the seminiferous tubules. Testicular peritubular and interstitial tissues interact in peritubular space and the basic components of this space that are vulnerable during pathological conditions are: small blood vessels, Leydig cells, macrophages and peripheric nerves [4].

In patients with severe testicular lesions in cases with azoospermia hyalinization process often occurred in small blood vessels and around seminiferous tubules. In patients with oligoastenozoospermia a complete tubular hyalinization was demonstrated [10]. According to the authors the hyalinization affects arterioles and venules, but not capillaries. Testicular small blood vessels revealed altered structure — they have narrow lumen and thick blood vessel's wall. This finding is supported by our previous examinations [6].

Harindel and Trainen [3] accented on testicular microvasculature, mainly because of the fact that it is modulator of endocrine and paracrine factors that are important in regulation of spermatogenesis. In turn, Leydig cells play a key role in regulation of blood transport.

Peritubular matrix is responsible for the function of endothelial cells and is also important for proliferation of these cells. On the other hand, matrix components are linked to intracellular structural and contractile filaments forming an interacting functional unit that is of great importance for testicular morphogenesis. In this respect the aim of present study was to examine: 1) the morphological changes in peritubular matrix under pathological conditions and 2) localization and distribution of desmin as a marker for peritubular myofibroblasts and of α-SMA — for terminal differentiation of vascular smooth muscle cells.

Material and Methods

Materials were obtained from testicular biopsies of 9 infertile men. In two of them the semen analysis showed oligoastenozoospermia III degree and in four — azoospermia on the base of pseudocrypthorchidism billateralis. In three men oligoastenozoospermia I degree is observed without any morphological alterations and they served as controls as usual.

Testicular biopsies for light microscopy (morphological and immunohistochemical examination) were fixed in Bouin's, dehydrated and embedded in paraffin. Sections (7μm) were processed according to the classical immunochemical techniques using monoclonal antibodies against desmin and α-SMA. Avidin-biotin-peroxidase method was applied and histostain kit from DAKO was used. Immunofluorescence for α-SMA was also performed. After the incubation with mouse anti-α-SMA the sections were incubated with FITC conjugated goat mouse antibody. The sections were mounted with Moviol and observed at POLYVAR immunofluorescent microscope.

Results

In cases of azoospermia disorganization of germinal epithelium was evident and basal membrane was folded at some places or destructed at others (Fig. 1). The results from immunohistochemical study showed positive reaction for desmin in basal membrane and peritubular myofibroblasts, as well. In cases of azoospermia and oligostenozoospermia III degree desmin was localized as dots or has fibrilar appearance and it was not homogeniously distributed in comparison with other matrix proteins (Fig. 1 and Fig. 2). Very strong immune reaction for desmin was also observed around blood vessels (Fig. 3).

Strong intensity of immune reaction of α-SMA was found in the testis in cases of oligoastenozoospermia. Strong reaction was observed in basal membrane of the seminiferous tubules and peritubular myofibroblasts. In basal membrane α-SMA was localized mainly in inner layer (Fig. 4). Strong immunoreactivity for α-SMA was found around small blood vessels in interstitium (Fig. 5).

The results form immunofluorescence confirm our immunohistochemical findings and showed very strong immune reaction in basal membrane of the seminiferous tubules, in peritubular and interstitial tissues, around Leydig cells and small blood vessels (Fig. 6).

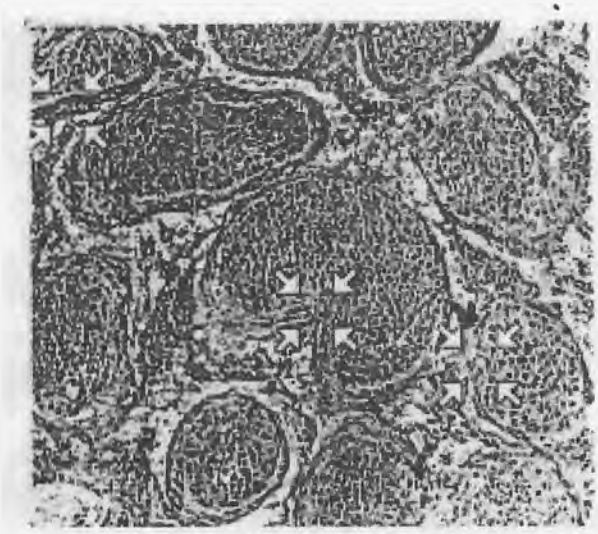

Fig. 1. Testicular biopsy. Azoospermia. Desorganization of germ epithelium. The basal membrane at some places is folded, at other is destructive (arrows). Desmin is localized in some places in basal membranes as dots or fine fibrils. Monoclonal anti-desmin antibody (× 200)

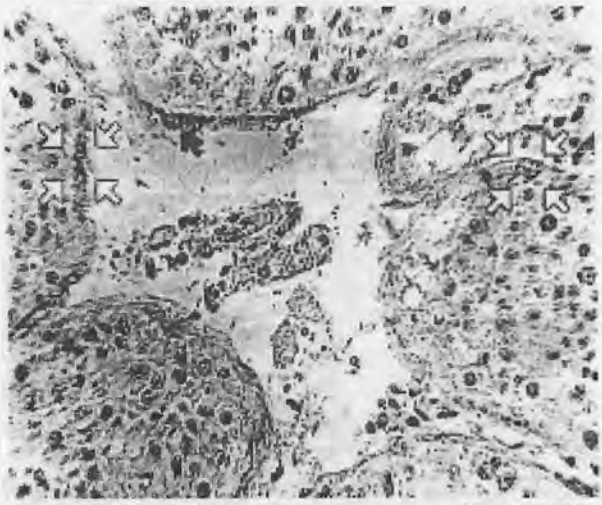

Fig. 2. Testicular biopsy. Oligoastenozoospermia III dg. Immune reaction for desmin, showing it's localozation as dots, and its fibrilar appearance, respectively. Monoclonal anti-desmin antibody (× 400)

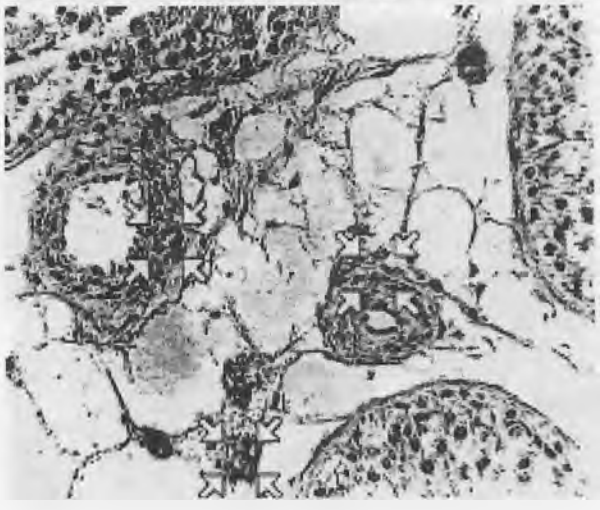

Fig. 3. Testicular biopsy. Oligoastenozoospermia III dg. Possitive immune reaction for desmin, showing it's localization around blood vessels. Monoclonal anti-dsmin antibody (× 400)

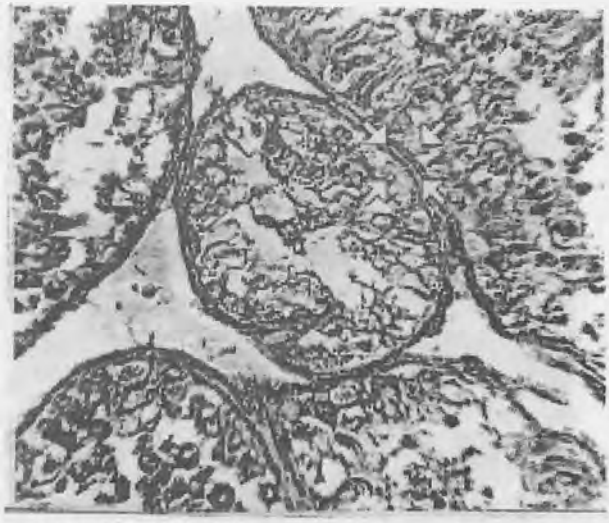

Fig. 4. Testicular biopsy. Oligo-astenozoospermia III dg. Strong immune reaction for α-SMA, showing it's localization in basal membranes of seminiferous tubules and in peritubular myofibroblasts. Monoclonal anti- α-SMA antibody (× 200)

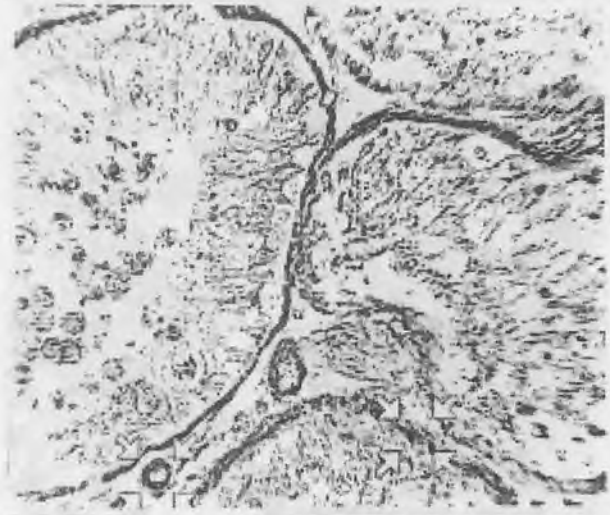

Fig. 5. Testicular biopsy. Oligo-astenozoospermia III dg. Strong immune reaction for α-SMA around blood vessels in interstitium. Monoclonal anti-α-SMA antibody (× 400)

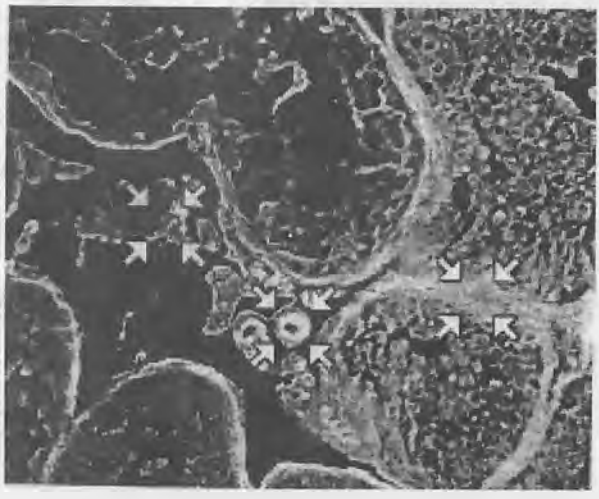

Fig. 6. Testicular biopsy. Oligoastenozoospermia III dg. Direct immunofluorescence for α-SMA. Strong immune reaction is observed in basal membranes of seminiferous tubules, in testicular interstitium, around Leydig cells and small blood vessels (× 200)

Discussion

Peritubular myofibroblasts produce α-SMA that is important for differentiation of vascular smooth muscle cells and myofibroblasts themselves are involved in cell to cell and cell to matrix interactions. All this is important for testicular morphogenesis [7, 8, 11]. Our results showed that myofibroblasts expressed α-SMA and desmin both are cytosceletal proteins characteristic for smooth muscle cells. It was reported that most myofibroblasts of the thickened lamina propria (as a result of pathological processes) lost their ability to express desmin suggesting that they may change their phenotype [1,2,5]. That was supported by Skalli [9] and Virtanen, Kollajoki [12]. In these circumstances we found that less myofibroblasts were immunoreactive for desmin. They were individual cells and are arranged in uncomplete, discontinuous layer.

Small blood vessels in testicular interstitium are this part of vascular system in testis that is responsible for the exchange of gases, nutritional substances and hormones [3] and is responsible for endocrine and paracrine regulation and normal testicular function.

In conclusion, our data brings additional support to the view that testicular peritubular matrix proteins play key role in endothelial organization especially in process of angiogenesis and testicular microvasculature is important for normal spermatogenesis.

References

1. Darby, J., O. Skalli, G. Gabbiani. α-Smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. — Lab. Invest., **63**,1990,21-29.
2. Davidoff, M. S., H. Breucker, A. F. Holstein, K. Seidl. Cellular architecture of the lamina propria of human seminiferous tubules. — Cell. Tiss. Rec., **262**,1990,253-261.
3. Ergun, S., J. Stingl, A. F. Holstein. Microvasculature of the human testis in correlation to Leydig cells and seminiferous tubules. — Andrologia, **26**,1994,255-262.
4. Herindel, I., K. A.Trainen. Physiology of the male reproductive system: endocrine, paracrine and autocrine regulation. — Toxicologic Pathology, **17**,1989,411-445.
5. Holstein, A. F., M. Davidoff. Myofibroblasts in the lamina propria of human seminiferous tubules are dynamic structures of the heterogeneous phenotype. — Arch. Histol. Cytol., **59**, 1996, No 2, 109-125.
6. Nanova, B. Morphological and immunohistochemical study of the testicular extracellular matrix in infertile men. — Human Reproduction., **12**, 1997, p.238.
7. Palomby, F. Development and cytodifferentiation of peritubular myoid cells in the rat testis. — Anat. Rec., **233**, 1992, 32-40.
8. Schurch, W., T. A. Seemayer, R. Lagace, G. Gabbiani. The intermediate filament cytoskeleton of myofibroblasts:an immunofluorescence and ultrastructural study. — Virchows Arch.(A)., **403**,1984, 323-336.
9. Skalli, O. A monoclonal antibody against α-SMA: a new probe for smooth muscle cells differentiation. — J.Cell.Biol., **103**,1986, 2787-2796.
10. Soderstrom, K. O. Tubular hyalinization in human testis. — Andrologia., **18** (1), 1986, 97-103.
11. Virtanen, I., M. Miettinen. Diagnostic application of monoclonal antibodies to intermediate filaments. — Ann. N. Y. Acad. Sci., **455**, 1985, 635-648.
12. Virtanen, I., L. M. Kollajoki. Peritubular myoid cells of human and rat testis are smooth muscle cells that contain desmin-type intermediate filaments. — Anat.Rec., **215**,1986,10-20.

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Immunohistochemical Studies on Human Testicular Peritubular Matrix — Comparative Data

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The importance of testicular peritubular matrix is well established but only few data have been reported on significance of basic matrix components and interstitial microvasulature as well as the relationship between small blood vessels and Lyedig cells. In this respect we aimed: 1) to study morphological changes in basal membrane of seminiferous tubules under pathological conditions; 2) to investigate comparatively immunohistochemical localization and distribution of basic matrix proteins. Biopsical materials from 16 men were processed for immunohistochemistry according to classical technique and monoclonal antibodies against collagen type IV, laminin and fibronectin were used. The results showed strong immunoreactivity of matrix proteins that revealed specific localization and distribution. The intensity of immune reaction corresponds to the degree of testicular lesions. In conclusion, our results indicate that the peritubular matrix with its basic components and functional complex Sertoli cells — peritubular myofibroblasts are of great importance for the normal process of testicular morphogenesis.

Key words: peritubular matrix, immunohistochemistry, matrix proteins.

Introduction

Different forms of disturbance in spermatogenesis are accompanied with thickening of testicular lamina propria. It is composed of basal membrane (2-6 layers elongated myofibroblasts), that support the seminiferous epithelium and layers of connective tissue fibres and amorphous substance as well [1, 3]. Alterations in lamina propria as a result of pathological processes were reported by S a l o m o n e and H a d i n g e r [7]. In men with disturbances in fertility some adverse changes in testicular extracellular matrix have been seen. To what extent myofibroblasts and fibroblasts are involved in process of formation of lamina propria in normal and under pathological conditions was investigated immunohistochemically by D a v i d o f f et al. [2]. An increase in intercellular components that include collagen and "basal membrane-material" surrounding myofibroblasts were observed [1] and abnormally thickened basal membrane was demonstrated [4, 5].

In infertile men increased peritubular matrix has been seen not only in peritubular tissue, but also in interstitial tissue, around Leydig cells and small blood

vessels so called testicular microvasculature [2]. Leydig cells are surrounded by more or less increased amount of peritubular matrix components. In these cases spermatogenesis was arrested at stage of primary spermatocytes and in some cases at spermatogonial stage [6]. It is supposed that male infertility is connected with abnormal thickening of peritubular tissue — lamina propria. [2, 3].

In men testicular interstitial and peritubular tissues interact in intertubular space and the basic components of this space are: ✓small blood vessels (microvasculature); ✓Leydig cells;✓macrophages and ✓peripheric nerves.

The present study focused on structural and functional integrity between the elements of peritubular tissue and testicular interstitium that is interrupted under pathological conditions. Abnormally thickened peritubular matrix is a real obstacle for transport not only of nutritions, but also of regulatory substances.The aim of the present study was to study morphological changes in basal membranes under pathological conditions and immunohistochemical localization and distribution of collagen type IV, laminin and fibronectin.

Material and Methods

Material was obtained from testicular biopsies of 16 infertile men. Data obtained from semen analysis showed that in 5 of them oligoastenozoospermia II-III degree was observed and in 3 — azoospermia. In two of the patients normospermia was established and they served as controls.

Biopsical materials for routine light microscopy and immunohistochemistry were fixed in Bouin's and classical immunohistochemical technique was used. Paraffin sections (7 μm) was prepared and on some of them adhesive technique with Poly-L lysine was applied. For the purposes of immunohistochemistry avidin-biotin-peroxidase method was used. Sections were deparaffinized and incubated with monoclonal antibodies against collagen type IV (Clone COL-94), laminin (Clone Lam-89) and fibronectin (Clone Fn-3E2). After the incubation with second biotinylated antibody the avidin-biotin-peroxidase complex was applied and aminoethyl carbasole (AEC) was used as substrate. Histostain kit from DAKO was used.

Results

Results from present study showed morphological alterations in seminiferous tubules that are expressed with: cessation of germ cell maturation at stage of primary spermatocytes (in patients with oligoastenozoospermia II-III dg.) and in cases with azoosprmia — at spermatogonial stage. Basal membrane is abnormally thickened, increased production of peritubular matrix and initial fibrosis are observed. Small blood vessels in interstitium are with thickened wall and process of fibrosis is evident. Some seminiferous tubules are with diminished lumen or lost their lumen. Dislocation and proliferation of myofibroblasts are observed (Fig. 1, Fig. 2 and Fig. 3)

The results obtained from immunohistochemical examination showed positive immune reaction for collagen type IV and its distribution in peritubular matrix (Fig.4). It was localized in basal membranes. Positive immune reaction is observed not only in peritubular connective tissue, but also in interstitial tissue — around Leydig cells and small blood vessels (Fig. 5 and Fig. 6).

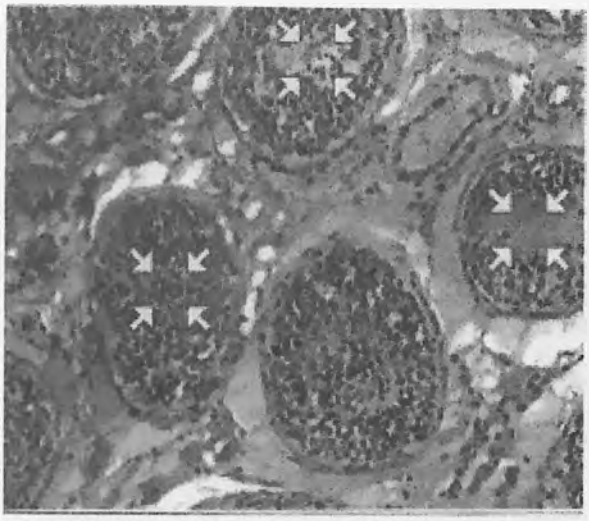

Fig. 1. Testicular biopsy. Azoospermia. Increasing production of peritubular matrix. The seminiferous tubules are with diminished lumen or lost completely their lumen (white arrows). Haematoxyline- Eosine staining (× 200)

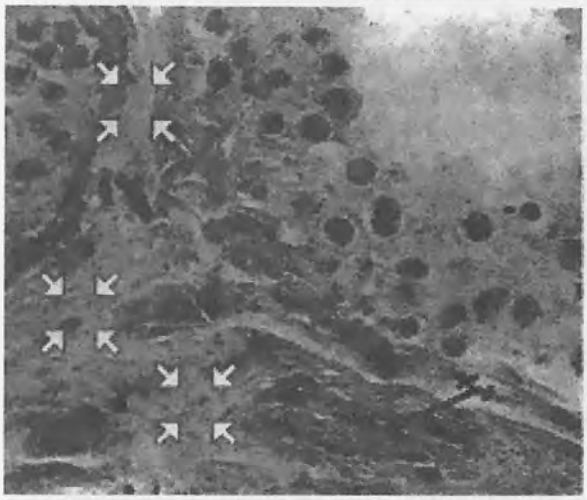

Fig. 2. Testicular biopsy. Oligoastenozoospermia III dg. Dislocation of myofibroblasts and increased production of collagen in peritubular matrix, basal membrane is fragmented and folded (arrows). Haematoxyline-Eosine staining (× 400)

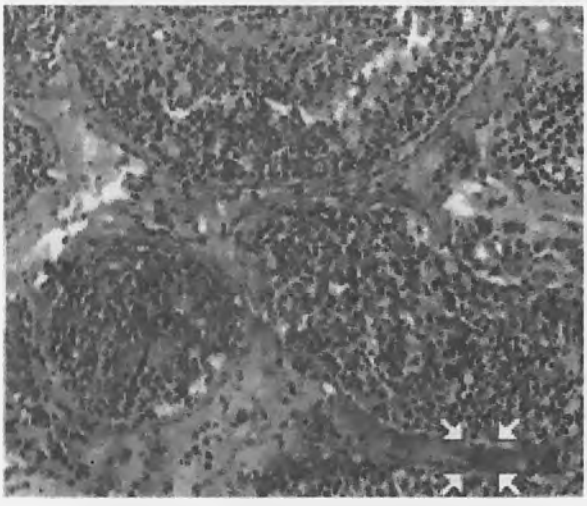

Fig. 3. Testicular biopsy. Azoospermia. Disorganization of germinal cells with intertubular cessation of maturation. The basal membrane is thickened (arrows). Haematoxyline-Eosine staining (× 200)

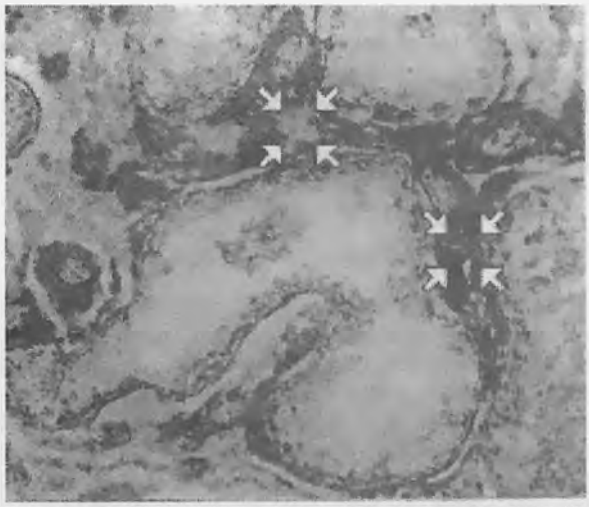

Fig. 4. Testicular biopsy. Azoospermia. Complete disorganization and massive loss of germ cells. Possitive immune reaction showing localization of collagen type IV in peritubular tissue, interstitium and around blood vessels and Leydig cells (arrows) (× 400)

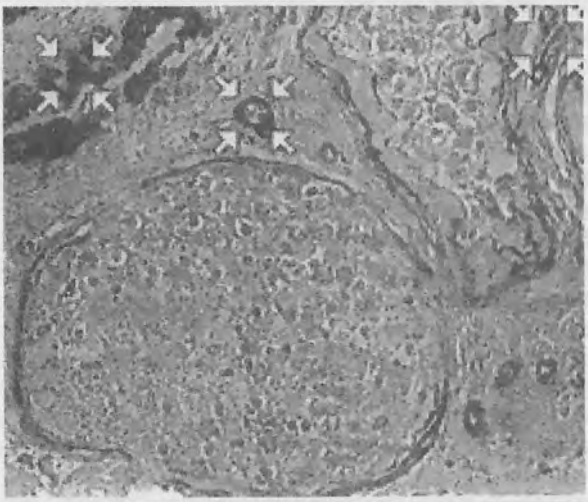

Fig. 5. Testicular biopsy. Oligoastenozoospermia III dg. Positive immune reaction showing localization of collagen type IV in basal membrane. The positive immunostaining for collagen type IV is observed in interstitium as well as around blood vessels (× 400)

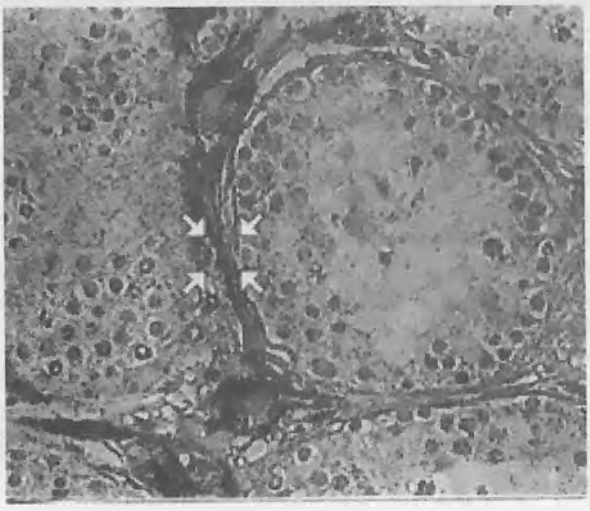

Fig. 6. Testicular biopsy. Oligoastenozoospermia III dg. Positive immune reaction for collagen type IV, showing its localization in both layers of basal membrane of seminiferous tubules and around Leydig cells in the interstititum (arrows) (× 400)

Positive immunostaining for fibronectin was observed in peritubular tissue. It was localized in fibroblasts, myofibroblasts and in Sertoli cells of the seminiferous tubules (Fig. 7 and Fig. 8). A strong immune reacion for laminin was found in basal membrane of seminiferous tubules, especially in inner layer of basal membrane (Fig. 9). Positive immunostaining is also observed in interstitial tissue and around blood vessels.

Discussion

The increased peritubular matrix is an obstacle for transport of nutritious and of regulatory substances as well. As an element of haematotesticular barrier the basal membrane is vulnerable in pathological processes. Our results clearly indicate fragmentation and folding of basal membrane in patients with oligoasthenozoospermia III degree and azoospermia on the base of pseudocryptorchidism. Matrix proteins ensure normal proceeding of spermatogenesis and contribute for structural integrity, for cell-cell and cell-matrix interactions and thus they are important for testicular morphogenesis. As main structural component of basal membrane collagen type IV is involved in tissue differentiation, morphogenesis and in the process of repair. It also plays an important role in tissue structure, metabolism and cell differentiation and migration. Collagen type IV is accumulated on sheafs and is presented in all layers of basal membrane. Collagen serves for connection of myoid cells one to one and sets them firmly to elastic and collagen fibrils, presenting in basal membranes [2]. The authors showed the relation of myoid cells with the elements of connective tissue. We have established thickened and folded basal membrane associated with its invagination and initial fibrosis in patients with pseudocryptorchidism. Sertoli cells and peritubular myoid cells are in close relationship during different stages of spermatogenesis and they influence on basal membrane structure and components of extracellular matrix [7]. This hypothesis was supported by our previous data [6]. An extraordinary expansion of the intercellular layers of matrix components of the connective tissue that was observed by D a v i d o f f et al. [2] is probably responsible for the thickening of the lamina propria of the seminiferous tubules. The results of the current study demonstrate that laminin as a highly-specific, tissue-specific and cell-specific factor is localized mainly in basal membrane of the seminiferous tubules, as in capillaries of the interstitial tissue and around Leydig cells. Other earlier studies [4] pointed out the fact that laminin is a major component in all the basal membranes and it is involved in cell migration and cell differentiation. We found that laminin is localized in outer layer of basal membrane, collagen type IV is observed in both layers and most intensively laminin is accumulated in the layer of peritubular myofibroblasts. On the base of these results we postulate that laminin may play an important role in morphogenesis and structural stability of seminiferous tubules and peritubular myofibroblasts are surrounded by collagen type IV [6] and laminin. The immunohistochemical data showed the localization of fibronectin in inner layer of lamina propria. Our results suggest the possible role of fibronectin in Sertoli cell-peritubular myofibroblast complex that is essential for cell adhesion and formation of basal membrane and functional units in testicular peritubular matrix.

In conclusion, our results indicate that the peritubular matrix with its basic components and functional complex Sertoli cells-peritubular myofibroblasts are of a great importance for the normal process of testicular morphogenesis. In patho-

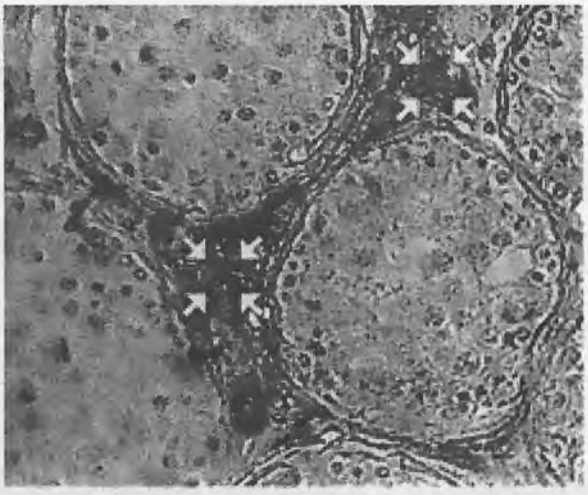

Fig. 7. Testicular biopsy. Azoospermia. Positive immune reaction for fibronectin, showing its localization in myofibroblasts and in Sertoli cells (black arrow) (× 400)

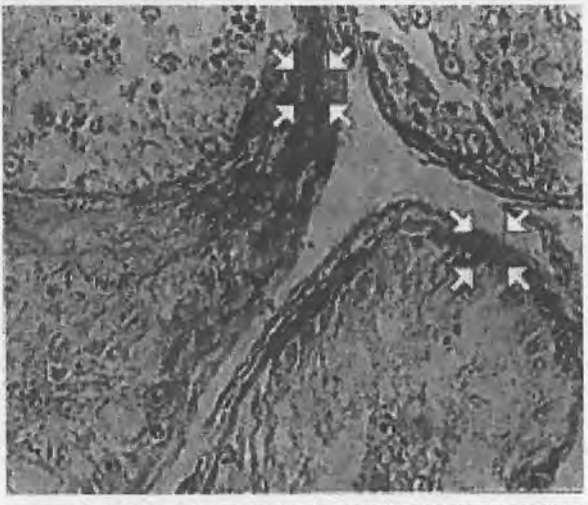

Fig. 8. Testicular biopsy. Azoospermia. Positive immune reaction for fibronectin, showing its localization in peritubular tissue (myofibroblasts and at some places in inner layer of basal membranes of seminiferous tubules) (× 400)

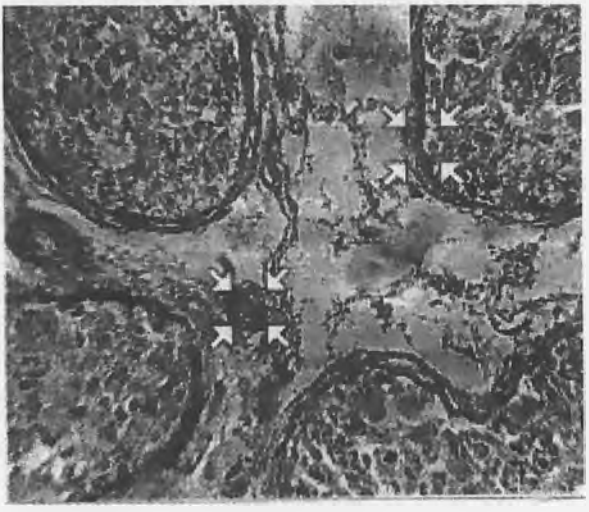

Fig. 9. Testicular biopsy. Oligoastenozoospermia III dg. Positive immune reaction for laminin. It is localized in interstitial tissue, around blood vessels. In particular, the localization of laminin is seen very well in inner layer of basal membranes of seminiferous tubules (× 400)

logical conditions this complex together with basal membrane (as one of the constituents of haemato-testicular barrier) are vulnerable to different ethiological factors and that makes worse the prognosis of male fertility.

References

1. Bustos-Oregon, E., A. F. Holstein. On structural pattern of lamina propria of human seminiferous tubules wall. — Z. Zellforsh. Mikrosk. Anat., **141**, 1973, 413-425.
2. Davidoff, M. S., H. Breuker, A. F. Holstein, K. Seidl. Cellular architecture of the lamina propria of human seminiferous tubules — Cell. Tiss. Rec., **262**, 1990, 253-261.
3. De Kretser, D. M., J. B. Kerr, C. A. Paulsen. The peritubular tissue in the normal and pathological human testes. An ultrastructral study. — Biol. Reprod., **12**, 1975, 317-324.
4. Hermo, L., M. Lalli., J. Clermont. Arrangement of Connective Tissue Components in the Walls of Seminiferous Tubules of Men and Monkey. — Am. J. Anat., **148**, 1976, 433-446.
5. Gay, E. D. Extracellular matrix. — J. Cell Biol., **91**, 1981, 205-223.
6. Nanova, B. Clinical and morphological studies on testicular extracellular matrix in men with disturbances in spermatogenesis. — Anatomischer Anzeiger Anals of Anatomy, **94**, 1999, p.142.
7. Salomone, F., C. E. Hadinger. Abnormal membrane structures of the seminiferous tubules in infertile men. — Lab. Invest., **47**, 1982, 543-554.

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Apoptotic State of Colostral/Milk Cells

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It has been proposed that the population of leukocytes released in the mother's milk penetrates the newborn's body through the gut and populates it's lymphoid organs. In order to test this hypothesis we first studied the state of apoptosis of the cells in the milk population. For this purpose we used a method measuring the fragmentation of DNA (by means of the ^{3}H-thymidine incorporation in DNA located in the different cellular compartments — nucleus and cytoplasm). When working with whole milk cell population we found different percent of DNA fragmentation from 40 to 70%. We next separated the milk cell population into nearly pure fractions of macrophages and lymphocytes. The level of the DNA fragmentation in these experiments was much better. The macrophages and the lymphocytes were enough viable to penetrate the mucosal immune system of the newborn and to influence its development and maturation.

Key words: — milk cells, apoptosis, DNA fragmentation, lymphocyte, macrophage.

Introduction

In the colostrum and milk of all mammalian species a huge number of viable cells are released consisting of macrophages, neutrophils and lymphocytes. It has been shown for different species including monkeys that these cells penetrate the gut of the newborn and populate its lymphoid organs[2]. On the basis of these results a hypothesis has arisen that the cells of the milk population, especially lymphocytes and macrophages are immunocompetent cells and they could influence the immature mucosal immune system of the new born[3]. To test this hypothesis we have to be sure that these otherwise vital cells do not undergo an apoptotic process.

The aim of the present study was to investigate the apoptotic state of the cells in the human colostrum and milk.

Materials and Methods

Apoptosis assay

Milk samples were taken from 14 healthy human donors. They were washed 3 times in PBS and resuspended in 1 ml RPMI 1640 containing 5% FCS. 1 μl ^{3}H- thy-

midine was added to each sample. The next step was an incubation of the samples at 37 °C for approximately 16 hours. After spinning down the cells were washed 3 times with PBS (or serum free media) to remove any free radiolabel, then the cells were resuspended at a concentration of 5×10^5 cells/ml in serum free medium. After 1 hour the cells were gently pelleted and supernatant count: supernatant — A. The pellet was resuspended in lysis buffer (1× PBS; 0.2% Triton-X100; 2Mm EDTA) and centrifuged. The collected pellet and supernatant were counted in scintilation counter (Beckman): supernatant — B and pellet — C. To The per cent fragmentation was calculated after the equation:
Per cent fragmentation = (A + B) / (A + B + C)
All the samples were in triplicates and the results were expressed as mean per cent DNA fragmentation[6, 1].

Separation of the milk cells

In respect to the significant differences observed after performed DNA fragmentation test with whole milk cell population and for more precise image of apoptotic events among the different subpopulation of milk cells, we separated the milk cells to macrophage and lymphocyte fractions. The separation protocol includes the next chain of laboratory manipulations: washing 3 times in PBS; resuspension of cells in RPMI 1640 (serum free media), preparation of Ficol-RPMI 1640 gradient (11%, 20%, 30% Ficol); centrifugation on this gradient; collection of macrophage or lymphocyte fractions and performance of the assay for apoptosis.

Microscopic approach used

For a visual demonstration of the apoptosis in the milk cells the microscopic observations of the fresh milk samples was performed. After 3 times washing in PBS, ethidium bromide was added to the cells at 0.5 μg/ml final concentration and the cells were observed under microscope at UV and visible light. The photos of stained cells were taken in parallel at UV and visible light [6].

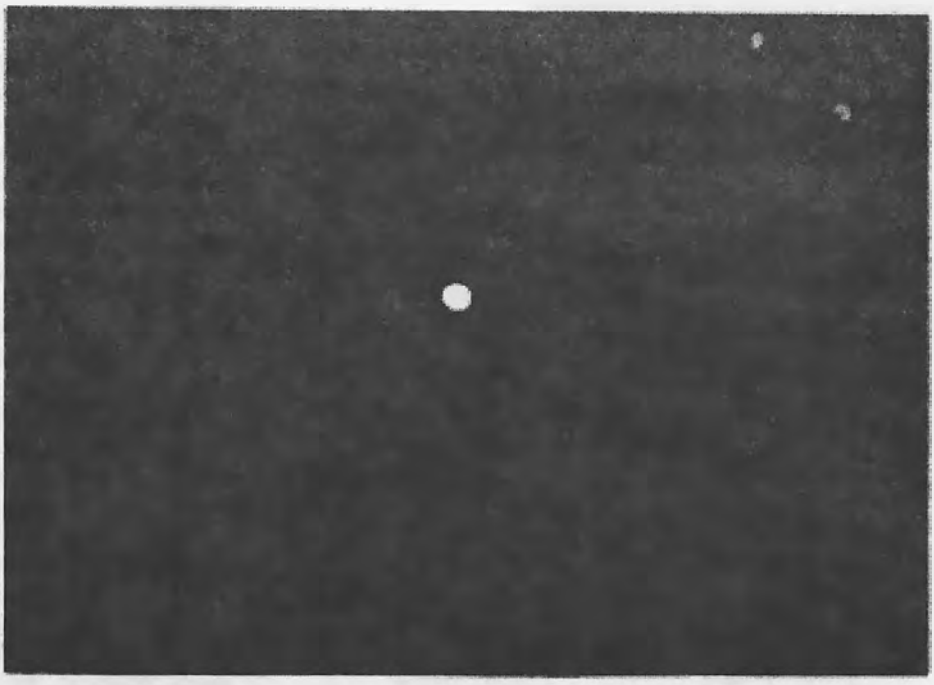
Results and Discussion

Apoptosis is widely distributed process affecting all animal cells, organs and tissues. Here we present another approach into the application of knowledge about the apoptosis. The hypothesis, that we have tested in the present study, is that the leukocytes, that are released in the mother's milk penetrate the gut of the newborn and populate its lymphoid organs. It is possible that these cells could influence the maturation of the mucosal immune system of the newborn[4]. To be involved in a transfer of immunological competence, a certain level of viability of the leukocyte cells is required[5]. We examined the apoptotic status of cells isolated from human mother's colostrum and milk. A variable count of DNA fragmentation was observed. As Table 1 shows, the per cent of apoptotic events (presented as a mean percent fragmentation of DNA in whole milk population) varied from 40 to 70% approximately. We might explain these differences with

Table 1. Per cent fragmentation in whole milk leukocyte population

Dónor, No	Per cent fragmentation
1	43
2	87
3	97
4	95
5	70
6	40

A



B

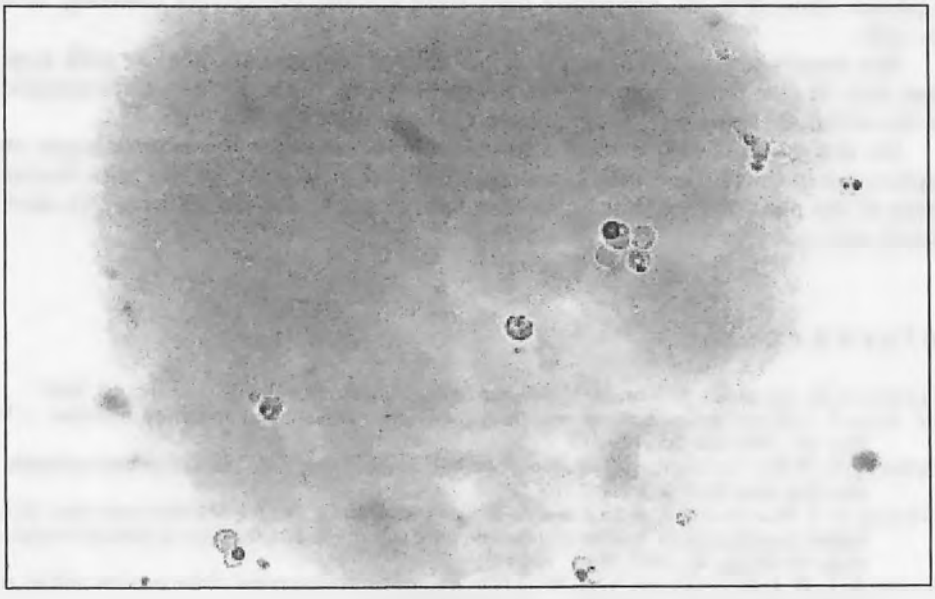


Fig. 1. Apoptotic cells from milk's population, treated with ethidium bromide under UV light (*A*) and visible light (*B*)

Table 2. Per cent fragmentation in mother's milk after separation of macrophage and lymphocyte subpopulations

Donor, No	Fraction	Percent fragmentation	Fraction	Percent fragmentation
1	Macrophages	42	Lymphocytes	59
2	Macrophages	94	Lymphocytes	91
3	Macrophages	83	Lymphocytes	74
4	Macrophages	74	Lymphocytes	68
5	Macrophages	80	Lymphocytes	71
6	Macrophages	80	Lymphocytes	65
7	Macrophages	62	Lymphocytes	77
8	Macrophages	69	Lymphocytes	83

genetic and environmental influences on the donor.

For more precise investigation of apoptotic status of milk cells a separation of milk population into macrophage and lymphocyte fractions was performed. The level of DNA fragmentation in these experiments was much better as shown in Table2. The macrophages showed about 40% fragmentation and lymphocytes showed about 60% fragmentation.

For visualizing apoptotic cells in mother's milk, the ethidium bromide inclusion test was used. As the photos show (Fig. 1), apoptotic cells were stained intensively contrary to viable cell.

Our results suggest that the higher per cent of fragmentation in the milk population may be due to the neutrophils, which are in the final stage of differentiation and die within 24 hour after their release in the mother's milk.

On the grounds of our results we might conclude that the macrophages and lymphocytes in the mother milk are enough viable to penetrate the mucosal immune system of the newborn and not apoptotic enough so they could influence its development and maturation.

References

1. Jayadev, S., Apoptosis: Miniassay. Molecular Biology Protocols online, September 13, 1993.
2. Le Jan, C. Cellular components of mammary secretions and neonatal immunity: a review. — Vet Res, **27**, 1996, No 4-5, 403-417.
3. Tuboly, S., S. Bernath. Intestinal absorption of colostral lymphoid cells in newborn animals. — Adv Exp Med Biol, **503**, 2002, 107-114.
4. Tuboly, S., S. Bernath, R. Glavits, A. Kovacs, Z. Megyeri. Intestinal absorption of colostral lymphocytes in newborn lambs and their role in the development of immune status. — Acta Vet Hung, **43**, 1995, No 1, 105-115.
5. Webb, S. J., D. J. Harrison, A. H. Wyllie. Apoptosis: An overview of the process and its relevance in disease. — Adv. Pharmacol., **41**, 1997, 1-34.
6. Willingham, M. Cytochemical methods for the detection of apoptosis. — J. Histochem. Cytochem., **147**, 1999, No 9, 1101-1109.

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Acta morphologica et anthropologica, 8
Sofia•2003

Kariometrical Studies on the Effect of Sex Hormones on the Adrenal Cortex in Rats Acclimated to Different Environmental Temperatures

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The effect of testectomy and sex hormones treatment on the nuclear volume and area in adrenocorticocytes of adult male rats, acclimated to different ambient temperatures (20±2°C and 35±1°C) was examined. The obtained results indicated that testectomy provoked increase of the nuclear volume and area in all adrenocorticocytes, regardless of the previous thermal acclimation, but it was statistically significant only for nuclei of zona reticularis'cells in rats from room temperature. The effect of testosterone depended on the previous thermal acclimation. Namely, testosterone didn't provoke significant changes of the examined parameters in animals from room temperature, but in heat acclimated rats the significant increase in these parameters was noted. The estradiol treatment, in both environmental temperatures, provoked statistically significant increase in nuclear parameters in all zones, which was more expressed in animals from room temperature.

Key words: kariometry, adrenocorticocytes, sex hormones, hyperthermia, rat.

Introduction

Numerous kariometrical studies on the endocrine glands, especially those concerning the adrenal gland, show that the nuclear volume of adrenocorticocytes represents a very important sign of their activity [11]. Many of them have also shown that estradiol provokes increase and testosterone decrease of cell and nuclear volumes in adrenal cortex, as well as volumes of mitochondria and SER — two organelles involved in steroid production [6].

The acute exposure of rats to a high environmental temperature provokes an increase of the nuclear volume of adrenocorticocytes, which is associated with the increased activity of the gland [12]. On the other hand, the chronical exposure of rats on elevated ambient temperature provokes decrease of nuclear volume in adrenocortical cells of the inner zones, which is correlated with the decreased activity of the gland [10].

Regarding the fact that there are no data about the effect of testectomy and sex hormones on the kariometrical parameters in adrenocorticocytes under elevated

ambient temperature, the aim of this study was to find the effects of sex hormones on the nuclear volume and area in heat acclimated rats, compared the same at room temperature.

Material and Methods

The experiments were performed on adult male laboratory rats, Wister strain, weighing 203-309 g. The access to food and water was ad libitum. The animals were divided into two groups: heat-acclimated group kept 30 days at 35±1°C and the control group kept at 20±2°C for several weeks. Each group was divided into 4 subgroups: 1) Control; 2) testectomized; 3) testectomized + testosterone; 4) testectomized + estradiol.

Animals were subjected to bilateral testestomy and 14 days after the operation they were hormonally treated. The hormonal application (testosteron propionate—(+)-17β-propionil-oksi-4-androsten-3-ona, and estradiol dipropionate—(+)-3,17-β-dipropionil-oksi-2,3,5-estratriena, "Galenika"-Belgrade) on testectomized rats was performed i.m. in doses of 1mg/100g b.w./daily for last 4 days. The animals were sacrificed 24 hour following the last dose. The adrenal glands were fixed in Bouin's solution, embedded in paraffin and cut at 3-4 μm. Paraffin sections were stained with hematoxylin and eosin. For kariometrical analyses specific software for digitalization and analysis "Lucia G" (Nikon) was used. The nuclear volume and area of 150 spherically nuclei from every zone of the cortex were measured. The nuclear area was determined automatically, but the nuclear volume was measured according to the formula:

$V=\pi/6*EqDia^3$.

EqDia is a mean diameter of the circle, which has same area as the measured nuclei.

Results were statistically evaluated by the Student's t-test.

Results

The results from our investigation were given as graphs illustrating the mean volume and area of nuclei in zona glomerulosa (Table 1), zona fasciculata (Table 2) and zona reticularis (Table 3) cells.

T a b l e 1. Mean value of nuclear volume and area in zona glomerulosa cells in rats from both ambient temperatures.

Nuclear Parameters	20±2°C				35±1°C			
	Intact (control)	test.	test.+T	test.+E	control	test.	test.+T	test.+E
Nuclear volume (μm^3)	94.27 ±2.04	95.26 ±2.54	92.11 ±2.70	106.68± 2.13^{c,B}	78.92 ±3.81	81.84 ±1.12	88.50 ±0.58^{a,A}	96.75 ±2.60^{c,C}
Nuclear area (μm^2)	24.98 ±0.37	25.17 ±0.43	24.58 ±0.48	27.14 ±0.37^{c,B}	22.17 ±0.72	22.74 ±0.21	23.96 ±0.11^{a,A}	25.41 ±0.45^{c,C}

Notes. Cont. (control); test. (testectomized); test+T (testectomized and treated with testosterone); test+E (testectomized and treated with estradiol). Values are given as mean±stand. dev. Significance of the values is given with the superscripts, compared with the control (a: $p<0.05$; b: $p<0.005$; c: $p<0.001$) and compared to the testectomized animals (A: $p<0.05$; B: $p<0.01$; C: $p<0.005$)

T a b l e 2. Mean value of nuclear volume and area in zona fasciculata cells in rats from both ambient temperatures: cont

Nuclear Parameters	20±2°C				35±1°C			
	Intact (control)	test.	test.+T	test.+E	control	test.	test.+T	test.+E
Nuclear volume (μm^3)	127.64 ±3.06	132.84 ±4.50	124.04 ±2.97	184.52± 2.88[b]	111.37 ±1.30	113.55 ±1.34	124.21 ±4.75[a]	150.84 ±2.49[b]
Nuclear area (μm^2)	30.59 ±0.49	31.41 ±0.71	30.01 ±0.48	39.12 ±0.40[b]	27.89 ±0.23	28.27 ±0.21	30.03 ±0.76[a]	34.18 ±0.37[b]

Notes. Cont. (control); test. (testectomized); test+T (testectomized and treated with testosterone); test+E (testectomized and treated with estradiol). Values are given as mean±stand. dev. Significance of the values is given with the superscripts, compared with the control as well as testecomized animals (a: $p<0.05$; b: $p<0.001$)

T a b l e 3. Mean value of nuclear volume and area in zona reticularis cells in rats from both ambient temperatures

Nuclear Parameters	20±2°C				35±1°C			
	Intact (control)	test.	test.+T	test.+E	control	test.	test.+T	test.+E
Nuclear volume (μm^3)	79.22 ±0.72	83.36 ±0.57[b]	79.96 ±5.51	108.32± 6.31[b,c]	74.32 ±5.08	73.70 ±1.41	88.55 ±5.84[a]	101.59 ±4.39[c]
Nuclear area (μm^2)	22.26 ±0.13	23.02 ±0.10[b]	22.34 ±1.06	27.41 ±1.08[b,c]	21.29 ±0.99	21.19 ±0.27	23.97 ±1.04[a]	26.25 ±0.77[c]

Notes. Cont. (control); test. (testectomized); test+T (testectomized and treated with testosterone); test+E (testectomized and treated with estradiol). Values are given as mean±stand. dev. Significance of the values is given with the superscripts, compared with the control as well as testectomized animals (a: $p<0.05$; b: $p<0.01$; c: $p<0.001$)

Two weeks after testectomy a slight increase in volume and area of cellular nuclei was observed in all the zones, regardless of the previous thermal acclimation. Nevertheless, the increase of these parameters was significant only for zona reticularis' cells (Table 1, 2 and 3).

In testectomized rats from room temperature, testosterone provoked a slight decrease in measured parameters compared to the testectomized animals. In heat-acclimated testectomized and testosterone treated rats a significant increase ($p<0.05$) of nuclear parameters can be observed (Table 1, 2 and 3).

The introduction of estradiol into testectomized animals from both ambient temperatures, showed a remarkable increase in volume and area of cellular nuclei in all zones (Table 1, 2 and 3), although these parameters in heat-acclimated rats were considerably smaller than those from room temperature.

Discussion

Testectomy provokes increase of cellular, nuclear and mitochondria volumes, SER proliferation, as well as activity of enzymes involved in steroid production [6,11]. The significant increase in nuclear volume and area of zona reticularis cells in testectomized rats from room temperature, obtained in our study, corroborates the data that testectomy affects mainly zona reticularis, which partly undertakes the hormonal function of testes [4,5,7]. Similarly, [15] found an increased activity of zona reticularis during first 4 weeks after testectomy, while substitution therapy with testosterone preserves further increase or brings them back to normal levels, due to the inhibitory effect of testosterone to HPA axis [6].

Biochemical studies [16] have shown that the adrenal glands of testectomized, heat-acclimated rats had an increased activity. This finding doesn't correspond with our results of a slight (not significant) increase of measured nuclear parameters. We presume that the period of 14 days after the testectomy (used in our studies) is probably not long enough to show significant increase of the nuclear volume and area of adrenocortical cells, although we have found some other signs (supracortical nodules, decreased lipid content), which indicate an elevated stimulation.

It is well known that testosterone inhibits RNA synthesis [8], as well as volumes of nuclei and organelles involved in steroid production and the activity of their enzymes [6]. In our study, the administration of testosterone in testectomized rats from room temperature showed only numerical decrease of measured nuclear parameters, besides evidently enlarged lipid vacuolisation of the adrenocorticocytes, and probably longer period of application is needed to provoke nuclear changes. On the other hand, our results of the increase in nuclear parameters of cells in all the zones in heat-acclimated testectomized rats is opposite with the well-known effect of this hormone in animals at room temperature. On the other hand, we've found a large destruction of the histoarchitecture in the two inner zones in all heat-acclimated testectomized and treated with testosterone animals. Therefore, we presume that the increase of nuclear parameters in this experimental group is probably a result of a compensation for the great destruction of the cortex and therewith, steroid secretor cells in above-mentioned regions, but we have to do more researches on this issue.

The administration of estradiol in testectomized rats from both ambient temperatures provokes significant increase in nuclear parameters in cells of all the zones. This corroborates the data of other authors [4, 5, 6], who found that estradiol doesn't stimulate only HPA axis, but also has a direct effect on adrenocorticocytes, as well as stimulating effect on thyroid gland [2]. Nevertheless, the effect of estradiol was less expressed at elevated ambient temperature, due to a decreased activity of other glands: pituitary [9], thyroid [13, 14] and gonads [2, 9] in rats acclimated to moderately high ambient temperature.

Conclusion

From the obtained results can be concluded that testectomy has a stimulating effect on the activity of adrenal cells of zona reticularis in animals from room tem-

perature, but has no effect on nuclear volume and area of heat-acclimated animals. We think that this is probably due to a shorter period after testectomy for analysis the gland, compared to data obtained from other authors.

The effects of sex hormones depend on the previous thermal acclimation of the animals. Namely, testosterone treatment of testectomized rats from room temperature doesn't show any effect of nuclear parameters in adrenocortical cells, while in heat-acclimated testectomized rats, kariometrical parameters point to an increased adrenocortical activity.

Estradiol treatment in testectomized rats from both ambient temperatures provokes kariometrical changes that indicate a stimulation of adrenocorticocytes and therewith increased cortical activity. These changes are more pronounced in animals from room temperature.

References

1. Baniska, A. The effect of temperature as an external factor upon the plasma levels of some hormones and their evaluations. — Mag. thesis (Institute of Biology, Skopje), 2001.
2. Buzalkov, R., S. Mitev, S. Dinevska. The effects of oestradiol and testosterone administration on liver glycogen and some other parameters in gonadectomized rats acclimated to various environmental temperatures. — God. Zb. Biol., **45**, 1992, 57-68.
3. Cure, M. Plasma corticosterone response in continuous versus discontinuous chronic heat exposure in rat. — Physiol. Behav., **45**, 1989, No 6, 1117-1122.
4. Malendowicz, L. K. Kariometrical studies on the effect of castration, stilboestrol and both castration and stilboestrol on adrenal cortex of adult male rats. — Endokrinologie, **56**, 1970, No 3, 270-279.
5. Malendowicz, L. K. Sex differences in adrenocortical structure and function. I. The effect of post pubertal gonadectomy and gonadal hormone replacement on nuclear volume of adrenocortical cells in rat. — Cell Tissue Res., **151**, 1974, 525-536.
6. Malendowicz, L. K. Cytophysiology of the mammalian adrenal cortex as related to sex, gonadectomy and gonadal hormones. — Poznan, Widawnictwo PTPN, 1994.
7. Malendowicz, L. K., C. Robba, G. G. Nussdorfer. Sex differences in adrenocortical structure and function. XXII. Light electron-microscopic morphometric studies on the effect of gonadectomy and gonadal hormone replacement on the rat adrenal cortex. — Cell Tissue Res., **244**, 1986, No 1, 141-145.
8. Mazzocchi, G., L. K. Malendowicz, C. Robba, P. Rebuffat, G. Gottardo, G. G. Nussdorfer. Effects of testosterone on the zona fasciculata of the male rat adrenal cortex — correlated stereological and biochemical study. — J. Submicrosc. Cytol., **15**, 1983, No 4, 991-1005.
9. Mitev, S. About the effect of hyperthermic environment and some endocrine factors on liver glycogen and some other parameters in white rat. — Doctoral thesis (Institute of Biology, Skopje) 1983.
10. Miteva, N. Cytomorphological characteristics of the adrenal cortex, liver and testes in rats under the hyperthermia. — Doctoral thesis (Institute of Biology, Skopje) 1986.
11. Nussdorfer, G .G. Cytophysiology of the adrenal cortex. — Int. Rev. Cytol., **98**, 1986.
12. Pugachev, M. K. Changes in adrenocorticocyte nuclear volume of white rats under their overheating. —Tsitologii, **22**, 1980, 868-872.
13. Rousset, B., M. Cure, D. Jordan, A. Kervran, H. Bornet, R. Mornex. Metabolic altherations induced by chronic heat exposure in rats: the involvement of thyroid function. — Pflugers. Arch., **401**, 1984, No 1, 64-70.
14. Simova, N., J. Dimovska, R. Buzalkov. 131J-Thyroid clearance in rats exposed to high environmental temperature. — Iugoslav. Physiol. Pharmacol. Acta, **21**, 1985, suppl. 4, 305-306.
15. Setoguti, T., Y. Inoue, M. Shin. Effect of the orchiectomy on the adrenal zona reticularis of adult rats: an ultrastructural morphometric study. — Okajimas Folia Anat. Jpn., **58**, 1982, 649-660.
16. Shvareva, N., J. Kaplanski, L. Abramovich, U. A. Sod - Moriah. Testosterone modifies response to chronic heat exposure in rats. — Comp. Biochem. Physiol. A Mol. Integr. Physiol., Aug. **120**, 1998, No 4, 575-578.

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Ultrastructure of the New Spermatogonia of Ohrid Trout (*Salmo Letnica* Ka R.) In the Postspawning Period

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The new spermatogonial population on ultrastructural level of Ohrid trout (*Salmo letnica* K a r.) in postspawning period has been analysed. The new spermatogonial population in Ohrid trout is represented by different generation of spermatogonia, the primary hypertrophied spermatogonia of type A and secondary spermatogonia of type B, of smaller dimensions. Its multiplication starts in course of the postspawning period and is a representative of the new spermatogenesis for the following year.

Key words: Ohrid trout (*Salmo letnica* K a r.), testis, ultrastructure, spermatogonia of type A and B.

Introduction

In previous investigations which concern the ultrastructure of the new spermatogonial generation in different Teleostei there are many explanations.

In *Oryzias latipes* the structure of the "nuages" particles during the differentiation of the spermatogonia was especially investigated by C l e r o t [2] and H a m a g u c h i [3].

In *Ciprinus carpio* L. an intensive mitosis of spermatogonia of type B was noticed by B i l l a r d et al. [1].

The ultrastructural characteristics of the spermatogonial population with Salmo gairdneri was described by H u r k et al. [4]; S c o t t & S u m p t e r [6] and generally about the trout by L o i r [5].

Material and Methods

Testes of sexually mature Ohrid trout (*Salmo letnica* K a r.) males caught in Ohrid Lake in a period of 3 years (1993—1996) have been analysed. Analyses have been done with electronic microscope. Small parts of testes 1-2 mm big have been used for electronic microscopy. The material has been fixed according to following procedure: Immediately after the tissue sections have been taken, they are fixed in

3% glutaraldehyde and then conserved in 0.1 M phosphate buffer. After adequate fixation the material has been subunitted to postfixation in 1 % osmium tetraoxid (OsO_4). In the further treatment the material has been washed in phosphate buffer, dehydrated in series of acetone and uranil acetate. The tissue parts have been 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 tickness have been prepared, with the help of glass knives, on Reichert-Yung: Ultracut" ultramicrotom, installed on copper nets, contraste with uranil acetatae and lead cytrate. The sections have been observed on Tesla BS 500 and OPTON (Zeis) EM 109 electronic microscope.

Results

The new spermatogonial of Ohrid trout (*Salmo letnica* K a r.) comes from latent spermatogonia located in the wall of the seminiferous lobules individually or in groups, the number of which increases a lot in the postspawning period and is a representative of the new spermatogenesis, i. e. the new reproductive cycle.

On the ultrathin sections in the cytoplasm of some spermatogonia of type A we have noticed presence of thick particles, i.e. complex of mitochondria ("nuages") of perinuclear location (Fig. 1) which represents a characeristic material for the germinative cells.

In contrast of these spermatogonia in the wall of some seminiferous lobules spermatogonia of the second generations can be seen, i.e. groups of spermatogonia of small dimensions, that is of smaller diameter of the nucleus and presence of more heterogeneous chromatin. i. e. secondary spermatogonia of type B (Fig. 2).

On the ultrastructural level completely well can be noticed that the new spermatogonial generation is represented by young cells. These young cells possess darker cytoplasm (Fig. 3, 4, 5) in which mitochondria with electron dense matrix and lamelar crusts (Fig. 5) in phase of formation, can be noticed.

In the cytoplasm of some spermatogonia "lamelae anulate" (Fig. 4), a lot of ribosomes and polyribosomes (Fig. 2, 5) can be noticed, which points to an intensive synthesis of proteins which is characteristic of the young cells which grow up.

These cells have a clearly seen nucleus with prominent nucleolus (Fig. 3, 4, 5, 6).

It is noticed that spermatogonia which are organised in groups (cysts) at ultrastructural level are connected among themselves with desmosomes (Fig. 7, 8).

Discussion

Our results which concern the ultrastructure of the new spermatogonial generation of Ohrid trout (*Salmo letnica* K a r.) correspond with the findings of other authors, as well as our previous results which concern the same or other teleost species [7, 8, 9].

The ultrastructural characteristics of the spermatogonial population with Salmo gairdneri was described by H u r k et al. [4]; S c o t t & S u m p t e r [4] and generally about the trout by L o i r [5].

Our investigations of the spermatogonial population in Ohrid trout (*Salmo letnica* K a r.) point to intensive mitotic divisions of spermatogonia of type B, especially in the period of regeneration.

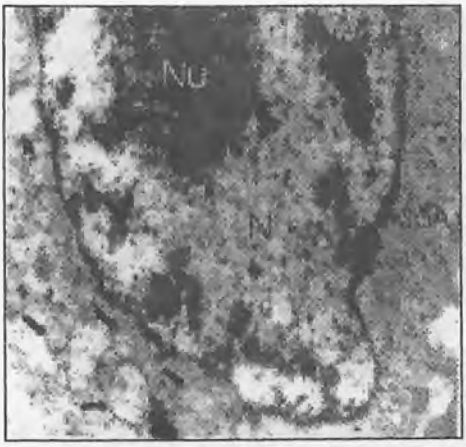

Fig. 1. A part of spermatogonium of type A (SpA) with prominent nucleus (N), nucleolus (Nu) and "nuages" particles with perinuclear location (arrows). Ultrathin section (× 12 000)

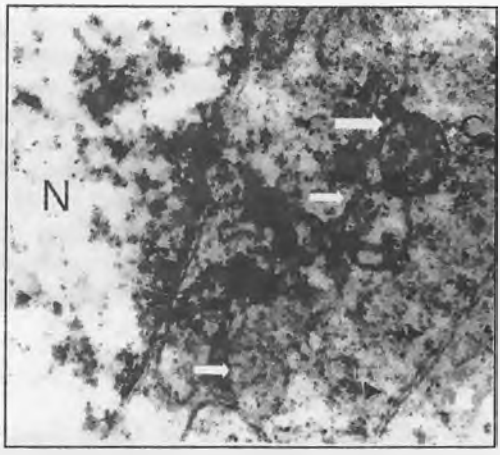

Fig. 2. A part of nucleus (N) and darker cytoplasm (C) of young spermatogonium. Mitochondria (arrows) with electron dense matrix and tubular crusts in the phase of formation, ribosomes and poluribosomes (small arows). Ultrathin section (× 20 000)

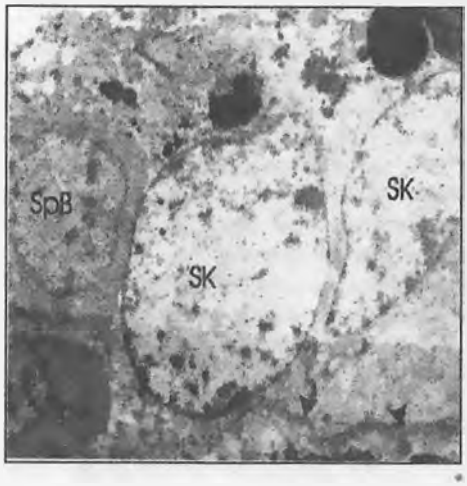

Fig. 3. Two Sertoli cells (SK) in degeneration. Two spermatogonia of type B (SpB). Visible basal lamina of the lobule (smal arrows). Ultrathin section (× 4 400)

Fig. 4. A part of nucleus (N) and cytoplasm (C) of two young spermatogonia with mitochondria (arrows) and "lamelae anulate" (small arrows). Ultrathin section (× 12 000)

In *Cyprinus carpio* L. an intensive mitosis of spermatogonia of type B was noticed by B i l l a r d et al. [1].

In the cytoplasm of this salmonid fish from Ohrid Lake presence of "nuages" particles with perinuclear location were noticed, which is characteristic material for the germinal cells.

The structure of "nuages" particles during the differentiation of spermatogonia in Oryzias latipes was investigated by C l e r o t [2] and H a m a g u c h i [3].

Also, these particles with the spermatogonial population of Dojran perch (*Perca fluviatilis macedonica* K a r.) were noted by Tavciovska-Vasileva (1992, 1994).

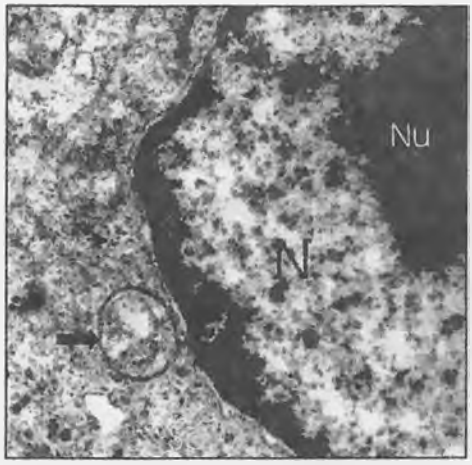

Fig. 5. A part of young spermatogonium (Sp) with nucleus (N) and prominent nucleolus (Nu). In the cytoplasm presence of ribosomes and polyribosomes (small arrows), mitochondrium with lamelar crusts which are in the phase of formation (big arrow). Ultrathin section (× 20 000)

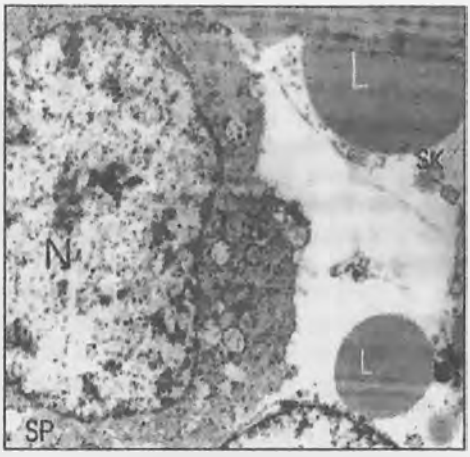

Fig. 6. Young spermatogonium (Sp) with visible nucleus (N) and darker cytoplasm (C) with mitochondria (small arrows). A part of Sertoli cell (SK) in degeneration with big lipids (L). Ultrathin section (× 7 000)

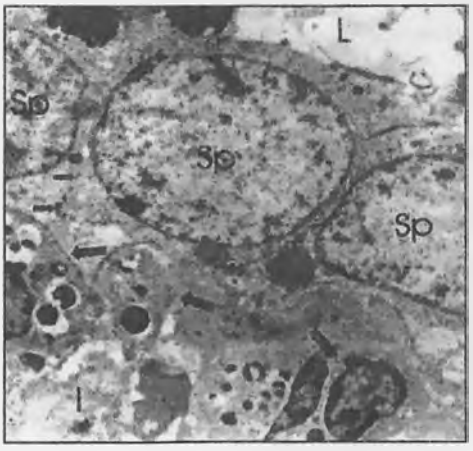

Fig. 7. Spermatogonia (Sp) connected among themselves with desmosomes (small arrows). Presence of polymorphonuclears (neutrophils) (big arrows) in the interstitium (I). Visible lumen (L) of the lobule. Ultrathin section (× 4 400).

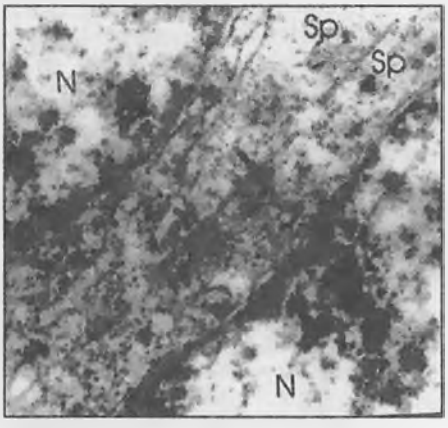

Fig. 8. A part of two spermatogonia (Sp) connected with desmosomes (arrows). Visible nuclei (N). Ultrathin section (× 3 000)

Conclusions

The ultrastructural characteristics of spermatogonial population of Ohrid trout (*Salmo letnica* K a r.) in the postspawning period can be defined like this:

1. "Nuages" particles with perinuclear arrangement can be noticed in the cytoplasm of spermatogonia of type A.

2. The young spermatogonia of type B possess darker cytoplasm in which mitochondria with with electron dense matrix and lamelar crusts which are in the phase of formation, can be noticed.

3. In the cytoplasm of some spermatogonia "lamelae anulate", a lot of ribosomes and polyribosomes can be noticed, which points to an intensive synthesis of proteins which is caracteristic of the young cells which grow up.

4. The young spermatogonia have a clearly seen nucleus with prominent nucleolus.

5. The spermatogonia which are organized in cysts are connected among themselves with desmosomes.

References

1. B i l l a r d, R., C. W e i l, K. B i e n i a r z, T. M i k o l a j c z y k, B. B r e t o n, P. E p l e r, M. B o n g o u s s a. Testicular and some hormonal changes during the first four years of life in the mirror carp, *Cyprinus carpio L.* — J. Fish Biology, **41** (3), 1992, No 3, 473-487.
2. C l e r o t, J. C. Les groupements mitochondriaux des cellules germinales des poissons Teleosteens Cyprinides. I. Etude ultrastructurale. — J. Ultrastruct. Res., **54**, 1976, No 3, 461-475.
3. H a m a g u c h i, S. The structure of the germinal dense bodies (nuages) during differentiation of the male germ line of the Teleost, *Oryzias latipes.* — Cell Tiss. Res., **248**, 1987, 375-380.
4. H u r k, R. V a n d e n, J. P e u t e, J. A. J. V e r m e j. Morphological and enzyme cytochemical aspects of the testis and vas deferens of the rainbow trout, Salmo gairdneri. — Cell Tissue Res., **186**, 1978, 309-325.
5. L o i r, M. Trout Sertoli and germ cell in primary culture: I. Morphology and ultrastructural study. — Gamete Res., **24**, 1989, 151-169.
6. S c o t t, A. P., J. P. S u m p t e r. Seasonal variations in testicular germ cell stages and plasma concentration of sex steroids in male rainbow trout (*Salmo gairdneri*) maturing at 2 years old. — Gen. Comp. Endocrinol., **73**, 1989, 46-58.
7. T a v c i o v s k a-V a s i l e v a, I. Histološka struktura na semenikot na dojranskata perkija (*Perca fluviatilis macedonica* K a r.) vo periodot po mrestenjeto. — Magisterski trud, Skopje, 1992.
8. T a v c i o v s k a-V a s i l e v a, I. Spermatogonijalna degeneracija kako propraten fenomen na spermatogenezata vo postmrestitelniot period kaj dojranskata perkija (*Perca fluviatilis macedonica* K a r.). — God. Zb., Biol., Skopje, **47**, 1994, 189-198.
9. T a v c i o v s k a-V a s i l e v a, I. Komparativni strukturni i ultrastrukturni karakteristiki na semenicite kaj *Salmonidae* (Pisces: Teleostei) od Ohridskoto Ezero vo postmrestitelniot period. — Doktorska disertacija, Skopje, 1999.

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Tetrazolium Salts Method for the Histochemical Demonstration of Aminopeptidase A

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A new substrate for aminopeptidase A (APA; EC 3.4.11.7) — L-Glutamic acid-1-(1-hydroxy-4-naphthylamide) (Glu-HNA) is synthesized and applied for the histochemical localization of the enzyme by a tetrazolium salt procedure with Nitro Blue Tetrazolium (NBT) or Tetra Nitro Blue Tetrazolium (TNBT) as visualization agents. Using the newly developed tetrazolium method, the enzyme is successfully demonstrated in tissue sections from rat organs. The histochemical technique proposed here escapes the disadvantages of the diazonium salts method and permits the precise localization of the enzyme in all the organs studied.

Key words: Aminopeptidase A, Tetrazolium salts method, Enzyme histochemistry

Introduction

Aminopeptidase A (Angiotensinase A; APA, EC 3.4.11.7) is a membrane-associated enzyme, hydrolyzing α-glutamyl or α-aspartyl residues from the N-terminal of different peptides, e.g. angiotensins. The enzyme activity level depends on the presence of calcium ions and is optimal at pH 7.6. Formerly, APA has been identified by G l e n n e r and F o l k [5]. Its activity distribution in the kidney of different mammalian species has been thoroughly studied by Kugler [7, 8, 9, 10, 11] and in the other mammalian organs — by L o j d a and G o s s r a u [12]. The APA activity is usually demonstrated using α-Glu-2-naphthylamide or α-Glu-4-methoxy-2-naphthylamide as substrates according to the azo-dye method with different diazonium salts (Fast Blue B, freshly hexazotized pararosaniline or new fuchsin) as visualization agents [12, 11, 9]. Though the tetrazolium salts are generally considered to be better auxiliary reagents than the diazonium salts [13], no tetrazolium method for the demonstration of APA has been proposed thus far.

Previously, we introduced a new tetrazolium principle for the histochemistry of aminopeptidases, based on newly designed substrates, bearing 1-hydroxy-4-naphthylamine (HNA) as a chromogenic leaving group. The method was successfully

applied for the histochemical visualization of dipeptidyl peptidase IV and gamma-glutamyl transpeptidase [2, 3]. In the present study we describe the synthesis of L-glutamic acid-1-(1-hydroxy-4-naphthylamide) (Glu-HNA) and its application as a new substrate for the histochemical demonstration of APA according to the tetrazolium salts principle.

Material and Methods

Synthesis of the substrate

The synthesis of 1-hydroxy-4-naphthylamine (HNA) was performed by the reduction of 4-sulfophenyl-azo-4-hydroxynaphthalene (the azo dye Orange I) with sodium hydrosulfite after [4]. The protected amino acid Z-Glu-OtBu (purchased from Bachem, Heidelberg, Germany) and HNA were coupled by the dicyclohexyl carbodiimide method [1] using N-ethylmorpholine as a hydrogen chloride acceptor in an inert atmosphere (argon). The tert-butyl-ester protection of the amino acid was cleaved using 6 N hydrogen chloride in dioxan and, after that, the Z-protection was cleaved by a catalytic hydrogenation in ethanol/ HCl with Palladium on activated carbon as a catalyst. Thus, the APA substrate — L-Glu-1-(1-hydroxy-4-naphthylamide) (Glu-HNA) was obtained as a hydrochloric salt.

Tissue treatment and incubation media

Mature Wistar rats of both sexes were killed by decapitation under ether anaesthesia and pieces of different organs (kidney, liver, heart and jejunum) were frozen immediately in liquid nitrogen. Ten micrometers thin sections were prepared on a cryotome 2800N, Reichert Jung, Nussloch, Germany at -20°C. The sections were mounted on glass slides. Freeze-dried sections from the liver were also prepared and mounted on albuminized glass slides. Then, all the sections were fixed in acetone for 5 min at -20°C, air-dried and incubated. The incubation solution consisted of 1 mM substrate (Glu-HNA), 2 mg/ml tetrazolium salt — NBT (Fluka, Switzerland) or TNBT (Serva, Heidelberg, Germany) and 10 mM calcium dichloride, all dissolved in 0.1 M cacodylate buffer, pH 7.6. The substrate and the tetrazolium salt were predissolved in minimal amounts of dimethylformamide. The substrate medium was filtrated before use. The incubation was performed for 20 to 45 minutes at 37°C. After that, the sections were fixed in neutral formaldehyde and embedded in glycerol-jelly.

Control sections were prepared in the same way, but incubated in the lack of substrate (only in buffer, containing the tetrazolium salt).

Results

Control sections did not show any non-specific diformazan precipitations.

The APA substrate — Glu-HNA was hydrolyzed quickly by the enzyme, so that an incubation time of 20—45 minutes was enough to reveal the enzyme locations in all the organs studied. The 1-hydroxy-4-naphthylamine, released upon the enzyme hydrolysis, reduced immediately the tetrazolium salt to give deeply coloured diformazan deposits exactly on the APA locations. The enzyme visualization was equally good with both tetrazolium salts. In the case of NBT, the

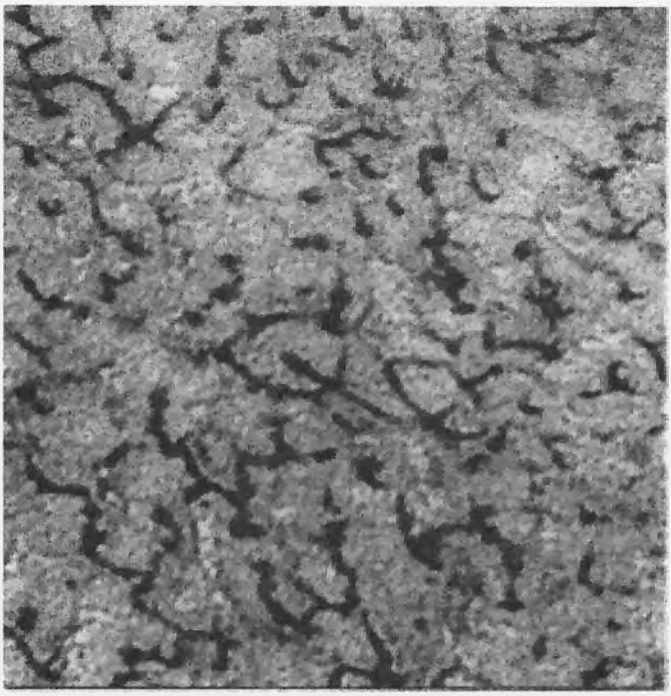

Fig. 1. APA in the rat liver. Glu-HNA and NBT. Freeze-dried, acetone fixed section. Diformazan deposits in the bile capillaries (× 500)

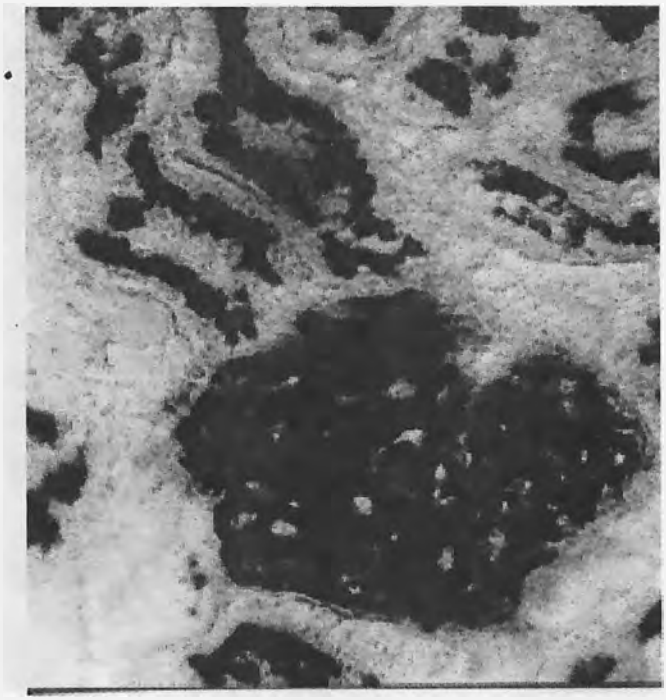

Fig. 2. APA in the kidney. Glu-HNA and NBT. Fresh-frozen acetone fixed sections. Reaction for APA in the brush borders of the convoluted tubules, glomerular endothelium and podocytes (× 500)

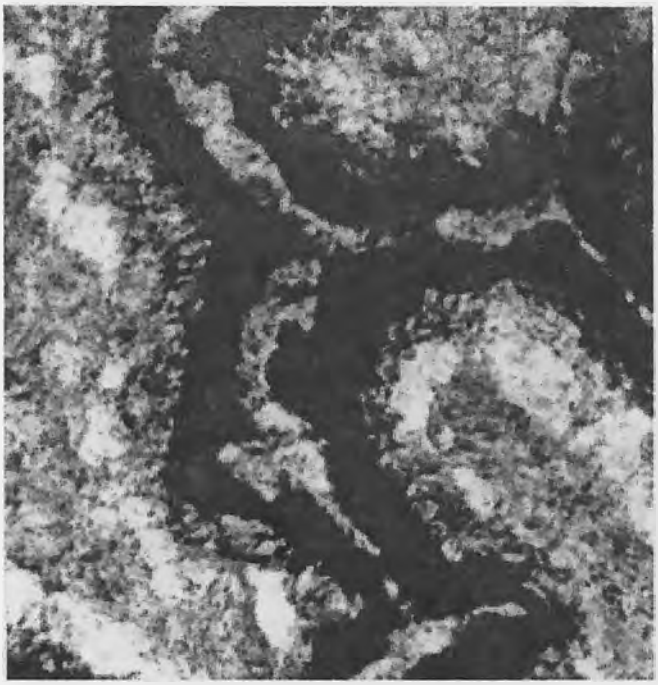

Fig. 3. APA in the jejunum. Glu-HNA and NBT. Fresh-frozen acetone-fixed section. High APA activity in the brush borders of the entherocytes (× 500)

Fig. 4. APA in the heart muscle. Glu-HNA and NBT. Fresh-frozen acetone-fixed section. Diformazan deposits in the heart capillaries (× 500)

diformazan precipitate was bluish-violet in colour. When TNBT was used, the diformazan was reddish-brown.

Good results were obtained in non-freeze-dried sections from all the organs except the liver, for which the freeze-drying appeared to be obligatory. In the liver, the final reaction product (the NBT or TNBT diformazan) was deposited in the bile capillaries (Fig. 1). In the kidney, APA activity was visualized in the brush borders of the convoluted renal tubules as well as in the glomerular endothelial cells and podocytes (Fig.2). In the jejunum, the brush borders of the enterocytes were highly APA-positive (Fig.3). In the myocardium, the diformazan precipitates were visible on the capillary endothelium (Fig.4).

Discussion

Aminopeptidase A is widely distributed in mammalian organs and tissues, where it plays an important role in peptide metabolism (for review see [12]). For example, it is present in the brush borders of the small intestinal enterocytes and proximal renal tubules, where an active absorption take s place. Furthermore, the enzyme is found in the capillary endothelium of every organ studied and is considered a marker enzyme for capillary bed endothelium [6, 12]. The only method for the histochemical determination of APA still remains the azo-coupling procedure, though it suffers a lot of drawbacks, caused by the use of diazonium salts as visualization agents. The diazonium salts are enzyme inhibitors. They decompose especially at pH above 7, not permitting the incubation at the optimal pH of the membrane-bound alkaline peptidases. In contrast, the tetrazolium salts are known to inhibit the enzymes to a much lesser extent [13]. The ditetrazolium salts, such as NBT and TNBT are very convenient for use in alkaline media. However, no tetrazolium method for APA has been proposed thus far. Here we propose the first tetrazolium method for APA, based on the new substrate L-Glu-1-(1-hydroxy-4-naphthylamide). The substrate is readily hydrolyzed by the enzyme to release a highly active reducing agent — 1-hydroxy-4-naphthylamine, which reduces quickly and quantitatively NBT or TNBT to the respective diformazans. The last compounds, being highly water- and buffers insoluble, precipitate on the enzyme locations, marking them accurately by a deep colour (bluish-violet or reddish-brown). A very important advantage of the method is that it permits incubation at the optimum pH of the enzyme (7.6). Thus, the new method escapes the disadvantages of diazonium salts methods and allows the precise localization of the enzyme.

With the present study, we expand our work on the new tetrazolium principle for the histochemical visualization of aminopeptidases, introduced recently by us [2, 3].

Acknowledgement. This work was supported by Deutsche Forschungs-gemeinschaft, Bonn — Germany.

References

1. Bodanszky, M., A. Bodanszky. The dicyclohexylcarbodiimide method. — In: The practice of peptide synthesis. Berlin, Academie Verlag, 1985, 143-144.
2. Dikov, A., M. Dimitrova, I. Stoineva, K.-J. Halbhuber. New tetrazolium method for the histochemical localization of dipeptidyl peptidase IV. — Cell. Mol. Biol., 45, 1999, 225-232.
3. Dikov, A., M. Dimitrova, R. Krieg, K.-J. Halbhuber. New tetrazolium method for the his-

tochemical demonstration of gamma-glutamyl transpeptidase. — Cell. Mol. Biol., **45**, 1999, 241-248.
4. F i e s e r, L. F. 1,4-Aminonaphthol hydrochloride. — Org.Synth.Coll. **II**, 1943, 39-41.
5. G l e n n e r, G. G., J. E. F o l k. Glutamyl peptidases in rat and guinea pig kidney slices. — Nature (London), **192**, 1961, 338-340.
6. G o s s r a u, R. Cytochemistry of membrane proteases. — Histochem. J., **17**, 1985, 737-771.
7. K u g l e r, P. Localization of aminopeptidase A (Angiotensinase A) in the rat and mouse kidney. — Histochemistry, **72**, 1981, 269-278.
8. K u g l e r, P. Aminopeptidase A is Angiotensinase A. I. Quantitative histochemical studies in the kidney glomerulus. — Histochemistry, **74**, 1982, 229-245.
9. K u g l e r, P. Ultracytochemistry of aminopeptidase A (Angiotensinase A) in the kidney glomerulus and juxtaglomerular apparatus. — Histochemistry, **74**, 1982, 199-212.
10. K u g l e r, P. Angiotensinase A in the renomedullary interstitial cells. — Histochemistry, **77**, 1983, 105-115.
11. K u g l e r, P., G.Wolf, J. Scherberich. Histochemical demonstration of peptidases in the human kidney. — Histochemistry, **83**, 1985, 337-341.
12. L o j d a, Z., R. Gossrau. Study on aminopeptidase A. — Histochemistry, **67**, 1980, 267-290.
13. L o j d a, Z., E. Havrankova. The histochemical demonstration of aminopeptidase with bromoindolyl leucinamide. — Histochemistry, **43**, 1975, 355-366.

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Glycyl-L-Prolyl-1-Anthraquinonyl hydrazide — a New Substrate for the Histochemical Detection of Dipeptidyl Peptidase IV

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Glycyl-L-Prolyl-1-anthraquinonylhydrazide (Gly-Pro-AH) was synthesized and used as a new substrate for the histochemical detection of dipeptidyl peptidase IV (DPP IV). The enzyme hydrolysis liberates the insoluble dark red 1-anthraquinonyl hydrazine, which marks the enzyme sites. After a post-incubation with some aromatic aldehyde in an acid medium, 1-anthraquinonyl hydrazine is converted to the respective hydrazone. The most convenient aromatic aldehyde is selected to give a deeply colored and amorphous hydrazone, thus improving the histochemical picture considerably. The new method is used to demonstrate the enzyme in tissue sections from different rat organs. It is found to allow an accurate DPP IV localization in all its locations.

Keywords: Dipeptidyl peptidase IV, 1-anthraquinonyl hydrazine, hydrazone formation, enzyme histochemistry.

Introduction

Dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) — a serine type aminoexopeptidase [3] is found in the cell surface membranes in many tissues [8, 12]. It cleaves aminoacyl-Pro-dipeptides from the amino-terminal of oligopeptides at pH optimum 7.8. DPP IV is identical to T-cell activation marker CD 26 [19]. Presently, this enzyme is a subject of a vast interest, due to the variety of biological functions it has in the organism, such as digestion of natural polypeptides [17] and collagen [1], activation or inactivation of peptide hormones [16], activation of T- and B-lymphocytes [18, 20], etc.

Histochemically, DPP IV is usually determined using Gly-L-Pro-4-methoxy-2-naphthylamide as a substrate with either diazonium salts according to the azo-coupling method [9, 13] or 5-nitrosalicylaldehyde as a fluorogenic reagent [10, 11]. However, the above methods suffer a lot of drawbacks [12]. Recently, we developed a convenient chromogenic method for the histochemical detection of DPP IV, based on the substrate Gly-L-Pro-1-hydroxy-4-naphthylamide and tetrazolium salts as visualization reagents [4].

In the present paper, we propose a new chromogenic substrate for DPP IV — Gly-L-Pro-1-anthraquinonyl hydrazide (Gly-Pro-AH), which we use to localize the enzyme in tissue sections of different rat organs. The new technique is developed to escape most of the disadvantages of the methods in use and to provide a tool for the precise determination of the enzyme.

Material and Methods

Synthesis of the substrate

1-Anthraquinonyl hydrazine was synthesized from 1-chloroanthraquinone (Merck, Darmstadt, Germany) and hydrazine in pyridine as described by Moehlau [15]. Boc-Gly-L-Pro-OH (Bachem, Heidelberg, Germany) was coupled to 1-anthraquinonyl hydrazine by the dicyclohexylcarbodiimide method after [2]. The Boc-protective group of Gly was cleaved with hydrogen chloride in dioxane to obtain the substrate Gly-L-Pro-1-anthraquinonyl hydrazine (Gly-Pro-AH) as a hydrochloric salt.

Tissue treatment and incubation media

Mature Wistar rats of both sexes were killed by decapitation and pieces of different organs were immediately frozen in liquid nitrogen. Tissue sections, 10 μm thin, were cut on cryotome 2800M Reichert-Jung, Nussloch, Germany. They were mounted on glass slides and fixed in acetone for 5 min at —20°C or, otherwise, they were freeze-dried, mounted on albuminized glass slides and covered by 1 % celloidin for a minute at room temperature. All the sections were incubated in a substrate medium, containing 0.5 mM substrate (Gly-Pro-AH), pre-dissolved in a minimal amount of dimethyl formamide, and 0.1 M Tris/HCl buffer or phosphate buffer, pH 7.8. The incubation was carried out at 37°C for 20 to 60 min (longer incubation was necessary for the celloidin-embedded sections). After that, the sections were transferred into a second incubation medium, consisting of acetate buffer, pH 4.5, and 1 mg/ml aldehyde — 4-dimethylaminobenzaldehyde, 4-methoxybenzaldehyde, 4-nitrobenzaldehyde or 4-diethylaminobenzaldehyde. The second incubation was performed at room temperature for 4 to 6 h (or, better, overnight). Then, the sections were post-fixed in neutral formaline and embedded in glycerol-jelly.

Control sections were prepared in the same manner, but heated to 80°C for 10 min in water before the incubation (thermally inactivated controls). Other controls, incubated only in buffered aromatic aldehyde at pH 4.5 were also prepared.

Results

Thermally inactivated controls as well as the controls, incubated only in aromatic aldehyde solutions, were free of any non-specific staining.

The newly synthesized DPP IV substrate — Gly-Pro-AH was hydrolyzed readily by the enzyme to release 1-anthraquinonyl hydrazine. The last compound, being practically insoluble in aqueous media, precipitated on the sites of the enzyme activity, marking them precisely by a deeply red color. In the acetone fixed sections, the hydrazine deposits were grainy to microcrystalline. Much better results for all the organs studied were obtained in the freeze-dried celloidin-mounted sections,

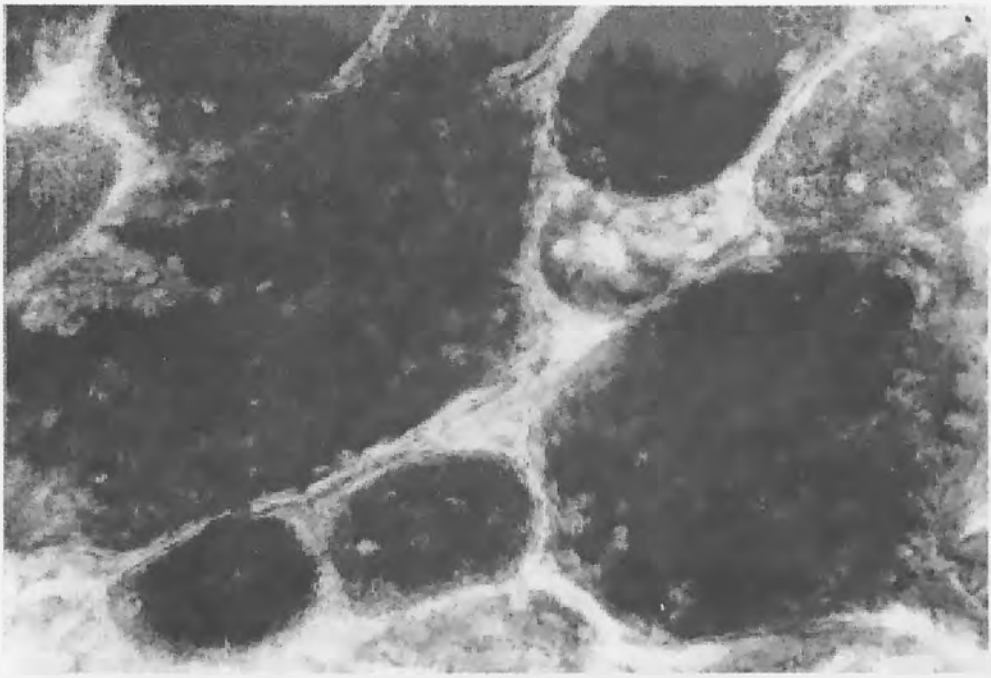

Fig.1. DPP IV in the kidney. Gly-L-Pro-AH and 4-dimethylaminobenzaldehyde. Reaction product in the glomeruli and in proximal renal brush border (× 500)

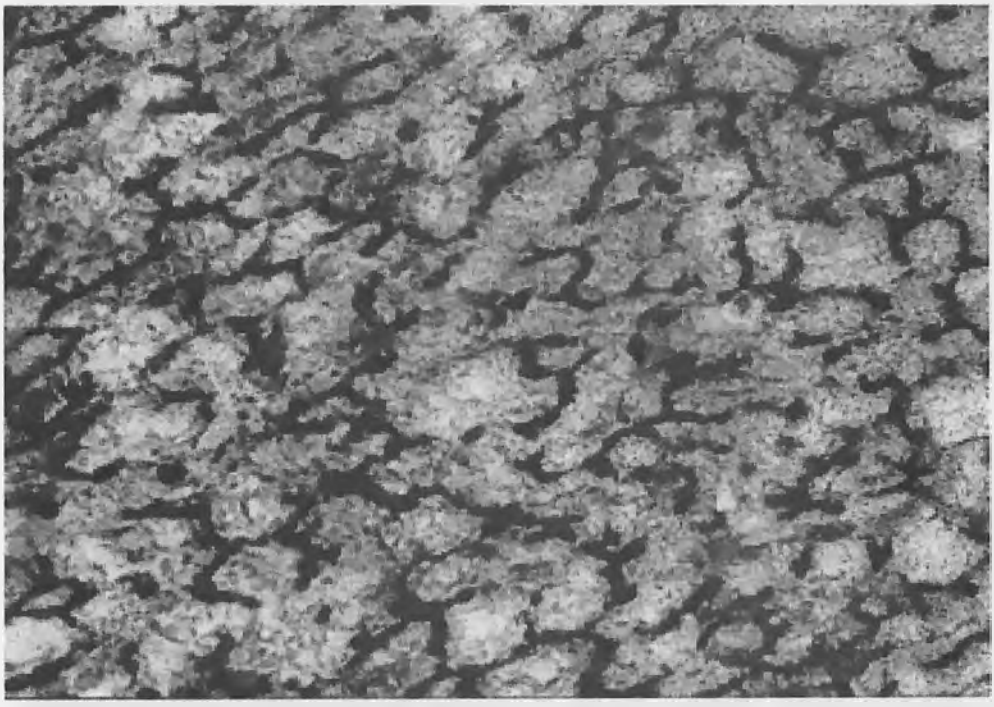

Fig.2. DPP IV in the liver. Gly-L-Pro-AH and 4- dimethylaminobenzaldehyde. High enzyme activity in the bile capillaries and sinus endothelial cells (× 500)

where the hydrazine deposits were amorphous. Better results were obtained using Tris/HCl buffer. As it was described above, the sections were transferred into acetate buffer, supplied with an aromatic aldehyde for the post-coupling procedure. During the second incubation, 1-anthraquinonyl hydrazine was converted into hydrazone, which color was different, depending on the aromatic aldehyde employed: dark blue with 4-dimethylaminobenzaldehyde, dark violet with 4-methoxybenzaldehyde, red-orange with 4-nitrobenzaldehyde and blue to black with 4-diethylaminobenzaldehyde. The last two aldehydes were rejected for further use, since with them granular to crystalline hydrazones were formed. In contrast, 4-methoxybenzaldehyde and 4-dimethylaminobenzaldehyde were found appropriate, because they gave finely amorphous and very dark in color hydrazones with 1-anthaquinonyl hydrazine. The histochemical contrast was much improved by this conversion. In the kidney (Fig.1), a high DPP IV activity was visualized in the proximal renal brush border and in renal corpuscles. In the liver (Fig. 2), the final product was visible in bile capillaries as well as in sinus endothelial cells. In the epididymis (Fig. 3), it was observed in stereocilia and apical parts of the duct epithelial cells. In the seminal vesicles (Fig. 4), DPP IV was highly active in the epithelial cells.

Discussion

Recently, it is widely recognized, that the mostly used method for the histochemical detection of DPP IV — the azo-coupling method — suffers a lot of disadvantages. Diazonium salts are powerful enzyme inhibitors. They decompose in the incubation media, especially at pH > 7.0, thus causing precipitation artifacts and not permitting the incubation at the optimal pH of the enzymes [12]. In a previous study [4] we introduced a tetrazolium method for the histochemical detection of DPP IV. Tetrazolium salts are known to inhibit the enzymes to a much lesser extent than the diazonium salts [14]. No histochemical method for DPP IV without auxiliary reagent in the incubation solution is still available.

We recently established the use of 1-anthraquinonyl hydrazine—based substrates in the histochemistry of peptidases. Using such substrates we managed to localize dipeptidyl peptidases I and II (DPP I, II) and tripeptidyl peptidase I (TPP I), the last enzyme had not been demonstrated histochemically before that [5, 6, 7]. Though the above substrates are hydrazides, not amides, they are readily hydrolyzed by peptidases. With the present work we expand the newly developed method to the demonstration of a membrane-associated peptidase. For the lysosomal enzymes, which have acidic pH optima, the second reaction step — the conversion of 1-anthraquinonyl hydrazine into hydrazone with some aromatic aldehyde, can be performed in the incubation medium (simultaneous coupling). The DPP IV optimal pH (7.8) does not permit a hydrazone production in a single step. Thus, a second incubation is needed. Anthraquinonyl hydrazine is highly water- and buffers- insoluble and almost amorphous in celloidin, but its color is not deep enough and it tends to form crystals upon a time. If it is converted to a hydrazone, the precipitations become very deeply colored, perfectly amorphous and do not change upon storage. We made a screening of several aromatic aldehydes to choose the most convenient one for the hydrazone formation step. The aldehydes of choice proved to be 4-methoxybenzaldehyde and 4-dimethylaminobenzaldehyde. The second incubation step proposes more advantages, since no auxiliary reagents are present in the incubation medium. Without the use of a second reagent one escapes all the disadvantages of the azo-coupling and tetrazolium methods (see above). Using the new method, we localized precisely DPP IV activity in all the organs studied.

Fig.3. DPP IV in the epididymis. Gly-L-Pro-AH and 4- dimethylaminobenzaldehyde. Hydrazone final reaction product in the stereocilia and the apical part of duct epithelial cells (× 500)

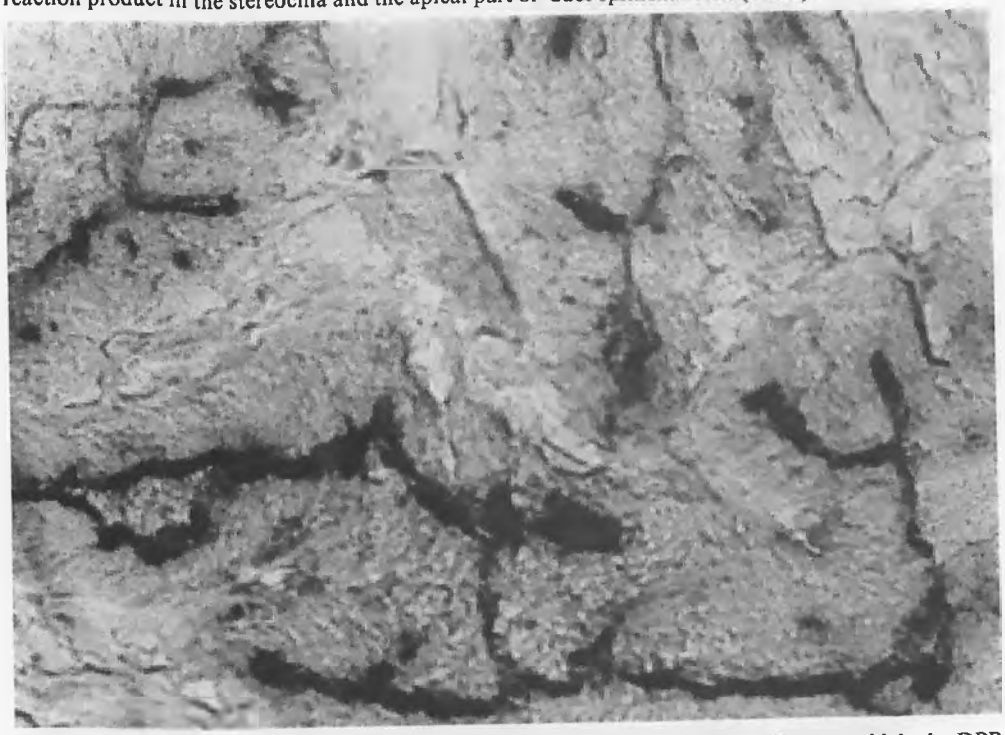

Fig.4. DPP IV activity in the seminal gland. Gly-L-Pro-AH and 4- dimethylamino-benzaldehyde. DPP IV activity in the seminal gland epithelial cells (× 500)

The novel histochemical method, proposed here, proved to be very appropriate for the visualization of the membrane-associated alkaline peptidase DPP IV. The synthesis of other aminopeptidases substrates, based on 1-anthraquinonyl hydrazine are in progress.

Acknowledgment. This work was supported by Deutsche Forschungsgemeinschaft, Bonn — Germany.

References

1. Bermpohl, F., K. Loester, W. Reutter, O. Baum. Rat dipeptidyl peptidase IV (DPP IV) exhibits endopeptidase activity with specificity for denatured fibrillar collagens. — FEBS Lett., **428**, 1998, 152-156.
2. Bodanszky, M., A. Bodanszky. — In: The practice of peptide synthesis. Academie-Verlag Berline, 1985, pp.182.
3. David, F., A. M. Bernard, M. Pierres, D. Marguet. Identification of serine 624, aspartic acid 702 and histidine 734 as the catalytic triad residues of mouse dipeptidylpeptidase IV (CD 26). A member of a novel family of non-classical serine hydrolases. — J.Biol.Chem., **268**, 1993, 17247-17252.
4. Dikov, A., M. Dimitrova, I. Stoineva, K.-J. Halbhuber. New tetrazolium method for the histochemical localization of dipeptidyl peptidase IV. — Cell.Mol.Biol., **45**, 1999, 225-231.
5. Dikov, A., I. Ivanov, M. Dimitrova, R. Krieg, K.-J. Halbhuber. New method for the histochemical demonstration of dipeptidyl aminopeptidase I activity using a novel anthraquinonyl hydrazide substrate. — Cell.Mol.Biol., **45**, 1999, 1229-1235.
6. Dikov, A., M. Dimitrova, I. Ivanov, R. Krieg, K.-J. Halbhuber. Original method for the histochemical demonstration of tripeptidyl aminopeptidase I. — Cell.Mol.Biol., **46**, 2000, 1219-1225.
7. Dikov, A., M. Dimitrova, T. Pajpanova, R. Krieg, K.-J. Halbhuber. Histochemical method for dipeptidyl aminopeptidase II with a new anthraquinonyl hydrazide substrate. — Cell. Mol. Biol., **46**, 2000, 1213-1218.
8. Gossrau, R. Peptidasen II. Zur Lokalisation der Dipeptidylpeptidase IV (DPP IV). Histochemische und biochemische Untersuchung. — Histochemistry, **60**, 1979, 231-248.
9. Gossrau, R. Investigation of proteinases in the digestive tract using 4-methoxy-2-naphthylamine (MNA) substrates. — J. Histochem. Cytochem., **29**, 1981, 464-480.
10. Gossrau, R. Fluorescence histochemical detection of hydrolases in tissue sections and culture cells. — Histochemistry, **79**, 1983, 87-94.
11. Gossrau, R. Cytochemistry of membrane proteases. — Histochem.J., **17**, 1985, 737-771.
12. Hartel, S., R. Gossrau, C. Hanski, W. Reutter. Dipeptidylpeptidase IV (DPP IV) in rat organs. Comparison of immunohistochemistry and activity histochemistry. — Histochemistry, **89**, 1988, 151-161.
13. Kugler, P., G. Wolf, J. Scherberich. Histochemical demonstration of peptidases in the human kidney. — Histochemistry, **83**, 1985, 337-341.
14. Lojda, Z., E. Havrankova. The histochemical demonstration of aminopeptidase with bromoindolyl leucinamide. — Histochemistry, **43**, 1975, 355-366.
15. Moehlau, R. Ueber eine neue Synthese der Anthrachinonyl-hydrazin. — Chem.Ber., **45**, 1912, 2244-2247.
16. Nausch, I., R. Mentlein, E. Heymann. The degradation of bioactive peptides and proteines by dipeptidyl peptidase IV from human placenta. — Hoppe-Seyler Biol. Chem., **371**, 1990, 1113-1118.
17. Neymann, E., R. Mentlein. Beeinflußt Dipeptidylpeptidase IV Blutdruck und Gerinnung? — Klin.Wchenschr., **61**, 1984, 2-10.
18. Schoen, E., H.-U. Demuth, E. Eichmann, H.-J. Horst, I.-J. Korner, J. Kopp, T. Mattern, K. Neubert, F. Noll, A. J. Ulmer, A. Barth, S. Ansorge. Dipeptidyl peptidase IV in human T-lymphocytes: impaired induction of interleukin 2 and gamma interferon due to specific inhibition of dipeptidyl peptidase IV. — Scand. J. Immunol., **29**, 1989, 127-135.
19. Ulmer, A. J., T. Mattern, A. C. Feller, E. Heymann, H.-D. Flad. CD 26 antigen is a surface dipeptidyl peptidase IV (DPP IV) as characterized by monoclonal antibodies clone TII-19-4-7 and 4EL1-C7. — Scand.J.Immunol., **31**, 1990, 429-443.
20. Wesley, U. V., A. P. Albino, S. Tiwari, A. N. Houghton. A role for dipeptidyl peptidase IV in suppressing the malignant phenotype of melanocytic cells. — J. Exp. Med., **190**, 1999, 311-322.

Institute of Experimental Morphology and Anthropology

Acta morphologica et anthropologica, 8
Sofia • 2003

A Novel Anthraquinonyl Hydrazine — Based Substrate for the Histochemical Detection of Gamma-Glutamyl Transpeptidase

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A new substrate for gamma-glutamyl transpeptidase (GTP; EC 2.3.2.2) — L-Glu-4-(1-anthraquinonyl hydrazide) is synthesized and applied for the histochemical detection of the enzyme in tissue sections from rat and human organs. The enzyme hydrolysis liberates the water insoluble deeply red 1-anthraquinonyl hydrazine, which marks the enzyme locations. The newly developed method for GTP does not use any auxiliary reagents in the incubation medium and permits the incubation to be performed at the optimal pH of the enzyme. A second reaction step — incubation in an acid medium with 4-dimethylamino benzaldehyde is performed to improve the quality of the preparations. The new method is used with success for the visualization of GTP activity in the organs studied.

Key words: Gamma-glutamyl transpeptidase, anthraquinonyl hydrazine, enzyme histochemistry.

Introduction

Gamma glutamyl transpeptidase (GTP; EC 2.3.2.2) catalyses the transfer of gamma-glutamyl moieties from different peptides, including glutathione, to other peptides, amino acids or water. It is a membrane-associated transferase with an alkaline pH optimum of about 8.2. GTP is widely distributed in mammalian organs and tissues (for review see [5]) and is found to increase its activity in some tumor cells [8, 9, 11]. Histochemically, GTP is usually demonstrated using gamma-L-Glu-2-naphthylamide or gamma-L-Glu-4-methoxy-2-naphthylamide as substrates with different diazonium salts as visualization agents according to the azo-dye method [4, 10]. Previously, we introduced a convenient tetrazolium method for the histochemical visualization of GTP [3]. However, no histochemical method without any auxiliary reagents in the incubation solution is still available for GTP.

In the present study we describe the synthesis of a novel substrate for GTP - L-Glu-4-(1-anthraquinonyl hydrazide) (Glu-AH) and its use for the histochemical detection of the enzyme in a procedure not using auxiliary reagents in the incubation medium. We give also a possibility of improvement of the histochemical picture by a second incubation step and present the localization of GTP in several rat and human organs, obtained by the newly developed method.

Material and Methods

Synthesis of the substrate

The colorful compound 1-anthraquinonyl hydrazine was synthesized by boiling 1-chloroanthraquinone (Merck, Darmstadt, Germany) and hydrazine hydrate in pyridine after M o e h l a u [7]. The 1-anthraquinonyl hydrazine was coupled with Z-L-Glu-OBzl (Bachem, Heidelberg, Germany) by the dicyclohexylcarbodiimide method [1]. The two protective groups of the amino acid (Z-group and the benzyl ester group) were cleaved simultaneously by a catalytic hydrogenation in ethanol/HCl with Pd on activated carbon as a catalyst. Thus the substrate - L-Glu-4-(1-anthraquinonyl hydrazide) (Glu-AH) was obtained as a HCl-salt.

Tissue treatment and incubation media

Mature Wistar rats of both sexes were killed by decapitation and pieces of different organs were immediately frozen in liquid nitrogen. Pieces of human kidney cortex were taken at autopsy from a person, killed in an accident, 12 hours after the death and were frozen in liquid nitrogen too. Tissue sections, 10 μm thin, were cut on cryotome 2800M Reichert-Jung, Nussloch, Germany. They were mounted on glass slides and fixed in acetone for 5 min at —20°C or, otherwise, they were freeze-dried, mounted on albuminized glass slides and covered by 1 % celloidin for a minute at room temperature. All the sections were incubated in a substrate medium, containing 1 mM substrate (Glu-AH) (pre-dissolved in a minimal amount of dimethyl formamide), 5 mM glycyl-glycine and 0.1 M Tris/HCl buffer, pH 8.2. The incubation was carried out at 37°C for 30 to 90 minutes (longer incubation was necessary for the celloidin-embedded sections). After that, the sections were transferred into a second incubation medium, consisting of acetate buffer, pH 4.5, and 1 mg/ml 4-dimethylamino benzaldehyde. The second incubation was performed at room temperature for 4 to 6 hours. Then, the sections were post-fixed in neutral formaline and embedded in glycerol-jelly.

Two types of controls were prepared — thermally inactivated control sections were heated at 80°C for 10 min in water and incubated in the complete substrate solution, whereas the other controls were incubated only in buffered 4-dimethylamino benzaldehyde at pH 4.5.

Results

Thermally inactivated controls did not show any colorful reaction product. The other controls also had no precipitation artifacts.

The substrate — Glu-AH was quickly hydrolyzed by the enzyme to release the deeply colored reaction product — 1-anthaquinonyl hydrazine, which precipitated on the sites of the enzyme activity and marked them in a red color. However, those preparations showed a tendency to distort upon storage, because the colorful precipitation in them used to become crystalline in time. So, we intended a second incubation with 4-dimethyldminobenzaldehyde in an acid medium (acetate buffer, pH 4.5), which was aimed to convert the 1-anthaquinonyl hydrazine into the respective hydrazone (4-dimethylaminobenzylidene-1-anthraquinonyl hydrazone) — a dark blue highly amorphous compound. Thus, the preparations became more contrasted and were stable on storage (permanent preparations). Good results were obtained

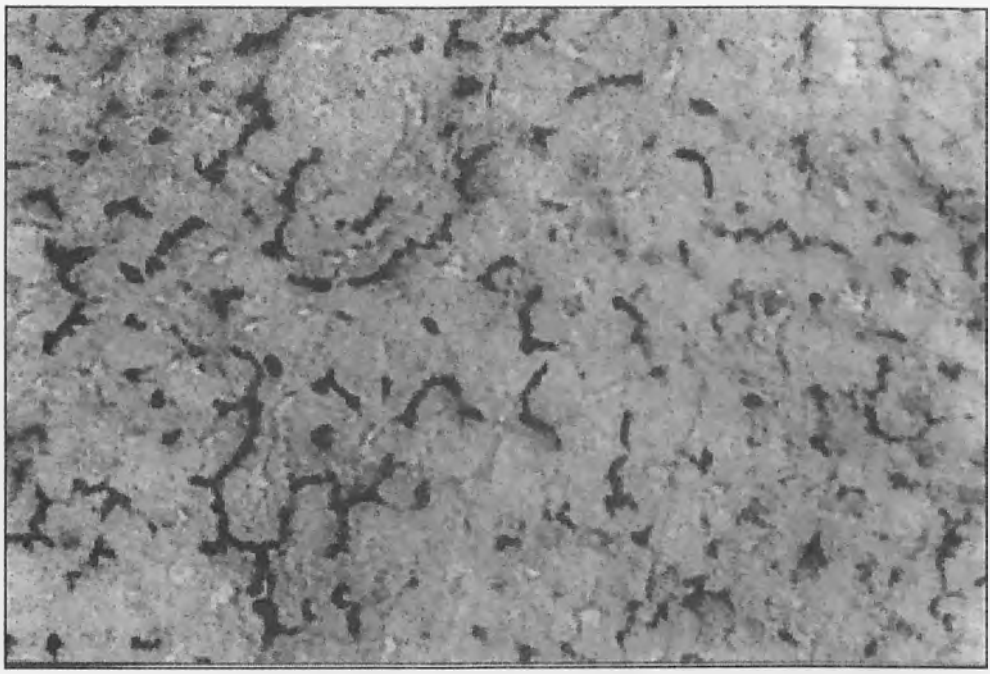

Fig. 1. GTP in the rat liver. Freeze-dried celloidin-mounted section. A small quantity of the final reaction product is visible in the bile capillaries (× 500)

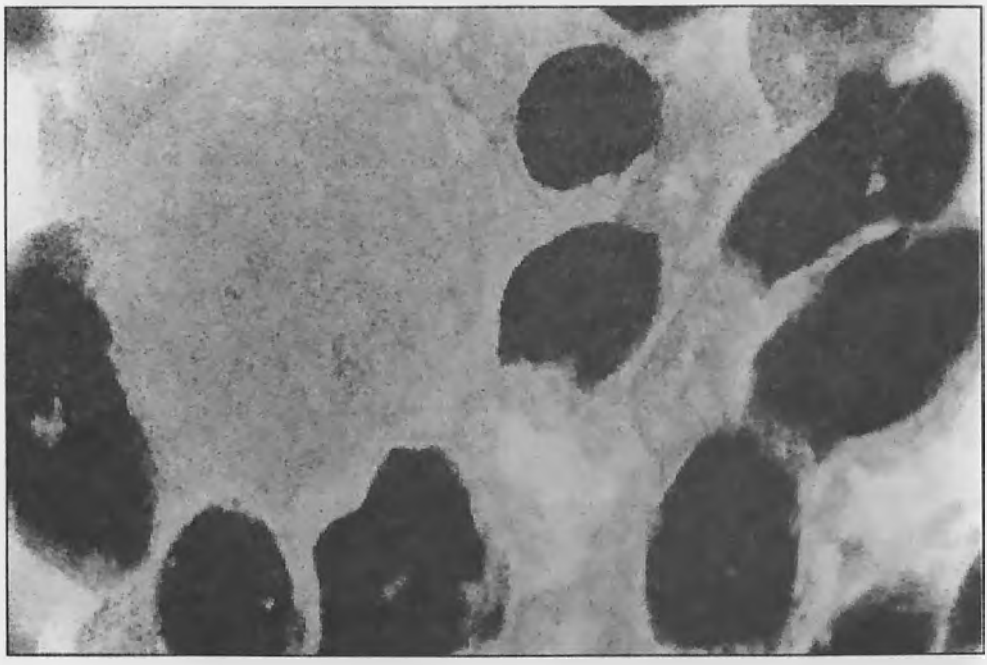

Fig. 2. GTP in the rat kidney. Fresh-frozen acetone-fixed section. A very strong reaction for GTP in the brush borders of the convoluted tubules. No reaction product in the glomeruli (× 500)

Fig. 3. GTP in the human kidney. Fresh-frozen acetone-fixed section. Large quantities of hydrazone in the brush borders and the basal membranes of the renal convoluted tubules (× 500)

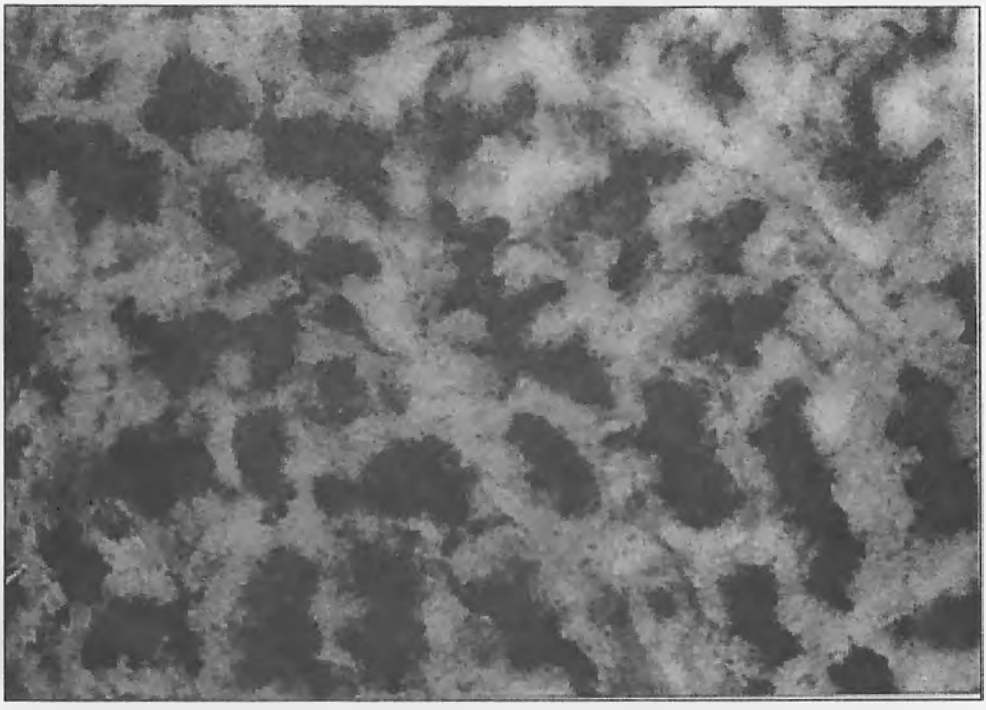

Fig. 4. GTP in the rat pancreas. Freeze-dried celloidin-mounted section. The main reaction for GTP is visible in the apical poles of the acinar cells (× 500)

in the freshly frozen acetone fixed sections for all the organs studied except for the liver and pancreas, where the results were better in the freeze-dried celloidin-mounted sections. In the liver (Fig. 1) the small quantity of the final reaction product was observed in the bile canaliculi and only single sinusoidal cells were GTP-positive. These results are in agreement with the previous findings, that GTP has a low activity in the rat liver [5]. In the rat kidney (Fig. 2) a very strong reaction for GTP was seen in the epithelial cells of the renal convoluted tubules, whereas the glomeruli were perfectly negative. In the human kidney (Fig. 3) the same pattern of GTP was observed, however, the basal membranes of the tubules were well accentuated. In the rat pancreas (Fig. 4) a considerable quantity of hydrazone was visible in the apical parts of the acinar cells. In the epididymis (Fig. 5) the enzyme activity was located in the principal cells of the tubules, including stereocilia and the sperms within the channels were also GTP-positive.

Discussion

Gamma-glutamyl transpeptidase is an enzyme of a vast scientific interest, since it plays a significant role in degradation of natural polypeptides, the most important of which are glutathione and its derivatives. Other functions of the enzyme have also been suggested (for review see [6]). However, in order to understand the multiple functions of GTP it is important to know its precise localization in mammalian organs, the main tool for that purpose still remains the activity histochemistry. Till now, the mostly used histochemical method for the visualization of GTP is the azo-dye procedure, which suffers a lot of drawbacks (for review see [2]). The most im-

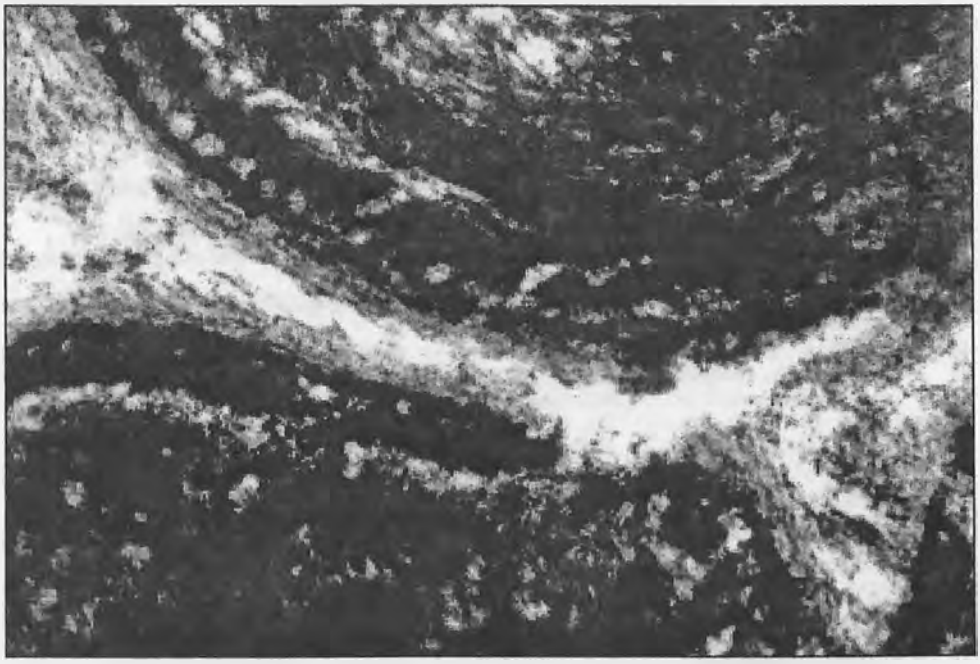

Fig. 5. GTP in the rat epididymis. Fresh-frozen acetone-fixed section. A very strong activity of GTP in the stereocilia and the whole principle cells of the channels, as well as in the spermatozoa within the channels (× 500)

portant disadvantage of this method is that the incubation can not be carried out at the optimal pH of the membrane-bound alkaline peptidases.

In the present work we propose a new method for GTP, which does not use any auxiliary reagent in the incubation medium. This point might be regarded as a very important advantage, because any additional compound in the solution may inhibit the enzyme at a certain extent (even tetrazolium salts, which are usually considered to be very weak enzyme inhibitors). Furthermore, the newly developed method permits the incubation at the optimal pH of GTP — 8.2. The novel GTP substrate — Glu-AH was readily hydrolyzed by the enzyme to release the deeply red water-insoluble 1-anthaquinonyl hydrazine, which marks the enzyme locations. We performed also a second incubation, in which 1-anthraquinonyl hydrazine is converted into a hydrazone with 4-dimethylaminobenzaldehyde, but this additional step is only for the sake of improvement of the preparations quality. The finally obtained preparations are of a very good contrast with the dark blue perfectly amorphous hydrazone. Moreover, they are permanent, i.e. they do not change upon storage.

With the newly developed method we managed to localize GTP in the same locations in the rat and human organs as it was described before. So, the new method, proposed here, is very promising for future application in other membrane-bound peptidases histochemistry as well.

Acknowledgement. This work was supported by Deutsche Forschungsgemeinschaft, Bonn — Germany.

References

1. Bodanszky, M., A. Bodanszky. The dicyclohexylcarbodiimide method. — In: The practice of peptide synthesis. Academie-Verlag Berlin, 1985, 143-144.
2. Dikov, A., R. Gossrau, H.-G. Frank. Menadiol diphosphate, a new substrate for non-specific alkaline phosphatase in histochemistry and immunohistochemistry. — Histochemistry, **94**, 1990, 217-223.
3. Dikov, A., M. Dimitrova, R. Krieg, K.-J. Halbhuber. New tetrazolium method for the histochemical demonstration of gamma-glutamyl transpeptidase. — Cell. Mol. Biol., **45**, 1999, 241-248.
4. Gossrau, R. Investigation of proteinases in the digestive tract using 4-methoxy-2-naphthylamine (MNA) substrates. — J.Histochem.Cytochem., **29**, 1981, 464-480.
5. Gossrau, R. Cytochemistry of membrane proteases. — Histochem. J., **17**, 1985, 737-771.
6. Meister, A., S. S. Tate. Glutathion and related gamma-glutamyl compounds: biosynthesis and utilization. — Ann. Rev. Biochem., **45**, 1976, 559-604.
7. Moehlau, R. Ueber eine neue Synthese der Anthrachinonyl-hydrazin. — Chem.Ber., **45**, 1912, 2244-2247.
8. Power, C. A., S. A. Griffiths, J. L. Simpson, Y. Laperche, G. Guellaen, M. M. Manson. Induction of gamma-glutamyl transpeptidase mRNA by aflatoxin B 1 and ethoxiquin in rat liver. — Carcinogenesis, **8**, 1987, 737-740.
9. Russell, J. J., E. F. Staffeldt, B. J. Wright, A. Prapuolenis, B. A. Carnes, C. Peraino. Effects of rat strain, diet composition and Phenobarbital on hepatic gamma-glutamyl transpeptidase histochemistry and on the induction of altered hepatocyte foci and hepatic tumors by diethylnitrosamine. — Canser Res., **47**, 1987, 1130-1134.
10. Ruttenburg, A. M., H. Kim, J. W. Fishbein, J. S. Hanker, A. L. Wassercrug, A. M. Seligman. Histochemical and ultrastructural demonstration of gamma-glutamyl transpeptidase activity. — J. Histochem. Cytochem., **17**, 1969, 517-526.
11. Sepulveda, A. R., G. M. Habib, A. Damjanov, S. Matacic, I. Damjanov, R. M. Lebovitz, M. W. Lieberman. Expression of gamma-glutamyl transpeptidase in midgestation mouse yolk sac and mouse visceral yolk sac carcinoma cells. — Exp. Cell Res., **219**, 1995, 494-498.

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Acta morphologica et anthropologica, 8
Sofia • 2003

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Anthropometrical Nutritional Status of Adult Bulgarian Population at the End of the 20th Century

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Characteristics of anthropometrical nutritional status in adult Bulgarian population at the end of the 20th century is made, as well as, the frequency of individuals with different anthropometrical nutritional status according the WHO classification norms is assessed. It is established that during the steadiest biological age (30-40 years), the Bulgarians have an anthropometrical nutritional status between the categories "healthy" and "overweight". The individuals with BMI over 30 kg/m^2, which marks the obesity, are 15.8% from the males and 17.6% from the females. According to the WHO data about obesity in Europe, the frequency of obesity Bulgarians at the end of the 20th century is near to the mean frequency for the European population.

Key words: Anthropometrical nutritional status, anthropometry, BMI, Bulgarian adult population.

Introduction

During the past years more and more often data about the alarming obesity of the population in the well-developed countries are published [1,4]. Publications about the increase of the frequency of obesity people in Bulgaria are not an exception [5, 8].

As an appraiser of the anthropometrical nutritional status (ANS) when making large-scale investigations, the WHO still recommends the data of BMI to be used instead of the critics concerning its informative possibilities [2, 3]. More often the applied and recommended by the WHO demarcation cut off values about ANS categorization are those worked out by Garrow in 1981 [2].

The aim of the present work is to assess the anthropometrical nutritional status in adult Bulgarian population (30-40 years old) at the end of the 20th century.

Material and Methods

For the evaluation of ANS in the present work are used data about BMI of a representative for Bulgaria subpopulation of 30-40 years old males and females. These

data are a part from the National program "Anthropological characteristics of the Bulgarian population". The program is confirmed by the Educational and Scientific Ministry, as well as by the Bulgarian Academy of Sciences and is the only representative for Bulgaria as a complex anthropological investigation at the end of the 20th century. The study includes 2413 males and 2842 females at the age 30-40 years from 120 settlements (showed in the Bulgarian map) in the 9 regions of the country. The districts division corresponds to the administrative regions of the country during the period in which the program took place and includes representative subpopulations from the 9 regions in this time (Fig. 1).

For the categorization of ANS are used the cut off values of BMI recommended by the WHO and worked by Garrow in 1981, and the classification is made according to the scheme of Balabansky [5]. (Tabl. 1).

Results and Discussion

The mean values of BMI give a mostly general idea about ANS of the investigated individuals. The data from the representative for the country investigation (Table 2, Figure 1) show that at the end of the 20th century, the 30-40 years old Bulgarians have mean values for BMI between 25.3 and 26.8 kg/m^2. These data assign their ANS to the upper limit of the category "norm" and the lower limit of the category "overweight". By the regional comparison is established that biggest BMI have the males and females from Sofia and Montana regions (more well expressed in males), and lowest is the BMI for the females from Sofia town and the males and females from Bourgas and Rousse regions.

Next to this common idea about body nutritional status of the adult Bulgarian population at the end of the 20th century, received from the mean values of BMI, we shall analyse the frequency of the individuals from the different categories ANS, as for the whole country, so in a comparative aspect between the separate regions (Tables 3 and 4). In Fig. 3 is presented the frequency of the individuals from the different categories ANS totally for the whole country. The data show that 1.6% of the males and 4.2% of the females are with weight below the norm. Healthy ANS have 35.7% from the males and 43.9% from the females. Overweight are 46.8% from the males and 34.4% from the females, and with obesity, commonly for its three degrees (BMI > 30 kg/m^2) are 15.8% from the males and 17.6% from the females. The com-

T a b l e 1. BMI cut off values for the classification of anthropometrical nutritional status

Body mass index (BMI)			
categories	by L. Ballabansky, 1990	categories	WHO by Garrow, 1995
thin	x – 19.9	thin	x – 19.9
normal	20.0 – 24.9	normal	20.0 – 24.9
overweight	25.0 – 29.9		
obesity grade I	30.0 – 34.9	overweight grade I	25.0 – 29.9
obesity grade II	35.0 – 39.9	overweight grade II	30.0 – 39.9
obesity grade III	40.0 – x	overweight grade III	40.0 – x

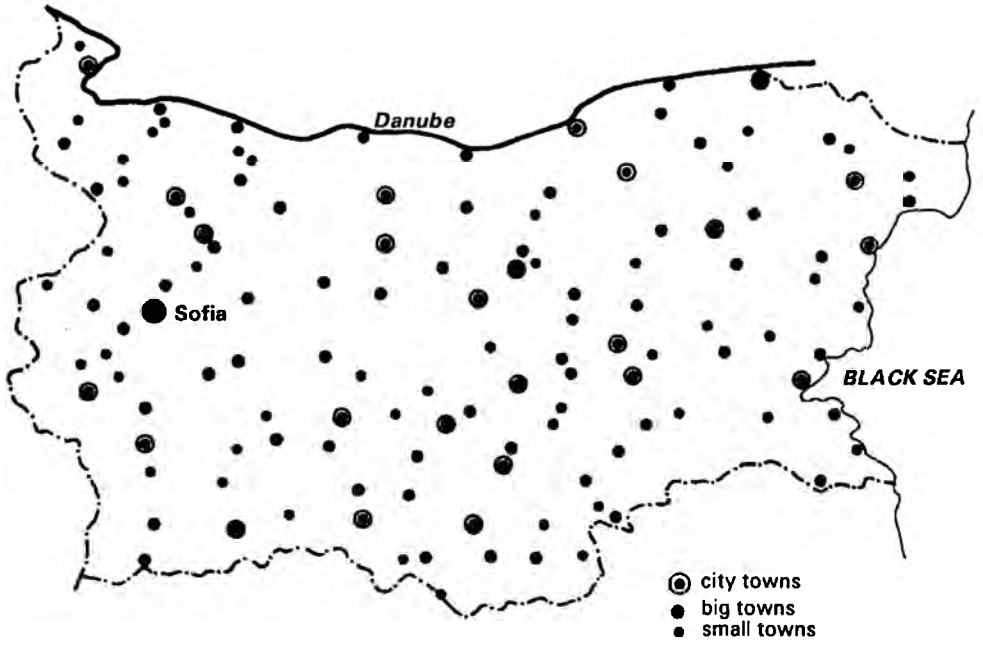

Fig. 1. Investigated settlement in Bulgaria

parison of these data with the data of WHO for the European countries in which the frequency of obesity individuals is between 10% and 20% in males, and between 10% and 25% in females shows that the frequency of the obesity Bulgarians at the end of the 20th century is near to the mean frequency for the European population [4, 8].

Of special interest is the sexual comparison of the frequency of individuals with healthy and overweight ANS in males and females. The frequency of females with healthy ANS is higher commonly for the whole country with 8.2 % than it is for males. The males, however, from the category "overweighed" are considerably more frequent — with 12.4% than the frequency of the females in this category.

T a b l e 2. Anthropometrical nutritional status of the Bulgarian population (30 — 40 years old)

No	Territorial division		Males							Females						
			n	mean	SD	SEM	V	min	max	*n*	mean	SD	SEM	V	min	max
1	Sofia city		234	26.3	3.8	0.2	14.2	19.1	41.7	275	25.3	4.9	0.3	19.3	18.3	43.8
2	Districts	Sofia	310	26.8	3.7	0.2	13.9	18.0	39.2	370	26.4	4.7	0.2	17.8	15.5	44.8
3		Plovdiv	276	26.4	3.6	0.2	13.5	19.2	40.7	339	26.1	4.3	0.2	16.6	17.9	41.8
4		Haskovo	288	26.4	3.2	0.2	12.1	18.7	37.2	351	26.0	4.1	0.2	15.9	17.2	46.2
5		Bourgas	247	26.4	3.9	0.2	14.8	18.2	45.6	336	25.3	4.2	0.2	16.6	17.6	43.6
6		Montana	344	26.7	4.1	0.2	15.4	16.4	41.5	357	26.6	4.9	0.3	18.4	16.8	46.8
7		Lovech	266	26.3	3.9	0.2	14.7	18.1	47.9	316	26.0	4.6	0.3	17.8	17.0	45.2
8		Rousse	186	25.6	3.4	0.2	13.5	17.4	36.0	204	25.9	4.3	0.3	16.7	18.7	41.0
9		Varna	262	26.0	3.9	0.2	14.9	16.1	39.9	294	25.8	4.4	0.3	17.3	17.0	41.2
10	Whole country		2413	26.4	3.8	0.1	14.3	16.1	47.9	2842	26.0	4.5	0.1	17.4	15.5	46.8

Table 3. Distribution of the investigated population according to anthropometrical nutritional categories in males

No	Territorial division		Total number	Categories											
				thin x-19.9		normal 20.0 – 24.9		overweight 25.0-29.9		obesity I 30.0-34.9		obesity II 35.0-39.9		obesity III 40.0-x	
				n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
1	Sofia city		234	3	1.3	84	35.9	111	47.4	30	12.8	5	2.1	1	0.4
2	Districts	Sofia	310	5	1.6	100	32.3	144	46.5	52	16.8	9	2.9	0	0
3		Plovdiv	276	3	1.1	96	34.8	141	51.1	29	10.5	6	2.2	1	0.4
4		Haskovo	288	1	0.3	96	33.3	153	53.1	34	11.8	4	1.4	0	0
5		Bourgas	247	1	0.4	98	39.7	108	43.7	33	13.3	6	2.4	1	0.4
6		Montana	344	8	2.3	115	33.4	144	41.9	64	18.6	11	3.2	2	0.6
7		Lovech	266	7	2.6	90	33.8	136	51.1	26	9.8	6	2.3	1	0.4
8		Rousse	186	6	3.2	74	39.8	87	46.8	18	9.7	1	0.5	0	0
9		Varna	262	4	1.6	109	41.6	105	40.1	37	14.1	6	2.3	1	0.4
10	Whole country		2413	38	1.6	862	35.7	1130	46.8	323	13.4	54	2.2	6	0.2

Table 4. Distribution of the investigated population according to anthropometrical nutritional categories in females

No	Territorial division		Total number	Categories											
				thin x-19.9		normal 20.0 – 24.9		overweight 25.0-29.9		obesity I 30.0-34.9		obesity II 35.0-39.9		obesity III 40.0-x	
				n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
1	Sofia city		275	12	4.4	146	53.1	84	30.5	22	8.0	8	2.9	3	1.1
2	Districts	Sofia	370	14	3.7	144	38.9	134	36.2	54	14.6	21	5.7	3	0.8
3		Plovdiv	339	12	3.5	148	43.7	116	34.2	46	13.6	16	4.7	1	0.3
4		Haskovo	351	11	3.2	146	41.6	139	39.6	47	13.4	6	1.7	2	0.6
5		Bourgas	336	19	5.7	158	47.0	120	35.7	28	8.3	10	3.0	1	0.3
6		Montana	357	16	4.5	144	40.3	119	33.3	52	14.6	21	5.9	5	1.4
7		Lovech	316	13	4.1	140	44.3	99	31.3	50	15.8	12	3.8	2	0.6
8		Rousse	204	5	2.5	92	45.1	71	34.8	28	13.7	7	3.4	1	0.5
9		Varna	294	15	5.1	131	44.6	97	33.0	38	12.9	10	3.4	3	1.0
10	Whole country		2842	117	4.2	1248	43.9	977	34.4	367	12.9	112	3.9	21	0.7

Table 5. Secular trend of anthropometrical nutritional status

Sex	Parameters	Literature data			Our data
		1970 by Janev et al. [9]	1975 by Mutafov et al. [8]	1980 by Slanchev et al. [7]	1989 - 1993
males	n	1201	425	744	2413
	x	25.64	25.78	25.98	26.37
females	n	1199	425	741	2843
	x	26.76	27.15	26.05	25.96

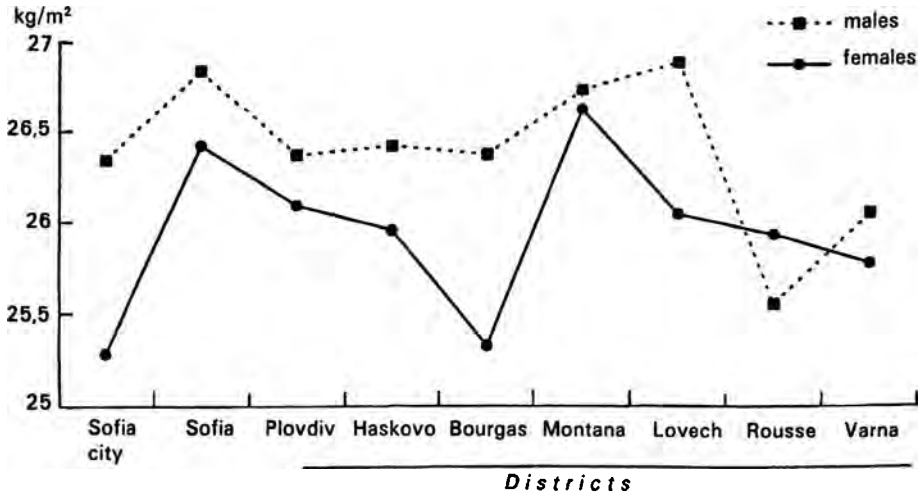

Fig. 2. Mean values of BMI

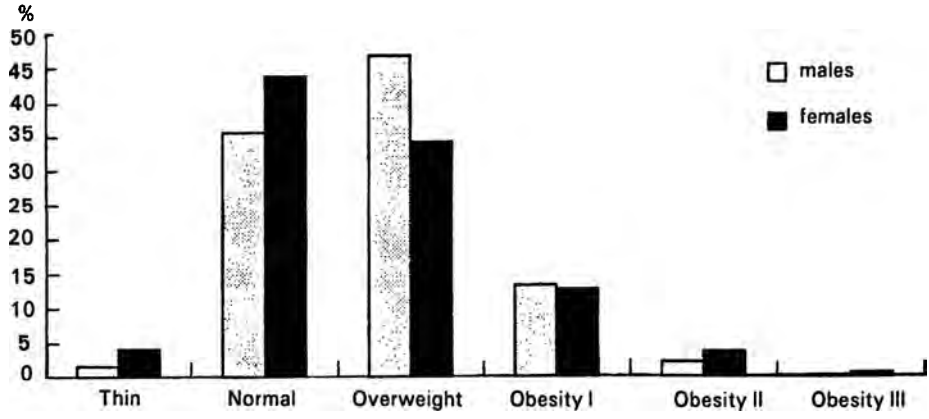

Fig. 3. Distribution of the population in the whole country according to the categories of anthropometrical nutritional status

More detailed information about this tendency could be received from the regional comparison (Fig. 4 and 5). The data show that from all regions in the country higher is the percentage of males with overweight (between 40.1% and 53.1%) than of males with healthy ANS (between 32.3% and 41.6%). Most frequent are the overweighed males in Haskovo, Lovech and Plovdiv regions and those with healthy ANS are most frequent in Varna, Rousse and Bourgas regions. In females the proportion is opposite of those in males. The frequency of females with overweight is lower (between 30.5% and 39.6%) than of females with healthy ANS, whose frequency is between 40.3% and 53.1%. Highest is the frequency of females with over-

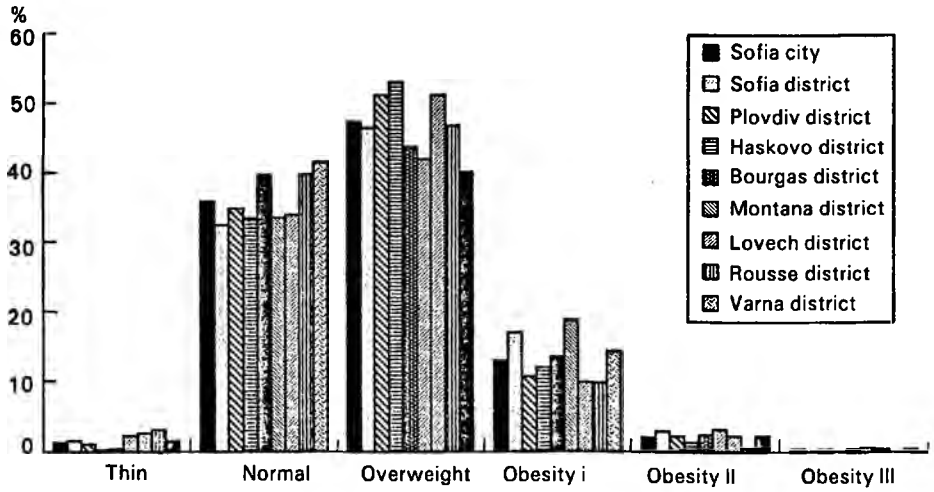

Fig. 4. Distribution of the population in the separate 9 regions according to the categories of anthropometrical nutritional status (*males*)

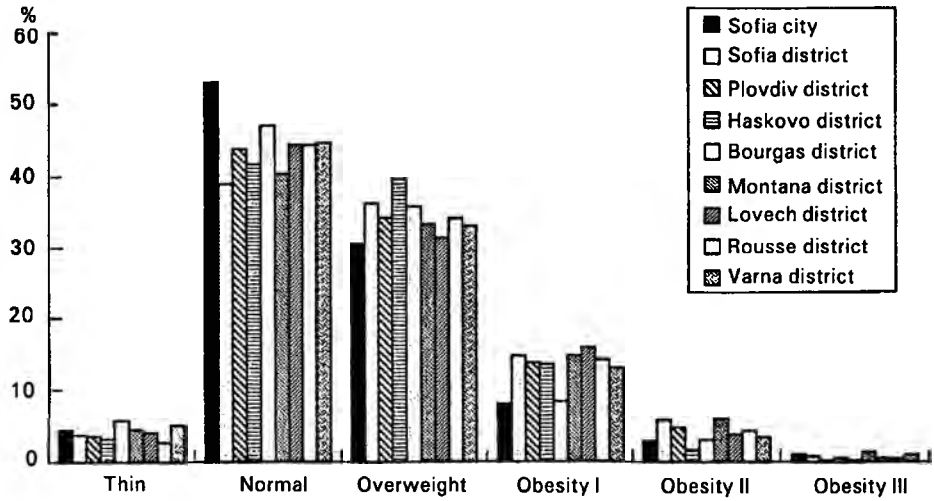

Fig. 5. Distribution of the population in the separate 9 regions according to the categories of anthropometrical nutritional status (*females*)

weight in Haskovo region, and most frequently such with healthy ANS are observed in the capital city of Sofia and Bourgas region.

The data analyses about frequency of individuals with the three obesity degrees are of a special interest (Fig. 6 and 7). Most obese males with BMI > 30.0 kg/m^2 are found in Montana and Sofia region, and lowest is their frequency in Rousse region. The frequency of obese females is highest again for Montana and Sofia regions, as in the category obese Ist degree with BMI between 30.0 and 34.9 kg/m^2 their frequency is highest for Lovech region.

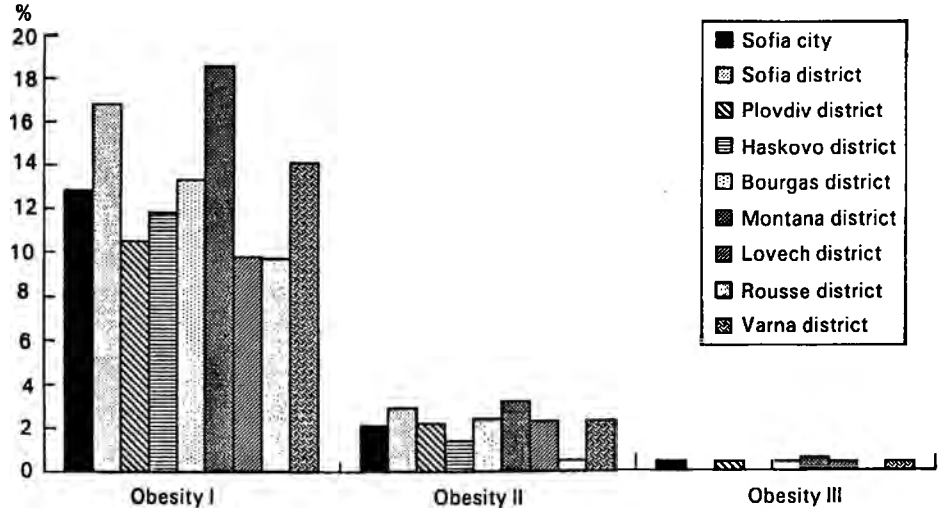

Fig. 6. Frequency of the population from the different obesity categories (*males*)

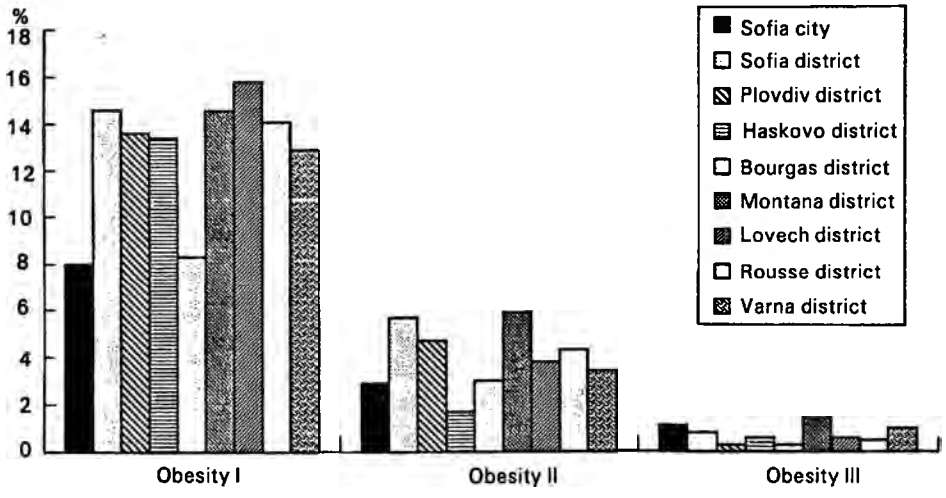

Fig. 7. Frequency of the population from the different obesity categories (*females*)

To study if there are changes in ANS for different generations of the Bulgarian population during the 20th century, i.e. if there are data about secular changes in this period, we made at the end a comparison between our study and the existing similar investigations for Bulgaria in 1970, 1975 and 1980. These are the only representative studies of 30-40 years old Bulgarians which could be used for objective comparisons.

It is obvious from Table 5 and the illustrations in Fig. 8 that there are not big changes in ANS for the Bulgarians from the 70s till the end of the century. Nevertheless, in the sexual aspect it could be accounted a tendency of a BMI increase in

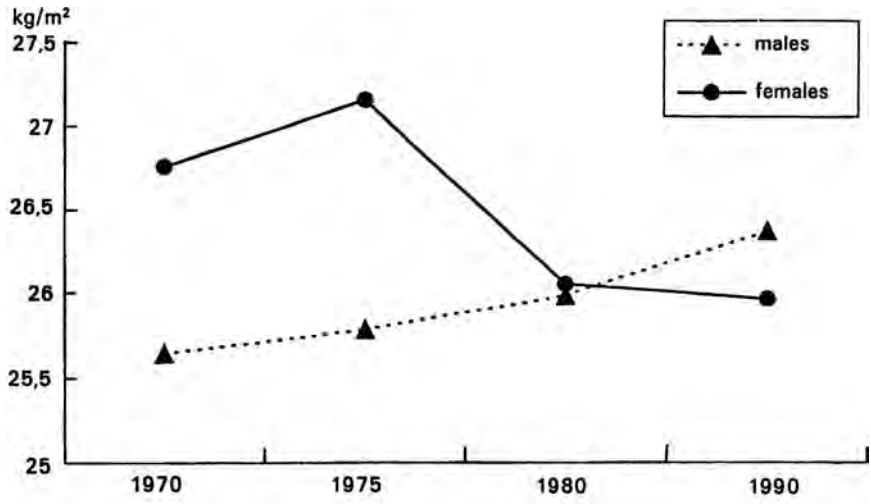

Fig. 8. Secular trend of anthropometrical nutritional status

males for 20 years period with 0.73 points. In females, highest is the BMI for the generation studied in 1975 and lowest — for the studied by the National Anthropological Program (1989-1993). The decrease of the BMI mean values between 1975 and the end of the century is with 1.19 points, and could be discussed as a deceleration tendency about body nutritional status of the Bulgarian females during the last two decades of the 20th century.

Conclusion

The results in the present study show that at the end of the 20th century the 30-40 years old Bulgarians, i.e. the ones in the steadiest biological ages, have anthropological nutritional status between the upper limit of norm and the lower limit of overweight, as in males the frequency of overweighed individuals is higher. It is very important to be verified that in the Bulgarian population, the individuals with BMI over 30 kg/m ^{2} which is indicative for the obesity are 15.8% from the males and 17.6% from the females. According to the WHO data, the frequency of obesity for Bulgarians at the end of the 20th century is near to the mean frequency for the European population.

References

1. Kuczmarski, R., C. Johnson. National Nutritional Surveys Assessing Anthropometric Status. — In: Anthropometric Assessment of Nutritional status (ed. Himes), 7,1991, 319-335.
2. Measuring obesity — classification and description of anthropometric data. Report on a WHO Consultation on the Epidemiology of Obesity. EUR /ICP/ NUT 125, 0612 v., 1989, 1-21.
3. Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee. Technical Report Series 854, Geneve, 1995, 4-33; 312-340; 345-370.
4. Seidell, J. Obesity in Europe. Obesity research, 3, 1995, Suppl. 2, 89-93.
5. Балабански, Л. Хранене и обменни заболявания. С., Медицина и физкултура, 1990, 176.
6. Мутафов, С., И. Горанов, Д. Сепетлиев, С. Торньова-Рандслова, А. Начева.

Антропологично-ергономична характеристика на българското население. С., БАН, 1985, 35—36.
7. Слънчев, П., Б. Янев, Ф. Генов, П. Щерев, П. Боев, Д. Сепетлиев, Б. Захариев. Физическо развитие, физическа дееспособност и нервно-психическа реактивност на населението на България (1980—1982). С., Национална спортна академия, 1992, 211—212, с. 262.
8. Хаджиев, Св. Затлъстяването в нова светлина. — Наука Кардиология, 1, 2000, 37—41.
9. Янев, Б., П. Щерев, П. Боев, Ф. Генов, Д. Сепетлиев, И. Попов, Б. Захариев. Физическо развитие, физическа дееспособност и нервно-психическа реактивност на населението в България от раждането до шестдесетгодишна възраст. С., Медицина и физкултура, 1982, 55—58, 70—75.

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Sexual Differences in Body Composition and Distribution of Subcutaneous Fat Tissue in Newborns

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The aim of the present study is to analyze and evaluate the sexual differences in the body composition and the topic distribution of subcutaneous fat tissue in newborns on the base of data about body weight and six standard skinfolds on body and extremities.

Totally 219 full-term and healthy newborns (110 boys and 109 girls) in the first 24 hours after birth are measured. The following six skinfolds: triceps, forearm, thigh, calf, subscapular and abdomen are measured by standard methods with the caliper of Holtain. The total thickness of the measured subcutaneous fat tissue as a sum from the six skinfolds is calculated in addition. The relative share of every skinfold is calculated regarding the sum of six skinfolds. The body composition is assessed by Guihard — Costa indexes.

It is established that the newborn boys have smaller quantity of subcutaneous fat tissue, but their body weight is bigger opposite to the newborn girls.

Key words: newborns; body composition; subcutaneous fat tissue.

Introduction

The body composition, body weight and subcutaneous fat tissue thickness are basic characteristics for human anthropometrical nutritional status and physical development [1, 5]. These characteristics are relatively well studied and estimated in children, adolescents and adults, when their evaluation in newborns is rare in the specialized literature.

Many authors [1, 6, 7, 8, 9] are working in the fields of physical development and methodological trends, and concrete results about metrical data for different ages, sexes, nationalities and other groups of children are published, as well. Studying the malnutrition during the last months of the intrauterine development, O w e n [4] reports that the thickness of subcutaneous fat tissue (SFT) gives more reliable information about malnutrition of newborns than body weight. G u i h a r d - C o s t a et al. [2] analyze the sexual differences in the subcutaneous fat tissue thickness in newborns and fetuses during the last months of pregnancy, as they search relation with body weight of the pregnant woman during this period. The authors use the ratio between thickness of two skinfolds (subscapular skinfold (SF) and triceps skinfold) and body weight for the estimation of well nutrition/malnutrition.

Guihard - Costa et al. [2] established, that the newborn boys have lower values for the two ratios compared to the newborn girls, i.e. they have smaller quantity of subcutaneous fat tissue per body weight unit. The authors found that the thickness of subcutaneous fat tissue on back is smaller in boys, than in girls, and the subcutaneous fat tissue thickness on arm is relatively equal for both sexes.

The aim of the present work is to estimate the body composition in newborns by the ratio thickness of subcutaneous fat tissue/body weight (SFT/BW) and to study the sexual differences in the quantity and distribution of subcutaneous fat tissue on their body and extremities.

Material and Methods

The data presented in the study are part of a detailed transversal anthropological investigation realized during the period April — May 2001. Totally 219 full-term (38 — 42 G. W.) and healthy newborns (110 boys and 109 girls) are studied during the first 24 hours after birth from the neonatal ward of the IInd Obstetrical Hospital "Sheinovo" in Sofia. The anthropometrical features are defined by the Martin-Saller [3] classical method and are measured by the first author in the present publication. The data about body weight and six standard skinfolds on body and extremities are analyzed. The body weight is measured in gram by a balance and the skinfolds — in mm by the standard method with Holtain caliper on the right side of the body. The body composition is estimated by using the two indexes introduced by Guihard - Costa et al. [2]:

Subscapular SF (mm x 10^3) / Body weight (g)

Triceps SF (mm x 10^3) / Body weight (g)

The authors mentioned above use as estimation criteria these indexes because subscapular and triceps skinfolds are in lowest relation with body weight ($r = -0,08$ and $-0,12$, respectively). This is very important requirement from mathematical point of view the compound features of indexes to be independent variables.

The total thickness of the measured subcutaneous fat tissue is calculated as a sum from six skinfolds. The relative share of every skinfold is determined regarding the sum of the six skinfolds. The sexual differences are assessed comparing the mean values of the investigated features and calculating the Index for sexual difference (ISD) by the formula: $ISD = X_{♀} \times 100 / X_{♂}$.

This index provides a possibility for a quantitative evaluation of the sexual differences. The values over 100 % show priority for girls and below 100 % — for boys. The metrical data are statistically analyzed by SPSS program and the reliability of the established differences is assessed by Students t-criterion at $P < 0,05$.

Results and Discussion

Regardless the body weight data are not subject of independent discussion in the present work, we should emphasize that the investigated newborn boys have average body weight of 3390 g and they are heavier than girls, who have average body weight of 3290 g (Table 1). It is established that the values of both body composition indexes in the investigated newborns are lower for boys compared to girls (Table 2, Fig. 1). The sexual differences in newborns investigated by Guihard-Costa et al. [2] are identical. These results show a presence of sexual differences in the body composition of the investigated newborns. The newborn boys have

T a b l e 1. Data about skinfolds and body weight of newborns (2001)

Features	Boys, n = 110				Girls, n = 109				t ♂/♀	ISD (%)
	mean	SD	Sx	V	mean	SD	Sx	V		
Subscapular SF (mm)	4.6	0.9	0.4	20.0	5.0	1.0	0.5	20.2	3.08*	108.70
Abdomen SF (mm)	4.4	0.7	0.4	16.7	4.6	0.8	0.4	17.9	2.0*	104.55
Triceps SF (mm)	4.9	0.9	0.4	18.5	5.2	1.0	0.4	18.5	2.31*	106.12
Forearm SF (mm)	5.1	0.8	0.4	16.1	5.3	0.9	0.4	17.3	1.82	103.92
Thigh SF (mm)	6.2	1.2	0.5	19.5	6.7	1.2	0.5	17.4	3.57*	108.06
Calf SF (mm)	5.8	0.9	0.4	15.9	6.3	0.9	0.4	14.8	4.17*	108.62
Sum of 6 SF (mm)	31.0	-	-	-	33.1	-	-	-	-	-
Body weight (g)	3390	0.38	0.0	11.2	3290	0.38	0.0	11.6	1.95	97.05

* Statistically significant differences (P<0.05)

T a b l e 2. Body composition according to the ratio SFT / BW

Sex	Body Weight (g) mean	Subscapular SF (mm . 10^3) mean	Triceps SF (mm . 10^3) mean	Sc SF/ BW mean	Tric.SF/ BW mean
Boys	3390	46.00	49.00	13.57	14.45
Girls	3290	50.00	52.00	15.20	15.81

smaller quantity of subcutaneous fat tissue, but their body weight is bigger opposite to the newborn girls.

The SFT thickness determined by the skinfolds gives specific information about the type of anthropometrical nutritional status in newborns.

The absolute values of thickness of the measured six skinfolds and their sum give general idea about the investigated thickness of the SFT layer on body and extremities in newborns.

The sum of the six skinfold thickness, which characterize the total quantity of the studied SFT have bigger values in the newborn girls — 33.1 mm, than in newborn boys — 31.0 mm. Since the six skinfolds characterize the SFT thickness on every body and extremities area (on back, abdomen, upper extremities and lower extremities) we can make the conclusion, that the newborn girls have thicker layer of SFT, than boys (Table 1, Fig. 2). Identical are also the sexual differences in the thickness of the six measured skinfolds separately. All skinfolds with the exception of the forearm SF thickness are statistically significant thicker in the newborn girls than in the newborn boys.

The evaluation of the sexual differences about the every skinfold thickness by ISD shows that the newborn girls have thicker SFT layer by 8 % — 9 % on back, thigh and calf, and by 4,5 % — 6 % on triceps and abdomen compared with the newborn boys. The sexual differences are smallest in the forearm SFT thickness, where the priority for the girls is approximately 4 % (Table 1, Fig.3).

Objective notion about distribution of SFT on the different parts of body and extremities are received by calculating the relative share of every SF thickness. Our data shows comparatively slight sexual differences (Table 3, Fig. 4). Nevertheless, it should be marked that in newborn girls the relative share of the SFT thickness is

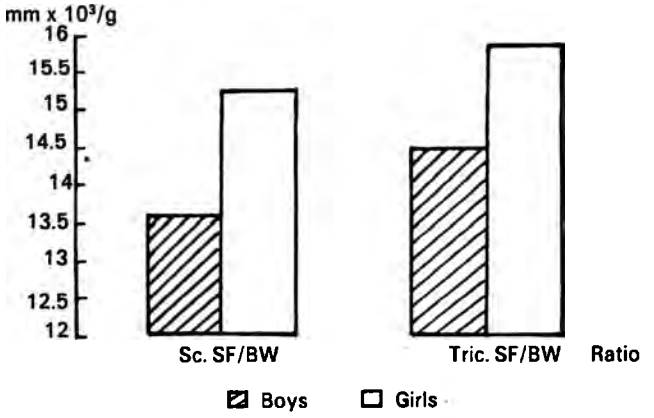

Fig. 1. Ratio SFT / BW

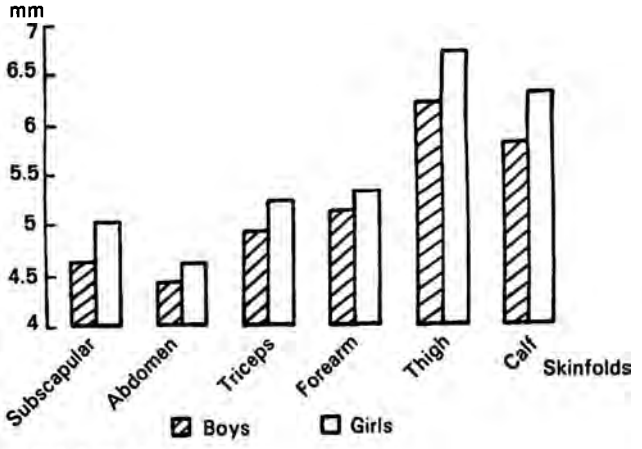

Fig. 2. Skinfold thickness

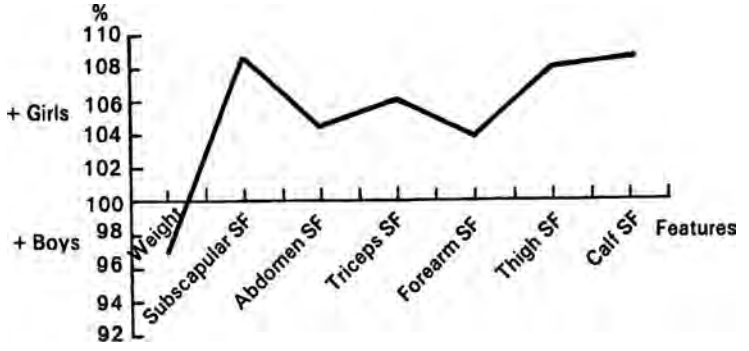

Fig. 3. Sex differences according to the ISD data

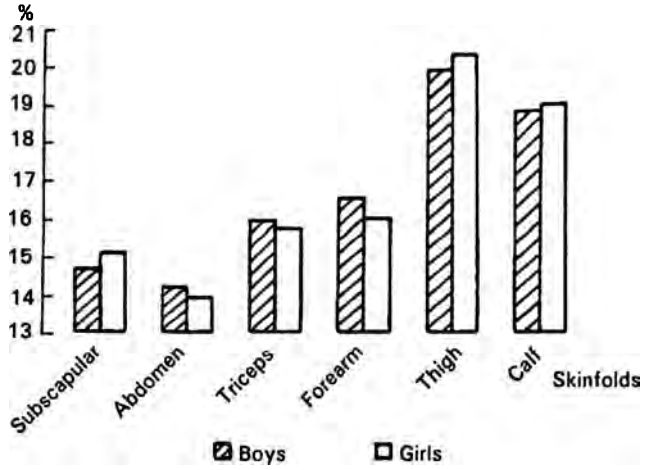

Fig. 4. Percentage fat distribution

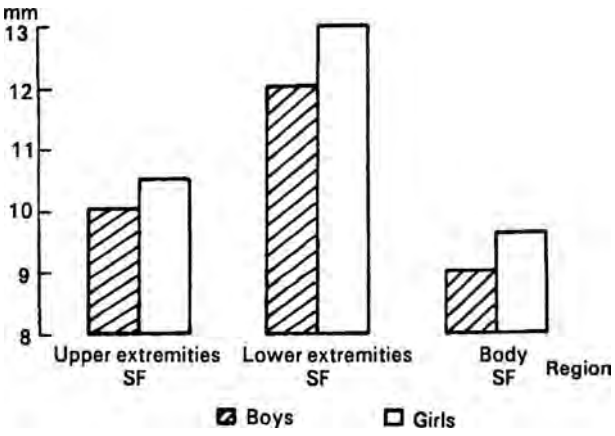

Fig. 5. Regional fat distribution

bigger on thigh, calf and back, and in newborn boys the relative share of the SFT thickness is bigger on triceps, forearm and abdomen.

A general notion about the type of anthropometrical nutritional status could be obtained by the assessment of the relative share of the two skinfolds on the upper extremity, the two skinfolds on the body and the two skinfolds on the lower extremity (Table 4, Fig 5). Our data shows slightly expressed sexual differences in the type of anthropometrical nutritional status. For both sexes the relative share of SFT thickness on body is equal (29 %), on upper extremities in the newborn boys it is 32.3 %, and in the newborn girls it is lower with 0.6 % (31.7 %). On lower extremities the ratio is opposite — the newborn boys have 39.3 % from the measured quantity of SFT, and the newborn girls — 38.7 % (i.e. lower with 0.6 %). These data show that the relative share of SFT thickness on lower extremities dominate in girls and— on upper extremities in boys.

T a b l e 3. Percentage fat distribution according to the sum of 6 skinfolds

Skinfolds	Boys (%)	Girls (%)	t ♂/♀
Subscapular	14.8	15.1	0.07
Abdomen	14.2	13.9	0.14
Triceps	15.8	15.7	0.10
Forearm	16.5	16.0	0.07
Thigh	20.0	20.3	0.15
Calf	18.7	19.0	0.16

T a b l e 4. Percentage of regional fat distribution

Region	Boys		Girls	
	mean	%	mean	%
Upper extremities SF	10.0	32.3	10.5	31.7
Lower extremities SF	12.0	38.7	13.0	39.3
Body SF	9.0	29.0	9.6	29.0

Conclusion

- The results obtained show that even by birth exist underlined sexual differences of the body composition, the thickness and the relative share of subcutaneous fat tissue.
- The newborn boys have thinner subcutaneous fat layer, but their body weight is bigger opposite to the newborn girls in whom the subcutaneous fat layer is thicker and the body weight is lower.
- The newborn girls have statistically significant thicker skinfolds than the newborn boys have.
- Concerning the topical distribution of subcutaneous fat tissue on body and extremities, the relative share of subcutaneous fat tissue on upper extremities and abdomen is bigger in the newborn boys, and it is bigger on lower extremities and back in the newborn girls.

References

1. F o l k n e r, F., J. M. T a n n e r. Human growth. New York, Plenum Press, 1987, 1—537.
2. G u i h a r d - C o s t a, A.-M., E. P a p i e r n i k, G. G r a n g e, A. R i c h a r d. Gender differences in neonatal subcutaneous fat store in late gestation in relation to maternal weight gain. — Annals of Human Biology, 29, 2002, No 1, 26-36.

3. M a r t i n, R., K. S a l l e r. Lehrbuch der Anthropologie in sistematischer Darstellung. T. I. Stuttgart, Gustav Fischer Verlag, 1957, 322-324.
4. O w e n, P. Foetal growth velocities in pregnancies with neonatal features of intrauterine malnourishment. — Clinical and Experimental Obstetrics and Gynecology, 24, 1997, 8-10.
5. T a n e r, J. M., R. H. W h i t e h o u s e, M. T a k a i s h i. Standards from birth to maturity for height, weight, height velocity and weight velocity; British children 1965. — Arch. Dis. Child., 41, 1966, 454-471; 613-635.
6. С л ъ н ч е в, П., Б. Я н е в, Ф. Г е н о в, П. Щ е р е в, П. Б о е в, Д. С е п е т л и е в, Б. З а х а р и е в. Физическо развитие, физическа дееспособност и нервно-психическа реактивност на населението на България (1980-1982). С., Национална спортна академия, 1992, с. 211, с. 212, 242 — 244.
7. Ц и р о в с к и, М. Върху някои страни от физическото развитие на деца от гр. Пловдив, изследвани лонгитудинално от раждането им до навършване на три годишна възраст. Дисерт. труд (Пловдив), 1976.
8. Ц и р о в с к и, М. Медико-антропологично изследване на деца от периодите кърмачество и ранно детство. Дисерт. труд (Пловдив), 1987.
9. Я н е в, Б., П. Щ е р е в, П. Б о е в, Р. С е м е р д ж и е в а, Д. С е п е т л и е в. Физическо развитие и дееспособност на населението в България от раждане до двадесет и шест години. С., БАН, 1965, 31—44.

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Women's Pool — the Metabolites and Hormonal Mechanisms

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The mechanisms, controlling lipid storage, lipid mobilization and utilization are interesting. Adipose tissue metabolism varies from one region of the body to another. The fat on *women's pool (thighs, hips, belly)* is more difficult to be mobilized due to increased α-2-adrenergic receptor activity induced by estrogen and another hormones and enzymes. The obesity of gynoideous type and typical cellulite in women are caused by a number of factors. The increasing adipocytes during growing fat, as well as the retention of liquid during the monthly cycle are becoming a reason for the development of local hypoxy. The hypoxy appears to be a constant irritating factor for these tissues and they make attempts to adapt to it. Then the hypoxy includes the vicious circles which forming the women's pool in girls and its sustainment in women.

Key words: women's pool, hypoxy, free radicals.

Introduction

Obesity of gynoideous type and cellulite have a common histological predisposition. They are most commonly found in women effecting prevailingly the thighs, hips and belly (*the women's pool).* During the puberty in these processes are included very important endocrine and metabolite mechanisms, which control the lipid storage, lipid mobilization and lipid utilization [25, 26]. The increasing of estrogens during the puberty leads to an increase in α-2-adrenoreceptors. This increase the lipogenesis and the adipocytes in the gluteofemoral region (women's pool). In the same time, the fat in the women's pool is more difficult to be mobilized due to an increased α-2-adrenergic receptor activity induced by estrogen and another hormones and enzymes. Thus the retention of fats and liquids in girls during the monthly cycle are becoming a reason for the development of local hypoxy. The local hormones can determine body fat distribution. Women, through the effect of estrogen, have more α-2-receptors on the fat cells of their hips and thighs. This gives a higher lipolytic threshold and causes concentration of fat in the area in women [1, 2, 15, 20].

The adipocytes have 3 different types of receptors in its outer membrane: β-adrenergic receptors, α-2-adrenergic receptors and proteinkinase (pkC)-receptors. β-receptors block or inhibit phosphodiesterase production, leading to increased levels of cyclic-AMP which are known to trigger lipolysis. A β-receptor stimulator or activator Isoproternol (β-agonist), inhibit phosphodiesterase increase cyclic AMP and the lipolysis [8]. α-2-receptors increase the level of phosphodiesterase, reduce the level of cyclic AMP and reducing lipolysis. Thus, α-2 inhibition is desirable. α-2-inhibitors are Yohimbine, Gincko biloba etc. [11, 28]. Protein-kinase receptors increase phosphodiesterase and reduce cyclic AMP-level and inhibiting lipolysis. Thus inhibition of the pkC- receptors is desirable. Xanthine, Caffeine and Theophilline inhibit pkC-receptors [12].

Local hypoxy and oxidative processes

The increasing adipocites during growing fat, as well as the retention of liquid during the monthly cycle are becoming a reason for the development of local hypoxy. The process is lasting for a long time, sometimes during the whole life. Many other normal mechanisms are effected by it, like the oxygenasis, local hormones (Leucotriens, Thromboxan — A2), NO, prostaglandines (PGE, PGI-2) etc. [13, 18, 19]. The accumulated not thoroughly oxidated products stimulate the oxidative modification of Low density cholesterol (LDL-C), the result of which is ox-LDL-C. They oxidate and in the blood appear lipoprekises and H_2O_2. A part of the metabolites are stimulating the fibroblasts, which is leading to growth of the connective tissue [22]. It fatness the *tela subcutanea* of the fats particles and embarrasses the metabolism and circulation. The lower activity of the oxygenasis promotes the delaying of the speed of the oxidating phosphoriling and for the lower activity of Glutathion peroxidasis (GPX). The accumulated reactive metabolites (H_2O_2, $^{\cdot}OH$ etc.) are leading to degradation of the hialuronous acid and of the collagen in the hypoderma — morphological changes [7, 14].

During the reduction of electrons from the oxygen molecule is generated a Superoxide anion radical ($^{\cdot}O_2^-$), a Hydroxil radical ($^{\cdot}OH$) and a Hydrogen peroxide (H_2O_2). All of them, together with the other familiar reactive oxygen species (ROS) are damaging the cellular membranes, the cells and tissues, with which they come into contact. Their harmful action is not only on the place of their generation, but reaches the neighboring tissues as well [14].

This is very characteristic in conditions of local hypoxy, where the possibility for neutralizing the metabolites is lower due to the lower activity of the oxygenases. This slows down the speed of the oxidative phosphoriling and as its result the resynthesis of ATP. The concentration of NADP is changing, and it affects the interrelation between the restoring and oxidated glutation (GSH: GSSG). The disturbed restoration of GSH is reflecting negatively to the activity of the glutathion peroxidasis, which makes harmless the generated during the neutralization of the superoxide anion radical hydrogen precis. There is a possibility for accumulation of H2O2.

During the enzyme detoxication of $^{\cdot}O_2^-$ with the participation of superoxidismutasis (SOD), is formed the intermediate product H_2O_2 the neutralization of which is taking place on the following two ways: through catalasis (CAT) and through glutathion peroxidasis (GPX). The insufficient activity of GPX could become a reason for the accumulation of H_2O_2, which is leading to a tissue acidosis. The huge amount of H_2O_2, and the availability of Fe^{2+} generate a lot of $^{\cdot}OH$. This is the most potent oxidant know, has an extremley short half-life, reacting at the site

of its formation through its ability to attack most biological molecules resulting in the propagation of OFR chain reactions. Due to the lower possibilities of the hypoxical tissues of detoxication, in the hypoderma are accumulated other OFR, as well as products of the precis oxidation of the lipids, including conugates of dien, malon dialdehide, etc. [6, 17, 28]. Their accumulation causes a degradation of the hialironic acid and the collagen, damaging of the cells through peroxidation of the polynotsaturated fat acids and destruction of the permeability of the cells membranes and the structural elements. It is even possible to be got to acidosis with the additional after-effects for the tissue [16, 24]. Due to its less significance for the survival of the organism in comparison with the internal organs, the brain and the hypoxy of the women's pool region appears to be a constant irritating factor for these tissues and they make attempts to adapt to it. This is connected with the appearance of cellulite in girls and obesity of gynoideous type in women [23,27].

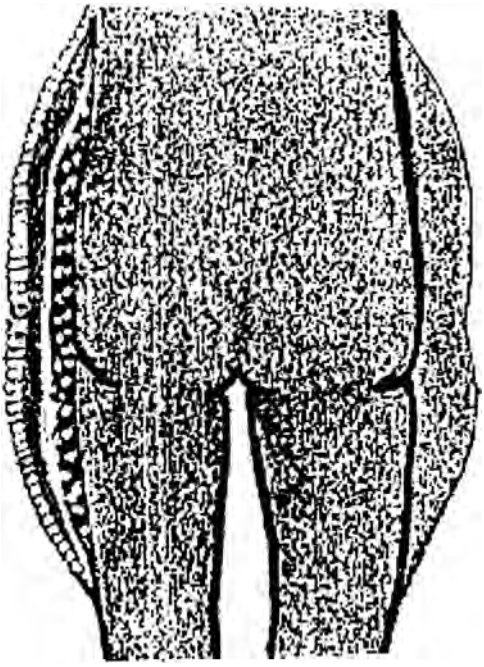

Fig. 1. Women's pool and its layers — cellulite and adipose tissue

The aim of this study is to present the importance of the adipose tissue, monthly cycle, local hypoxy and oxidative processes and their connections as mechanisms forming the women's pool.

Lipolysis

Adipose tissue metabolism varies from one region of the body to another. The authors demonstrated that fat was absorbed more slowly in the femoral region in

women losing weight after the jeuno-ileal bypass operation for server obesity [10, 21]. These observation suggested regional differences in the lipolytic processes that might respond to the local application of lipolytic agents [5].

The lipolytic process has been described in great detail in the past 20 years. Lipolysis, the process of hydrolyzing triacilglycerol into glycerol and fatty acids, is mediated by the enzyme Hormone sensitive lipase (HSL). HSL is active in the phosphorylated form. This activation is produced by proteinkinase-A which is activated by cyclic AMP. Membrane-bound adenylate cyclase can be inhibited or stimulated by the action of inhibitory or stimulatory GTP binding proteins (Gs-proteins) acting on adenylate cyclase [8, 9].

A number of hormones react with cell surface receptors on the adipocyte to influence lipolysis. Stimulation of the β-adrenergic receptors stimulated the Gs-proteins which activates adenylate cyclase which, in turn, activates cyclic AMP. The α-2-adrenergic receptor and the adenosine receptor, on the other hand, stimulate GTP inhibitory binding proteins (Gi — proteins) which inhibit adenylate-cyclase and thus inhibit the lipolytic process. The relative number of β- and α-2-adrenergic receptors on the surface of the fat cells determine the lipolytic balance of those cells [2, 3, 11].

Hormones can have long-term effects on the lipolytic processes by influencing the number of α-2- and β-receptors on the fat cell. Thus by controlling lipolysis,muscles, the hipoderma, and the whole skin too have adopted to catch the harmful metabolites, when they are a huge amount and to store them as in a depot, thus protecting the vitally-important organs and systems. Thus the human organism acts according to the Law for common biological adaptation, by storing them and gradually organizing them in its hypoderma. There are formed cellulite nodules, new morphological structures which worsen the common state and outside look of the skin. *Nature sacrifices esthetics for the save of the survival of the organism.*

Two vicious circles

The accumulating metabolites gradually organize and form cellulite formations in the hipoderma — *cellulite.* This is connected with the **1st vicious circle** *cellulite — obesity — cellulite:* Cellulite — Local hypoxy with peroxidation — Disturbed permeability of the cellular membranes — Entrance of Ca^{2+} ions in the cells — Activation of the phosphoinozitid—specific phospholipase-C — Stimulation of the phosphoinozitides — Activation of the proteinkinases — Desensitisation of the β-adreno-receptors — Embarrassed lipolyse — Accumulation of triglicerides — gynoideous type Obesity — Local hypoxy and Cellulite.

The increased lipogenesis is leading to local (gynoideous) obesity, which includes the **2nd vicious circle** *obesity — cellulite — obesity:* Increased lipogenesis — Enlargement of adipocites (obesity) — Higher interssue pressure — Worsened draining — Retention of liquids and metabolites — Growth of connective tissue (cellulite) — Local hypoxy — Enzyme degradation — Embarrassed lipolyse — Gynoideous type of obesity.

Than the hypoxy includes the vicious circles which forming the women's pool in girls and its sustainment in women.

Conclusion

In conclusion, the hormonal and metabolite mechanisms lead to stagnation of liquids and increase the body fatty depots in thighs, belly, hips and the transition zone between them. Many women like selectively lose these undesirable fats from this specific area of the body as it is their most frequent concern. To obtain good results of the remedial procedures it is necessary to know well the mechanisms, related to the forming and sustaining the women's pool.

References

1. Ando, Y., Y. Yamaguchi, K. Hamada, K. Yoshikawa, S. Itami. Expression of mRNA for androgen receptor, 5-reductase and 17-hydroxysteroid dehydrogenase in human dermal papilla cells. — Br. J. Dermatol., **141**, 1999, 840—845.
2. Arner, P. Beta-adrenoreceptor expression in human fat cells from different regions. — J. Clin. Invest., **86**, 1990, 5, 1595—1600.
3. Arner, P. Adrenergic receptor function in fat cells. — Amer. J. Clin. Nutr., **55**, 1992, 228—236.
4. Belmonte, C., F. Cervero. Neurobiology of Nociceptors. — New York, Oxford University Press, 1996.
5. Curri, S. B. Local Lipodistrophy and Distructural Microcirculation. — Cosmetics & Toiletries, **109**, 1994, 51-53.
6. Dencheva, D., Iv. Topouzov. Cellulitogenesis as attempt for adaptation to local hypoxy and its vicious circles. — In: European S. Conference (Berlin, 23-26.11.2000), p. 146.
7. Draelos, Z., K. Marenus. Cellulite: Etiology and purported treatment. — Dermatol. Surg., **23**, 1997, 12, 1177-1181.
8. Greenway, Fr., G. Bray. Regional fat loss from the tight in obese women after adrenergic modulation. — Clin. Ther., **9**, 1987, 6, 663—669.
9. Greenway, Fr., G. Bray, D. Heber. Topical fat reduction. — Obesity Res., 3, 1995, Suppl. 4 Nov. 561—568.
10. Kral, J., P. Bjorntorp, T. Schesten, L. Sjostorm. Body composition in adipose tissue cellularity before and after jejuno-ileostomy in several obese subjects. — Eur. J. Clin.Invest., 7, 1977, 414—419.
11. Kong, W. C. Herbal cellulite treatment. US Pat 5705170, 1998.
12. Lafontan, M., A. Bousqet-Melou, J. Gallitzki, P. Barbe, Ch. Carpene, D. Langan. Adrenergic receptors and fat cells: differencial recruitment by physiological amines and homologous regulation. — Obesity Research, **3**, 1995, Suppl. 4, 507-514.
13. Monkada, S. Nitric oxide: discovery and impact on clinical medicine — J. Royal Soc. Med., **92**, 1999, No 4, 164-169.
14. Piyerard, G., J. Nizet, C. Piyerard-Franchimont. Cellulite: from standing fat herniation to hypodermal stretch marks. — Amer. J. Dermatopathol., **22**, 2000, No 1, 34-37.
15. Presta, E., R. Leibel, J. Hirsch. Regional changes in adrenergic receptor status during hypocaloric intake do not predict changes in adipocyte size or body shape. — Metabolism, 39, 1990, 307— 315.
16. Ronsard, N. Cellulite : Those Lumps, Bumps, and Bulges You Couldn't Lose Before. New York, Beauty and Health Publishing Co, 1973.
17. Rosenbaum, M., V. Prieto, J. Hellmer, M. Boschmann, J. Krueger, R. Leibel, A. Ship. An exploratory investigation of the morphology and Biochemistry of cellulite. — Plast. Reconstr. Surg., **101**, 1998, No 7, 1934-1939.
18. Rubanyi, G. M., P. M., Vanhoutte. Hypoxia releases a vasoconstrictor substance from the canine vascular endothelium. — J. Physiol. (L), **346**, 1985, 45-46.
19. Rubanyi, G. M The role of endothelium in cardiovascular homeostasis and diseases — J. Cardiovasc. Pharmacol., **22**, 1993, Suppl. 4, 1—14.
20. Sanigovski, A., D. C. Smith, P. Lewandowski, K. Walder, A. de Silva, G. Morgan, G. Collier. Impact of obesity and leptin treatment on adipocyte gene expression in Psammomis obesus. — J. of Endocrinology, **164**, 2000, 45-50.
21. Smith, J., J. Hammersten, P. Bjorntorp, J. Kral. Regional Differences in the effect of weight reduction in human fat cell metabolism. — Eur. J. Clin. Invest. **9**, 1979, 327-332.
22. Slominski, A., J. Wortsman. Neuroendocrinology of the skin. — Endocrine Reviews, **21**, 2000, No 5, 457—487.

23. Topouzov, Iv. The cellulite — characteristics and complex treatment. (Ed. Gracia M). Sofia, 2000.
24. Topouzov, Iv. The sports cellulite and the microtraumatism as a result of oxidative stress in athletes (Hypothesis). — Acta morphologica and anthropologica, 6, 2001, 104—108.
25. Topouzov, Iv. Pubertal cellulitis in girls — precursor of gynoideous type of obesity in women. — Pediatria, 41, 2001, No 2, 10—12.
26. Topouzov, Iv. The mechanisms forming the women pool in girls and its sustainment in women. — Endocrinology, 4, 2001, 14—20.
27. Topouzov, Iv. Rehabilitation of obesity of gynoideous type and cellulite with complex kinesitherapy. Dissertation (Sofia), 2002.
28. Vernon, R. G. Effect of diet on lipolysis and its regulation. — Proc. Nutr. Soc., 51, 1992, 397—408.

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Appearance of Menarche in Actively Training and Non-training Girls

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The article summarizes the data about the important role of sport on the sexual maturity in girls. The survey encompasses totally 481, 11-17 years old girls. The active training girls are 326. Non-training girls (155) at the same age (controls), 73 with obesity have been studied, too. Their sexual maturity, the appearance of menarche and the interdependence of these from the level of the fat tissue is examined. No acceleration has been found in their sexual maturity and in the appearance of menarche. The menarche in the actively training girls delays from 2 months up to 3 years in comparison with the non-training ones. Depending on the appearance of menarche, the authors offer the following classification of the girls: Non-training girls with normal body weight — menarche at 12 years and 3 months (12, 3); Non-training girls with overweight and obesity — menarche at 12 years and 1 months (12,1): Actively training girls: Sports Shooting and Volleyball — menarche at 12 years and 10 months (12,8); Basketball — menarche at 13 years and 1 month (13, 1); Feld athletics — menarche at 13 years and 3 months (13,3); Rowing and swimming — menarche at 13 years and 10 months (13,8); Sports gymnastics — menarche at 15 years and 8 months (15,7).

A scheme of the hormonal and enzyme mechanisms, explaining the puberty development and the menarche in the girls, is suggested and their dependence from the level of the fat tissue is proven.

Key words: menarhe, actively training and non-training girls.

Introduction

The intensive physical exercises exert a versatile impact on young girl's organism. Her adaptation to big physical efforts is connected with series of changes in her organs and systems and it is an object for study in age physiology, sport medicine and other sciences.

The aim of the present study is to determine relationship between systematically physical loadings and the appearance of menarche in actively training girls.

Materials and Methods

The survey encompasses totally 481, 11-17 years old girls (Table 1). The active training girls are 326. Non-training girls (155) at the same age (controls), 73 with obesity have been studied, too. The appearance of menarche was specified by inquiry.

The per cent of the fat mass was measured with body fat monitor "Tanita".

Comparisons between the different girls-group were made by Student's t-test ($p<0.05$).

Results and Discussion

In the present study, we paid attention especially to the appearance of menarche in young sportswomen because this indicator is considered the most objective in the evaluation of sex development in females. The results for the time of appearance of the first menstruation in sportswomen actively training different sports are presented in Table 1. No significant difference between these groups (for the appearance of menarche) was observed (the per cent of body fat mass was from 11 to 23 for different sports).

The appearance of menarche in the actively training girls in comparison with the non-training ones with normal body weight shows that the first menstruation in young sportswomen in various sports shows a delay. The delay is 2-5 months in Marksmanship, Volleyball and Basketball, 7-10 months in Track-and-field athletics and almost a year in Rowing, and Swimming. The delay of menarche appearance (three years) is established in Sport gymnastics and we suppose that other yet uninvestigated by us sports will be added to this group. In order to describe the influence of sport on sex development and appearance of menarche we should know their features in non-training girls where the differences in the biological development are of utmost importance.

T a b l e 1. Age of menarche in actively training girls and non-training girls depending on their body weight

No	*n*	Groups	Menarche
1	73	Control group with obesity	12.1
2	82	Control group with normal body weight	12.3
3	34	Sport Shooting	12.8
4	48	Volleyball	12.8
5	57	Basketball	13.1
6	69	Field and track events	13.3
7	39	Rowing	13.8
8	46	Swimming	13.8
9	33	Sport gymnastics	15.7

It is difficult to explain the arrangement of the sports groups in the table. Perhaps, together with the per cent of the fat mass in the girls for the appearance of menarche is important also the total body mass, and another factors, connected with kind of physical activity, nutrition and specifical characteristics of the concrete sport.

Stanimirova (1998) determined that in Bulgaria menarche appears at the mean age of 12.7 years, body weight 47.7 kg and fat weight 12.22 kg (25.5%), in case of obesity — at the age of 12.1 years, body weight 57.2 kg and fat weight 18.4 kg (32.1%). In case of emaciation, it appears at the age of 13.3 years, body weight 40.6 kg and fat weight 7.3 kg (18%). These data coincide with those of foreign authors who establish that the critical body weight for appearance of menarche is about 47 kg [5]. This body weight supposes the presence of the critical fat weight, which is a precondition for a normal metabolism of female sex hormones to take place [13]. This necessity explains the early appearance of menarche in girls with obesity and its delay in girls with emaciation [10, 11 and 12].

The lower level of fat weight is a factor for the delay of appearance of menarche in actively training girls with advanced biological development independently from their high stature and enough body weight. Our studies showed that the fat weight of actively training girls seldom exceeds 20 % with a mean value of 11-17 % [10].

The comparison shows that these per centages are even lower than the critical 18 % for appearance of menarche in the group of the girls with emaciation [10 and 11]. The low per cent of fat weight in the actively training girls is due to increased energy consumption. The trained muscles show a higher activity of the enzymes lipoprotein lipase, muscle lipase and creatine-acyltransferase, which lead to an easy metabolism of fatty acids [3]. Besides, the physical exercises and systematic trainings make в-adrenergic receptors more sensitive, thus increasing the lipolysis in the adipocyte [6 and 7] (Fig. 1). All these mechanisms are in the base of better use of fats by actively training girls and are the reason for the low percent of their fat weight [8].

When a girl's organism reaches the critical adipose depot, the adipocytes already synthesize enough quantity of the hormone leptin. It is the necessary signal for the brain (hypothalamus) to produce the first quantities of the gonadotropin-releasing hormone (GnRH) [9]. GnRH stimulates the anterior part of the pituitary gland to secrete the gonadotropic hormones: the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH). The cells of theca interne in the ovaries possess many receptors for LH. LH increases cAMP and accelerates the conversion of cholesterol into androstendion. The enzyme aromatase catalyzes the conversion of androstendion into estrone as well as that of testosterone into 17 — β-estriol [1]. The granulosa cells in the ovaries have receptors for FSH. The latter increases cAMP, which stimulates estradiol secretion there due to aromatase activity increase. The granulosa cells have receptors for LH, which stimulates estradiol secretion, too. Estradiol and the other estrogenic hormones (estrone and estriol) switch on the menstrual cycle with the appearance of the first menstruation and at the same time increase the density of the б-2-receptors in the adipocytes. This is a precondition for an intensive lipogenesis and a local accumulation of fatness in the region of the "woman's pool" in girls [1 and 14].

B r a y [2] and D u c l o s [4] showed that the leptin level decreases after physical training and it is this hormone from the periphery, which modulates estrogen expression and serves as a signal from adipocytes to the brain [2 and 4].

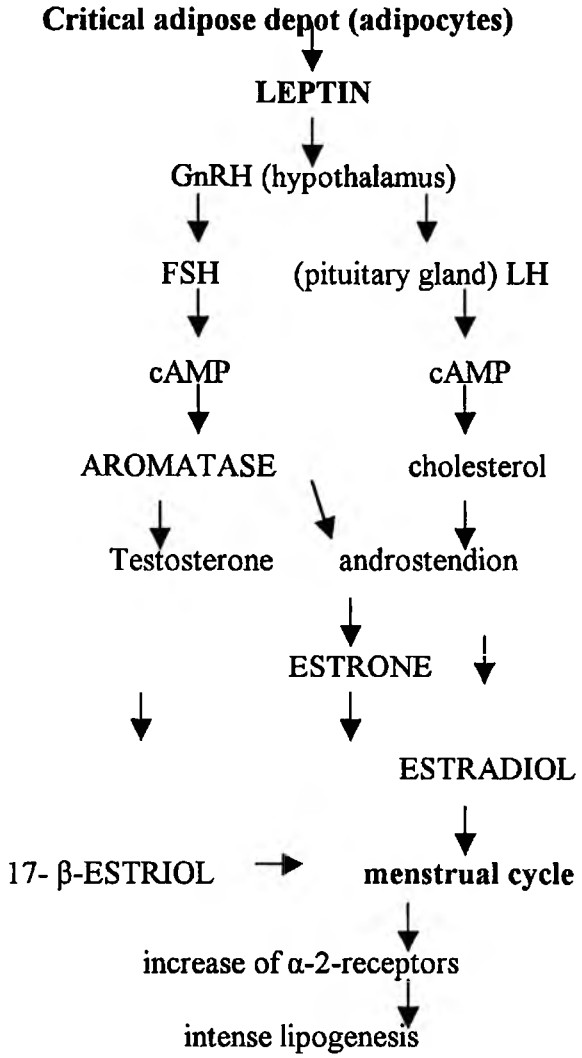

Fig. 1. Hormonal and enzyme mechanisms related to puberty development and menarche appearance in girls.

F r i s c h [5] noticed the dependence of estrogen activity from adipose tissue 20 years before the discovery of leptin.

Clinical practice constantly proves the dependence of the menstrual cycle on the level of the peripheral adipose tissue. The excessive loss of weight due to different factors, which include intensive physical training and hypo energy diets, is a frequent reason for disturbances in normal menstrual cycle.

In our practice, we have encountered similar cases with sportswomen who were on a strict diet in order to maintain low body weight. We would like to share one case. The acrobat A. M. (a peak in a pyramid) at the age of 17 and body weight of 33-34 kg and stature 141 cm had not yet received menarche. At the preparation for the next competition, she got a tetanic convulsion and was transported to the

Endocrinology clinic by emergency. The tests showed serious disturbances of the ionic and hormonal balance. Due to the timely infusions and appropriate feeding, she recovered very fast and after increasing her body weight to 41 kg, she received menarche. Later she resumed her sport but with another part in the pyramid, which allowed her a higher body weight and thus she went on competing for a long time. We think that this particular case proves once more the significance of body and fat weight for insuring a normal metabolism of female sex hormones and normal homeostasis of the organism as a whole.

Conclusion

The systematic physical training exerts a serious impact on the organism of sportswomen. It influences their puberty development and especially the appearance of menarche by delaying it. These processes in young girl's organism depend directly upon the levels of their fat weight, which as a rule is lower for actively training girls. These girls reach later the critical level of fat weight, which switches on female sex hormone metabolism thus causing a delaying menarche. The decrease of fat weight under defined levels can cause hormonal and metabolic disturbances even in cases of normal menstrual cycle. The trainers and sport doctors should not ignore this fact but should try to combine it with the ever lasting striving for new sport attainments.

References

1. Arner, P. Adrenergic receptor function in fat cells. — Amer. J. Clin. Nutr., **55**, 1992, 228-236.
2. Bray, G. Leptin and leptinomania. — Lancet, **348**, 1996, No 1, 140.
3. Brouns, Fr., Ger J. van der Vusse. Utilization of lipids during exercise in human subjects: metabolic and dietary constraints. — Brit. J. of Nutrition, **79**, 1998, 117-128.
4. Duclos, M. Rapid Leptin decrease in immediate post exercise recovery. — Clin. Endocr., **50**, 1999, No 3, 337-342.
5. Frisch, R. E. Delayed menorrhea and amenorrhea in balet dancers. — N. Engl. J. Med., 1980, 303, 17-19.
6. Saltin, B., P. O. Astrand. Free fatty acid and exercise. — Amer. J. of Clininical Nutrition, **57**, 1993, 752-758.
7. Wahenberg, H., P. Engfeldt, J. Bolinder, P. Arner. Acute adaptation in adrenergic control of lipolysis during physical exercise in humans. — Amer. J. of Physiology, **253**, 1987, 383-390.
8. Wendling, P. S., S. J. Peters, G. F. Heigenhauser, L. L. Spriet. Epinephrine infusion does not enhance net muscle glycogenolysis during prolonged aerobic exercise. — Canadian J. Appl. Physiology, **21**, 1996, 271-284.
9. Бонева, Ж., Д. Николовска, П. Йовчевски. Лептинът — отправни точки, Ендокринни заболявания, 28, 1999, № 4, 8—14.
10. Савов, Сл., Ив. Топузов, Ив. Наков. Особености в половото съзряване при момичета, активно-занимаващи се със спорт. — В: Научно-практ. конференция на НДСМЛФК (Ловеч), 13—15 октомври 1989 г. Сборник резюмета, с. 56.
11. Станимирова, Н., И в. Топузов, Ч. Петрова. Сравнително проучване на затлъстяването в детско-юношеската възраст в Плевен, 1973—1988. IV симпозиум по затлъстяване. Варна, 13 — 15 .V. 1988. Сборник резюмета, с. 39.
12. Станимирова, Н. Растеж и пубертетно развитие — норми и физиологични отклонения. Дисерт. труд (Плевен, 1998 г.).
13. Топузов, Ив. Затлъстяването при подрастващи — разпространение, характеристика и лечебен подход. Дисерт. труд (София, 1997 г.).
14. Топузов, Ив.. Целулитът — характеристика и комплексно лечение. С., Грация-М, 2000.

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Sexual Maturation in Schoolpupils in The Town of Smolyan in 1980s, in their Mothers and its Sociofamilial Differentiation

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In 1986 in the district center Smolyan a transversal study of sexual maturation in 192 girls and 169 boys aged 9 to 17 and a retrospective study of age at menarche in 220 their mothers were carried out on the background of their sociofamilial conditions. A relatively late sexual maturation is found. Sexual maturation terms are close to these in small towns and villages, not to these in the big cities. This phenomenon is due to the specific sociofamilial structure and the ruralization in the way of life. The withingroup analysis shows significant social differentiation in pubertal terms both in children and in their mothers. Fathers social status seems to be more important than mothers one in this differentiation. Schwidetzky-Pavilonis' sexual maturation index is found to be a good measure of the influence of the environment on the maturation in boys (as the age at menarche in girls is).

Key words: puberty, age at menarche, adolescence in boys, sexual maturation index, sociofamilial differenciacion

Introduction

The majority of the investigations on sexual maturation in Bulgaria are carried out in the cities (predominantly Sofia) and other highly urbanized areas [4]. The aim of this study is to throw some light on this process in a less urbanized environment.

The town of Smolyan is located in the Rhodope mountains in South Bulgaria on an altitude of about 900-1000 metres. It was formed in 1958 by consolidation of two small towns (Smolyan, Ustovo), two big villages (Upper and Lower Raykovo) and a few smaller ones. Even today it is not a compact built area, but a 16 kilometres long series of settlements. Before 1975 its population increased quickly because of high rural-urban migration. Then the migration balance became negative. About one third of the population is Bulgarian-speaking, but connected with the Islam (mostly by family tradition), with a relatively conservative way of life. Because of all the above mentioned reasons in 1980s Smolyan was a district center, but the way of life of its 30 000 inhabitants was similar to that in the small towns and villages (the phenomenon of "ruralization").

Material and Methods

In the autumn of 1986 a transversal study of sexual maturation with an investigation on sociofamilial conditions was carried out in 192 schoolgirls and 169 schoolboys, aged 9 to 17 years at last birthday, in the town of Smolyan. In 220 women, their mothers, data of age at menarche and of sociofamilial origin have been collected.

The following signs of sexual maturation are traced: axillary and pubertal hair and breast development in both sexes, beard hair in boys and menarche in girls. The secondary sexual signs are evaluated by a scale from 0 to 3 points, excluding pubic hair and breast development in boys — from 0 to 4 and from 0 to 2 respectively [9]. If needed, intermediate marks are used. For evaluation of the stage of puberty development the total index of sexual maturation after Schwidetzky and Pavilonis is used. This index is a generalisation of the particular sexual maturation signs and varies from 0 to 12. On the basis of it the following stages of sexual maturation are distinguished: 0 — juvenile stage; 0.5 — first puberty signs or juvenile-2 in girls, prepuberty in boys; 1.0 — prepuberty in girls; 2.0 (1.5) — early puberty or phasis cetera in girls (boys); 6.5 (5.0) — late puberty or phasis lenta in girls (boys); 10.5 — postpuberty in both sexes; 12.0 — adult stage [1].

Median age at menarche and other stages of puberty is evaluated by probit analysis. In the subsamples with different sociofamilial conditions three-year moving sums and average ages are used to estimate the proportion of adolescents of the traced stage of puberty by age (the basic data for probit analysis).

Results and Discussion

The sexual maturation of the investigated boys and girls is relatively late [Table 1]. The median age at menarche is 13.44 ± 0.17 years against 12.84 ± 0.11 in Sofia ones in the same time [6] — a delay of 0.6 years. When calculated excluding breast development (as in Sofia adolescents) the sexual maturation index 5.0 (the peak moment in the puberty in the boys) appears at 15.0 ± 0.2 years against 14.3 ± 0.1 in Sofia ones [6], i.e. also by 0.7 years later. Thus the sexual maturation index in boys demonstrate its value as an indicator of the differentiation of the sexual maturation terms.

The explanation of the relatively late maturation in Smolyan adolescents against Sofia ones is found to be in their different sociofamilial characteristics: lower parental education, more manual workers among the parents, higher number of children in the family etc. (Table 2). These characteristics are usually connected with a later sexual maturation [2].

Since most of the investigations on sexual maturation in Bulgaria are carried out in urbanized areas, the reported ages at menarche (from late 1960s to 1980s) are close to these found in Sofia. Only in the small mountain town of Kostenets and in the rural population in the district of Haskovo the ages at menarche (13.4 and 13.8 years respectively) are similar to this found in Smolyan [7, 8]. Retrospective studies in Rhodopian local rural samples show even higher mean age at menarche — about 13.8 years in traditionally Christians and 14.6 in traditionally Muslims in 1960s and 1970s [4]. The figures obtained in retrospective study in Sofia university students are also close to Smolyan one — 13.6 in the students from small towns and 13.3 in these ones from medium towns (mostly district centers) in the late 1970s [10].

The withingroup analysis shows that the dwelling conditions show the stronger connection with the sexual maturation in the Smolyan boys and girls (Table 3). In

T a b l e 1. Age at some stages of sexual maturation in Smolyan adolescents (years)

Sign	Girls				Boys			
	Stage	Mean	*m*	*s*	Stage	Mean	*m*	*s*
Menarche		13.44	0.17	1.21				
Breast	Ma 0.5	10.14	0.38	1.45	C 0.5	12.72	0.28	2.43
	Ma 1.5	12.68	0.20	1.35	C 1.0	13.57	0.20	1.68
	Ma 2.5	13.82	0.19	1.37	C 1.5	14.66	0.17	1.21
	Ma 3.0	15.31	0.20	1.65	C 2.0	14.99	0.17	1.08
Pubic hair	P 0.5	11.31	0.23	1.32	P 0.5	12.44	0.20	1.58
	P 1.5	12.46	0.14	0.91	P 1.5	13.45	0.16	1.30
	P 2.5	13.03	0.19	1.37	P 2.5	14.78	0.17	1.23
	P 3.0	13.72	0.22	1.67	P 3.5	16.24	0.37	1.65
					P 4.0	(17.6)	(1.7)	(2.6)
Axillary hair	Ax 0.5	11.71	0.22	1.42	Ax 0.5	13.95	0.20	1.78
	Ax 1.5	13.56	0.18	1.27	Ax 1.5	15.10	0.21	1.40
	Ax 2.5	14.12	0.16	1.11	Ax 2.5	16.82	0.51	1.91
	Ax 3.0	14.69	0.17	1.40	Ax 3.0	(17.3)	(0.8)	(1.3)
Beard					Ba 0.5	14.27	0.32	2.43
					Ba 1.5	16.27	0.36	1.53
					Ba 2.5	(17.6)	(0.8)	(1.8)
					Ba 3.0	(19.0)	(3.8)	(2.4)
Juvenile 2	SMI 0.5	10.09	0.37	1.43				
Prepuberty	SMI 1.0	11.26	0.24	1.31	SMI 0.5	11.93	0.47	1.82
Phasis cetera	SMI 2.0	11.86	0.20	1.04	SMI 1.5	12.78	0.21	1.75
Phasis lenta	SMI 6.5	13.09	0.16	1.08	SMI 5.0	14.61	0.17	1.17
Postpuberty	SMI 10.5	14.44	0.16	1.18	SMI 10.5	16.67	0.37	1.28
Adult	SMI 12.0	15.72	0.19	1.45				

(17.) uncertain values because exceeding the limits of the period under study

Sofia the most important role plays the income per capita [6]. The explanation can be that only regular money incomes were reported in the investigations. However, in Smolyan (as in the villages and the small towns) the small private farming played an important role in family economy in this period. Thus the dwelling conditions reflect better the social status of a family in Smolyan (in the big cities housing was centralized and relatively more independant from the family well-being).

The mean age at menarche in pupils' mothers is 14.24±0.09 years against 13.32±0.07 years in Sofia schoolchildren mothers of the same generation [5]. It reflects the situation around 1960 (1950s and 1960s were the period of adolescence in these women).There are strong sociofamilial differences in their age at menarche. The most important of them are the differences by the education of the father and by the number of sibs (Table 4). In the adolescents father's education and occupation are also more important than mother's ones. The first present statistically significant relations with 7 markers of sexual maturation, the second — only with one (mother's education with age at menarche). Differences by education of father and by number of sibs were also the higher ones in Sofia university students menarcheal age, which reflects the situation in late 1970s [10] and in Sofia adolescents in mid 1980s [6]. The investigations about 15 years later in Smolyan adolescents also demonstrate, that father's education influences stronger the growth than mothers, despite the well pronounced acceleration of the sexual maturation in the period 1960-2000 — age at menarche decreased from 14.2 to 12.8 years [3].

T a b l e 2. Comparison between the families of Smolyan and Sofia adolescents

Sociofamily conditions:	Smolyan			Sofia			
	N	*M*	*m*	*N*	*M*	*m*	$p \leq$
Children in the family	361	2.04	0.03	902	1.87	0.03	0.001
Father's education, years	271	11.8	0.2	540	13.8	0.1	0.001
Mother's education, years	264	11.9	0.2	544	13.6	0.1	0.001
Monthly income per capita, levs	263	118.3	2.4	511	126.3	2.0	0.02
Dwelling surface per capita, sq.m.	233	21.6	0.5	492	19.2	0.4	0.001
Inhabitants per room	254	1.59	0.05	502	2.00	0.05	0.001
	n	%		*n*	%		$p \leq$
Father's occupation:							
manual work	185	56.1		174	31.4		
nonmanual work	145	43.9		381	68.6		0.001
Mother's occupation:							
manual work	95	34.5		81	14.8		
nonmanual work	179	65.1		461	84.3		0.001
Muslim confessional tradition	143	39.6		3	0.3		0.001

T a b l e 3. Sexual maturation and dwelling conditions

Dwelling conditions	Age (years) at:					
	SMI 5.0 in boys			menarche in girls		
	N	*M*	*m*	*N*	*M*	*m*
Dwelling surface per capita, sq.m.						
less than 21	59	14.95	0.19	59	13.88	0.15
over 22	54	14.02	0.15	51	13.07	0.17
Inhabitants per room:						
less than 1.6	75	14.26	0.13	79	13.47	0.12
over 1.67	53	14.96	0.23	47	14.14	0.26

T a b l e 4. Age at menarche in Smolyan women by number of sibs and parental education

Family characteristics		*n*	Age at menarche (years)		
			M	*m*	*s*
Number of sibs					
0-1		121	13.90	0.12	1.36
2-3		72	14.50	0.14	1.21
4-8		26	15.08	0.23	1.20
Father's education, years	0-4	48	14.70	0.23	1.58
	8	124	14.30	0.11	1.27
	10+	43	13.61	0.16	1.09
Mother's education, years	0-4	56	14.74	0.18	1.36
	8	138	14.13	0.11	1.32
	10+	25	13.71	0.25	1.25

Conclusions

1) A relatively late sexual maturation is found in Smolyan, due to the specific sociofamilial structure and the ruralization in the way of life.

2) Even in these relatively small samples statistically significant differences in pubertal terms in different sociofamilial environment can be traced.

3) The sexual maturation index is nearly such a good indicator of the influence of the sociofamilial environment on the sexual maturation in boys, as the menarche in girls.

4) Father's social status seems to be more important factor in the differentiation of sexual maturation terms as in Smolyan and in Sofia, i.e. Bulgarian society is still "patriarchal".

References

1. Cieslik, J., M. Drozdowska, A. Malinowski. Norma rozwojowa — teoretyczne i praktyczne aspekty oceny rozwoju biologicznego czlowieka. — In: Antropologia fizyczna (Editors: A.Malinowski, J.Strzalko). Warszawa-Poznan, PWN, 1986, 511-536.
2. Laska-Mierzejewska, T. , E.Luczak. Biologiczne mierniki sytuacji spoleczno-ekonomicznej ludnosci wiejskiej w Polsce w latach 1967, 1977, 1987. Wroclaw, Zaklad Antropologii PAN, 1993, 1-127.
3. Mladenova, S., M. Nikolova. Intragroup differences in morphological characteristics in children and adolescents from Smolyan region based on social and economic factors. — Anthropology, 4, 2003 (in print).
4. Stoev, R. Sexual maturation in girls from Bulgaria (a review). — In: Growth and Ontogenetic Development in Man IV, Prague, Dept. of Anthropology, Charles Univ., 1994, 197-200.
5. Stoev, R. Age at menarche in women in Sofia. — Papers of Anthropology, VI, 1995, 218-220.
6. Stoev, R., T. Kazakova, L. Zhelkova, L.Tsacheva. Sexual maturation and sociofamilial conditions in school boys and girls in Sofia. — Anthrop. Kozl., 32, 1989/90, 131-135.
7. Бъчварова, М., Й. Великова. Менархе при момичетата от Костенец. — Научн. Труд., Пловд. Унив., 22, 1984, № 1, Биол., 287-296.
8. Дамянова, Цв. Показатели за нормален пубертет при момичета. Дисерт. труд (София), 1—238.
9. Миклашевская, Н. Н. Биологический возраст. — В: Морфология человека. Москва, МГУ, 1983, 20—25.
10. Стоев, Р., Цв. Казакова, Л. Желкова, Л. Цачева. Възраст при менархе и някои специфични за човека екологични фактори при студентки в град София. — В: Приносът на младите научни работници при решаването на екологичните проблеми. Враца, СНРБ — клон Враца, 1988, 151—156.

Institute of Experimental Morphology and Anthropology

Acta morphologica et anthropologica, 8
Sofia • 2003

Fluctuating Asymmetry in Patients with Down's Syndrome

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The fluctuating asymmetry level of four dermatoglyphic features (palmar ridge count, finger ridge count, *atd* angle and patterns' type on the homologous digits) is studied. The investigation encloses 116 boys and girls with Down's syndrome, as well as a control group of 260 healthy boys and girls. Generalized the Down's patients showed a higher level of fluctuating asymmetry compared to the controls. In boys with Down's syndrome, the highest level of fluctuating asymmetry is established for the ridge count and the type of pattern on the 4th homologous digits. In girls with Down's syndrome the highest level is founded for "b-c" and "c-d" palm ridge count and the pattern's type on the 4th homologous digits. The data obtained can give an interpretation to the results from the disturbances in the ontogenetic development of the individuals with Down's syndrome.

Key words: dermatoglyphic features, fluctuating asymmetry, patients with Down's syndrome.

Introduction

The fluctuating asymmetry of bilateral morphological structures is an indicator of homeostasis in the development of individuals. Basis for this understanding is the idea that the genetical and environmental factors, confusing the normal development of individuals, have a negative effect upon the control in the formation of bilateral structures. Therefore, the disturbances' level in the perfect bilateral symmetry gives possibility for the preciseness of the mechanisms in the homeostatic control, as well as the general capability of the organism to resist the negative genetical and environmental factors to be assessed [2, 3, 4, 5, 6, 7, 10].

The aim of the present study is to evaluate the fluctuating asymmetry level of four dermatoglyphic features (fingerprint patterns and finger ridge counts on pair fingers; palmar *a-b, b-c, c-d* and *a-d* ridge counts, and *atd* angle on pair palms) in patients with Down's syndrome.

Material and Methods

The investigation includes 116 patients (64 boys and 52 girls) with Down's syndrome. The dermatoglyphic prints are taken by the typographical method [9]. The

finger papillar patterns are read by the method of C u m m i n s and M i d l o [1] and the *atd* angle is evaluated by the criterion of S h a r m a [8].

The fluctuating asymmetry level about finger and palm ridge counts and *atd* angle is determined by the coefficient of indetermination ($1-r^2$). The square of the product-moment correlation coefficient (r^2) of the two variables is a measure of their common variance, and the coefficient of indetermination ($1-r^2$) is an estimate of their unshared variance and thus of fluctuating asymmetry [6]. In our study, this unshared variance, regarding the finger and palmar ridge counts and *atd* angle on both hands, determines the fluctuating asymmetry level for the three investigated features. The measure of fluctuating asymmetry for finger patterns is estimated by the degree of pattern discordance [6]. The fluctuating asymmetry level of four dermatoglyphic features in the group of patients is compared to analogical data for a control group of 260 healthy children (129 boys and 131 girls).

Results and Discussion

The mean values of finger ridge count, palmar ridge count and *atd* angle in right and left for the patients with Down's syndrome and the controls are given in Table 1. Summarized the boys from the control group have bigger values of finger ridge count on both hands compared to the patients. Statistical significant are the differences of ridge counts between patients and controls in boys with the exception of those for the III finger in left and II finger in right. The girls from the control group have bigger values for ridge counts on IV and V fingers in left and on I, IV and V fingers in right compared to the girls with Down's syndrome. Statistical significant are only the ridge counts' differences for the V fingers in left and right, as well as for the IV finger on the right hand.

The control boys and girls have greater measures for palmar ridge counts *a-b, b-c, c-d* and the total *a-d* ridge count on both hands compared respectively to the Down's boys and girls. The established differences of palmar ridge counts are statistical significant for the boys of both groups. In girls, statistical significant are only the differences for palmar *a-b* and *c-d* ridge counts and the total *a-d* ridge count on the right hand.

The *atd* angle on the right and left hands in the Down's patients is considerably larger compared to the controls for both sexes. All the established differences are statistical significant.

The correlation coefficients between ridge count on homologous fingers, palmar ridge count and *atd* angle for the right and left hands are given in Table 2. The finger and palm ridge count analyzed shows predominantly smaller values of correlation coefficient in Downs than in the controls for both sexes. The differences between correlation coefficient for both boys and girls, and Down's patients and controls are comparatively great, but statistical significant is only the difference for IV pair fingers in boys.

The patients with Down's syndrome display smaller correlation for palmar ridge counts compared to the controls. Statistical significant differences are not established for the correlation coefficients of palmar ridge count between boys and girls in both studied groups.

Considerably greater are the correlation coefficients for *atd* angle in the patients with Down's syndrome for both sexes compared to the healthy subjects. The difference in boys is statistical significant, and in girls it comes nearby, but is not statistical significant.

T a b l e 1. Finger ridge counts, palmar ridge counts and palmar atd angles of persons with Down's syndrome and controls

Features	Boys						Girls					
	persons with Down's syndrome (n=64)		controls (n=129)		t	P	persons with Down's syndrome (n=52)		controls (n=131)		t	P
	X	SD	X	SD			X	SD	X	SD		
Finger ridge counts												
I left	15.19	5.25	18.03	5.29	3.530	<0.01	13.81	6.01	13.34	5.41	0.491	>0.05
II left	10.59	4.91	12.38	6.27	2.168	<0.05	10.31	5.20	9.05	5.88	1.423	>0.05
III left	11.59	4.67	12.88	5.53	1.697	>0.05	10.90	4.83	9.44	5.73	1.746	>0.05
IV left	10.86	4.69	16.29	5.22	7.289	<0.01	10.29	5.62	11.81	6.31	1.592	>0.05
V left	8.48	4.11	13.33	4.27	7.618	<0.01	8.29	4.21	10.08	4.51	2.541	<0.05
I right	17.16	5.56	20.43	5.14	3.943	<0.01	15.44	6.21	16.06	5.86	0.619	>0.05
II right	11.34	4.86	12.98	6.88	1.912	>0.05	10.93	4.94	10.31	5.89	0.724	>0.05
III right	11.34	4.53	13.35	5.75	2.646	<0.01	10.52	4.23	9.89	5.53	0.829	>0.05
IV right	10.66	4.66	17.15	5.33	8.676	<0.01	11.27	5.25	13.12	5.53	2.117	<0.05
V right	8.75	3.34	13.40	5.07	7.608	<0.01	8.29	4.27	10.63	5.30	3.113	<0.01
I–V left	56.75	19.14	72.94	20.36	5.416	<0.01	53.60	19.99	53.49	21.62	0.033	>0.05
I–V right	59.25	18.13	77.66	21.58	6.225	<0.01	56.44	18.24	59.91	22.21	1.088	>0.05
Palmar ridge counts												
a-b left	30.75	8.36	37.91	7.68	5.622	<0.01	31.46	7.04	33.76	5.95	1.928	>0.05
b-c left	19.43	5.72	24.30	5.72	5.448	<0.01	21.28	5.62	22.11	5.08	0.860	>0.05
c-d left	28.48	8.12	32.91	6.74	3.678	<0.01	30.70	6.28	31.08	6.05	0.347	>0.05
a-d left	78.88	18.50	95.45	15.32	6.041	<0.01	83.91	14.70	86.88	12.47	1.196	>0.05
a-b right	30.98	7.04	39.02	7.60	7.124	<0.01	31.42	5.65	34.64	5.88	3.210	<0.01
b-c right	21.10	5.27	24.85	5.88	4.386	<0.01	21.91	5.49	22.08	5.22	0.178	>0.05
c-d right	30.22	8.25	33.60	6.27	2.818	<0.01	29.67	6.64	32.01	5.64	2.078	<0.05
a-d right	82.30	15.52	97.74	14.99	6.435	<0.01	83.54	12.91	88.65	12.68	2.262	<0.05
atd angles												
left	73.43	18.93	43.91	7.23	12.020	<0.01	72.43	16.48	45.08	9.42	11.083	<0.01
right	73.38	17.96	44.13	7.31	12.493	<0.01	70.18	14.83	44.91	8.64	11.351	<0.01

T a b l e 2. Correlation (r) between the left and right finger ridge counts, palmar ridge counts and palmar *atd* angles of persons with Down's syndrome and controls

Features	Correlations (r)							
	Boys				Girls			
	persons with Down's syndrome	controls	t	P	persons with Down's syndrome	controls	t	P
Finger ridge counts								
I	0.7636	0.6715	1.189	>0.05	0.7710	0.6963	0.911	>0.05
II	0.6217	0.7108	1.040	>0.05	0.5979	0.7294	1.403	>0.05
III	0.6304	0.7275	1.201	>0.05	0.7169	0.6455	0.788	>0.05
IV	0.5370	0.7600	2.513	<0.05	0.6937	0.7651	1.026	>0.05
V	0.7480	0.7124	0.550	>0.05	0.7091	0.7969	1.258	>0.05
Palmar ridge counts								
a-b	0.7000	0.7027	0.001	>0.05	0.5120	0.5877	0.635	>0.05
b-c	0.6689	0.7561	1.162	>0.05	0.4663	0.6632	1.561	>0.05
c-d	0.6983	0.6923	0.121	>0.05	0.5148	0.6992	1.682	>0.05
a-d	0.8177	0.8486	0.623	>0.05	0.6260	0.7405	1.154	>0.05
atd angles	0.8187	0.6485	2.419	<0.05	0.7476	0.5650	1.889	>0.05

Table 3. Fluctuating asymmetry measure ($1-r^2$) of finger ridge counts, palmar ridge counts and palmar *atd* angles of persons with Down's syndrome and controls

Features	Coefficient of indetermination ($1-r^2$)					
	Boys			Girls		
	persons with Down's syndrome [D]	controls [C]	difference [D-C]	persons with Down's syndrome [D]	controls [C]	difference [D-C]
Finger ridge counts						
I	0.4169	0.5491	-0.1322	0.4056	0.5152	-0.1096
II	0.6135	0.4948	0.1187	0.6425	0.4680	0.1745
III	0.6026	0.4707	0.1319	0.4861	0.5833	-0.0972
IV	0.7116	0.4224	0.2892	0.5188	0.4146	0.1042
V	0.4405	0.4925	0.0520	0.4972	0.3650	0.1322
Palmar ridge counts						
a-b	0.5100	0.5062	0.0038	0.7379	0.6546	0.0833
b-c	0.5526	0.4283	0.1243	0.7826	0.5602	0.2224
c-d	0.5124	0.5207	-0.1183	0.7350	0.5111	0.2239
a-d	0.3314	0.2799	0.0515	0.6081	0.4517	0.1564
atd angles	0.3297	0.5794	-0.2497	0.4411	0.6808	-0.2397

Table 4. Coincidence of finger patterns of homologous fingers of persons with Down's syndrome and controls

Homologous fingers	Boys						Girls					
	persons with Down's syndrome		controls		*t*	*P*	persons with Down's syndrome		controls		*t*	*P*
	X	SD	*X*	SD			*X*	SD	*X*	SD		
I	0.8308	0.378	0.7132	0.454	1.900	>0.05	0.7647	0.424	0.7752	0.419	0.152	>0.05
II	0.8923	0.312	0.5581	0.499	5.689	<0.01	0.7451	0.436	0.4961	0.502	3.335	<0.01
III	0.7846	0.414	0.7519	0.434	0.508	>0.05	0.7647	0.424	0.7209	0.450	0.619	>0.05
IV	0.6462	0.482	0.7519	0.434	1.482	>0.05	0.6667	0.471	0.7519	0.434	1.128	>0.05
V	0.8769	0.331	0.8295	0.378	0.893	>0.05	0.8235	0.381	0.8605	0.348	0.607	>0.05

Table 5. Discordance of finger patterns of homologous fingers of persons with Down's syndrome and controls

Homologous fingers	Boys			Girls		
	persons with Down's syndrome [D]	controls [C]	difference [D-C]	persons with Down's syndrome [D]	controls [C]	difference [D-C]
I	0.1693	0.2868	-0.1175	0.2353	0.2248	0.0105
II	0.1077	0.4419	-0.3342	0.2549	0.5039	-0.2490
III	0.2154	0.2481	-0.0327	0.2353	0.2791	-0.0438
IV	0.3538	0.2481	0.1057	0.3333	0.2481	0.0852
V	0.1231	0.1705	-0.0474	0.1765	0.1395	0.0370

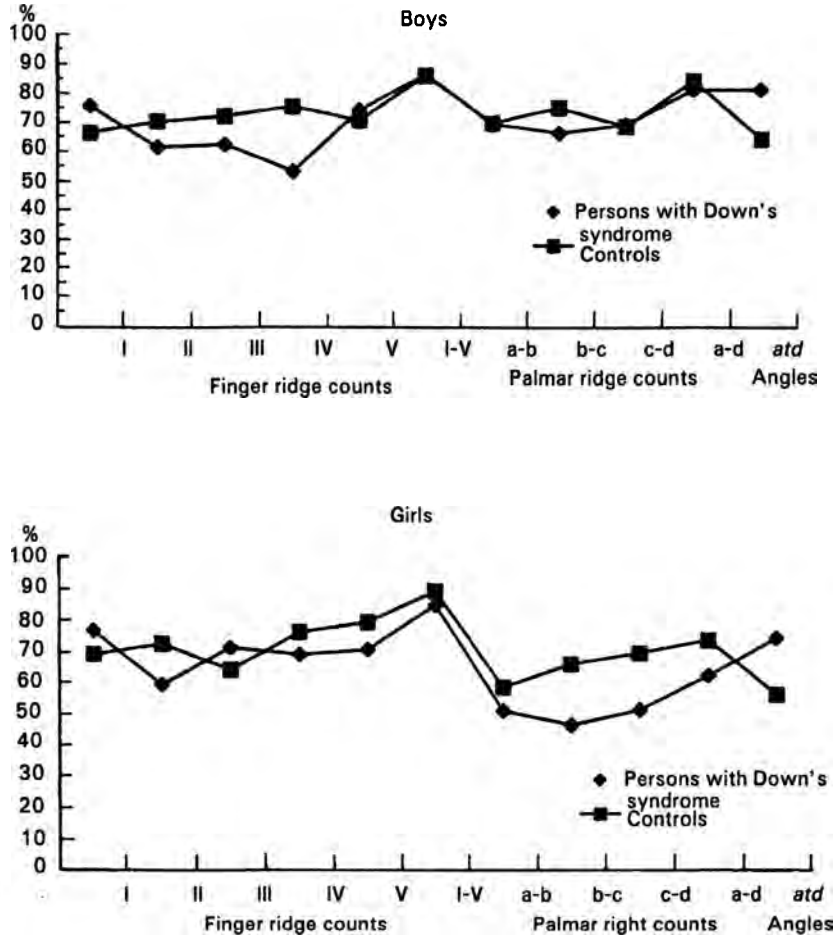

Fig. 1. Correlation between the left and right finger ridge counts, palmar ridge counts and palmar *atd* angles of persons with Down's syndrome and controls

The fluctuating asymmetry of the ridge count on pair fingers, palmar ridge counts and *atd* angle are given in Table 3. The fluctuating asymmetry measures are greater for II, III and IV finger pairs in the Down boys, and for II, IV and V finger pairs in the Down girls compared to the normal children. In the Down boys the fluctuating asymmetry level decrease in the direction IV > II > III > V > I pair fingers, and in the Down girls — II > IV > V > III > I pair fingers. Opposite to the patients' group, the fluctuating asymmetry for the controls decrease in the direction I > II > V > III > IV pair fingers for boys and III > I > II > IV > V pair fingers for girls. Greatest is the differences of fluctuating asymmetry between Downs and controls on the IV digit pairs in boys and on the II digit pairs in girls.

The Down's patients have greater measures of fluctuating asymmetry for palmar ridge count excepting the *c-d* ridge count in boys. Greatest is the difference of fluctuating asymmetry between Downs and controls for the *b-c* ridge count in both sexes.

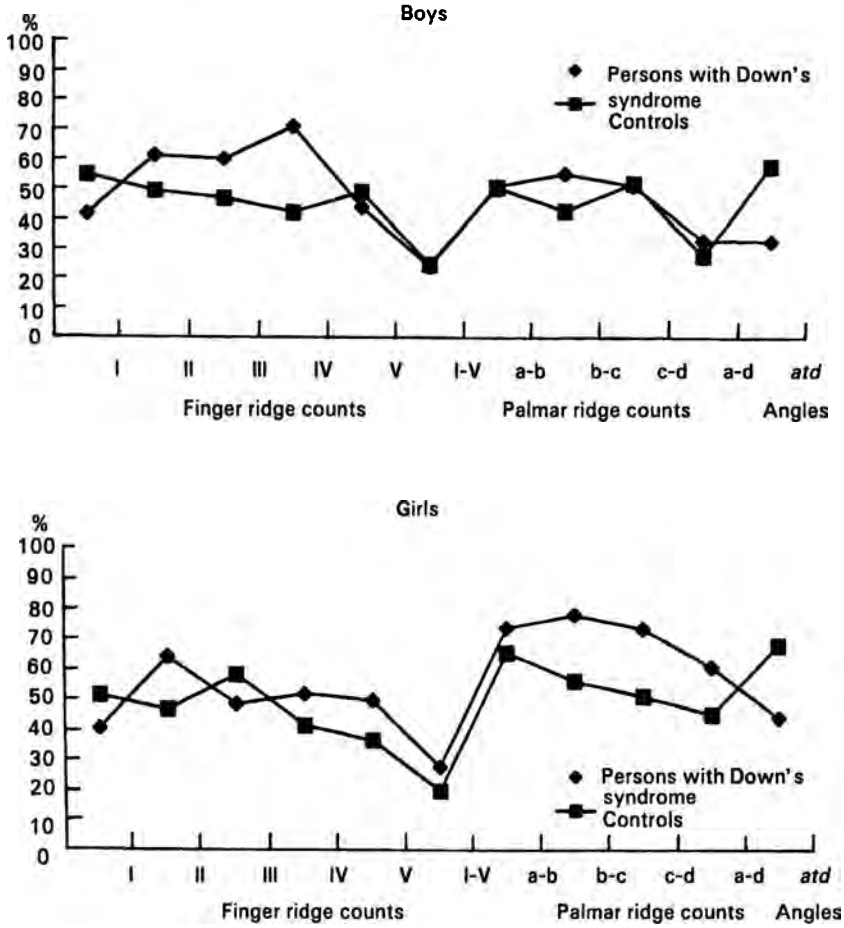

Fig. 2. Fluctuating asymmetry of finger ridge counts, palmar ridge counts and palmar *atd* angles of persons with Down's syndrome and controls

Opposite to the correlation coefficients' data, the fluctuating asymmetry level is smaller for the *atd* angle in both sexes of Downs compared to the controls.

The percentage variability of correlation coefficients and fluctuating asymmetry about finger and palmar ridge counts and *atd* angle are given in Fig. 1 and 2. The comparative analyses of the separate features indicate predominantly greater measures of fluctuating asymmetry for finger ridge count in the Down boys compared to the palmar ridge count and *atd* angle. Different are the findings for the Down girls. In them greater is the fluctuating asymmetry for palmar ridge count compared to the finger ridge count and *atd* angle. For the normal subjects, highest is the fluctuating asymmetry level for *atd* angle in both sexes.

The data about concordance of papillar patterns on homologous fingers for the right and left hand in the patients and controls are presented in Table 4. The proportion of concordant pairs belonging to the Down boys is highest for II and V pair fingers, and belonging to the Down girls — for V, III and I pair fingers. For the controls highest is the concordance for V, III and IV pair fingers in boys and for V

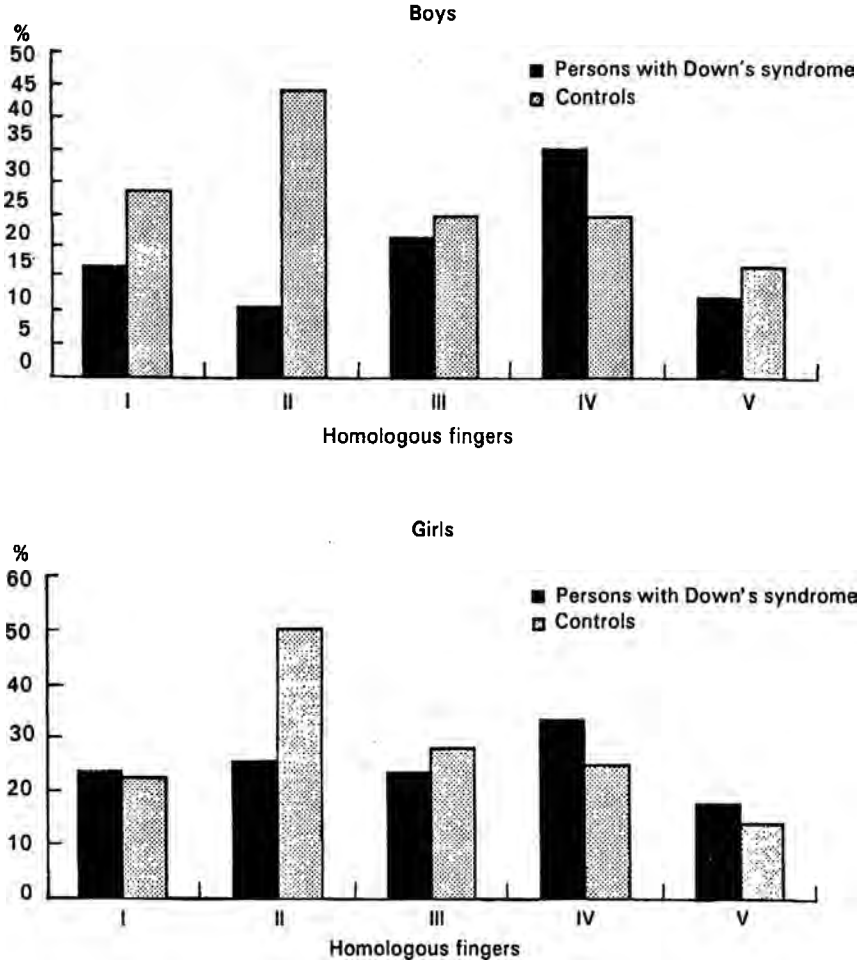

Fig. 3. Fluctuating asymmetry of finger print patterns of homologous fingers of persons with Down's syndrome and controls

and I pair fingers in girls. From all the established differences for the proportion of concordant pairs both for the investigated groups, and sexes statistical significant ($P<0.01$) are only the differences about II-nd homologous digits.

The data about proportions of discordance for finger papillar patterns, as well as the fluctuating asymmetry measures are given in Table 5. For the patients with Down's syndrome, the fluctuating asymmetry decrease in the direction IV > III > I > V > II pair fingers in boys, and in the direction IV > II > I = III > V pair fingers in girls. The fluctuating asymmetry measures for the controls decrease in the direction II > I > III = IV > V pair fingers in boys and in the direction II > III > IV > I > V pair fingers in girls.

The data analyzed about measures of fluctuating asymmetry for the type of finger patterns on the pair fingers in the Down's patients and controls are presented in Fig. 3. Very high is fluctuating asymmetry level for the II pair fingers, as in both investigated groups (patients and normal), so in boys and girls.

Conclusion

The summarized analyzes of the investigation shows considerable differences of fluctuating asymmetry level in the patients with Down's syndrome and the norm.

The fluctuating asymmetry degree is predominantly greater for the Down's girls than it is for the Down's boys, which causes the assumption, that possibly the sexual chromosomes exert some influence on the dermatoglyphic fluctuating asymmetry level.

The fluctuating asymmetry measures are greater for finger and palmar ridge counts then they are for *atd* angle in the patients with Down's syndrome. These data give reason, to be determined approximate correctly the period, during which the Downs gestation development is a subject of enormous disturbances (the formation of finger ridge count is during the period 10.5—13 gestation week, of palmar ridge count — till the 15 gestation week and of *atd* angle — after the 15 gestation week [10]).

The dermatoglyphic fluctuating asymmetry measures are considerably greater for the Down's patients than for the controls. As we know the disorder in the gene fund caused by the gene mutation — trisomie 21 leads to the manifestation of heavy morphological, physiological and neuro-psychological deviations in the individuals with Down's syndrome. The high level of fluctuating asymmetry in the Down's patients laid the assumption that this disorder leads also to an enormous reduction of the common stability in their individual development against the negative environmental factors.

References

1. Cummins, H., C. Midlo. 1943 Finger prints, palms and soles. Ann. Introduction to Dermatoglyphics. Philadelphia, Blakinstone. 1943. Reprinted: New York., Dover., 1961.
2. Koleva, M. A. Nacheva, M. Boev. Somatotype and Disease Prevalence in Adults. Reviews on environmental health., 17, 2002, No 1, 65-80.
3. Koleva, M. A. Nacheva. Somatotype, overweight and obesity. — Journ. of Anthr., 3, 2000, 83-91.
4. Livshits, G., E. Kobyliansky. Fluctuating asymmetry as a possible measure of developmental homeostasis in humans: a review. — Human Biology., 63, 1991, No 4, 441-466.
5. Livshits, G., P. Smouse. Multivariate fluctuating asymmetry in Israeli adults. — Human Biology., 65, 1993, № 4, 547-578.
6. Mellor, C, S. Dermatoglyphic evidence of fluctuating asymmetry in schizophrenia. — British Journal of Psychiatry, 160, 1992, 467-472.
7. Moore, S., B. Munger. The early ontogeny of the afferent nerves and papillary ridges in human digital glabrous skin. — Dev. Brain Res., 48, 1989, № 1, 119-141.
8. Sharma, A. Comparative methodology in dermatoglyphics. Delhi, 1964.
9. Гладкова, Т. Кожные узоры кисти и стопы обезьян и человека. М., Наука, 1966.
10. Сивков, С. Сравнителнo антропологично проучване на шизофренно болни от гледна точка на невроонтогенетичната хипотеза за шизофренията. Дисерт. труд (Пловдив), 2000, 140 с.

Institute of Experimental Morphology and Anthropology

Acta morphologica et anthropologica, 8
Sofia • 2003

Functional Asymmetry of the Upper Limbs in Bulgarians from North Bulgaria

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The hand clasping and arm folding types of 770 men and 741 women, aged 30-39, from North-West, North Central and North-East Bulgaria have been studied. In the males the L-hand clasping and arm folding types are prevalent while in the women it is the R-hand clasping and the L-arm folding types that are more common. The territorial variations in both sexes with regard to both types of asymmetry are statistically insignificant. The intersexual differences are more clearly pronounced in hand clasping. A statistically insignificant correlation between hand clasping and arm folding has been established.

Key words: functional asymmetry, hand clasping, arm folding, intersexual differences.

Introduction

Hand clasping (interlocking the fingers of the hands) and arm folding (crossing of arms on the chest) represent some of the main tests for studying functional asymmetry in the human as an expression of the functional asymmetry of the brain. The study of the functional asymmetry of the upper limbs is of major importance since it is related to active perception which is to a large extent attributed to hands. It is one of the main sources of sensory cognition and hence of abstract thinking and psychological development of the human as a whole.

L u t z [14] was the first to describe two types of interlocking of the fingers. In one case the right thumb is situated over the other fingers and is defined as the R-type and in the other it is the left thumb that is in the same position and this is the L-type. Analogous are the cases in crossing the arms noted by W i e n e r [19]. Quite a number of authors show an interest towards this problem starting from the early 20th century up to our day. Their interests have been directed towards the determination of the factors accounting for these asymmetries, their interrelation, the sex, age and racial influences on them. A unified explanation as to their nature is not found even now, however. L u t z himself [14] as well as a variety of other authors [5, 6, 15, 20] support the hypothesis of their genetic predetermination. Others state that there are no proofs of such a control [4, 19]. According to L o u r i e [13] arm folding as opposed to hand clasping is of a lesser value as an anthropological marker and that the genetic control is more evident in hand clasping. L a i a n d

W a l s h [11] suggest that these are more of a habit rather than genetic predetermination. L e g u e b e [12] mentions about a combined influence of the factors in determining the hand clasping type. According to some authors sex and age do not exert an influence on the asymmetries under study [1, 7] while according to others they do [15]. A number of authors do not find correlation between the hand clasping and arm folding types [1, 16, 17, 19]. Others insist on a significant correlation between them [9, 10]. S a r n a et al. [18] based on literature data prove statistically that asymmetry in hand clasping and arm folding is not an arbitrary phenomenon.

In our country there is a scanty amount of studies on this problem. B o e v and T o d o r o v [2] have investigated a total of 2638 persons of three ethnic groups and have found a very high percentage of the R-hand clasping type especially in the Bulgarians. M u t a f o v [21] has studied a control group of 1500 healthy children with a predominant L-hand clasping type. K a r e v [8] has surveyed 2100 students later and has also found a higher per cent of the L-type in both asymmetries.

The aim of the present study is to establish the frequency of the hand clasping and arm folding types in Bulgarians from the three parts of North Bulgaria — North-West, North Central and North-East Bulgaria, to take into account the territorial variability, the intersexual differences and looking for a correlation between these two types of asymmetry.

Material and Methods

A total of 1511 Bulgarians of both sexes (770 men and 741 women) aged 30-39, from the three regions of North Bulgaria — North-West, North Central and North-East Bulgaria have been studied. In the boundaries of the regions under study four administrative districts — Montana, Lovech, Rousse and Varna are situated (according to the administrative-territorial division of Bulgaria since 1987). The studied persons originate from these districts and live there. This study was conducted in unison with the National Programme "Anthropological Characterization of the Bulgarian People" (1989 — 1993) carried by the Department of Anthropology in IEMA at the Bulgarian Academy of Sciences. It was performed using the conventional methods [3, 4, 12], with χ^2-test applied for comparing the populations under study.

Results and Discussion

Hand clasping

The L-type is prevalent in the males as a whole for North Bulgaria, while in women it is the R-type that is more common. The intersexual differences are statistically significant (Table 1). In the males from all three parts of North Bulgaria the L-type is predominant whose percentage grows from the West to the East. The territorial differences are statistically insignificant. In the women from North-West and North-East Bulgaria the L-type is prevailing while in those from the North Central Part the percentage of the R-type is higher. The territorial differences are statistically in significant (Table 2).

T a b l e 1. Percent distribution of hand clasping types among a population of North Bulgaria

Sex	R - type		L - type		Total
	n	%	*n*	%	
Males	344	44.68	426	55.32	770
Females	376	50.74	365	49.26	741
Both sexes	720	47.65	791	52.35	1511

$\chi^2 = 5.82 > 3.84$, $k=1$, $P < 0.05$

T a b l e 2. Comparison of the frequencies of hand clasping types among the three regions of North Bulgaria

Region	Sex	R –type		L - type		Total	Comparison groups	χ^2 0.05 $k=1$
		n	%	*n*	%			
1. North - West Bulgaria	Males	139	46.18	162	53.82	301	Males	
	Females	137	48.58	145	51.42	282	1 – 2	0.02
2.North Central Bulgaria	Males	97	46.41	112	53.59	209	1 – 3	1.04
	Females	109	55.33	88	44.67	197	2 – 3	0.93
3. North - East Bulgaria	Males	108	41.54	152	58.46	260	Females	
	Females	130	49.62	132	50.38	262	1 – 2	2.39
Total North Bulgaria	Males	344	44.68	426	55.32	770	1 – 3	0.11
	Females	376	50.74	365	49.26	741	2 – 3	1.52

T a b l e 3. Percent distribution of arm foldihg types among a population of North Bulgaria

Sex	R - type		L - type		Total
	n	%	*n*	%	
Males	342	44.42	428	55.58	770
Females	328	44.26	413	55.74	741
Both sexes	670	44.34	841	55.66	1511

$\chi^2 = 0.00 < 3.84$, k=1, P > 0.05

T a b l e 4. Comparison of the frequencies of arm folging types among the three regions of North Bulgaria

Region	Sex	R - type		L - type		Total	Comparison groups	χ^2 0.05 k=1
		n	%	*n*	%			
1. North - West Bulgaria	Males	142	47.18	159	52.82	301	Males	
	Females	120	42.55	162	57.45	282	1 – 2	3.62
2.North Central Bulgaria	Males	80	38.28	129	61.72	209	1 – 3	0.02
	Females	80	40.61	117	59.39	197	2 – 3	3.27
3. North - East Bulgaria	Males	120	46.15	140	53.85	260	Females	
	Females	128	48.86	134	51.14	262	1 – 2	0.11
Total North Bulgaria	Males	342	44.42	428	55.58	770	1 – 3	2.44
	Females	328	44.26	413	55.74	741	2 – 3	3.43

T a b l e 5. Percent distribution of hand clasping / arm foldihg combinations among the three regions of North Bulgaria

Sex	RR		RL		LR		LL		Total
	n	%	*n*	%	*n*	%	*n*	%	
Males	165	21.42	179	23.25	176	22.86	250	32.47	770
Females	153	20.65	222	29.96	175	23.62	191	25.77	741
Both	318	21.05	401	26.54	351	23.23	441	29.18	1511

$\chi^2 = 12.41 > 7.82$, $k=3$, $P < 0.05$

T a b l e 6. Comparison of the frequencies of hand clasping / arm folging combinations among the three regions of North Bulgaria

Region	Sex	RR		RL		LR		LL		Total	Comparison groups	χ^2 0.05 k=3
		n	%	*n*	%	*n*	%	*n*	%			
1. North - West Bulgaria	Males	67	22.26	72	23.92	74	24.58	88	29.24	301	Males	
	Females	50	17.73	87	30.85	70	24.82	75	26.60	282	1 – 2	4.68
2.North Central Bulgaria	Males	43	20.57	54	25.84	37	17.70	75	35.89	209	1 – 3	1.30
	Females	46	23.35	62	31.47	34	17.26	55	27.92	197	2 – 3	4.56
3. North - East Bulgaria	Males	55	21.15	53	20.39	65	25.00	87	33.46	260	Females	
	Females	57	21.76	73	27.86	71	27.10	61	23.28	262	1 – 2	4.98
Total North Bulgaria	Males	165	21.42	179	23.25	176	22.86	250	32.47	770	1 – 3	4.77
	Females	153	20.65	222	29.96	175	23.62	191	25.77	741	2 – 3	6.31

Table 7. Relationship between hand clasping and arm folging among a population of North Bulgaria

Hand clasping	Arm folding		
	right	left	total
Right	318	401	719
Left	351	441	792
Total	669	842	1511

$\chi^2 = 0.01 < 3.84$, k=1, $P > 0.05$

Arm folding

In both sexes for North Bulgaria as a whole the L-type is prevalent with percentages of distribution almost equal. Intersexual differences are not observed (Table 3). In both sexes from all three parts of North Bulgaria the L-type is prevalent with a highest percentage in North Central Bulgaria. The women from North-East Bulgaria are with close R- and L-types percentages in value. The territorial differences are statistically insignificant (Table 4).

The percent distribution of the combinations between the R- and L-types of hand clasping and arm folding has been traced. Very often a combination of one of two tests with right domination, and for others — with a left one is found. In the males as a whole for North Bulgaria the per cent of the combination between the L-type of hand clasping and the L-arm folding type is highest (LL). In the women it is the combination between the R-hand clasping type and the L-arm folding one that is more common (RL). The intersexual differences are statistically significant (Table 5). This tendency is preserved also in the territorial distribution of the combinations in both sexes where the differences are statistically insignificant (Table 6). The

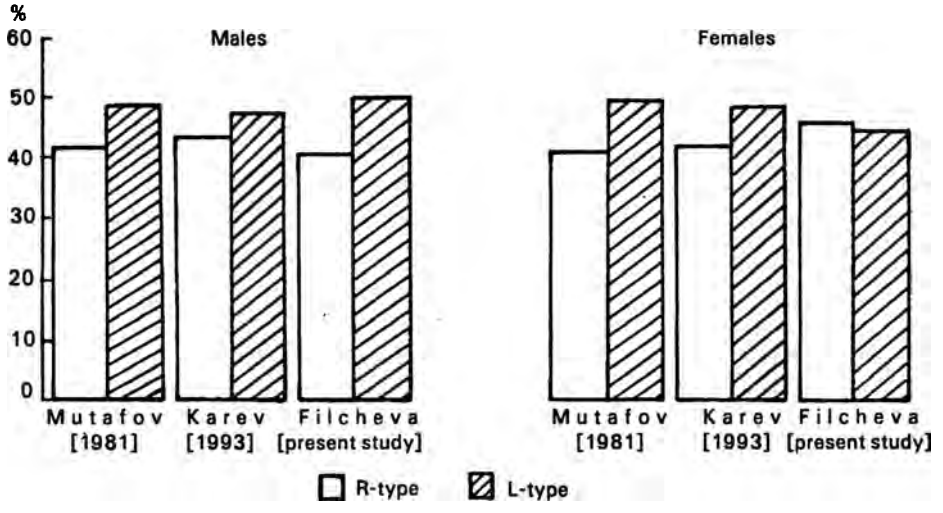

Fig. 1. Comparative data about the frequency of the hand clasping in Bulgarians

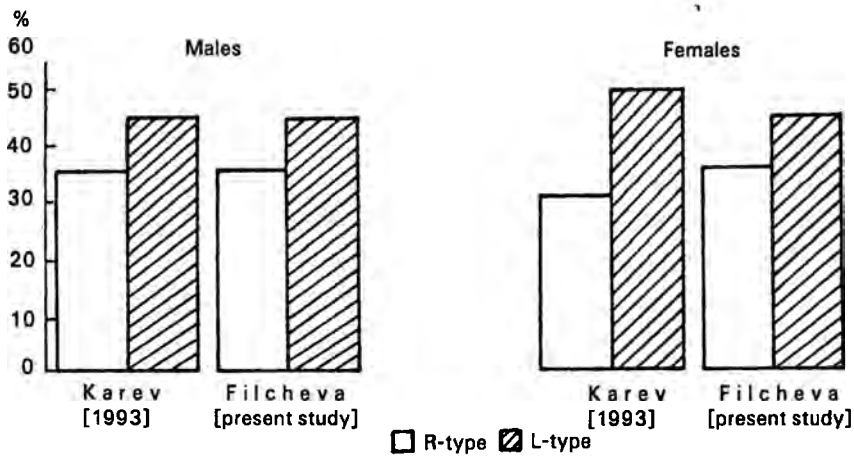

Fig. 2. Comparative data about the frequency of the arm folding in Bulgarians

sought correlation between hand clasping and arm folding is statistically insignificant (Table 7). The data yielded by the present study are closest to of the ones obtained by Mutafov and Karev (Fig.1, 2).

Conclusion

In the males from North Bulgaria the L-hand clasping and arm folding types are predominating, while in the women from North Bulgaria it is the R-hand clasping and the L-arm folding types that are more common. Intersexual differences are found in hand clasping and especially in the combinations between the hand clasping and arm folding types. The territorial variations of the frequency of the hand clasping and arm folding types are statistically insignificant in both sexes. A statistically insignificant correlation between these two types of asymmetry has been recorded.

References

1. Beckman, L. R.. Elston. Data on bilateral variation in man: handedness, hand clasping and arm folding in Swedes. — Human Biol., **34**, 1962, 99-103.
2. Boev, P., V. Todorov. Hand clasping bei den Bulgaren. — Anthropologie, **11**, 1973, No 1,2, 91-93.
3. Collins, E. The concept of relative limb dimance — Human Biol., **33**. 1961, 293-319.
4. Dahlberg, G. Twin births and twins from a hereditary point of view. Stockholm, Bukforlags A. B. Tidens Tryckeri, 1926.
5. Freire-Maia, N., A. Quelce-Salgado, A. Freire-Maia. Hand clasping in diffrent ethnic groups. — Human Biol., **30**, 1958, 281-291.
6. Freire-Maia, A., N. Freire-Maia, A. Quelce-Salgado. Genetic analysis in Russian immigrants. — Am. J. Phys.Anthrop., **18**, 1960, 235-240.
7. Freire-Maia, A., J. de Almeida. Hand clasping and arm folding among African Negroes. — Human Biol., **38**, 1966, 175-179.
8. Karev,G. B. Arm folding, hand clasping and dermatoglyphic asymmetry in Bulgarians. — Anthrop. Anz., **51**, 1993, No 1, 69-76.
9. Kawabe, M. A study on the mode of clasping the hands. — Trans. Sapporo Nat. Hist. Soc., **18**, 1949, 49-52.

10. Kobyliansky, E., S. Micle and B. Arensburg. Handedness, hand clasping and arm folding in Israeli males. — Annals of Human Biol., **5**, 1978, 247-251.
11. Lai, L. Y., R. J. Walsh. The patterns of hand clasping in different ethnic groups. — Human Biol., **37**, 1965, 312-319
12. Leguebe, A. Hand clasping: Étude anthropologique et génétique. — Bull. Soc. Roy. Belge Anthrop. Préhist., **78**, 1967, 81-107.
13. Lourie, J. A. Hand clasping and arm folding among Middle Eastern Jews in Israel. — Human Biol., **44**, 1972, 329-334.
14. Lutz, F. E. The inheritance of the manner of clasping the hands. — Am. Nat., **42**, 1908, 195-196.
15. Pons, J. Hand clasping (Spanish data). — Ann. Hum. Genet., **25**, 1961, 141-144.
16. Reiss, M. Händefalten — eine Übersicht — Anthrop. Anz., **57**, 1999, No 2, 165-184.
17. Rhoads, J., A. Damon. Some genetic traits in Solomon Island population. II. Hand clasping, arm folding and handedness. — Am. J. Phys. Anthrop., **39**, 1973, 179-184.
18. Sarna, J., A. Siniarska and A. Wokroj. Hand clasping, arm and leg folding in populations of Poland and other countries. — Coll. Antropol., **4**, 1980, No 1, 37-44.
19. Wiener, A. S. Observations on the manner of clasping the hands and folding the arms. — Am. Nat., **66**, 1932, 365-370.
20. Yamaura, A. On some hereditary characters in the Japanese race including the Tyosenese (Coreans). — Jap. J. Genetics, **16**, 1940, 1-9.
21. Мутафов, Ст. Психо-физически особености на децата с аномалии С.,Медицина и физкултура, 1981.

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Acta morphologica et anthropologica, 8
Sofia • 2003

Capitello-trochlear Complex and its Significance in the Anatomy and Function of the Distal Humerus

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The past two decades witnessed great improvement in the techniques and equipment used in medicine in general and widened the means of the diagnostic imaging [1, 2, 13].

However the classic anatomical techniques of observation and experimentation did not loose their value.

We reveal normal anatomy and function of the distal humerus in human in the light of the Bicolumn theory and add our view for the significance of the distal articular structures of the humerus.

Key words: Humerus, capitello-trochlear complex, distal humerus.

As it is known humerus is the bone of the arm [1, 2, 3, 4,19]. It is a long bone divided into three parts. The central part is known as humeral body. It consists of cortical bone. Other parts are proximal and distal end, structured mainly of spongy bone covered by thin cortical layer.

The distal end of the humerus is widened and the external edges of the humeral body pass into the epicondyles - rounded processes of bone. Bigger one is the medial epicondyle and lesser one — lateral epicondyle [1,12,10,11,].

The most distal parts of the humerus are trochlea and capitellum [18].

In newborn the distal humeral epiphysis is completely built of cartilage. It does not differ from the distal humerus in the adult, in macro anatomic view [8].

Humerus ossifies from a single primary center and additional centers. They are visible at a different age, as it is shown on the figures 3 and 4.

There is a difference in the time of ossification of the distal humeral structures. In males lateral condyle ossifies during the 12^{th} year and in female during the 11^{th} year. Medial condyle in male - 7^{th} year and in female - 5^{th} year. Capitellum — male 5^{th} month, female — 4^{th} month. Trochlea in male — 9^{th} year and female — 5^{th} year [5,6]. This is the so-called "cross" rule.

Medial condyle lies extremely extra-articularly (Fig 3). The cortical layer of bone is thin. It differs from the adults by not passing over the entire anterior surface. It is deficient in the zone of coronoid fossa. This "defect" is covered by cortical bone during the growth [7, 9].

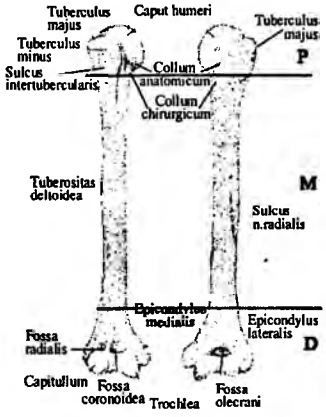

Fig. 1. Humerus — anatomy

Fig. 2. Skeletal development of a 6 months old fetus

Like an answer of the new trends entering the orthopedic surgery, Jupiter J. and Mehne D., created the Bicolumn theory in 1991 [15, 16, 17].

The idea is, when viewed from a posterior approach, the humeral shaft divides into medial and lateral columns longitudinally. These columns terminate distally where the transversely oriented trochlea connects between both columns.

By interconnecting with these divergent columns, the terminal part of the elbow joint most resembles triangle, which is fundamental in understanding of the proper mechanics of joint motion and the intra-articular fracture treatment.

With disruption of anyone of the three arms of the triangle, the entire construct is weakened, but it is more weakened if the disruption is on the base of the triangle.

Some authors believe that the trochlea and the capitellum are the articular surfaces of the condyles [9, 12, 19].

According to others, they are absolutely independent parts of the distal humerus [5, 7]. However,

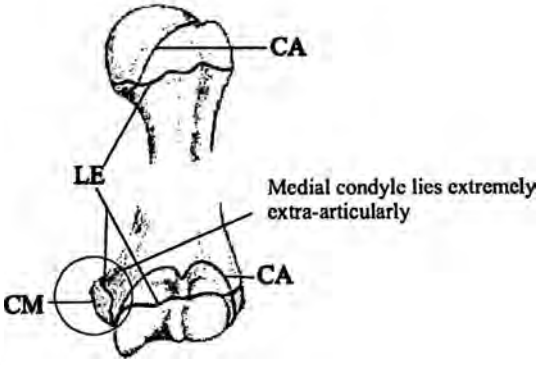

Fig. 3. Epiphyseal lines of the humerus — anterior view. CA — joint capsule line, LE — epiphyseal line, CM — medial condyle

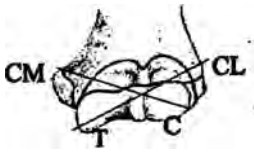

Fig. 4. "Cross rule" for the ossification of the distal humerus. CM — medial condyle, CL — lateral condyle, T — trochlea, C — capitellum

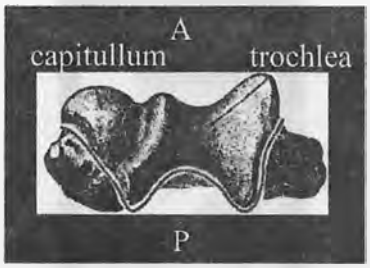

Fig. 5. "W" shaped outlook of intact distal end in coronal view

defining the distal humeral articulation as a triangle based in the trochlea ignores the capitellum.

As a result of long lasting observations, measurements and analyses, we reached the conclusion that the capitellum and the trochlea could be united in a complex — the capitello — trochlear complex (CTC) [14].

The CTC represents the intercolumnar "tie-rod". It has the form and comprises medial and lateral lips with an intervening sulcus. This sulcus articulates with the semi lunar notch of the proximal ulna. The adjacent lips offer medial and lateral stability to this articulation [15].

In coronary plan the CTC lies about 20° rotation in relation with the proximal part of the humerus. The angle is known as "torsion angle". It contributes for the total outlook of the humerus as a long bone.

We found that in the same coronary plan the outlines of the CTC resemble a "W" shape. This "W" sign is appearance of a norm and any disturbance of the shape speaks for intra-articular fracture (Fig. 5).

The CTC axis with the respect of the longitudinal axis of the humerus is approximately 94° in valgus in males and 98° in females. The normal valgus position of the elbow is commonly referred to as "carrying angle" of the elbow. Functionally, it allows the positioning to objects away from the body when they are held with the elbow in extension.

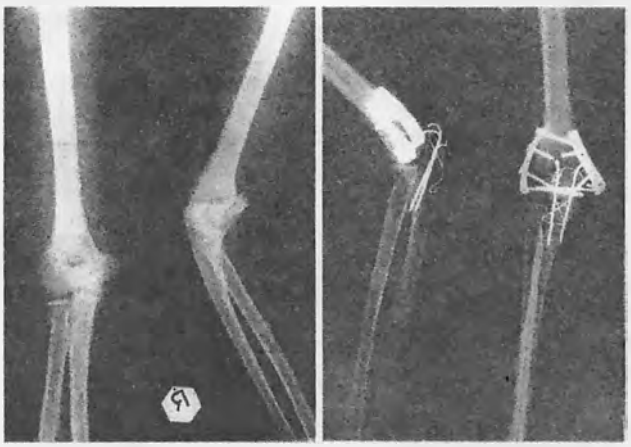

Fig. 6. Pre- and post-operational X-ray graphies of bicolumn fracture in 24 y.o. female

In addition the CTC axis is externally rotated between 3° and 8° with respect to a line connecting the medial and lateral epicondyles.

Traditional classifications of the fractures of the distal humerus have centered on the anatomic concept of the terminal end of the humerus, structured as condyles-hence the terms "condylar", "transcondylar" and "bicondylar" fractures.

The distal humerus could not be precisely described and understood as two diverging columns supporting an intercalary surface, rather as rounded projections (condyles) such as those found in the distal femur or head of the metacarpal bones.

Changing the term "condyle" to "column" more accurately describes as well as maintains the general categories.

As a confirmation of the Bicolumn theory created by Jupiter and Mehne, and the significance of the CTC, we show the diagnostic and post-operative X-ray graphies of a clinical case [17]. This is a bicolumnar fracture in 24 y.o. female and the CTC is separated.

Note that the two plates are oriented in perpendicular planes supporting the two columns and the situation of the transverse screws, which pass through the CTC in order to restore the base of the triangle (Fig. 6).

Intra-articular fractures of the distal humerus are big diagnostic and therapeutic problem. A new step in the solution of the problem is the Bicolumn theory, to which we add 3 supplements. We believe that will bring more serenity in the solution of the problem.

The so shown rational approach to the anatomy of the distal humerus is imposed because of the increasing need of adequate diagnostics and treatment of the fractures in this zone.

We hope that after mutual working of multidiscipline teams it will be sold in the nearest future.

References

1. Bergman, R., A. Afifi, R.Miyauchi. Illustrated Encyclopedia of Human Anatomic Variation: Opus V: Skeletal System, Electronic edition, 1999.
2. Clemente, C. Clemente Anatomy: A Regional Atlas of the Human Body. 4th edition. Lippincott, Williams & Wilkins, 1997, 83 — 192.
3. Grant, J. C. Boileau, Grant's Atlas of Anatomy. 9th edition. Springer, 1991, 299 — 482.
4. Gra,y H. Anatomy of the Human Body. Philadelphia: Lea & Febiger, , Renewed 20th edition, 1996, 123-157.
5. Jacobson, K. Hughston Clinic Sports Medicine book. Williams & Wilkins, 1995, 305 — 348.
6. McMinn, R. M. H., R.T. Huthchings, McMinn. Color Atlas of Human Anatomy, 4th edition Mosby-Year Book, 1998.
7. Moore, K., A. Dalley. Clinically oriented anatomy. II, Appleton & Lange, QS 4 M, 1999, 822 — 953.
8. Morrey, BF. Anatomy of the elbow joint. — In: The Elbow and its Disorders, Philadelphia, W.B. Saunders, Co., 1995, 16-52.
9. Sobotta, J. Atlas of Human Anatomy. 12th English edition. Lippincott, Williams & Wilkins, 1996, 120-204.
10. Terry, R. J. New data on the incidence of the supracondyloid variation. — Am. J. Phys. Anthropol., 9, 1926, 265-270,
11. Terry, R. J. A study of the supracondyloid process in the living. — Am. J. Phys. Anthropol., 4, 1921, 129-139.
12. Trotter, M. Septal apertures in the humerus of American whites and Negroes. — Am. J. Phys. Anthropol., 34, 1919,213 — 217
13. Walter, J. Basic atlas of sectional anatomy with correlated imaging, Lippincot. Williams&Willkins, 1998, 65-109.
14. Katsarov, A. Morphometric and morphologic characteristic of the distal humerus in recent Bulgarian. Acta mporphologica et anthropologica, 6, 2001, 98 — 103.
15. Jupiter, J. B., D. Mehne. Fractures of the distal humerus. — Journal of orthopedics, 15, 1992, No7, 825-833
16. Mehne, D K., J. Matta. Bicolumn Fractures of the adult Humerus. Presented at the 53rd Annual Meeting of the American Academy of the orthopedic surgeons (New Orleans, La), 1986.
17. Jupiter, J. B., D. Mehne. Fractures of the distal humerus. — In: Skeletal Trauma. Philadelphia, PA: W.B. Saunders; 1991, 1146-1176.
18. Синельников, Р. Д. Атлас Анатомии Человека, Второе издание. Т. I. М., Госуд. изд. мед. литературы, 1963, 122 — 125.
19. Ванков, В., Г. Гълъбов. Анатомия на човека. С., Медицина и физкултура. 1990, 125 — 185.

Institute of Experimental Morphology and Anthropology

Acta morphologica et anthropologica, 8
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Cytomegalovirus Infection — Cause of Death at Infancy

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The purpose of this study is to draw attention on cytomegalovirus infection as one of the most frequent infections among infants and most frequent infection in foetus. Its generalized forms could be the basic factor in thanatogenesis at neonatal and infant age and its localized forms — favourable area for the development of other infections with fatal outcome. We introduce 7 children at the age of up to one year, three of them with generalized form of cytomegalovirus infection and the rest with localized ones. We examine specific morphological changes in the salivary glands and in the affected by the virus organs as well as the rest of the pathomorphological changes.

Key words: Human Cytomegalovirus (HCMV) infection, morphological changes, cause of death.

Introduction

The Human Cytomegalovirus (HCMV), together with the Herpes simplex virus belongs to the Herpesvirudae family, genus Cytomegalovirus. The foetus, newborn babies and infants up to one year are the most sensitive to the virus. It is well known that the HCMV infection is widely spread among pregnant women [1]. The number of children born in Europe and in USA every year with congenital HCMV infection is 40 000 [2]. In Bulgaria the in utero infection is also the most frequent case [5]. This infection in itself could be the cause of abortion or premature birth with heavy damages of various degrees [3]. The proving of the HCMV infection is achieved through IgM-tests (proving an antivirus antibodies in the serum) [5].

The Human Cytomegalovirus destroys the epithelial cells, since it has a definite affinity for gland epithelium, but also organs of different structures could be affected such as nerve and mesenchyme elements [4]. In the damaged cells appear typical intranuclear and cytoplasmatic inclusions usually between the 10^{th}-72^{nd} day after the infection [3]. Around the intranuclear inclusion setts in an area of brightness in the nucleoplasma, wich gives the cell a typical likeness of an "owl's eye".

Clinical manifestation of HCMV infection after birth are numerous: icterus prolongatus and hepatosplenomegalia, transitory thrombocytopenic purpura, hepatitis, encephalitis or other damages of the central nervous system with cerebral calcifications, pneumonia, haemolitic desease etc. [6]. The removal of HCMV from

the human organism is through the urine, saliva, sweat and faeces (when gastroenteral tract is infected).

Morphological changes with HCMV infection are so very specific that no virous confirmation of the diagnosis is necessary. We observe a specific giant-cell metamorphosis (transformation) of contaminated by virus cells (cytomegali) and lymphoid-histiocid infiltration in the stroma. The cytomegali have the same structure and staining properties, independently of their localization in tissues and organs [7, 8, 9].

There are two forms existed of HCMV infection: localized and generalized but this division is relative because under the influence of unfavourable factors the localized form could become generalized [7].

The *purpose* of this study is to accentuate upon the most frequent herpes virus infection especially at neonatal and infant age where it could be the independent cause of sudden death or suitable basis for development of the other infections. We present specific and nonspecific changes in children examined.

Materials and Methods

We present 7 children of age between 4.5 and 11 months, dissected in the Department of Forensic Medicine — MU Varna. Four of the children were boys and three girls. Five children had died in their homes in apparently good health, one of them had been in hospital for cautery and died on 11th day after the accident. The 7th child had been hospitalized for spasmodic bronchitis a week before exitus letalis and had a sudden death without visible symptoms. All children were born in due time with the exception of one first degree prematurely born. Five of the children were Gipsies, one was Bulgarian and one was Turk. Their mothers offered no data about other disease problems during their pregnancy and after birth.

All children underwent histological investigation of internal organs and salivary glands. The materials were stained with by haematoxylin eosin.

Results and Discussion

The external examinations of the children reveal only hypotrophy I-II degree in two of them and no disease changes in five. The internal examination reveals inflammatory changes in the lungs, hepatomegalia (in six children), almost wholly moderately expressed increasing of the lymph nodulae, dystrophic changes in parenchim organs, and thymus atrophy.

The histological investigation showed that in all cases there was cytomegal damage of the salivary glands in the form of giant-cell transformation of the epithelium of the ducts and round-cell infiltration of the part of the stroma, hyperplasia of the lymph foliculi, sometimes (when quantity of the cytomegali is not big) — fibrosis, kystosis and increasing of fat tissue (Fig. 1).

In all children there are established inflammatory changes expressed in various degrees in the lungs and respiratory tract. Five of the children had intersticial pneumonia, the child with cautery was with massive confluencing and abscessing bronchopneumonia, abscessing bronchitis and sepsis and the last of the children was with scattered catarrhal bronchopneumonia and bronchiolitis. Depending on the etiology of the pneumonia the morphological changes in the lungs have some peculiarities in the form of massive haemorage, growing of the bronchial epithelium

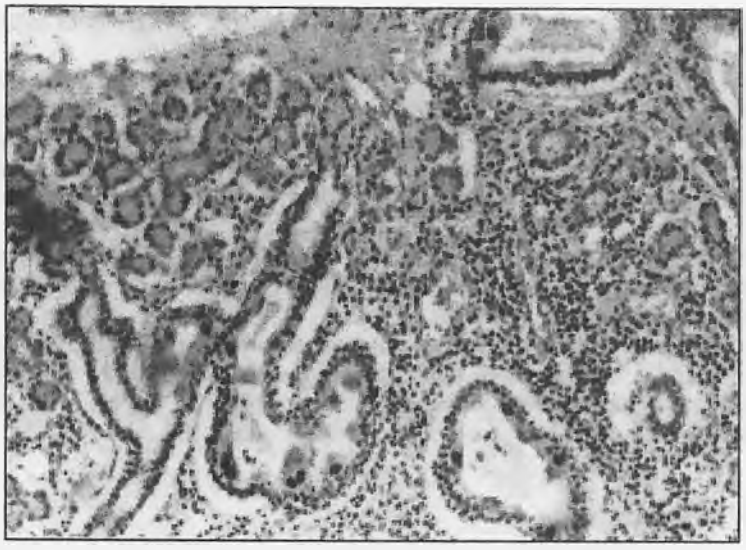

Fig. 1. Salivary gland with cytomegali in channels and infiltration in the stroma (stain HE, × 150)

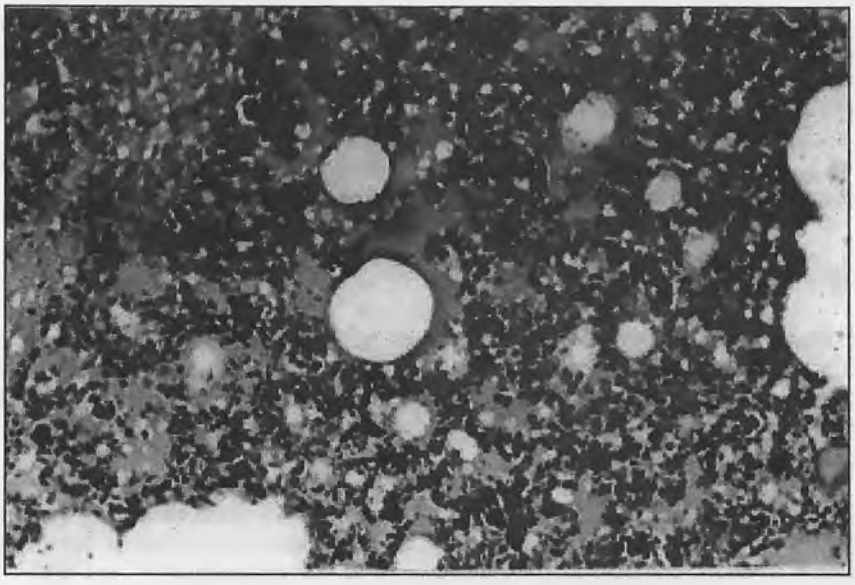

Fig. 2. Lung — dispersed intersticial and intersticial-desquamative pneumonia, irregular airiness, and oedema, cytomegals (stain HE, × 100)

resembling small cushions with paragrippe etc. In all cases there are irregular airiness with dispersed athelectasis, disorder in blood circulation and in some places serosive exudate in the alveoli mixed with scaled cells of the alveolar epithelium and macrofagi and scaling of the bronchial epithelium.

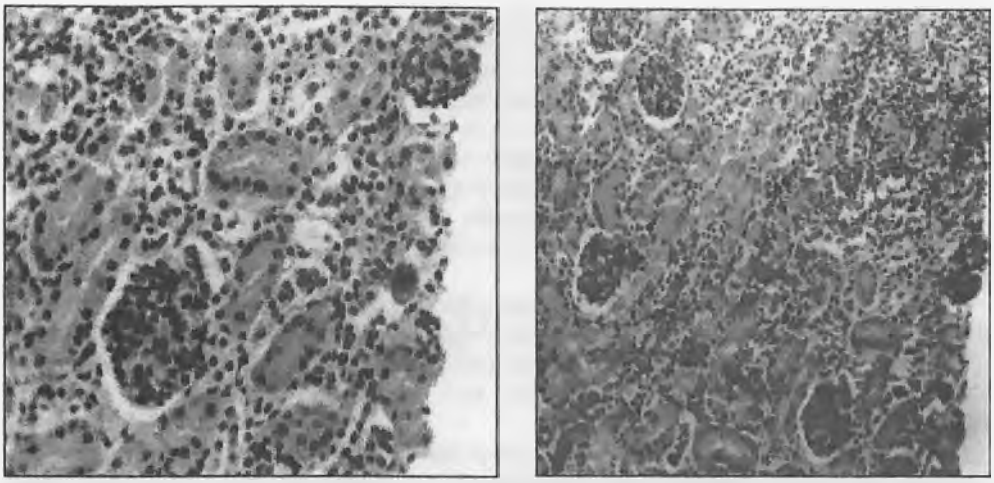

Fig. 3, 4. Kidney with cytomegal transformation cell in the channels (stain HE, × 200) and dispersed cytomegal nephritis (stain HE, × 100)

In three of the seven children there was obseved generalized HCMV infection in the lungs, kidneys, salivary glands and small intestines and in one of them also in the liver. In the lungs was seen cytomegal transformation of the epithelium of the small bronchi and bronchioli, as well as of the bronchial glands. At the same time bronchitis, peribronchitis, intersticial and intersticial-desquamative pneumonia was developed (Fig. 2). Often the cytomegali in the lungs were placed among not big mono-lympho-histocyte infiltrations.

In the kidneys (Fig. 3, 4) giant-cell transformation was observed in the epithelium channels, sometimes around them there was round-cell infiltrations, but it is possible for the damaged organ not to react. In two of the three cases of generalized HCMV infection in the kidneys was established dispersed cytomegal nephritis, dis-

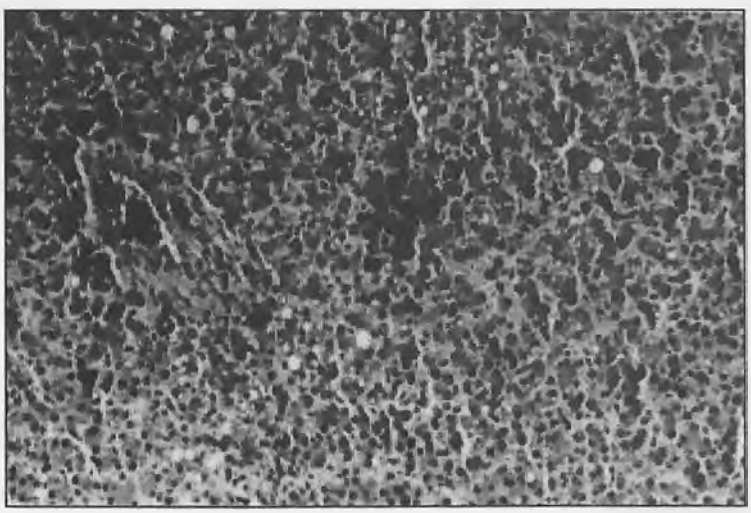

Fig. 5. Liver — parenchim and fat dystrophy with round-cell infiltration

persed membraneous glomerulonephritis with sclerosis of single glomeruls and in the third case we observed focuses with many eosinophiles, epitheloid cells etc.

In the rest of the organs: in all children were observed hyperplasia of the lymph apparatus — lymph nodulae in mesenterium, small and large intestines, peribronchial, perihilar, lymph nodes in the lien. Usually atrophy of the thymus in different degree was established, which means chronic exhausting of the immunity system. Only in one of the children we observed atrophy of the Hassall' bodies with ample increasing the number of lymphocytes. Parallelly to this in all cases there was observed a considerable atrophy of the suprarenal glands (leaf-like, mainly with atrophy of their cortex).

In the other internal organs we had almost always changes of the same type: parenhim and/or fat distrophy of the liver (Fig. 5), sometimes with preserved focuses of extramedular haemopoesis, superficial inflammatory changes in the small and in the large intestines, parenchim distrophy and intersticial oedema of the myocard and cerebral oedema.

Discussing thanatogenesis in the three cases of generalized HCMV infection we take it as a basic disease in the background of which some acute respiratory virus infection might develop and prove to be the direct cause of death. In the remaining four cases we accept HCMV infection to be an accompanying disease, terrain, which strongly favours death because of the general influence of the virosis. One should not forget that the limitation of the HCMV infection is in all cases greater than the limitation of the acute respiratory disease and sometimes is congenital which is proved by the morphological changes in the salivary glands. These opinions disagree with those of N a z a r o v [7], who does not accept the generalized HCMV infection as a basic disease. Contemporary authors [3, 5] agree that this infection is a leading link in the cause of death, in whose background there could also be other ordinary virus infection.

Conclusion

The HCMV infection is diagnosed really easily and should be sought for actively and taken into account in all cases with sudden death especially in infant age. The investigation of the salivary glands gives the quickest direction for detecting HCMV infection in other organs. This diagnosis could lead to specifying the thanatogenesis in any concrete case. On the other hand establishing of HCMV infection is also important for the retrospective searching and proving of a HCMV infection in a mother, respectively a change of behaviour during later pregnancy etc. In a more general sense the exact forensic diagnosis is a part of the fight against numerous complications of congenital HCMV infection.

R e f e r e n c e s

1. D r ö s z u s, J., M. V o g e l. Placentitis fetalis bei generalisierter Cytomegalie. — Verh. Dtsch. ges. Path., **60**, 1976, 463-469.
2. F o w l e r, K. B., S. S t a g n o, R. F. P a s s, W. J. B r i t t, T. J. B o l l and C. A. A l f o r d. The outcome of congenital cytomegalovirus infection in relation to maternal antibody status. — N. Engl. J. Med., **326**, 1992, 663-667.
3. P l a c h t e r, B. Strategies for the control of congenital cytomegalovirus infection. — Biotest Bulletin, **6**, 2000, 129-138.

4. Демидова, С. А., Е. И. Семенова, В. М. Жданов. Цитомегаловирусная инфекция человека. Москва, Медицина, 1976.
5. Иванова, Л. Сероепидемиологични и лабораторно-диагностични проучвания върху разпространението на някои човешки херпесни вирусни инфекции в североизточна България. Дисерт. труд (Варна), 2000.
6. Ивановский, Т. Е., Б. С. Гусман. Патологическая анатомия болезней плода и ребенка. Т. 2. Москва, Медицина, 1981.
7. Назаров, В. Ю. О цитомегалии у скоропостижно скончившихся детей. — Суд. Мед. Эксп. 4, 1978, 31-33.
8. Самохин, Н. А. Некоторые закономерности генерализации цитомегалии и ее патоморфологической диагностики. — Арх. пат., 11, 1978, 51—57.
9. Чарный, А. М. Инклюзионная цитомегалия. Москва, Медицина, 1972.

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Infant and Juvenile Mortality in Populations from Bulgarian Lands in the Osman Empire (XV-XVII c.)

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Data about age distribution into five years age intervals based on anthropological material from necropolises dated to XV-XVII c. in present Bulgaria have been analyzed using methods of paleodemography. Valuable results about infant mortality in the period have been obtained, which have been correlated with data for Central European and Balkan contemporary populations.

Key words: infant mortality, populations XV-XVII, Bulgaria.

Introduction

Infant mortality is one of the most sensitive demographic indicators of changes in survival conditions of social and nature envirommment. Individuals of infant age are most vulnerable of negative influences like infections deseases, malnutririon, decrease in hygiene. These factors cause high variations in human life span in the first age after birth, which appears as an important indicator of social development. Thestudy of infant martality and survival in the period of XV-th-XVII-th c. is of importance for understanding the demographec processes in Bulgarian populations during and after Ottoman conquest of Balkans.

Material and Methods

The present study makes use of data about age distribution of the anthropological material from the following necropolises: by town Kavarna and cape Kaliakra, which represent Christian populations of the flourishing trade centers of North Black Sea cost of Early Osman Empire, inheritors of Bulgarian populations from XIII-XIV c. [2, 14, 15, 17, 18, 21]; by villages of Krivina, Vladimirovtsi, Iliyantsi, Balsha, some of them dated in more wide chronological limits to XVIII c., which represent Bulgarian, Christian, rural populations [20, 22], by village Gradishte, near Shoumen, which represent Islamic population from the epoch [16, 19, 23]. The relative number of infant skeletons in the material from these necropolises exceeds 30 % (with a single exception of the big series from Iliyantsi with 29,5 % of infant skel-

etons) and the relative number of skeletons, assigned to the individuals in the first age interval of 0-4 years surpasses that of individuals of the second age interval of 5-9 years of age. The portion of infant skeletons exceeds even 40 % in more of the studied populations — Kavarna, Kaliakra, Krivina, Vladimirovtsi, Gradishte.

Data about age distribution of anthropological material are used in construction of life tables after the methods of paleodemography, using five years age intervals [1]. In analysis parameters from life tables, that directly describe mortality, are used, namely relative number of dead and risk of death in five-year age intervals of infant (Infans I, 0-7 years and Infans II, 7-14 years) and juvenile (Juvenis, 15-19 years) ages.

Results and Discussion

The relative number of dead in the first age interval (0-4 years) (Table 1, Fig. 1) exceeds 25 % in populations from necropolises Kavarna, Kaliakra, Gradishte and Krivina. The population from Vladimirovtsi approaches to this group with a value of this parameter of 24.14 %. The population from Krivina is distinguished with the value of 41.94 %. The populations from Kaliakra, Gradishte and Krivina with this mortality frequency in first age interval show fast decrease in mortality for the second age interval of 5-9 years of age. On the contrary, in population of Kavarna such decrease has not been observed. It seems that some unfavorable factors have been acting in this case, which led to increase of mortality in the second age interval, instead of decrease as it has been more often observed. An increased infant mortality in the second age interval in the population from Kavarna is established as well in values of risk of death (Table 1, Fig. 2), being higher in the second age interval. Similar unfavorable distribution of values of relative number of dead and risk of death is established also for population from Vladimirovtsi. In populations from Kaliakra, Gradishte and Krivina values of risk of death for the first age interval are higher than calculated for second age interval.

Table 1. Values of relative number of dead and risk of death

	Kavarna		Ilienzi		Kaliakra		Gradiste		Balsha		Krivina		Vladimirovzi		Mean	
	dx	*qx*	*dx*	*qx*	*dx*	*qx*	*dx*	*qx*	*dx*	*qx*	*dx*	*qx*	*dx*	*qx*	*dx*	*qx*
0-4	27,30	0,27	14,24	0,14	26,85	0,27	26,56	0,27	16,67	0,17	41,94	0,42	24,14	0,24	19,61	0,20
5-9	22,37	0,31	12,08	0,14	14,79	0,20	9,90	0,13	10,00	0,12	6,45	0,11	20,69	0,27	14,36	0,18
10-14	6,26	0,12	3,18	0,04	3,89	0,07	5,73	0,09	6,67	0,09	6,45	0,13	3,45	0,06	4,64	0,07
15-19	2,66	0,06	7,59	0,11	3,11	0,06	6,77	0,12	3,33	0,05	6,45	0,14	10,34	0,20	4,92	0,08

Values of relative number of dead and risk of death (Table 1, Fig. 1, 2), calculated for first (0-4 years) and second (5-9 years) age intervals, are also close for the population from Iliyantsi. In this case this feature is due to the reduced mortality in the first age interval.

The lowest mortality is established in all of studied populations in the age interval of 10-14 years. In this age interval values of relative number of dead are low and values of risk of death are the lowest in populations from Ilientsi, Gradishte, Vladimirovtsi, and very close to the lowest values in the remaining studied populations (Table 1, Fig. 1, 2).

Mean values of the relative number of dead in the first and the second age intervals for the period are estimated to be 19.61 % and 14.36 %, respectively, so they have a relatively low difference of 5.25 % in-between. These values are strongly influenced by data from small series, where the relative number of infant skeletons is

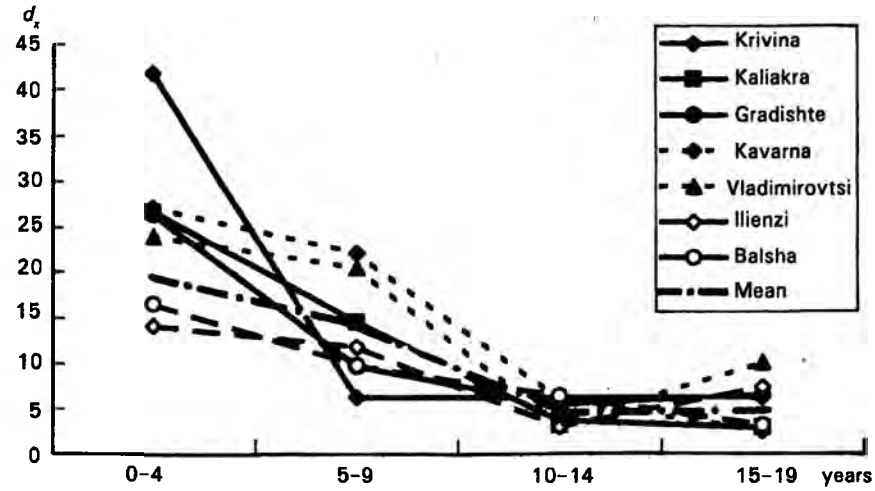

Fig. 1. Distribution of values of relative number of dead (d_x)

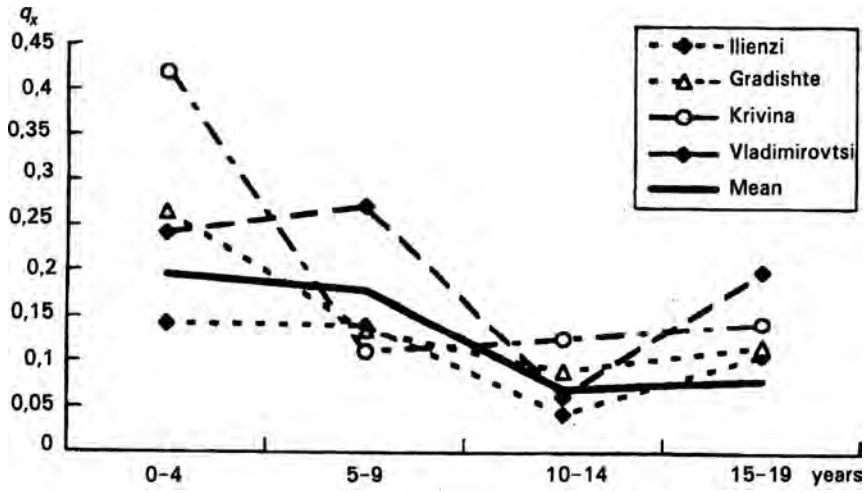

Fig. 2. Distribution of values of risk of death (q_x)

reduced, or from bigger series from Chiprovtsi, Bozhenishki Urvich and Debnevo, where infant mortality is not satisfactorily represented in the anthropological material from these necropolises.

A peculiarity of the studied populations is the increased juvenile mortality, established from the relatively high values of relative number of dead in the age interval 15-19 years (Table 1), in a large number of populations exceeding 6 % — Iliyantsi (7.59 %), Gradishte (6.77 %), Krivina (6.45 %), и Vladimirovtsi (10 %). The high juvenile mortality is associated with Christian populations, as with the studied Islamic population from the period. This feature could be caused by the premature physical and social overloading of individuals from this age group, expressed in in-

creased role of their labor in the agriculture and handicrafts, increased economical and military obligations to the state, premature marriages and subordinate position in the extended patriarchal families. Increased juvenile mortality is a negative tendency in demographic development of Bulgarian populations from the period.

Results obtained for infant and juvenile mortality in the populations from Bulgarian lands in the Osman Empire are comparable to the features of contemporary Balkan and Central European populations. In most of investigated skeletal populations, where infant mortality is satisfactorily represented in the first age interval it remains at a high level and does not strongly differ from the preceding period. At the beginning of the period in Rumanian populations from Măicăneşti-Străuleşti I (end of XIV-XV c.) [7, 8, 9] infant mortality exceeds 40 %, and the relative number of dead in the first age interval is the highest with 25.69 %. The relatively high infant mortality is kept in populations from XV-XVII c. In the population from the necropolis Străuleşti II (XV-XVI c.) infant mortality is reduced (34.75 %), but the relative number of dead in the first age interval remains the highest with 20,30 % [9]. In Borlad (XVI-XVII) infant mortality reaches 53.85 % [4], in Bucov — 45.16 % [10], in Radovanu XV-XVII c. Infant and juvenile skeletons (Infans I and II and Juvenis) to 19 years of age are 51.5 % from the anthropological material [11]. Lower relative number of infant skeletons (0-14 years of age) is established for populations from Brigadiru-Zimnicea — 25 % and Căscioarele — 18.18 % [10]. Summing up in the period preserves the pattern of age distribution of relative number of dead, where in the first age interval 0-4 years or in the group of Infans I (0-7 years) fall the highest values of relative number of dead. This is observed in Cernica, with 20.48 % skeletons from Infans I [3], Sînnicolau de Beiuş, with 36.5 % and Borlad 30.77 %. No strong changes in infant mortality in Rumanian populations are established from the end of the period XVII-XVIII c. In Sonnicolau de Beiuş infant mortality reaches 52.37 % [12], in Cernica — 2.58 %. Only population from Strehaia exhibits much reduced relative number of skeletons of infant individuals (0-14 years of age) (8.75 %) [10]. Considering data from the other sites this value could be due to the lack of material.

High infant mortality is established for population from the necropolis Nova Rača, Croatia. Here the relative number of dead in the first age interval reaches 50 % [13]. In the series from Kranj, Sloveniathe relative number of infant skeletons is reduced — 31.1 %, which is estimated by researchers as to low to be a representative one [6]. These two populations from Slavonic territory of the Habsburgian Empire, on the border with the Osman Empire show close parameters for infant mortality to the studied Bulgarian populations from the period. In population from Czech territories of the Habsburgian Empire in Dukovo infant mortality is estimated to be 30.9 % in spite of the upper chronological limit of the necropolis in the XIX c. [5].

Conclusions

Three groups of necropolises with similar mortality pattern of infant and juvenile mortality can be derived from the demographic situation in the XV-XVII c. — first group — Krivina, Gradishte and Kaliakra with very high and high mortality in the first age interval, second group — Kavarna and Vladimirovtsi with high mortality in the first age interval and specifically high mortality in the second age interval, where in most of the studied populations the mortality is reduced and third group of populations from Iliyantsi and Balsha with favorable low values of mortality parameters below the mean values for the period. For most of the populations from

the period it is established an increased juvenile mortality.

The infant mortality rate in the studied Bulgarian populations correlates to that established for the Second Bulgarian Kingdom. A significant increment is established for the juvenile mortality.

Results about studied Bulgarian populations are close to that obtained from the investigations in the Balkans and Central Europe.

References

1. Acsádi, G., J. Nemeskéri. History of Human Life Span and Mortality. Budapest, Akadémiai Kiadó. 1970.
2. Boev, P., N. Kondova, Sl. Čolakov. Données anthropologiques sur la population médiévale dans la Bulgarie du Nord-Est. — Dobrudza. Etudes ethno-culturelles, **1**, 1987, 208-219.
3. Botezatu, D., Gh. Ştefănescu. Caracterizarea antropologică a la sceletelor din cimitirul feudal de la Cernica (sec. XVII-XVIII e.n.). — St. Cerc. Antropol, **7**, 1970, 2, 181-191.
4. Botezatu, D., G. Miu, P. Cantemir. Studiul paleoantropologic al materialului osteologic descoperit la Borlad, datond din perioada feudalr (secolele XII-XVIII e.n.). — St. Cerc. Antropol., **26**, 1989,3-9.
5. Hanáková, H., A. Secáčová, M. Stloukal. Pohřebiště v Dukovém. Praha, Narodni Muzeum v Praze, 1984
6. Leben-Seljak, P. Paleodemografska analiza nekropole pri farni cerkvi v Kranju (Paleodemographic Analysis of the Necropolis at the Parish Chirch in Kranj). — Antropološki Zvezki, **4**, 1996.95-107.
7. Popovici, I. Données anthropologiques concernant la population d'une petite comunauté villageoise (cimetière I — Străuleşti — XIV^{e}-XV^{e} siècles). — Ann. Roum. Antropol., 7, 1970. 15-20.
8. Popovici, I. Notes Bearing on the Population Mortality of a Small Middle-Age Rural Settlement. — Ann. Roum. Antropol., **8**, 1971, 21-25.
9. Popovici, I. Cimitirele de la Străuleşti sec. XIV-XVI. Analiză demografică. — St. Cerc. Antropol., **10**, 1973, No 1, 15-21.
10. Popovici, I., M. Udrescu. Date demografice comparative asupra unor populaţii medievale din cîmpia Română în secolele XIV-XVIII. — St. Cerc. Antropol., 11, 1974, No 1, 3-6.
11. Popovici, I., L. Georgescu. Contribuţii antropologice la la studiul populaıoiei feudale. Cimitirul de la Radovanu. — St. Cerc. Antropol., **12**, 1975, 9-16.
12. Popovici-Bădărău, I., M. Udrescu. Contribuţii antropologice privind demografia unei aşezări din judeţul Bihor în secolele XVII-XVIII. — St. Cerc. Antropol., **18**, 1981, 9-11.
13. Šlaus, M. Biocultural Analysis of Sex Differences in Mortality Profiles and Stress Levels in the Late Medieval Population from Nova Rača. — Croatia. Am. J. Phys. Antropol., **111**, 2000, 193-209.
14. Боев, П., Н. Кондова, Сл. Чолаков. В: 1982. Антропологично проучване на средновековни некрополи. — В: Чиракман-Карвуна-Каварна, С., 62—65.
15. Боев, П., Н. Кондова, Сл. Чолаков. 1989. Демографска структура, заболявания и расова типология на населението на Каварна през късното средновековие (по данни от некропола на Чиракман). — ИНМВ, **25** № 40, 156—166.
16. Йорданов, Й., Сл. Чолаков, Бр. Димитрова. Антропологична характеристика на късносредновековни ислямски популации от българските земи. — Год. Департ. Археол. НБУ, I, 1994, 218—227.
17. Йосифова, М. Калиакра през късното средновековие (XV-XVII в.). — ИНИМ, 2, 1978, с. 40.
18. Кондова, Н., П. Боев, Сл. Чолаков. 1982. Данни за средновековековното население на Чиракман. — В: Чиракман-Карвуна-Каварна, С., 66—69.
19. Петрова, П. Ислямски некропол при с. Градище, Шуменско. — АОР за 1989, 1990, 168.
20. Поборникова, Р. 1994. Нови археологически изследвания на три средновековни църкви от Софийско. — В: Сердика-Средец-София, 2, 117-143.
21. Радичков, Г. Късносредновековен некропол от XV-XVII век на Калиакра. — Добруджа, **10**, 1993, 168—181.
22. Чолаков, Сл. Антропологични данни за погребаните в късносредновековния некропол от кв. Илиянци — София. — Сердика-Средец-София, 2, 1994, 145—160.
23. Чолаков, Сл., Й. Йорданов, Бр. Димитрова. Антропологично проучване на костните останки от ислямски некропол при с. Градище, Шуменски район. — ИИМ Шумен, VIII, 1993, 101—164.

Institute of Experimental Morphology and Anthropology

Acta morphologica et anthropologica, 8
Sofia • 2003

Overview of the Results of Anthropological Research of Deformations of Human Bones Remains from the Neolithic and the Chalkolithic Periods in Bulgaria

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The present overview summarises the anthropological research results published up to now that describe deformations of human bones from the Neolithic and Chalkolithic periods in the current territory of Bulgaria caused by diseases, by deliberate action or by accident. It is based on the publications from the past 45 years in scientific research periodicals, popular science magazines or monographs. Three groups of bone deformations are distinguished — pathological deformations, traces from ritual manipulations and evidence for medical treatment or prophylactic activities. The pathological group includes diseases like fractures, tuberculosis, tumours, anaemia, etc. Ritual deformations are found more frequently on skulls, and include post-mortem trepanations, punctures with sharp objects, as well as artificial deformations of the skull. Medical treatment activities is the least represented group with data from only one necropolis, and few copper rings found on teeth of several skeletons.

Key words: Neolithic, Chalkolithic, pathological deformation, ritual manipulations.

Data about the diseases of the people who lived in the past can be derived from the traces that those diseases left on the bones and teeth remains of ancient people. These traces are studied by palaeoanthropology that on the other side, is part of anthropology, and on a larger scale is a part of medicine. Its research results contribute to different scientific areas, as is archaeology in this case.

The deformations of the studied bone and teeth material can be classified in three major categories:

- pathological deformations caused by diseases or traumas;
- deformations caused by manipulations of ritual character that the individuals survived or such that were conducted post-mortem;
- deformations caused by attempts for medical treatment or prophylactic activities.

Deformations of bones of the three categories were discovered in 18 Neolithic and Chalkolithic sites (necropoles and individual graves) in the current Bulgarian territory, namely Kremikovtzi — Sofia, Vaksevo — Kiustendil Distr., Tziganova Tell — Harmanli Distr., Malak Preslavetz — Silistra Distr., Chavdar — Sofia Distr., Slatina — Sofia, the necropolis at the village of Durankulak — Dobrich Distr.,

Iasatepe Tell — Plovdiv, Ruse Tell, Kubrat Tell, the necropolis at Liliak village — Targovishte Distr., Iagodinska Cave — Smolian Distr., the necropolis at Varna, the necropolis near Targovishte, Okrajna bolnitza Tell — Stara Zagora, Devetashka Cave — Pleven Distr., Polianitza Tell — Targovishte Distr., and the Pchelina necropolis near Omurtag. The distribution of the different types of deformations varies from site to site. The summary of research results reveals that some of the deformations are concentrated in a certain necropolis, several sites or a bigger region.

Pathological deformations

Diseases of the blood

Traces (cribra orbitalia) of blood diseases (probably haemolytic anaemia) are discovered in three necropoles on the total of seven skeletons — one Neolithic [18] and one Chalcolithic [19] skeletons from Durankulak, one from the necropolis at Targovishte [22], and four from the Pchelina Necropolis [5]. In the latter case two of the skeletons are of children, one of whom was 8—9 years old.

Diseases of the dentition and jaws

Dentition and jaw diseases, the most frequent of which is the tooth caries, are found in the majority of the enlisted sites (Table 1). In three cases (one form Kremikovtzi [10], one from Okrajna bolnica Tell [17], and one from Pchelina Necropolis [5]) traces of carious processes are found on the milk teeth — something untypical and rarely discovered in prehistoric populations. Caries' complications (dental-root cysts, pulpitis), as well as parodontal changes are found at most of the sites (Slatina [4], Chavdar [1], Vaksevo [21], Rousse Tell [8], Tziganova Tell [6], etc.). A bigger concentration of complications is observed in the necropolis at Targovishte [22].

Diseases of the dentitions and jaws, ranging from caries in early stages of development to heavy damages around dental roots and deformations of the alveolar parts of the jaws, are found on all seven skeletons, which also have other bone deformations. At one of the skeletons the damaged teeth amount to thirteenth. In one case (Okrajna bolnitza Tell [17]) mortification as result of caries is found on one of the molars. Against the background of this frequency of dentition and jaw diseases, we have to mention that at some of the biggest necropoles, such as the Varna Necropolis, these cases are just an exception [11].

Tartar is found on five skeletons (one from Vaksevo [21], two from Slatina [4], one from Iagodinska Cave [12], one from the necropolis at Targovishte [22]). The case from Targovishte is distinctive with the heavy coating of tartar, causing loss of the grinding qualities of the teeth.

Abrasion of the grinding and incisive dental surfaces is very frequent (Vaksevo [21], Malak Preslavetz [14], Slatina [4], Durankulak [18, 19], Iagodinska Cave [12], etc.). The level of abrasion varies ranging from loss of the surface layer of enamel to almost complete abrasion of the dental crown as is in one case from Slatina [4].

Bone and joint diseases

Pathological changes of the bones and joints are almost as common as those of the dentition and jaws (Table 1). Basically, they are represented by formations of bone growth at the edges and joint surfaces of the vertebrae (Vaksevo [21], Iasatepe Tell [9], Slatina [4], Durankulak [18, 19], Varna Necropolis [20], Pchelina Necropolis [5],

T a b l e 1. Pathological deformations of the bones and teeth

Site	Period	Diagnoses					
		traumas	diseases of the bones and the joints	diseases of the dentition and the jaws	diseases of the blood	tuberculosis	tumours
Kremikovtzi	Neolithic			1			
Vaksevo	Neolithic		1	1			
Tziganova Tell	Neolithic			1			
Malak Preslavec	Neolithic	1	2	1			
Iasatepe Tell	Neolithic		1				
Chavdar	Neolithic		1	1			
Slatina	Neolithic			3			
Durankulak	Neolithic		6	2	1	1	
Russe Tell	Chalkolithic	2		4			
Kubrat Tell	Chalkolithic	1					
Iagodinska Cave	Chalkolithic			3			
Varna Necropolis	Chalkolithic		1	1			1
Targovishte Necropolis	Chalkolithic		2	7	1		
Okrajna bolnica Tell	Chalkolithic		1	3			
Devetashka Cave	Chalkolithic			1			
Polianitza Tell	Chalkolithic			1			
Pchelina Necropolis	Chalkolithic	3	2	3	4		
Durankulak	Chalkolithic	1	12	1	1		2

etc.). In three cases (one from Iasatepe Tell [9], one from Varna Necropolis [20], one from Okrajna bolnitza Tell [17]) more serious damages are found that had probably impeded or caused loss of the motor abilities of the individuals.

Malignant formations

Tumour formations are found in individual cases — one from Varna Necropolis [20] and two skeletons from the Chalkolithic period in Durankulak [19]. In all three cases the tumour formations are at the inner side of the skull. This kind of formations could be a cause for epileptic attacks [20].

Tuberculosis

The only case of bone tuberculosis is found on two thoracic vertebrae of one Neolithic skeleton from Durankulak [18].

Traumas

Traumatic injuries are found in eight skeletons (one from Malak Preslavetz [14], two from Rousse Tell [8], three from Pchelina Necropolis [5], one from Kubrat Tell [8] and one from the Chalkolithic period of Durankulak [19]). In six of those cases the traumas are of the skull, which five of the individuals survived (two from Rousse Tell [8], one from Pchelina Necropolis [5], one from Kubrat Tell [8], and one from Durankulak Necropolis [19]). In the sixth case (Pchelina Necropolis [5]) the injury most probably caused death. At the seventh skeleton (Pchelina Necropolis [5]) the cervix of the left femoral bone was fractured, but later healed up. Fractures of two ribs of the skeleton from Malak Preslavetz were found, but they were survived and later healed up [15].

Ritual manipulations on the bones and the teeth

Intentional artificial deformation of the skull

One of the deliberate changes of bones, and especially of the skull, is the artificial deformation of single-band type. Unlike later periods, i.e. the Middle Ages, when single- as well as double-band deformations are known, in the Prehistoric periods the single-band type is the only one found in Bulgaria [16]. It was found both in Neolithic and Chalkolithic graves (Table 2). Most cases are from Durankulak — on eighteen Neolithic skeletons [18] and thirty-three Chalkolithic ones [19]. Such a kind of artificial deformation is found only in two other necropolises (five from Malak Preslavetz [14] and two from Okrajna bolnitza Tell [17]. Special attention should be given to the site distribution of artificial deformation and the fact that it is concentrated primarily in North Eastern Bulgaria. This distribution may be accidental and due to the fact that individuals with artificial deformation have not been discovered yet in other regions, but it may also be a characteristic of the prehistoric population inhabiting the territory mentioned above.

Unintentional deformation of the skull

One of the reasons for this kind of deformation could be a regular activity, for example carrying loads, such as heavy vessels, upon the head. Another similar effect

Table 2. Ritual manipulations and medical treatment

Site	Period	Ritual manipulations			Medical treatment	Unintentional deformation of the skull	Intentional deformation of the skull
		trepanations	punctures by sharp objects	ritual manipulations on the teeth			
Malak Preslavec	Neolithic			1		2	5
Durankulak	Neolithic			1			18
Rousse Tell	Chalkolithic	14	4				
Lijak Necropolis	Chalkolithic	1					
Okrajna bolnica Tell	Chalkolithic	1				1	2
Devetashka Cave	Chalkolithic	1					
Durankulak	Chalkolithic				19		33

may be a result of the continuous lying of a little child (baby) on a hard surface, such as a wooden cradle, this leading to permanent flattening of the occipital bone and of the skull [13].

Unintentional deformation of the skull was found in three cases (one from Okrajna bolnitza Tell [17], two from Malak Preslavetz [14]). The individual from Okrajna bolnitza Tell was 45-50 years old, and the two skeletons from Malak Preslavetz belonged to the children in Infans I.

Post-mortem trepanation of the skull

The post-mortem trepanation of the skull belongs to the ritual manipulations. Russe Tell is a good illustration with its big number of skulls with traces from post-mortem

trepanation; they are several per skull in some cases [13]. This kind of trepanation is also found on one skull from Devetashka Cave [7], one from Lilyak [2], and one from Okrajna bolnitza Tell [3]. For comparison, in Rousse Tell post-mortem trepanated skulls are fourteen (Table 2).

Puncture of the skull with a sharp object

Such punctures are found in four cases only in Rousse Tell. They are small round holes in the skulls probably inflicted post-mortem by an awl or another sharp object [8].

Ritual manipulations on the teeth

An unusual, probably post-mortem ritual manipulation is found at two sites — one in Malak Preslavetz [14] and one from the Neolithic period of Durankulak [18]. These are peculiar fractures of the dental crown of several molars, premolars and in one case (Durankulak) of an incisor. The fracture affects parts of the grinding surface, of the tubercles and of the dental crowns. It may be possible to cause such a fracture by an abrupt infliction along the vertical axis of the tooth [16]. The skeleton from Malak Preslavetz has four teeth broken in this way, while the total number of broken teeth of the Durankulak skeleton is eleven. It is believed that such a fracture could not be caused while a human is alive, and that it was inflicted post-mortem, probably, with some kind of a ritual purpose.

Traces from medical treatment

Data about probable medical treatment is found only in the Chalkolithic part of Durankulak [19]. Blue-green colouring is found on the teeth of nineteen skeletons, more often on the incisors, canines, and the first and second premolars of the lower jaw. The cause for this colouring was discovered only in several graves — rings made of copper wire with a diameter equal to the diameter of the necks of the corresponding teeth. One suggestion is that these rings were put on the teeth to exert an overall prophylactic influence over the organism [16].

References

1. Boev, P., S. Colakov. Anthropologische Untersuchung des neolithischen Skeletts von Cavdar, Bezirk Sofia. — Studia Praehistorica, 8, 1986, 217-224.
2. Boev. P., L. Kavgazova. Anthropological study of a chalkolithic cranium from Liljak village, Targovishte district. — Acta Morphologica, 6, 1987, Sofia, 47-51.
3. Boev, P., N. Kondova, D. Maslinkov. Anthropologische Untersuchung eines chalkolithischen skeletts aus Stara Zagora. — Anthropologie, XVI, No 1, 1978, 21-26.
4. Colakov, S. Anthropologische Untersuching der Knochenfunde aus dem I bauhorizont der fruhneolithischen Siedlung Slatina. — Acta praehistorica et archaeologica, 24, 1992, 235-244.
5. Cholakov, S., Y. Yordanov. Palaeoanthropological data about the chalcolithic populatin from the area of the town of Omourtag. — Хелис III, част 1, 1994, 9-26.
6. Leshtakov, K. Tsiganova. Mogila Soundage Excavation, 1993. — In: Maritsa project; Rescue arhcaeological excavation along Maritsa motorway in South Bulgaria. T. 1. Sofia, 1997, 15-28.
7. Боев, П. Енеолитични черепи от Деветашката пещера. — Изв. Инст. морфология, 2, 1957, 291—302.
8. Боев, П. Върху историческите трепанации. — Изв. Инст. морфология, 3, 1959, 197—231.
9. Боев, П. Неолитични антропологични материали от Ясатепе (Пловдив). — ГНАМ — Пловдив, 3, 1959, 87—92.

10. Боев, П., С л. Чолаков. Антропологично проучване на детска челюст от неолитното селище в Кремиковци. — Интердисциплинарни изследвания — V—VI, 1980, 51—56.
11. Йорданов, Й. За физическия облик и болестите на хората, населявали българските земи в древността. — Стоматология, 5, 1981, 318—322.
12. Йорданов, Й. Антропологични материали от Ягодинската пещера, Смолянски окръг. — Родопи, 4, април, 1985, 40—41.
13. Йорданов, Й. Наръчник по антропология за медици и стоматолози. — София, УИ „Св. Климент Охридски", 1997, с. 115.
14. Йорданов, Й., Бр. Димитрова. Антропологични данни за погребаните в интрамурален некропол в селище от ранния рисуван неолит — с. Малък Преславец, Силистренско. — Год. Департамент „Археология" — НБУ, II—III, 1996, 106—115.
15. Йорданов, Й., Бр. Димитрова — Човекът в миналото. каталог. София, АИ „Проф. Марин Дринов", 1999.
16. Йорданов, Й., Бр. Димитрова. Ритуални въздействия върху костни останки от археологически разкопки в България — Антропологически симпозиум в памет на E.M.Winkler — "Perspectives in Anthropology of past and present population" (29 Sep. — 03 Oct. 1995), Xanthi, Greece.
17. Йорданов, Й., Бр. Димитрова. Антропологично изследване на костните останки от раннохалколитни гробове в Стара Загора. — Изв. Старозагорския музей, I, 2002, 45—54.
18. Йорадданов, Й., Бр. Димитрова. Резултати от антропологично изследване на човешки костни останки на погребаните в праисторическия некропол край село Дуранкулак, Добричко, от неолита. — Journal of Anthropology, 2, 1999, 29—40.
19. Йорданов, Й., Бр. Димитрова. Резултати от антропологично изследване на човешки костни останки на погребаните в праисторическия некропол край село Дуранкулак, Добричко, от периода на енеолита. — Год. Археолог. инст. с музей — БАН, 1, 2002, 154—168.
20. Маринов, Г., Й. Йорданов. Антропологично проучване на костите от гроб №63 на Варненския халколитен некропол. — ИИМ-Варна, XVII, 1981, 47—50.
21. Чолаков, С л. Антропологично проучване на костни останки от раннонеолитното селище при село Ваксево, Кюстендилско. — ИИМ — Кюстендил, 3, 1991, 231—244.
22. Чолаков, С., П. Боев. Антропологично палеопатологично проучване на енеолитен некропол в град Търговище. — Интердисциплинарни изследвания — XIVA, 1986, 67—86.

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Acta morphologica et anthropologica, 8
Sofia • 2003

Review Articles

Regulation of Insulin Secretion by Means of Insulinoma Cell Lines

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The use of primary B cells in biochemical and molecular research is limited by the availability of pancreatic endocrine tissue. Numerous investigators have attempted to establsh an insulin-secreting cell line that retains normal regulation of insulin secretion. The most widely used insulin — secreting cell lines are RIN, HIT, MIN 6 and INS — 1. Insulin — secreting cell lines represent a potential source of transplantable tissue to overcome the limited availability of primary islets.

Key words: B cell, cell line, immortalization, insulin.

The transplantation of insulin — producing tissues in patients with diabetes (insulin — dependent — typel) offers an approach that is much more physiological to the restoration of the progressive diabetic neurovascular complications. While in the 1970s [2,14] the isolation and transplantation of Langerhans islets was only possible in experimental animals with diabetes the early 1990s gave rise to several teams working on diabetes treatment projects for transplantation of isolated pancreatic islets [11, 28, 29]. The recipients of these transplants maintain normal glucose levels without insulin therapy years end [16]. The limited opportunities for ensuring human donor pancreatic islets enhance the studies of a number of authors in search of means. For stimulating the proliferation of insulin producing endocrine cell types.

Insulinoma cell lines — definition and classification

Investigations of the differentiation and function of the B cells and elucidation of abnormalities associated with B cell dysfunction determine the necessity for establishment of insulinoma cell lines.

The insulinoma cell line is a cell line capable to grow in cultures and to synthesize and secrete insulin. These are spontaneously occurring and induced tumor lines even can be other than B cells [12]. To overcome the limited availability of primary B cells some authors have attempted to immortalize B cells and establish a stable insulin secreting cell line.

Insulin secreting cell lines can be classified according to the technique used for transformation of the original tissue:

Insulinomas

Naturally occurring insulinomas

Despite numerous attempts, derivation of stable cell lines from spontaneous insulinomas of human or animal origin is exclusively difficult [3, 26]. In principle naturally occurring B-cell tumors dedifferentiate very rapidly in vitro and lose their ability to synthesize and secrete insulin.

Induced insulinomas

Radiation-induced insulinomas — The first insulin-secreting cell line, the RIN cell line, was established in 1973 by Chick et al [4]. X-ray-irradiated rats developed pancreatic tumors 1,5 years after irradiation. The tumors were excised, sliced and transplanted under the skin of 6-week-old rats. Tumors were then propagated in vivo by serial transplantations into RIN rats or BAlb/C mice [8]. Endocrine cells were then purified from the tumors and seeded on a hepatocyte matrix. After several passages the cells begin to adhere spontaneously to the culture vessel. In 1992 [1] have derived INS-1 from parental RINm5f after the following procedure: In a co-culture of lymphocytes and RIN cells in the presence of 2-mercaptethanol free-floating cell aggregates were formed which appeared to be morphologically different from the parental cells. They gave origin to INS-cell line.

Virus-induced insulinomas

The first observation on the development of pancreatic tumors after injection of rats with a BK-virus suspension and investigation of the oncogenicity of the BK-virus and isolation of In-111 come from Uchida et al. [27].

Chemically-induced insulinomas

Insulinomas are obtained by administration of nicotinamid and streptozotocin after that they are serially transplanted in vivo, isolated and propagated in culture [5, 15].

BK- virus- transformation

By this method isolated rat Langerhans islets are transformed in vitro by incubation in the presence of BK virus. The problem that arises is that transformed cells do not secrete insulin after 50 days [10].

Morphological and functional characterization of insulin-secreting cell lines

Morphological characterization

Most insulin-secreting cell lines consist of several cell populations. Immuno-histochemically and with confocal microscopy it is demonstrated that βTC-6 cells contain insulin, glucagon and somatostatin. A colocalization of insulin and glucagon in the same cells is observed, but somatostatin — containing cells appear morphologically different [19]. HIT-T15 also contain the three hormones , but in this case most glucagon -containing cells do not contain insulin [13]. Probably synthesis of glucagon and somatostatin by transformed B-cells might represent a stage of dedifferentiation of all three hormones because the three hormones show a transiently coexpression in progenitor cells during pancreatic development. Most insulin-secreting cells tend to associate in clusters. Usually somatostatin- secreting cells segregate at the periphery, similarly to primary islets of Langerhans [1, 24, 19]. Electron microscopic analysis of insulin-secreting cells shows typical of the the B cell numerous secretory granules resembling those of primary B cells [19, 9].

Functional characterization

• Insulin content — Intracellular insulin content in insulinoma cell lines is lower than that of primary B cells by one to three orders of magnitude [1, 24, 9, 30]. Suppression of the decrease in insulin content of HIT-T15 and βTC-6 cells by a chronic culture in 0,8 mM glucose is shown [20, 23].

• Glucose-induced insulin secretion — Among the described cell lines only a few react to changes in glucose concentration. Regulation of insulin secretion by glucose is assessed by static incubation in the presence of increasing glucose concentrations. We must note that insulin release from all cell lines is lower than that of native islets.

Moreover, the magnitude of insulin secretion above the medium level is greatly diminished. Some of of RIN cell line subclones can retain the ability to secrete insulin in response to glucose [21]. HIT cells secrete insulin in response to glucose with a half-maximal stimulatory concentration of ~ 1,5 mM. Obviously differences found in the literature clearly suggest that assessment of glucose-induced insulin secretion in static incubation highly depends on the techniques used by investigators [6].

The transformed insulin-secreting cells lose some of the functional characteristics of the primary B cells. The described changes in the functional characteristics (reduction in intracellular insulin content, alteration in glucose-sensing, coexpression of glucagon and insulin) represent dedifferentiation of the cells.

Possibilities for application of insulin-secreting cell lines

Investigation on the molecular regulation mechanisms of B cell function and abnormalities associated with B cell dysfunction require the use of insulin-secreting cell lines. For example B cell lines are especially appropriate for the study of glucose-signalling pathways, ion channels, sulfonylurea receptor and regulation of synthesis and secretion of insulin. Investigation of gene regulation at the transcriptional level is extremely difficult in primary islets. Transfection of islets using traditional ap-

proaches are of a limited success: calcium-phosphate precipitation, electroporation of lipofectinusually provides a transfection efficiency of 1 to 10 per cent [25, 17, 7]. Alternatively, transfection of monolayered transformed cells is easier and more efficient. Moreover, the possibility for long-term cultures allow chronic exposure of HIT-T15 cells [21, 18] and BTC-6 cells [23] to high glucose concentrations. Another possibility for application of insulin-secreting cell lines is as a source of tissue for therapeutic applications. The availability of transplantable human islet tissue for the transplantation and for the treatment of diabetes mellitus is limited. Xenotransplantation of large animal islets encapsulated in a perm-selective membrane for protection from immune rejection could overcome the shortage of human tissue [22]. However, large-scale application of this approach is limited by the cost and inconsistency of large animal islet isolation and purification. Alternatively, transformed B cells could provide unlimited amounts of transplantable tissue of constant quality.

Although insulin-secreting cell lines are not so perfect as primary B cells in terms of regulation of insulin secretion, they represent an extremely valuable tool for studies of B cell function as well as dysfunction. The use of transformed insulin-secreting cell lines as transplantable tissues could solve the problem with limitations of primary islets and tissue rejection. It is reasonable to speculate that recent progress in cellular engineering and molecular manipulation will render possibilities for modification and stabilization of cell lines, so that their functional properties can closely mimic those of native B cells.

References

1. Asfari, M., D Janjic, P. Meda, G. Li, P. A. Halban, C. B. Wolheim. Establishment of 2-merkaptoethanol — dependent differentiated insulin-secreting cell lines. — Endocrinology, **130**, 1992, 167-178.
2. Ballinger, W. L., P. E. Lacy.Transplantation of intact pancreatic islets in rats. — Surgery, **72**, 1991, 175-186.
3. Chick, W. L., V. Lauris, J. S. Soeldner, M. H. Tan, M. Grinbergs. Monolayer culture of a human pancreatic beta-cell adenoma. — Metabolism, **22**, 1973, No 9, 1217-1224.
4. Chick, W. L., S. Warren, R. N. Chute, A. A. Like, V. Lauris, K. C. Kitchen. A transplantable insulinoma in the rat. — Proc. Natl. Acad. Sci. USA, **74**, 1977, No 2, 628-632.
5. Chick, W. L., M. C. Appel, G. C. Weir, A. A. Like, V. Lauris, J. G. Porter, R. N. Chute. Serially transplantable chemically induced rat islet cell tumor. — Endocrinology, **107**, 1980, 954-960.
6. Clark, S. A., B. L. Burnham, W. L. Chick. Modulation of glucose-induced insulin secretion from a rat clonal B-cell line. — Endocrinology, **127**, 1990, 2779-2788.
7. Gainer, A. L., G. S. Korbutt, R. V. Rajotte, G. L. Warnock, J. F. Elliot. Transfection of pancreatic islets using biolistics. — In: Fifth International Congress on Pancreas and Islet Transplantation, 1995. Miami Beach, USA (Abstract).
8. Gazdar, A. F, W. L. Chick, H. K. Oie et al. Continuous, clonal, insulin- and somatostatin secreting cell lines established from a transplantable rat islet tumour. — Proc. Natl. Acad. Sci. USA, **77**, 1980, No 6, 3519-3623.
9. Hashimoto, T., H. Kawano, S. Daikoku, K. Shima, H. Taniguchi, S. Baba. Transient coappearance of glucagon and insulin in the progenitor cells of the rat pancreatic islets.- Anatomy and Endocrinology, **178**, 1988, 489-497.
10. Haukland, H. H., B. Vonen, T. Travic. Transformed rat pancreatic islet-cell lines established by BK virus infection in vitro. — Int. J. Cancer, **51**, 1992, 79-83.
11. Hering, B. J. Insulin independence following islets transplantation in man a comparison of different recipient categories. — Int. Islet. Transpl. Registry, **6**, 1996, 5-19.
12. Kaiser, N., A. P. Corcos, I. Sarel, E. Cerasi. Monolayer culture of adult rat pancreatic islets on extracellular matrix: Modulation of B-cell function by chronic exposure to high glucose. — Endocrinology, **129**, 1991, No 4, 2067-2076.

13. Kendal, D. K., V. Poitot, L. K. Olson, R. L. Sorenson, R. P. Robertson. Somatostatin coordinately regulates glucagon gene expression and exocytosis in HIT-T15 cells. — J. Clin. Invest., **96**, 1995, 2496-2502.
14. Lacy, P., M. Kostanovsky. Method for the isolation of intact islets of Langerhans from the rat pancreas. — Diabetes, **16**, 1967, 1-7.
15. Massiello, P., C. B. Wollheim, B. Blondel, A. E. Renold. Studies in vivo and in vitro on chemically-induced primary islet cell tumors and non-tumour endocrine pancreatic tissue.- Diabetologia, **24**, 1983, 30-37.
16. Mevi, T., I. Otsu, J. J. O Neil, K. Dunleavy, C. J. Mullon, B. A. Solomon, A. P. Monaco. Treatment of diabetes by xenogenic islets without immunosupression. Use of a vascularized bioartificial pancreas. — Diabetes, **45**, 1996, 342-347.
17. Newgard, C. B. Cellular engineering and gene therapy strategies for insulin replacement in diabetes. — Diabetes, **43**, 1994, 341-350.
18. Olson, L. K., A. Sharma, M. Peshavaria, C. V. E. Wright, H. C. Towle, R. P. Robertson, R. Stein. Reduction of insulin gene transcription in HIT-T15 cells chronically exposed to a supraphysiologic glucose concentration is associated with loss of STF-1 transcription factor expression. — Proc. Natl. Acad. Sci. USA, 1995, 1279-1288.
19. Poitout, V., L. E. Stout, M. A. Armstrong, T. F. Walseth, R. L. Sorenson, R. P. Robertson. Morphological and functional characterization of bTC-6 cells, and insulin-secreting cell line derived from transgenic mice. — Diabetes, **44**, 1995, 306-313.
20. Poitout, V., L. K. Olson, R. P. Robertson. Chronic exposure of bTC-6 cells to supraphysiological concentrations of glucose decreases binding of the RIPE3b1 insulin gene transcription activator. — J. Clin. Invest., **89**, 1992, 202-213.
21. Praz, G. A., P. A. Halban, C. B. Wollheim, B. Blondel, A. J. Srauss, A. E. Renold. Regulation of immunoreactive — insulin release from a rat cell line (RINm5F). — Biochem J., **210**, 1983, 345-351.
22. Reach, G. Bioartificial pancreas. — Diabetic Medicine, **10**,1993, 105-109.
23. Robertson, R. P., H. G. Zhang, K. L. Pyzdrowski, T. F. Walseth. Preservation of insulin mRNA levels and insulin secretion in HIT cells by avoidance of chronic exposure to high glucose concentrations. — J. Clin. Invest., **90**, 1992, 320-325.
24. Santerre, R. F., R. A. Cook, R. M. D. Crisel, J. D. Sharp, R. J. Shmidt, D. C. Williams, C. P. Wilson. Insulin synthesis in a clonal cell line of simian virus 40-transformed hamster pancreatic beta cell. — Proc. Natl. Acad. Sci. USA, **78**, 1981, No 7, 4339-4343.
25. Seaquist, E. R., A. Robertson Neal, K. D. Shoger, T. F. Walseth, R. P. Robertson. G-proteins and hormonal inhibition of insulin secretion from HIT-T15 cells and isolated rat islets.- Diabetes, **41**, 1992, 1390-1399.
26. Thivolet, C. H., A. Demidem, M. Haftek, A. Durand, J. Bertrand. Structure, function, and immunogeniciti of human insulinoma cells. — Diabetes, **37**, 1988, 1279-1286.
27. Uchida, S, S. Watanabe, T. Alzawa, A. Furuno, T. Muto. Polyoncogenicity and insulinoma-inducing abiliti of BK virus, a human papovavirus, in syrian golden hamsters. — J. Natl. Cancer Inst, **63**, 1979, No 1, 119-126.
28. Warnock, G. L., R. V. Rajotte. Critical mass of purified islets that induce normoglicemia after transplantation into dogs. — Diabetes, **37**, 1988, 467-470.
29. Warnock, G. L., N. M. Kneteman, E. A. Ryan, A. Rabinovitch, R. V. Rajotte. Long-term-follow-up after transplantation of insulin producing pancreatic islets into patients with Type 1 insulin-dependent diabetes mellitus. — Diabetologia, **35**, 1992, 89-95.
30. Zhang, H. T., T. F. Walseth, R. P. Robertson. Insulin secretion and cAMP metabolism in HIT cells. Reciprocal and serial passage-dependent relationships. — Diabetes, **38**, 1989, 44-48.

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Quantitative DNA Analysis in Dysplastic Changes and Carcinoma of the Cervix Uteri

Mini-review

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It has been reported that dysplasias and neoplasias of the Cervix uteri occur as a result of changes in the genome and DNA quantity. These changes can be determined by measuring the cell's DNA quantity using two methods: Image Analysis and Flow Cytometry. The information obtained from the analysis could be used to predict the outcome of the observed genome change and it could also help in the medical treatment of patients.

Key words: DNA quantity, DNA analysis, Image Analysis, Flow Cytometry, dysplastic changes – neoplasias (Carcinoma cervix uteri).

The normal somatic cells of mammalian tissues have a big genetic stability and a conservative cell cycle. In most tissues the number of the dividing cells is less than 10% from the total cell number. The non-dividing cells have 23 pairs of chromosomes and a fixed DNA quantity that is about 7 pg. These cells are known as diploid or non-dividing cells (G_0). Their DNA index equals 1. Entering the synthesis phase (S-phase), diploid cells undergo a process of replication of the genetic information: the DNA quantity doubles, i.e., it is about 14 pg. Such cells are known as tetraploid or G_2 cells. Their DNA index is 2. During the S-phase the amount of DNA varies between 7 and 14 pg [2].

It has been established that genome changes could be different mutations – such as translocations, delisions, duplications etc., influencing the quantity of the cell's DNA. Cells registered as abnormal to their DNA quantity are known as aneuploid [2, 19].

It has been established that the biological development of the malignant cells is associated with their DNA ploidy status. The ploidy status depends on the type of genetic changes during ontogenesis and more specifically it reflects the degree and the stage of the genome changes [2, 11].

Benign tumors have the amount of DNA and the genetic stability of the euploid cells. Relative to DNA status, malignant tumors could be either euploid or

aneuploid. It's commonly accepted that euploid tumors have better prognosis than aneuploid ones. The identification of DNA aneuploidy in dysplatic squamous epithelia can increase the predictive value for malignant transformation to over 90% [3]. The registration of an aneuploid tumor branch suggests that significant changes have occured in the genome of these cells and they have an increased malignant potential [1, 12, 13, 14]. In that case multiple changes of the cell's genetic information could be provoked as well [2, 6]. The presence of aneuploidy usually signifies either recurrence or dysplasia. Polyploidy most frequently occurs in dysplastic processes, whereas diploid cells usually denote a benign disease course [4].

The results of Strang [18] show that DNA ploidy status depends on the patient's age: women at the age of 35.6 (+/- 11.7) or younger tend to develop diploid or polyploid squamous carcinoma, while the older one develop mainly aneuploid carcinoma (the data show that 80-90% of the solid tumors are aneuploid). Nasiell et al [17] established that progression of mild and moderate dysplasia (CIN I and CIN II) is due to serious genome changes. In Fu's [5] investigations all patients diagnosed as CIN III have aneuploid DNA amounts. Presented in per cent, the aneuploid DNA status in different types of dysplasia varies from 20% to 100%. That's why Hanselaar [7] considers aneuploidy as a marker for malignant progression. Kashyap et al. [12, 13] and Steinbeck [20] accept aneuploidy as a high risk factor for the development of neoplasia in women with mild and moderate dysplasia. According to Mariuzzi et al. [16] CIN I and CIN II changes could not be considered as neoplastic since aneuploidy wasn't found in the performed measurements of the DNA quantity. In CIN III, such changes could be observed, so that these lesions could be considered as neoplastic. In their investigations Shu and Gloor [18] determined that in CIN I lesions almost all cells were diploid. Most of the cells in CIN II were also diploid, but polyploid and even aneuploid cells could be found. In more than 80% of the CIN III lesions cells were aneuploid.

Leminen et al. [15] established that the patients with triploid cells had the worst prognosis for survival among those with aneuploid adenocarcinoma of the uterine cervix. The authors claimed that 69% of the tumors examined were diploid and 38% — aneuploid.

The cell fraction in S-phase is significant for the prognosis of tumor development [15]. In cases where the number of cells in S-phase is less than 14%, the survival prognosis of patients is better than in the cases with a high proliferation activity.

Using new methods such as Image Analysis and Flow Cytomerty it's possible to determine the ploidy status of the cell population and to register abnormal cells having deviations greater than 2% in their total DNA quantity [2].

DNA image cytometry, as an additional method, can be used to predict outcome in patients with CIN I and CIN II of the cervix. This is not a screening method but it can add further information for a treatment decision in doubtful cases [9].

Flow cytometry technique can examine thousands of cells within a very short time. Each cell is assessed for a variety of characteristics such as shape and size and cell compositions [8]. The principle of the flow cytometry is that the cells that will be examined are made into a cell suspension and stained by fluorescent dyes (fluorochromes). The cells are then sent into the examination region via a flowing liquid. There is a very strong light source, generally a laser beam to irradiate to the cells one by one. The irradiation will excite the fluorescently stained cells and emit a fluorescent beam, this signal is picked up and sent to the computer to process. The results will be printed out in histograms. It can measure the DNA content of different groups of the cells in a cell cycle. With the use of certain fluorescent labelled antibodies it can also measure different antigens in cells.

In this method fresh specimen could be used. The result of this measurement corresponds well with that of image analysis. E.g., Jacobsen [10] observed biopsy specimen of the pre-neoplastic lesions of the cervix by flow cytometry, and found that there are only 7% of CIN I and CIN II cases which are represented with aneuploid cells, but there are 79% of CIN III cases showing aneuploid.

The number of the samples is very big and it can yield a reliable representative results and qualified for statistics.

References

1. Костова, П. Организация, качествен контрол и ефективност на цервикалния скрининг в България. Дисерт. труд (София). 2001.
2. Auer, G. DNA — A Clinical Parameter. Denderstraat, Becton Dickinson, 1990, 1-9, 27-37.
3. Bocking, A., H. Motherby. Assessment of cervical dysplasia with DNA image cytometry. — Pathologe, **20**, 1999, No 1, 25-33.
4. Davey, D. D., S. Zaleski, M. Sattich, H. Gallion. Prognostic significance of DNA cytometry of postirradiation cervicovaginal smears. — Cancer, **84**, 1998, No 1, 1-16.
5. Fu, Y. S. DNA ploidy measurements in tissue sections. — Analyt. Quant. Cytol. Histol., **7**, 1985, 90-95.
6. Gonzalez-Oliver, A., O. M. Echeverria, R. Hernandez-Pando, G. H. Vazquez-Nin. Ultrastructural study of the nuclei of normal, dysplastic, and carcinomatous epithelial cells of the human cervix uteri. — Ultrastruct. Pathol., **21**, 1997, No 4, 379-392.
7. Hanselaar, A. G. J. M. DNA ploidy and cytometric analysis of cervical intraepithelial neoplasia grade III and invasive squamous cell carcinoma. — Cytometry, **11**, 1990, 624-629.
8. Hedley, D. W. Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. — J. Histochem. Cytochem., **31**, 1983, 1333-1335.
9. Hering, B., L. C. Horn, H. Nenning, K. Kuhndel. Predictive value of DNA cytometry in CIN 1 and 2. Image analysis of 193 cases. — Analyt. Quant. Cytol. Histol., **22**, 2000, No 4, 333-337.
10. Jakobsen, A. Prognostic impact of ploidy level in carcinoma of the cervix. — Am. J. Clin. Oncol., **7**, 1984, 475-480.
11. Khaled, A., Y. Imamura, S. Noriki, M. Fukuda. Early progression stage of malignancy of uterine cervical dysplasia as revealed by immunohistochemical demonstration of increased DNA-instability. — Eur. J. Histochem., **44**, 2000, No 2, 143-156.
12. Kashyap, V., U. K. Luthra. Predictive value of morphological nuclear parameters and DNA ploidy pattern in precancerous lesions of the uterine cervix. — Indian J. Pathol. Microb., **38**, 1995, No 2, 193-197.
13. Kashyap, V., D. K. Das, U. K. Luthra. Microphotometric DNA analysis in mild and moderate dysplasia of the uterine cervix: a retrospective study. — Indian J. Pathol. Microb., **33**, 1990, No 1, 30-34.
14. Kashyap, V., D. K. Das, U.K. Luthra. Microphotometric DNA analysis in cervical dysplasia of the uterine cervix: its relation to progression to malignancy and regression to normancy. — Neoplasma, **37**, 1990, No 5, 497-500.
15. Leminen, A. Deoxyribonucleic acid flow cytometric analysis of cervical adenocarcinoma: prognostic significance of deoxyribonucleic acid ploidy and S-phase fraction. — Am. J. Obstet. Gynecol., **162**, 1990, 848.
16. Mariuzzi, G. Cytometric evidence that cervical intraepithelial neoplasia I and II are dysplasias rather than true neoplasias. — Analyt. Quant. Cytol. Histol., **2**, 1992, 137-147.
17. Nasiell, K. Cytomorphologic and cytochemical analysis in the differential diagnosis of cervical epithelial lesions. — Analyt. Quant. Cytol., **6**, 1984, 196-200.
18. Shu, Y.-J., E. Gloor. Comprehensive cancer cytopathology of the cervix uteri. — In: Color atlas of cancer cytopathology v. 4. (Ed. O.A.N. Husain). New York, McGraw-Hill Book Company, 995, 96-107, p. 413.
19. Steinbeck, R. G., G. U. Auer. Genome instability in humane tumorigenesis: microphotometry of interphase nuclei and pathologic mitoses reveals dysplasia. — Eur J. Histochem., **44**, 2000, No 2, 133-42.
20. Steinbeck, R. G. Proliferation and DNA aneuploidy in mild dysplasia imply early steps of cervical carcinogenesis. — Acta Oncol., **36**, 1997, No 1, 3-12.

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New Views on the Role of Endothelin in the Regulation of Cell Functions

(Minireview)

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The endothelin family consists of isoforms endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3). ET-1 is the main isoform which was synthetized in endothelial cells, muscular cells in arterial walls and in heart, as well as in the kidney and the central nervous system. Endothelins affect functions of multiple cells, tissues and organ systems and are involved in the pathogenesis of many diseases.

Key words: endothelin (ET-1), endothelial cells, muscular cells of cardiovascular system, ovarian granulosa cells, cell function regulation.

Endothelin was detected as a cell factor produced by endothelial cells and causing smooth muscle contractions in 1985 [10]. Three years later (1988/1989), the biologically active substance — endothelin-1 (ET-1), has been isolated and identified by Yanagisawa et al. [16] from the *in vitro* cultivated pig arterial endothelial cells.

ET-1 is a peptide composed of 21 amino acids, including two disulphide bonds (the loss of these bonds between the amino acids 1—15 and 3 — 11, leads to the reduction of ET-1 biological activity).

In man, the endothelin gene encoding 212 aminoacid pre-pro-ET is localized in chromosome 6 and consists of 5 exons and 4 introns. Endothelin is formed by cleaving 164 amino acids from the pre-pro-ET by means of specific endopeptidase (s), resulting in big endothelin.

In further studies endothelin isomers, called endothelin-2 (ET-2) and endothelin-3 (ET-3) — encoded by independent genes were identified, which are different in their chemical structure and potency to contract the smooth muscle cells [11].

ET-1 is produced by vascular endothelial cells, vascular smooth muscle cells and less by astrocytes and neurons in the central nervous system (CNS), by Sertoli cells, hepatocytes in the liver and mesangium.

ET-2 is produced mainly within the kidney and intestines. The high levels of ET-3 have been found in the brain — probably involved in the regulation of neuronal functions.

Some cellular factors stimulate and others inhibite the endothelin synthesis and production by endothelial cells. The stimulating substances are thrombin, Ca ions, epinephrine, angiotensin II, vasopressin, dopamine, erythropoietin, cytokines (IL-1, IL-6), growth factors (fibroblastic, epidermal, insulin-like, GTF-beta), endothoxines, lipids (low density lipoproteis — LDL, and high density lipoproteis — HDL) and stress as well.

Inhibitors of the endothelin synthesis are nitrogen oxide (NO), cyclic guanosine monophosphate (cGMP), atrial natriuretic peptide (ANP), bradykinin, prostacyclin.

The blood levels of ET-1, AT-2 and ET-3 range from 0,3 to 3 pg/ml. [5, 14]. Endothelin concentrations in other body fluids, e.g. in milk, saliva, urine and cerebrospinal fluid are several times higher than the plasma concentrations.

Endothelin receptors are found in many internal organs and organ systems — heart, adrenal glands, kidneys, lungs, CNS [7]. The receptors to ET-1 are existing mainly in the vascular smooth muscle cells. Other type of receptors — responding equally well to ET-1 and to ET-3, are localized on the surface of endothelial cells.

Acting via the ET-1 receptors, ET-1 contributes to the blood vessels vasoconstriction and endothelial dysfunction [6, 9]. The mechanism of vasoconstriction (including of coronary blood vessels — coronary vasospasm), caused by endothelin, may be associated with calcium inflow into the cells and directly correlates with the functional and morphological status of endothelial cells [8, 12, 16]. Endothelin causes a marked and sustained vasoconstriction, exceeding in molar values the vasoconstricting properties of angiotensin II or catecholamines [16].

In this field, the involvement of endothelin in the ethiopathogenesis of arterial hypertension aroused the interest of medical scientists in ET-1 receptor blockers and their hypotensive effects [4, 9, 15]. These ET-1 antagonists also prevent the mitogenic effect of ET on vascular smooth muscle cells.

ET-1 is a potent cell growth factor for cardiomyocytes, exerting direct long term effects (such as myocardial hypertrophy) and causing cellular injury in the cardiac muscle cells [12].

ET-1 reduces the production of rennin in isolated juxtaglomerular cells *in vitro* [13]. Additionaly, endothelin controls some kidney functions: elevated blood concentrations of ET-1 have been observed in patient with acute and chronic renal failure [4]. Exerting a mitogenic effect, ET-1 increases as well a renal interstitial proliferation of fibroblasts in humans [15].

ET-1 stimulates also pulmonary fibroblasts to produce collagen, increases mucus secretion of bronchial mucous glands and contracts bronchial smooth muscles. ET-1 regulates the fibroblast functions in the process of wound healing. At the levels of cells and tissues, the involvement of ET-1 has been also demonstrated in the CNS pathology (cerebral vasospasm) as well as in the methabolic diseases such as atherosclerosis and diabetes mellitus [14]. Some cellular mechanisms of the ET-1 induced *in vitro* inhibition of progesterone synthesis in human ovarian granulosa cells have been demonstrated [1-3] by electron microscopical and histochemical methods.

References

1. Denkova, R., E. Yaneva, K. Baleva, B. Nikolov, V. Bourneva. The influence of endothelin-1 on progesterone production by porcine granulosa cells. — Macedonian J. Reproduction, 5, 1999, 181-185.

2. Denkova, R., V. Bourneva, B. Nikolov, L. Staneva-Dobrovski. Endothelin-1 as a regulator of granulosa cells in aging human ovary. — Hum. Reprod., **15**, 2000, 200-201.
3. Denkova, R., V. Bourneva, E. Zvetkova, E. Yaneva, K. Baleva, B. Nikolov, I. Ivanov, K. Simeonov, T. Timeva. Some mechanisms of in vitro endothelin-1 induced inhibition of progesterone synthesis in human ovarian granulosa cells. — Acta morphologica et anthropologica, **7**, 2002, 37-42.
4. Ding, S., C. Qui, P. Hess, J. P. Clozel, M. Clozel. Chronic endothelin receptor blockade prevents renal vasoconstriction and sodium retention in rats with chronic heart failure. — Cardiovasc. Res. **53**, 2002, 963-970.
5. Donkier, J., C. Hanet, A. Berbinschi, L. Galannti, A. Robert, H. Van Mechelin. Cardiovascular and endocrine effects of ET-1 at pathophysiological and pharmacological plasma concentrations in conscious dogs. — Circulation **84**, 1991, 2476-2484.
6. Gellai, M., T. Fletcher, M. Pullen, P. Nambi. Evidence for the existance of endothelin-1 receptor subtypes and their physiological roles in the rat. — Am. J. Physiol., **27**, 1996, R254-R261.
7. Giaid, A., S. Gibson, M. Herrero, S. Gentelmaon, S. Legon, M. Yanagisawa, T. Masaki, B. Ibrahim, G. Roberts, M. Rossi, J. Polak. Topographical localization of endothelin mRNA and peptide immunoreactivity in neurons of the human brain. — Histochemistry, **95**, 1991, 303-314.
8. Grossman, J., J. Morgan. Cardiovascular effects of endothelin. — News Physiol. Sci., **12**, 1997, 113-117.
9. Halcox, J. P., K. Nour, G. Zalos, A. Quyumi. Coronary vasodilatation and improvement in endothelial dysfunction with endothelin (ET) receptor blockade. — Circ. Res. **89**, 2001, 969-976.
10. Hicley, K., G. Rubanyi, R. Paul, R. Highsmith. Characterization of a coronary vasoconstrictor produced by cultured endothelial cells. — Am. J. Physiol., **248**, 1985, C550-C556.
11. Inoue, A., M. Yanagisawa, S. Kimura, Y. Kasuya, T. Miyauchi, K. Goto, T. Masaki. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. — Proc. Natl. Acad. Sci., USA, **86**, 1989, 2863-2867.
12. Kramer, B., K. Ittner, M. Beyer, H. Hoffmeister, G. Riegger. Circulatory and myocardial effects of endothelin. — J. Mol Med., **75**, 1997, 886-890.
13. Lin, H., S. Mariem, M. Smith, D. Young. Effect of endothelin-1 on glomerular hydraulic pressure and renin release in dogs. — Hypertension **21**, 1993, 845-85.
14. Migdalis, I., K. Kalogeropoulou, K. Karmaniolas, N. Varvarigos, G. Mortzos, P. Cordopatis. Plasma levels of endothelin and early carotid atherosclerosis in diabetic patients. — Res. Commun. Mol. Pathol. Pharmacol., **108**, 2000, 15-25.
15. Tian, X., G. Tang, Y. Chen. The effects of endothelin-1 selective endothelin receptor-type A antagonist on human renal fibroblasts in vitro. — Zhoghua Yi Xue Za Zhi, **82**, 2002, 5-9.
16. Yanagisawa, M., T. Masaki. Endothelin, a novel endothelium-derived peptide. — Biochem. Pharmacol., **38**, 1989, 1877-1886.