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INFLUENCE OF PROLONGED CENTRAL DEPRIVATION OF TESTOSTERONE SYNTHESIS ON PRODUCTION OF REACTIVE OXYGEN AND NITROGEN SPECIES AND MORPHOLOGICAL STRUCTURE OF RAT TESTES

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Some testicular diseases require long-term use of testosterone synthesis blockers. It is known that testosterone is a necessary component for the physiological cooperation of cell forms of the stroma and parenchyma of testes. The scientific literature contains a limited amount of data about the effect of long-term deprivation of testosterone synthesis on this cooperation. The aim of our study was to establish the effect of long-term (365 days) inhibition of hypothalamic-pituitary stimulation of spermatogenesis on morphological and biochemical parameters in rat testes. Central deprivation of testosterone synthesis leads to fibrosis with subsequent disruption of the structural organization of the convoluted seminiferous tubules, hemodynamic disorders, endothelial dysfunction, increased density of blood vessel walls and systemic congestion. A decrease in the activity of constitutive NO synthase isoforms plays a major role in the development of structural changes in the testes of rats.

Key words: testes, interstitial endocrinocytes, sustentocyte, NO-synthase, iNOS, cNOS, L-arginine, superoxide dismutase, rats, fibrosis.

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ВПЛИВ ТРИВАЛОЇ ЦЕНТРАЛЬНОЇ ДЕПРИВАЦІЇ СИНТЕЗУ ТЕСТОСТЕРОНУ НА ПРОДУКЦІЮ РЕАКТИВНИХ КИСНЕВО-АЗОТНИХ ФОРМ НА МОРФОЛОГІЧНУ СТРУКТУРУ СІМ'ЯНИКІВ ЩУРІВ

Деякі захворювання сім'яників у чоловіків вимагають тривалого використання блокаторів синтезу тестостерону. Як відомо, що тестостерон є необхідним компонентом для фізіологічної кооперації клітинних форм строми та паренхіми яєчка. У літературі наведено обмежена кількість даних про вплив тривалої депривації синтезу тестостерону на цю кооперацію. Метою нашого дослідження було встановлення впливу тривалої (365 днів) інгібіції гіпоталамо-гіпофізарної стимуляції сперматогенезу на морфологічні та біохімічні показники в сім'яниках щурів. Центральна депривація синтезу тестостерону призводить до фіброзу з подальшим порушенням структурної організації звивистих сім'яних канальців, порушень гемодинаміки, ендотеліальної дисфункції, збільшення щільності судинної стінки кровоносних судин і системному стазу. Зниження активності конституційних ізоформ NO-синтази відіграє основну роль у розвитку структурних змін в сім'яниках щурів.

Ключові слова: сім’яники, інтерстиційні ендокриноцити, сустентоцити, NO-синтаза, iNOS, cNOS, L-аргінін, супероксиддисмутаза, щури, фіброз.

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Aging is a crucial part of human life cycle. Some functions of our organism start to decrease in intensity with age. Sexual function is among functions which lower their activity. The reason for such decline in function is decreased production of testosterone [3]. Testosterone deficiency leads to oxidative damage to testicular tissue [6].

Testicular tissue contains antigens, which may be treated by our immune system as hostile antigens. In order to avoid formation of immune response to these antigens most of testicular macrophages express CD206 (anti-inflammatory marker) and rarely express activation marker (MHC class II), but even when macrophage expresses activation receptors ($CD206^{+}MHCII^{+}$) they have reduced ability to initiate specific immune responce [8]. Testosterone can cause shift of macrophage polarization through activin A signaling [7]. Therefore, testosterone is necessary to keep immune cells of testes in check and prevent their activation. Interstitial endocrinocytes as main testosterone producers play a vital role in intercellular cooperation and provide necessary activin A signaling for upkeeping the anti-inflammatory state of testicular macrophages [11,15]. The age-dependent decrease in testosterone synthesis may cause macrophage-mediated testicular damage.

On the other hand, some medical situations demand usage of drugs that block testosterone synthesis. For instance, prostate cancer requires either chemical of surgical castration as treatment, because this tumor is very sensitive to androgens and testosterone [12]. Prostate cancer is the second most frequent cancer diagnosis made in men and the fifth leading cause of death worldwide. In case of surgical castration there is no risk of autoimmune damage to testes because the antigens are removed from organism. However, in some cases

chemical castration may be used as treatment choice [9]. This is especially true if prostate cancer was diagnosed in rather young age or the patient still wishes to remain fertile. Influence of prolonged deprivation of testosterone synthesis on morphological, biochemical and immunological changes in testes is still poorly described in scientific literature.

The purpose of this research was to evaluate the microscopic organization of rat testes, to determine the sources of nitric oxide production and the intensity of oxidative stress in the rat testes during experimental central deprivation of testosterone synthesis by diphereline injection on the 365th day of the experiment.

Materials and methods. The experiments were carried out on 10 sexually mature male white rats of the Wistar line. Rats were divided into 2 groups with 5 animals in each group: the control group and the experimental group. Animals from the experimental group were injected subcutaneously with diphereline (Triptorelin embonate) at a dose of 0.3 mg of the active substance per kg [4]. Rats from the control group received injection of saline. Experiment lasted 365 days. Animals were kept in standard vivarium conditions of the Ukrainian Medical Stomatological Academy. Experimental animals were sacrificed in strict compliance with the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes"; (Strasbourg, 1986), as well as with the "General Ethical Principles of Animal Experiments" adopted by the First National Congress on Bioethics (Kyiv, 2001).

After an overdose of ketamine, the animals were decapitated, the prepared small pieces of the testes were fixed in a 2.5% glutaraldehyde solution (pH=7.2-7.4). Postfixation of the material was carried out with 1% solution of osmium (IV) oxide, followed by dehydration in propylene oxide and a sample was embedded into the epoxy resins mixture. Ultrathin sections made with an ultramicrotome were contrasted with a 1% aqueous solution of uranyl acetate and lead citrate according to the Reynolds' method and studied with an electron microscope [1].

Using standard methods, the material was imbedded in paraffin blocks, of which sections 4 μm thick were made and stained with hematoxylin and eosin. Histological preparations were examined using Biorex 3 light microscope with digital microfilter with software adapted for these studies (Serial No. 5604).

We carried out all biochemical studies in 10% homogenate of testis tissue using Ulab 101 spectrophotometer. General activity of NO-synthase (gNOS), activity of constitutive isoforms (cNOS), activity of inducible isoform (iNOS) was determined by increase of nitrite concentration after incubation in buffer solution (pH=7.4) containing 0.3 ml of 320 mM L-arginine solution and 0.1 ml of 1 mM NADPH+H solution [13]. Nitrite concentration was measured with help of Griess reagent [14]. Arginase activity was evaluated by increase of L-ornithine content after incubation in buffer solution (pH=7.0) containing 0.2 ml of 24 mM L-arginine solution [13].

Basic production of superoxide anion radical (SAR), its production by the mitochondrial electron transport chain (ETC) and microsomal ETC was determined by the growth of diformazan concentration, formed in the reaction of SAR with nitro blue tetrazolium [13]. Superoxide dismutase (SOD) activity was determined by inhibition of adrenaline autooxidation, while catalase activity was determined by the amount of hydrogen peroxide, remained after its catalase-dependent reduction [14]. The concentration of free malondialdehyde (MDA) was determined by reaction with 1-methyl-2-phenylindole resulting in formation of specific colored substance [13].

Statistical processing of the study results was carried out using the Microsoft Office Excel software and the Real Statistics 2019 extension to it. The nonparametric Mann-Whitney test was used to determine the statistical significance of differences between the groups. The difference was considered statistically significant at $p<0.05$.

Results of the study and their discussion. Visual examination of rat testes on 365th day of the experiment revealed that the weight and size of the testes varied greatly. The difference was apparent compared to control group and the previous term of the experiment [13, 14]. Replacement of testicular germ cell tissue with connective tissue leads to hardening and a decrease in the mass of the damaged organ [14].

Microscopic examination (semi-thin sections) of rat testes from the experimental group revealed clear changes in the interstitial tissue typical for fibrosis with a violation of the structural organization of the convoluted seminiferous tubules and the interstitial space between them (fig. 1).

In this experimental group, the increase in connective tissue spaces was associated with the qualitative and quantitative composition of the altered cells and intercellular substance of the interstitium. An increase in both the inflow and outflow units of the microcirculatory bed was significant, the number of capillaries was not changed.

Structural reorganization of the interstitium revealed changes in the structure of arterioles in the form of endothelial dysfunction and an increase in the density of the vascular wall. We observed vasodilation of

arterioles and venules, tortuosity of precapillaries in the field of view (fig. 2). Venule walls were enlarged and tightly woven into the structure of the interstitium. The capillaries were enlarged against the background of the general stasis. It is worth noting the following violation on the part of the microvasculature: perivascular fibrosis with reduction of the microvascular bed. The endothelium of the capillaries was thinned.

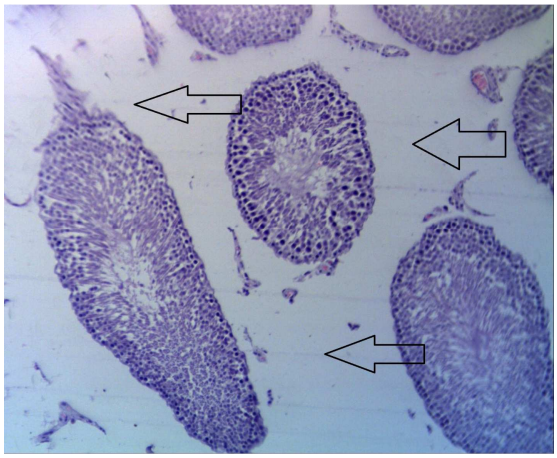

Fig. 1. Seminiferous tubules of experimental rat on the 365th day. Microimage. Stain: hematoxiline and eosine. Lens: 10: Ocular lens: 10. Interstitial space – fibrosis

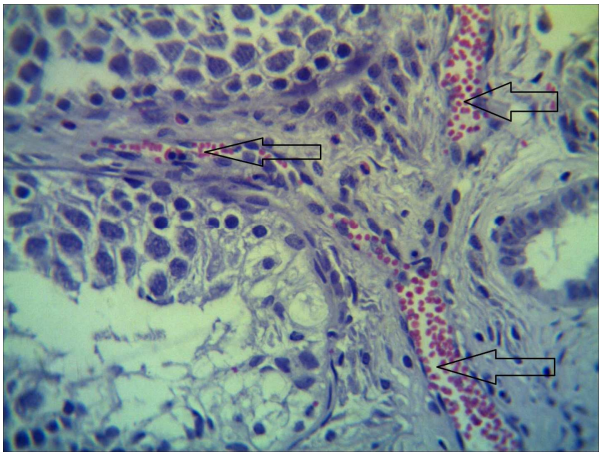

Fig. 2. Blood vessel in Interstitial space of experimental rat on the 365th day. Microimage. Stain: hematoxiline and eosine. Lens: 40: Ocular lens:10

In the stroma of the testes, there were almost no interstitial endocrinocytes or there were one or two in the field of view. We noted a tendency to their quantitative decrease in comparison with both the control group and the previous periods of the experiment [13, 14]. Interstitial endocrinocytes were reduced in size, had eosinophilic cytoplasm and heterochromic nuclei. There was a small amount of lipid granules in the cytoplasm (fig. 3).

In the interstitial tissue, macrophages were also detected, which we divided into two populations of cells. The first subpopulation was parietal macrophages, elongated and resembled myoepithelial cells in shape, but reduced in size and adjacent to the wall of the convoluted tubules. The second subpopulation was interstitial macrophages, which were located near the blood vessels, the number of parietal macrophages prevailed many times over interstitial (fig. 3).

We observed violation of all stages of spermatogenesis in convoluted seminiferous tubules of testes. The parenchyma of the testes was characterized by structural reorganization of the convoluted seminiferous tubules. The convoluted tubules were different in diameter and shape, the basal membrane was thickened and convoluted. There were tubules with a complete absence of cellular forms of the epithelial-spermatogenic layer. We noted reduced amount of spermatogonia types A and B in most tubules. Spermatogenic cells were swollen, disoriented in the tubule space. There were tubules with complete disorientation of cells (fig. 4).

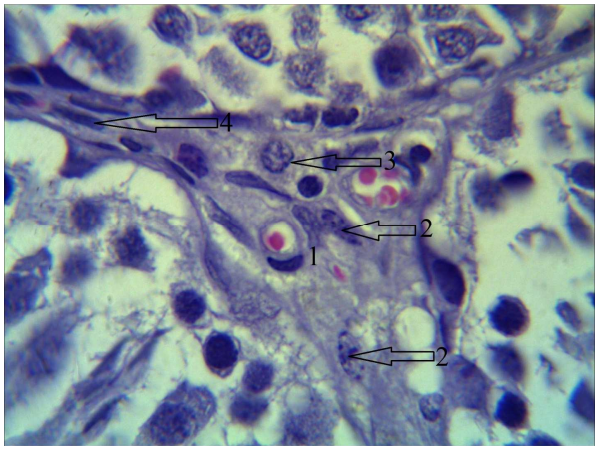

Fig. 3. Interstitial space of experimental rat on the 365th day. Microimage. Stain: hematoxiline and eosine. Lens: 40: Ocular lens: 15. 1. Interstitial space. 2. Interstitial endocrinocytes. 3. Interstitial macrophages. 4. Parietal macrophages.

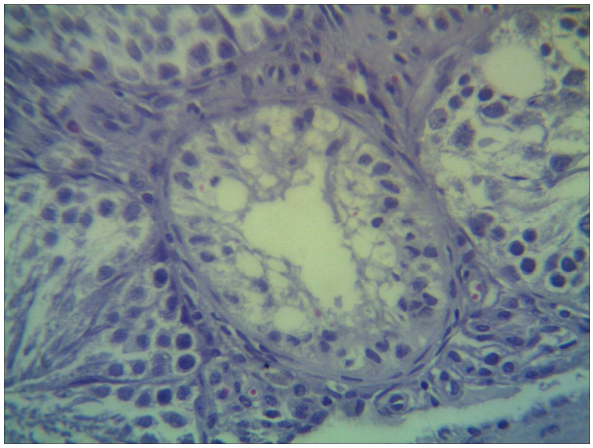

Fig. 4. Seminiferous tubules of experimental rat on the 365th day. Microimage. Stain: hematoxiline and eosine. Lens: 40: Ocular lens: 10.

Basic production of superoxide anion-radical in the experimental group was increased by 5.5 times compared to the control group of animals (tab. 1). Production of SAR from mitochondrial and microsomal ETC was elevated by 1.73 times and 1.25 times respectively. SOD activity was decreased in 1.38 times, while

activity of catalase is was also decreased in 1.54 times. There were no statistically significant changes in concentration of free MDA.

Table 1

Oxidative stress markers in rat testes during 365-day central testosterone synthesis deprivation (M±m)

Groups	Parameters					
	SOD activity, c.u.	Catalase activity, nkat/g of tissue	Basic $O_2^{\bullet-}$ production, nmol/s per g of tissue	Production of $O_2^{\bullet-}$ from mithochondrial ETC, nmol/s per g of tissue	Production of $O_2^{\bullet-}$ from microsonal ETC, nmol/s per g of tissue	Free MDA, µmol/g of tissue
Control	1.87 ±0.11	182.0 ±17	0.26 ±0.01	7.84 ±0.13	9.55 ±0.19	6.64 ±1.44
Experimental	1.36 ±0.14*	117.9 ±2.2*	1.43 ±0.04*	13.55 ±0.15*	11.95 ±0.15*	9.6 ±0.26

Note:* – indicates that the difference is statistically significant when compared with control group ($p<0.05$)

On the 365th day of the experiment we detected a decrease in gNOS activity by 42.6 % (tab. 2). There were no statistically significant changes in activity of iNOS in rats' testes after 365 days of central deprivation of testosterone synthesis. Activity of cNOS isoforms dropped by 8.2 times. Arginase activity decreased by 38.3 %. Concentration of nitrites did not change.

Table 2

Nitric oxide cycle function during 365-day central testosterone synthesis deprivation (M±m)

Groups	Parameters				
	gNOS activity, µmol/min per g of protein	iNOS activity, µmol/min per g of protein	cNOS activity, µmol/min per g of protein	Arginase activity, µmol/min per g of protein	NO_2^- concentration, nmol/L
Control	0.54 ±0.04	0.13 ±0.02	0.41 ±0.03	2.48 ±0.05	3.83 ±0.25
Experimental	0.31 ±0.04 *	0.26 ±0.04	0.05 ±0.003 *	1.53 ±0.15 *	3.47 ±0.16

Note:* – indicates that the difference is statistically significant when compared with control group ($p<0.05$)

Decreased cNOS activity may contribute to endothelial dysfunction observed in microvascular bed on 365th day of experimental central deprivation of testosterone synthesis. Endothelial dysfunction leads to insufficient blood flow in testes and hypoxia. Since spermatogenesis requires multiple divisions of the cells it is a highly energy dependent. At this stage of experiment lack of cNOS-derived nitric oxide leads to hypoxia, which in turn up-regulates hypoxia induced factor (HIF) signaling [11]. HIF signaling increases reactive oxygen species production, which may be the exact mechanism of increased SAR production observed in our study [5]. Up-regulated HIF signaling can also explain tortuosity of precapillaries observed in our research because HIF pathway also increases vascular endothelial growth factor (VEGF) production [5]. Ultimately, hypoxia in testes leads to fibrosis trough activation of transcription of transforming growth factor β (TGF- β) [10]. Hypoxia-induces testicular fibrosis was also shown in studies of Palatova T.V. et al. [2].

On previous terms of experiment, we observed acute development of oxidative damage to the testicular tissues and abundant production of nitric oxide [13]. On later terms of experiment, we observed beginning of fibrotic changes and decrease of nitric oxide production [14]. This suggests that at the beginning of testosterone deprivation there may be a change in macrophage polarization towards prevalence of M1 (proinflammatory) phenotype because iNOS/arginase ratio was high [13]. On the 365th day of central deprivation of testosterone synthesis iNOS/arginase ratio was low, which suggests prevalence of M2 (anti-inflammatory) polarization of macrophages.

The development of degenerative changes in rat testes, in our opinion, may be due to a violation of the cooperative interaction of cells of both the interstitial space and the parenchyma. Which in turn leads to changes in their metabolic processes, permeability of the blood-testicular barrier, changes that lead to a persistent defect in the quality of spermatogenesis. In our opinion, this system consists from macrophage, sustentocytes and interstitial endocrinocyte. Disruption of hypothalamic stimulus for testosterone production leads to change of macrophage polarization to M1 phenotype with subsequent testicular tissue damage and its replacement with fibrotic tissue. Further studies are necessary to evaluate precise changes in the cooperation between testicular macrophage, sustentocytes and interstitial endocrinocyte.

Conclusion

Prolonged central deprivation of testosterone synthesis by diphereline leads to disruption of spermatogenesis, endothelial dysfunction and fibrotic changes in rat testes. The main mechanism of

endothelial dysfunction observed during prolonged central deprivation of testosterone synthesis is a decreased activity of constitutive isoforms of NO-synthase.

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