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ROLE OF NITRIC OXIDE IN DEVELOPMENT OF FIBROTIC CHANGES IN RATS' TESTES AFTER 270 DAY CENTRAL DEPRIVATION OF TESTOSTERONE SYNTHESIS

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Disturbance in production of nitric oxide (NO) may lead to various changes in different organs and systems. Certain clinical situations require prolonged usage of inhibitors of testosterone synthesis. Scientific literature provides limited information regarding the influence of prolonged deprivation of testosterone synthesis on production of NO and microscopic organization of rats' testes. Prolonged central deprivation of testosterone synthesis leads to endothelial dysfunction, development of fibrosis, decreases the nitric oxide production and shifts pro-/antioxidant balance in favor of the pro-oxidants without increase in lipid peroxidation intensity. Central deprivation of testosterone synthesis leads to fibrosis with subsequent disruption of the structural organization of the convoluted seminiferous tubules, hemodynamic disturbances, endothelial dysfunction, increased density of the vascular wall of blood vessels and systemic stasis. Decreased production of NO from constitutive isoforms of NO-synthase plays major role in development of structural changes in the interstitial tissue of testes on the 270th day of the experiment.

Keywords: testes; rats; diphereline; oxidative stress; nitric oxide; fibrosis.

The study is a fragment of the research project "Experimental morphological study of cryopreserved placenta transplants action diphereline, ethanol and 1% methacrylic acid on the morphofunctional status in a number of internal organs", state registration No. 0119U102925.

Disturbance in production of nitric oxide (NO) may lead to various changes in different organs and systems. There is evidence that NO produced by inducible isoform of NO-synthase (EC 1.14.13.39, iNOS) may lead to liver fibrosis trough increase in peroxynitrite ($ONOO^-$) formation [1]. Liver is not the only organ in which excessive iNOS activity leads to fibrotic changes [2, 4].

Increased iNOS activity in rat testes during diabetes also leads to fibrosis and increased lipid peroxidation [12]. At the same time, decrease in endothelial NOS activity and testosterone content may also lead to disruption of erectile function [8]. Therefore, changes in quantity and source of NO production are important to testicular tissue metabolism.

Certain clinical situations require prolonged usage of testosterone synthesis inhibitors [7]. Testosterone metabolism is closely related to endothelial NOS activity, which is especially true during testosterone deficiency [5]. Testosterone synthesis is necessary for suppression of reactive nitrogen (RNS) and oxygen species (ROS) formation [10]. A decrease in testosterone synthesis, caused by intervention in autoregulation of its production by interstitial endocrinocytes, leads to decrease of nitric oxide formation from constitutive isoforms of NOS with simultaneous elevation of its production by iNOS [10]. This change in the source of NO production in turn causes two adverse events. First one is the uncoupling of constitutional NOS isoforms, which leads to increased production of ROS from microsomal electron transport chains. Second event is overproduction of NO by iNOS. The last event has two major consequences: increased nitrite accumulation (as the result of excessive NO oxidation be oxygen present in tissues) and peroxynitrite formation (in reaction between NO and superoxide anion radical). Therefore deficiency of testosterone synthesis may cause the development of nitritive and nitrosative stress, in addition to the oxidative stress.

Scientific literature provides limited information regarding the influence of prolonged deprivation of testosterone synthesis on production of NO and microscopic organization of rats' testes.

The purpose of the study was to the microscopic organization of rats' testes, to determine the sources of nitric oxide production and the intensity of oxidative stress in the rats' testes during experimental central deprivation of testosterone synthesis by diphereline injection on the 270th 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: the control group (5) and the experimental group (5). 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 of body weight for 270 days, while the control group received injection of saline [3]. 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).

All biochemical studies were carried out 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), activity of arginases and nitrite concentration was determined by methods described by Yelinska A.M. [11].

Basic production of superoxide anion radical ($O_2^{\bullet-}$), 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 $O_2^{\bullet-}$ with nitro blue tetrazolium [11]. 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 [11]. The concentration of free malondialdehyde (MDA) was determined by reaction with 1-methyl-2-phenylindole.

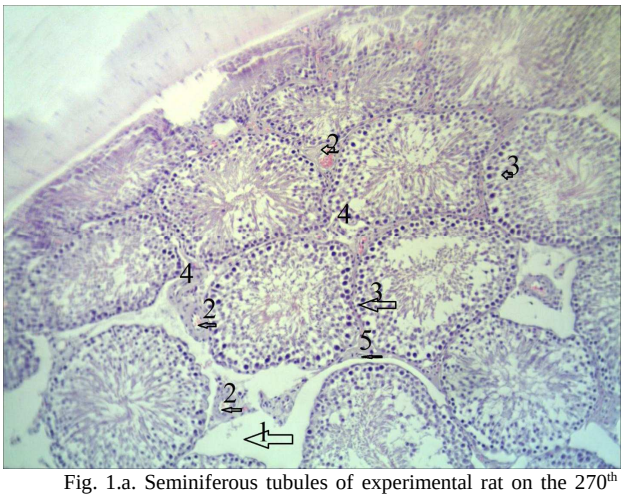

Fig. 1.a. Seminiferous tubules of experimental rat on the 270th day. Microimage. Stain: hematoxiline and eosine. Lens: 10: Ocular lens: 10. 1. Interstitial space - fibrosis. 2. Blood vessel 3. Spermatogenic epithelium of the tubule. 4. Interstitial cells. 5. The capillary in the interstitium.

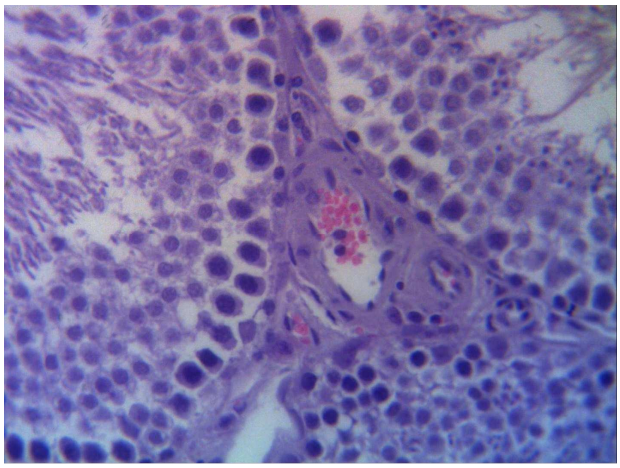

Fig. 1.b. Interstitial space of experimental rat on the 270th day. Microimage. Stain: hematoxiline and eosine. Lens: 40: Ocular lens:15.

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. When studying semi-thin sections of the testes on the 270th day of the experiment, we found changes in the interstitial tissue, which are very characteristic of fibrosis (fig.1a). We also observed violation of the structural organization of the convoluted seminiferous tubules. Disturbances in the microvasculature manifested as endothelial dysfunction and increase in density of the vascular wall.

Rats from the experimental group had an increase in connective tissue spaces associated with both the qualitative and quantitative composition of the altered interstitial cells and the microvasculature. Structural reorganization of the interstitial tissue manifested itself as a quantitative increase in arterioles, venules, and capillaries. We observed morphological signs of endothelial dysfunction, hemodynamic disturbances, perivascular fibrosis with a decrease in the volume of the microvascular bed with subsequent fibrosis of the interstitium in general. We determined vasodilation of arterioles and venules, tortuosity of precapillaries in the visual field. Capillaries were enlarged against the background of general stasis (fig. 1b).

From the side of interstitial endocrinocytes, there is a tendency to a quantitative decrease in comparison with the control group. Also, there are interstitial spaces between the convoluted tubules with a complete absence of interstitial endocrinocytes. The cells themselves are reduced in size, the nuclei are heterochromic, in the cytoplasm there is a small amount of lipid granules (fig. 1c).

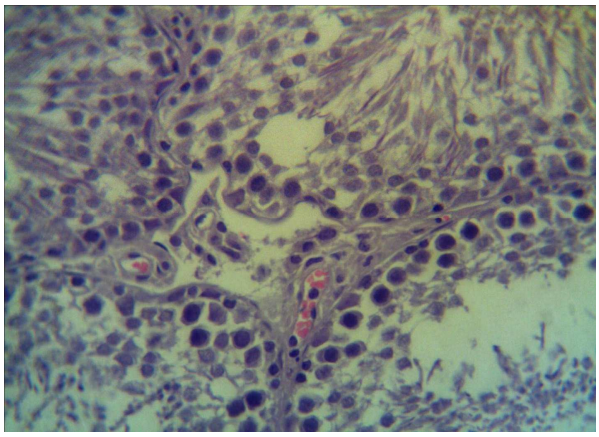

Fig. 1.c. Interstitial space of experimental rat on the 180th day. Microimage. Stain: hematoxiline and eosine. Lens: 40: Ocular lens:10.

In addition to interstitial endocrinocytes, macrophages were detected in the connective tissue, which had two populations of cells. The first population of cells were parietal macrophages, they were flattened and adjoined to the wall of the convoluted tubules. Second type of cells were interstitial macrophages located near the blood vessels. The number of parietal ones prevailed several times over interstitial (fig. 1.c).

Basic production of superoxide anion-radical in the experimental group was increased by 5 times compared to the control group of animals (tab. 1). Production of $O_2^{\bullet-}$ from mitochondrial and microsomal ETC was elevated by 63.9% and 22.62% respectively. SOD activity was decreased by 46.52%, while activity of catalase is was also decreased by 40.33%. There were no statistically significant changes in concentration of free MDA

Table 1

Oxidative stress markers in rats' testes during 270-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	0.26±0.01	7.84±0.13	9.55±0.19	6.64±1.44
Experimental	1.00±0.19*	108.6±9.3*	1.30±0.02*	12.85±0.13*	11.71±0.11*	9.15±0.26

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

On the 270th day of the experiment we detected a decrease in gNOS activity by 68.51% (tab. 2). There were no statistically significant changes in activity of iNOS in rats' testes after 270 days of central deprivation of testosterone synthesis. Activity of cNOS isoforms dropped by 8.2 times. Arginase activity decreased by 39.92%. Concentration of nitrites in testes lowered by 25.33%.

Table 2

Nitric oxide cycle function during 270-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	0.17±0.02*	0.13±0.02	0.05±0.0003*	1.49±0.11*	2.86±0.16*

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

Nitric oxide is synthetized in lower quantity and predominantly from inducible isoform of NO-synthase. Despite increased production of pro-oxidants and decreased activity of antioxidant enzymes, the intensity of lipid peroxidation is not elevated.

The reason for decreased production of NO is lowered activity of cNOS since activity of iNOS is within the values of the control group. This situation is the direct result of testosterone deficiency caused by experimental procedure. Also lowered activity of cNOS may contribute to the endothelial dysfunction observed in fig. 1b.

Lowered concentration of nitrites in rats' testes during prolonged central deprivation of testosterone synthesis can be the result of the increased activity of nitrite reductases, which is aimed at compensation of NO production deficiency.

In our previous study we observed increased iNOS activity and decreased activity of arginase [9]. Since iNOS and arginase can be considered as "marker enzymes" for definition of macrophage polarization, we can consider that most of the macrophages present in testes on the previous term of experiment were in M1 (proinflammatory) polarization [9].

The prevalence of parietal macrophages over the interstitial ones still remains on the 270th day of the experiment. However, we discovered that arginase activity is much greater than iNOS activity in this

term of experiment. This fact gives us grounds to speculate that a major part of macrophages, present in testes on the 270th day of experiment, have M2 (anti-inflammatory) polarization.

We observed a peculiar situation, when superoxide anion-radical production is increased, while SOD and catalase activities are decreased, however lipid peroxidation is not increased. Since free MDA levels were not changed after 270-day central deprivation of testosterone synthesis we can conclude that increased superoxide anion-radical is used for remodeling of the tissue and it does not cause cell damage. Without oxidative damage to the cell membrane SOD and catalase genes are not expressed hence the lowered activity of these enzymes. But we must also consider the possible influence of other antioxidants like glutathione system and non-enzymatic antioxidants.

Decreased cNOS activity may be the key factor contributing to increased production of superoxide anion from mitochondrial ETC since NO produced from neuronal isoform of NOS located in mitochondria has ability to regulate superoxide anion-radical production [13].

Conclusions

Central deprivation of testosterone synthesis leads to fibrosis with subsequent disruption of the structural organization of the convoluted seminiferous tubules, hemodynamic disturbances, endothelial dysfunction, increased density of the vascular wall of blood vessels and systemic stasis. Decreased production of NO from constitutive isoforms of NO-synthase plays a major role in development of structural changes in the interstitial tissue of testes on the 270th day of the experiment.

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Реферати

РОЛЬ ОКСИДУ АЗОТУ В РОЗВИТКУ ФІБРОТИЧНИХ ЗМІН СІМЯНИКІВ ЩУРІВ ПІСЛЯ 270 ДНІВ ЦЕНТРАЛЬНОЇ ДЕПРИВАЦІЇ СИНТЕЗУ ТЕСТОСТЕРОНА

Стецук Є.В., Акімов О.Є., Шепітько К.В., Борута Н.В., Гольцев А.Н.

Порушення виробництва оксиду азоту (NO) може призводити до різних змін в різних органах і системах. Певні клінічні ситуації вимагають тривалого використання інгібіторів синтезу тестостерону. У науковій літературі є обмежена інформація про вплив тривалого позбавлення синтезу тестостерону на продукцію NO і мікроскопічну організацію сім'яників щурів. Тривала центральна депривація синтезу

РОЛЬ ОКСИДА АЗОТА В РАЗВИТИИ ФИБРОТИЧЕСКИХ ИЗМЕНЕНИЙ В ТЕСТАХ КРЫС ПОСЛЕ 270 ДНЕЙ ЦЕНТРАЛЬНОЙ ДЕПРИВАЦИИ СИНТЕЗА ТЕСТОСТЕРОНА

Стецук Е.В., Акимов О.Е., Шепитько К.В., Борута Н.В., Гольцев А.Н.

Нарушение производства оксида азота (NO) может приводить к различным изменениям в разных органах и системах. Определенные клинические ситуации требуют длительного использования ингибиторов синтеза тестостерона. В научной литературе имеется ограниченная информация о влиянии длительного лишения синтеза тестостерона на продукцию NO и микроскопическую организацию семенников крыс. Длительная центральная

тестостерону призводить до ендотеліальної дисфункції, розвитку фіброзу, знижує вироблення оксиду азоту та зрушує про- / антиоксидантний баланс на користь прооксидантів без збільшення інтенсивності перекисного окислення ліпідів. 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 застою. Зниження продукції NO з конститутивних ізоформ NO-синтази відіграє основну роль у розвитку структурних змін інтерстиціальної тканини сім'яників на 270-й день експерименту.

Ключові слова: сім'яники, інтерстиціальні ендокріноцити, сустентоцити, NO-синтаза, iNOS, cNOS, L-аргінін, супероксиддисмутаза, щури.

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депривация синтеза тестостерона приводит к эндотелиальной дисфункции, развитию фиброза, снижает выработку оксида азота и сдвигает про- / антиоксидантный баланс в пользу прооксидантов без увеличения интенсивности перекисного окисления липидов. Центральная депривация синтеза тестостерона приводит к фиброзу с последующим нарушением структурной организации извитых семенных канальцев, нарушениям гемодинамики, эндотелиальной дисфункции, увеличению плотности сосудистой стенки кровеносных сосудов и системному застою. Снижение продукции NO из конститутивных изоформ NO-синтазы играет основную роль в развитии структурных изменений интерстициальной ткани семенников на 270-й день эксперимента.

Ключевые слова: семенники, интерстициальные эндокриноциты, сустентоциты, NO-синтаза, iNOS, cNOS, L-аргинин, супероксиддисмутаза, крысы.

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MORPHOMETRIC ASSESSMENT OF STRUCTURAL CHANGES IN THE DUODENAL WALL OF RATS CAUSED BY SKIN BURN INJURY UNDER CONDITIONS OF EXPERIMENTAL DIABETES

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This work is devoted to the morphometric assesment of structural changes in the duodenal wall of rats with skin burn injury under conditions of experimental diabetes mellitus. The control group included 21 intact animals without any signs of somatic pathology, experimental group I consisted of 21 rats with experimental skin burn injury, experimental group II consisted of 21 rats without skin burns but with experimentally simulated diabetes mellitus, and experimental group III consisted of 21 rats with both skin burn injury and experimentally simulated diabetes mellitus. The following morphometric parameters have been studied: mucosal thickness, villi height and thickness, crypt depth and width, thickness of lamina muscularis of mucosa, submucosa thickness, muscular layer thickness, serosa thickness, height of the epitheliocytes in the middle part of the villi, mitotic index of columnar epitheliocytes. The obtained data confirm the progredient course of changes characteristic of diabetic enteropathy, which gradually worsen after 7, 14 and 21 days of the experiment.

Key words: skin burn injury, streptozotocin-induced diabetes mellitus, duodenal wall, morphometric assessment.

The work is a fragment of the research project "Morphological features and changes of the digestive system organs in experimental skin burn injury", state registration No. 0119U101618.

Burn injuries and related complications are becoming more common in the current conditions of widespread use of thermal energy in production and everyday life [10, 11]. Pathogenesis of diabetes mellitus and related pathology of the digestive system are also a topical issue for present day medicine [7]. It should be noted that in the global breakdown of general injuries [12, 15] skin burn injuries accompanied by changes in the internal organs prevail [5, 1], and are the subject of current experimental studies [2, 3, 4, 6, 9]. In general, severe burns cause burn disease, with diabetic enteropathy being its component manifesting itself as intestinal dysfunction [6, 7]. However, the morphometric study of the structural features of the duodenal wall in skin burns in terms of its association with diabetes has not been the subject of special studies so far.

The purpose of the study was to perform morphometric assessment of structural changes in the duodenal wall of rats with skin burns in the conditions of experimental streptozotocin-induced diabetes mellitus.

Materials and methods. The study was performed on 84 laboratory white sexually mature male rats weighing 180-210 g. The control group was formed of 21 intact animals without signs of somatic pathology, experimental group I consisted of 21 rats with experimentally simulated skin burn injury, experimental group II - of 21 rats without skin burns with experimentally simulated diabetes mellitus, and experimental group III - of 21 rats with skin burns and experimentally simulated diabetes mellitus. The keeping of rats and all manipulations with them were carried out in full compliance with the recommendations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986); the provisions of the European Council