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INFLUENCE OF QUERCETIN ON BIOCHEMICAL AND MORPHOLOGICAL CHANGES IN RAT TESTES AFTER 30 DAYS LONG CENTRAL DEPRIVATION OF LUTEINIZING HORMONE

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Central blockade of luteinizing hormone synthesis by the injection of triptorelin acetate with parallel oral intake of quercetin on the 30th day of the experiment causes minor changes in the structure of the interstitial space of the rats' testes and is characterized by variability in the populations of interstitial endocrinocytes and macrophages, which in our opinion, affects the change in the parameters of spermatogenesis in the rats' testes. Quercetin protects tissue of rats' testes from oxidative damage induced by triptorelin injection on 30th day of the experiment by increasing the antioxidant protection and reducing reactive oxygen species formation. Quercetin increase the arginase-dependent arginine cleavage, which was inhibited by triptorelin injection.

Keywords: testes; interstitial endocrinocytes, macrophages, quercetin, NO synthase, iNOS, cNOS, L-arginine, superoxide dismutase, rats.

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ВПЛИВ КВЕРЦЕТИНУ НА БІОХІМІЧНІ ТА МОРФОЛОГІЧНІ ЗМІНИ В СІМ'ЯНИКАХ ЩУРІВ ПІСЛЯ 30-ДЕННОГО ЦЕНТРАЛЬНОГО ЗНИЖЕННЯ ЛЮТЕЇНІЗУЮЧОГО ГОРМОНУ

Центральна блокада синтезу лютеінізуючого гормону шляхом введення трепторелiна ацетату з паралельним введенням кверцитину досліджуваним тваринам на 30-й день експерименту викликає незначні зміни в структурі інтерстиціального простору сім'яників щурів і характеризується варіабельністю в популяціях інтерстиціальних ендокріноцітов і макрофагів, яка на наш погляд впливає на зміну параметрів сперматогенезу в сім'яниках щурів. Кверцетин захищає тканини яєчок щурів від окислювального пошкодження, спричиненого введенням трипторелiну, на 30-й день експерименту, збільшуючи антиоксидантний захист та зменшуючи утворення активних форм кисню. Введення кверцетину збільшує аргіназозалежне розщеплення аргініну, яке інгібується при введенні трипторелiну.

Ключові слова: сімяники, інтерстиціальні ендокріноціти, макрофаги, кверцитин, NO-синтаза, iNOS, cNOS, L-аргінін, супероксиддисмутаза, щури.

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.

The reproductive health of the population is an integral part of the demographic processes in society. Today the problems associated with its disorders go beyond medical and take on a social character. The reproductive function of humans and animals is the basis for the continuation of life on Earth, therefore any deviations in the reproductive sphere lead to serious, often global negative consequences. In recent decades, a sharp deterioration in semen analysis it is noted in men all over the world. According to the reproductive centers, the percentage of fertilization is decreasing, and cases of embryonic mortality are increasing [2]. During the period from 2015 to 2020, the increase in the absolute number of patients with prostate diseases in our country alone was 36.2 %. [10]. The number of infertile marriages has been on the rise. According to statistics, the proportion of the male factor is increasing.

Testosterone and follicle-stimulating hormone are two main and independent regulators of spermatogenesis, since the effects of each of them are realized at certain stages of the spermatogenic cycle [4]. The disorders in the hypothalamic-pituitary system are noted in rats under stress of various etiologies. This lead to a disruption in the production of releasing factors by neurosecretory cells of the hypothalamus, as a result the production of gonadotropic hormones by the anterior pituitary gland is inhibited [2]. A reducing of follicle-stimulating hormone in the blood serum of male rats was found under emotional pain stress, under zoosocial stress, under hypokinetic stress [3]. Similar results were obtained from studies about the influence of emotional stress and systematic physical activity on human sexual function. [2]. The authors found a decrease in the concentration of gonadotropic hormones and testosterone in men under emotional stress. This has been accompanied by changes in the main indicators of semen analysis [13].

The protective effects of the bioflavonoid quercetin were identified in an experimental model of cold adaptation. This makes it possible to use it for the correction of testicular dysfunction of various origins [14]. These facts highlight the necessity to clarify the causes of reproductive deviations, as well as study the mechanisms and search for preventive means.

Scientific literature provides limited information regarding the influence of prolonged deprivation of LH (luteinizing hormone) synthesis on production of NO and microscopic organization of rats' testes.

The purpose of the study was to establish the influence of quercetin on the microscopic organization of rat testes, production of nitric oxide and the intensity of oxidative stress in the rat testes on the 30th day of the experiment, during experimental central deprivation of testosterone synthesis, caused by the introduction of triptorelin acetate solution.

Materials and methods. The experiments were carried out on 20 sexually mature male white rats. Rats were divided into 2 groups with 10 animals in each group: the control group (I), the group with central deprivation of testosterone synthesis + quercetin (II). Animals from the group with central deprivation of testosterone synthesis were injected subcutaneously with triptorelin acetate at a dose of 0.3 mg of the active substance per kg [9] and quercetin 100 mg per kg body weight 3 times a week [8], while the control group was administered saline.

Experiment conducted for 30 days. Animals were kept in standard vivarium conditions of the Poltava State Medical University. 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 [7]. Nitrite concentration was measured with help of Griess reagent [7]. 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 [7].

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 [5]. 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 [5]. The concentration of free malondialdehyde (MDA) was determined by reaction with 1-methyl-2-phenylindole resulting in formation of specific colored substance [5].

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. On the 30th day of the experiment, convoluted seminiferous tubules (the parenchyma) were clearly determined in the structure of the testes. They had two types of cells lying on the basement membrane. Interstitial tissue with vessels of the microvasculature and a connective tissue component was located between the convoluted tubules, which does not contradict the general idea of the testes structure. So, when we calculated the area of the interstitial space to the total area of tissue, it was 17.4 %, in the control 16.4 %. The significant number of arterioles and numerous arterio-arterial anastomoses were observed in the interstitium of the rats' testes. In addition to the vessels of the microvasculature, each "triangle" of interstitial tissue contained interstitial endocrinocytes, morphologically

unidentifiable at low microscope magnification. They determined well against the background of connective tissue with oxyphilic color of the cytoplasm and light basophilic round or oval shape nuclei. With immersion magnification of the microscope, it can be seen that part of the interstitial endocrinocytes had an irregular polygonal shape, while most of the endocrinocytes were round or oval in shape. (fig. 1).

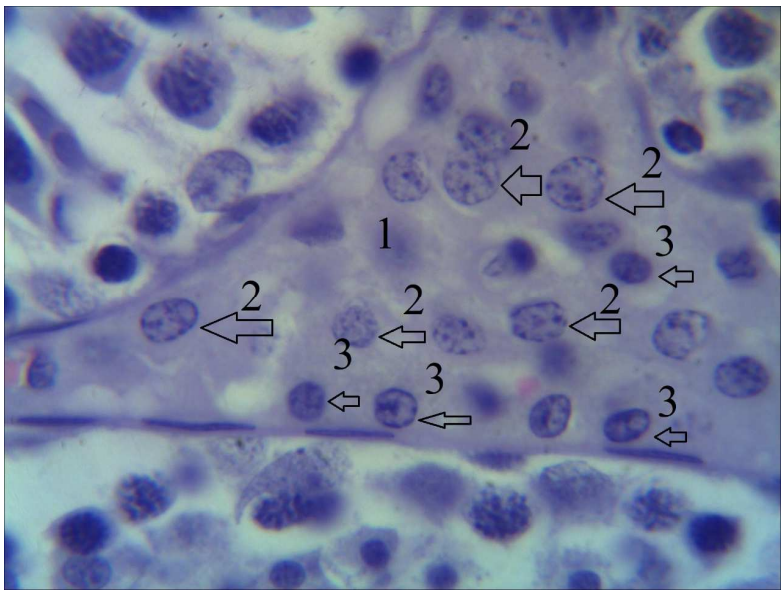

Fig. 1. Interstitial space of experimental rat on the 30th day (group II). Microimage. H&E stain: Lens: 40: Ocular lens: 15. 1. Interstitial space. 2. Interstitial endocrinocytes – large. 3.Interstitial endocrinocytes – small.

When we calculated the relative number of endocrinocytes of the experimental group (II) in the "triangle" of interstitium between the convoluted seminiferous tubules, the number of cells was 6.2±0.45, the average diameter of the cytoplasm of interstitial endocrinocytes was 7.9±0.04 μm, and the diameter of the nucleus was 5.9±0.02 μm. The morphological study has established that there were two different morphofunctional cell types in the population of interstitial endocrinocytes in the experimental group of rats (II), with a slight predominance of active forms above inactive by 8 %.

After mathematical studies, we identified three groups of interstitial endocrinocytes of the rats' testes, both in the experimental group (II) and in the control group (I). There are small, medium and large interstitial endocrinocytes, which, in our opinion, belong to different morphofunctional types. Small endocrinocytes are less active concerning steroidogenesis and represent involution forms. Cells of medium and large sizes actively produce steroid hormones, which can be seen on the typification of cells depending on their location to the convoluted seminiferous tubules in different phases of spermatogenesis. It was found that the percentage of the number of functionally active interstitial endocrinocytes, which include cells of large and medium size, in the control group of animals (I) was 65 % and in the experimental group (II) – 54 %.

The results of the quantitative analysis have confirmed morphological data that steroid-active endocrinocytes predominate in the intact rat testis, while in the experimental group (II), this percentage drops to 11 %.

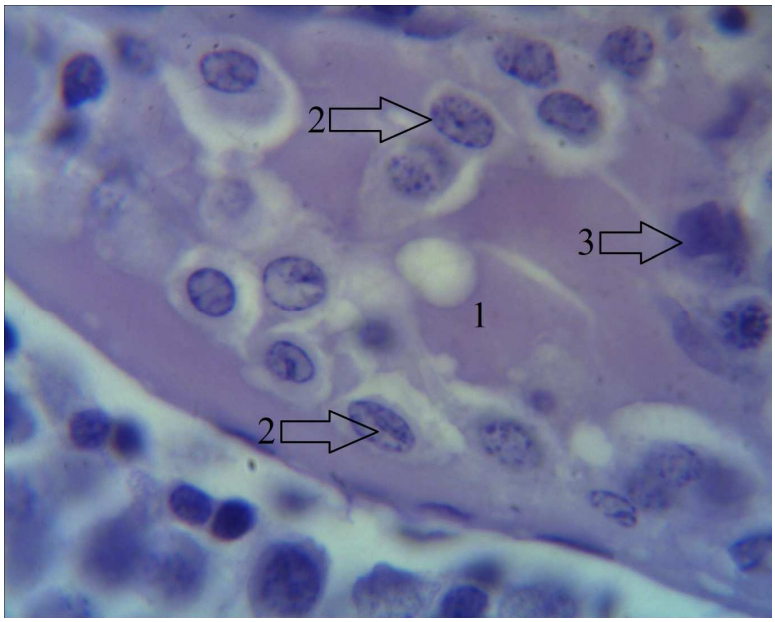

Fig. 2. Interstitial space of experimental rat testes (group II) on the 30th day. Microimage. H&E stain: Lens: 40: Ocular lens: 15. 1. Interstitial space. 2. Interstitial endocrinocytes. 3. Interstitial macrophages.

Testicular macrophages are visible in the interstitium of the testes. Two populations of cells can be found according to the type of localization in the connective tissue. Thus, parietal macrophages were identified near the basement membrane of the convoluted tubule, and interstitial were closer to the vessel wall. The number of macrophages was 4–7 in the structure of the interstitium "triangle". Macrophages were also detected in the lumen of the vessels in the testes interstitial space (fig. 2).

The ultrastructure of interstitial endocrine cells of the control group of animals under electron microscope was described in detail and presented in our protocols [15],

and there are no significant differences in comparison with ultrastructure of human interstitial endocrinocytes. Dark and light endocrinocytes are found in the testes of intact rats under electron microscope. Finely dispersed chromatin predominates in the nuclei of light and dark endocrinocytes, there are large nucleoli. The cytoplasm is characterized by well-developed vesicular type of smooth endoplasmic reticulum. The granular endoplasmic reticulum is poorly developed; it is represented by single flattened cisterns. Mitochondria in the cytoplasm of interstitial endocrinocytes are numerous and have a varied shape. The mitochondria of dark cells are characterized by an electron-dense matrix and lamellar cristae. Vesicular enlargement and matrix enlightenment are noted in the light cells between the lamellar cristae. The close contact of mitochondria with elements of the smooth endoplasmic reticulum is clearly visible in light cells. The number of lysosomes in the cytoplasm of cells is moderate, lipid droplets are not detected in light and dark endocrinocytes [15]. We did not find significant visible changes in the ultrastructural organization of interstitial endocrinocytes in the experimental group (II). There was a slight swelling of the mitochondrial cristae and a slight separation in the structure of the smooth endoplasmic reticulum. The nucleus, nucleolus and chromatin are unchanged.

These criteria have been choosen due to the fact that, according to a number of authors [15], the quality of morphological changes in the functional activity of endocrinocytes depends on the shape of cells and their nuclei, nuclear-cytoplasmic ratio and the state of nuclear chromatin, which correlates with the activity of sex steroid synthesis.

Triptorelin injection on the 30th day decreased SOD activity by 71.12 % (table 1).

Table 1.

Oxidative stress markers in rat testes after 30 days of central luteinizing hormone synthesis deprivation + quercetin (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
Central deprivation of testosterone synthesis	0.54±0.20*	89.0±1.0*	1,80±0.04*	15.60±0.11*	17.81±0.28*	23.82±0.39*
Central deprivation of testosterone synthesis + Quercetin	2.49±0.16**	251.0±2.0**	1.17±0.01**	10.59±0.17**	12.16±0.29**	19.79±0.28**

Note: * - indicates that the difference is statistically significant when compared with control group ($p<0.05$) ** - indicates that the difference is statistically significant when compared with group of central deprivation of testosterone synthesis ($p<0.05$).

At the same time catalase activity dropped by 51.1 %. Basic SAR production increased 6.92 times. Production of superoxide from microsomal and mitochondrial ETC increased 86.49 % and 98.98 % respectively. Concentration of free MDA raised by 258.73 %. Therefore, we can estimate that in rat testes during prolonged triptorelin acetate administration oxidative stress has developed. This oxidative damage is characterized by decrease in activity of antioxidant enzymes and increase in production of such oxidants as superoxide anion radical.

Administration of quercetin on the background of triptorelin acetate injection lowered basic SAR production by 35.0 %. Production of SAR from microsomal and mitochondrial ETC decreased by 31.72 % and 32.11 % respectively. SOD and catalase activity increased by 361.11 % and 182.02 % respectively. Concentration of free MDA decreased by 16.92 %. Quercetin partially prevented development of oxidative damage to the rat testes due to activation of antioxidant protection and decrease in production of SAR. However, as can be seen from the rather low decrease in MDA concentration on the background of several-fold increase in activity of antioxidant enzymes, quercetin has only partial effect on redox balance in rat testes. Studies of other parts of antioxidant system or reactive oxygen species are necessary to evaluate the exact mechanism of this effect.

Injection of rats with triptorelin acetate did not influence the general activity of NOS on the 30th day of the experiment (table 2).

Table 2

Nitric oxide cycle function after 30 days of central luteinizing hormone synthesis deprivation + quercetin (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
Central deprivation of testosterone synthesis	0.60±0.03	0.49±0.03*	0.11±0.02*	0.32±0.02*	3.83±0.21
Central deprivation of testosterone synthesis + Quercetin	0.26±0.02**	0.22±0.02**	0.036±0.0002**	1.04±0.07**	5.90±0.16**

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

However, it changed the sources of production of nitric oxide. Activity of cNOS dropped by 73.17 %, while iNOS activity increased by 296.72 %. This shift in the main oxidative producer of nitric oxide may explain the increase in microsomal SAR production. Since drop in cNOS activity may result in its uncoupling with the substrate leading to SAR production from microsomal ETC. Also, simultaneous overproduction of both SAR and nitric oxide can result in peroxynitrite formation, which is even more potent oxidizing agent than SAR and nitric oxide. Arginase activity decreased by 87.10 %. Administration of experimental rats with quercetin decreased gNOS activity by 56.67 %. Activity of iNOS and cNOS also decreased, by 55.1 % and 67.27 % respectively. Arginase activity increased by 225 %. Nitrite concentration increased by 54.04 %. Such decrease in nitric oxide production may have a controversial effect on tissue of rat testes. Decrease in iNOS activity may result in prevention of peroxynitrite formation (together with decrease in SAR production), but decrease in cNOS activity may lead to more severe uncoupling of cNOS. Such changes in cNOS activity may be explained by increase in arginase activity and intensification of these enzymes' competition for L-arginine. We can suspect development of L-arginine deficiency caused by triptorelin, but this statement demands further research. Increase in nitrite concentration in this case can be explained by intensification of nitrate and nitrite reduction as means of NOS-independent nitric oxide synthesis.

However, as we have seen from the results of our experiment, production of nitric oxide has no changed. Therefore, there must be another reason for decrease in arginase activity induced by prolonged injection of triptorelin. Decrease in arginase activity may result from lowered level of testosterone, since it acts as stimulator of arginases in testes [6].

There is a close functional relationship between Leydig cells and macrophages [12]. Under physiological conditions macrophages provide the production of growth factors and differentiation of interstitial endocrinocytes. Activated macrophages suppress the functional activity of endocrinocytes, releasing nitric oxide, reactive oxygen species, and a number of cytokines [13].

Therefore, our research of interstitial endocrinocytes and macrophages in the rats' testes has shown the heterogeneity of populations, variability of the structural and functional parameters of endocrinocytes and macrophages. The results are consistent with the literature, which set out the basic principles and patterns of the cell population organization in the interstitial space of the testes [12, 13].

Concentration of nitrites in rat testes did not change. Arginase is necessary for tissue repair and proliferation because it leads to formation of L-ornithine, which is transformed by ornithine decarboxylase to putrescine. Putrescine is than converted to spermidine and spermine. These polyamines necessary for stimulation of mitotic and meiotic cycles. Arginase activity can be regulated by nitric oxide concentration [11].

Conclusion

Central blockade of LH synthesis by the injection of triptorelin acetate with parallel oral intake of quercetin on the 30th day of the experiment causes minor changes in the structure of the interstitial space

of the rats' testes and is characterized by variability in the populations of interstitial endocrinocytes and macrophages, which in our opinion, affects the change in the parameters of spermatogenesis in the rats' testes.

Quercetin protects tissue of rats' testes from oxidative damage induced by triptorelin injection on 30th day of the experiment by increasing the antioxidant protection and reducing reactive oxygen species formation. Quercetin increases the arginase-dependent arginine cleavage, which was inhibited by triptorelin injection.

The results provide a theoretical basis for the development of correction methods of generative and endocrine testicular dysfunctions under extreme influences on the body. Data on the functional morphology of the testes at the stages of adaptation to changes in the endocrine function of the testes expand the understanding of the causes of spermatogenesis disorders and its regulation. The data can be used in research work and teaching at the departments of medical universities and biological faculties of universities.

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