

**THE EFFECT OF QUERCETIN ON THE STRUCTURAL ORGANISATION
OF THE TESTICULAR INTERSTITIAL SPACE IN THE DYSHORMONAL STATE INDUCED
BY TRYPTORELIN AT DAY 270 IN THE EXPERIMENT**

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The objective of this study was to assess the morphological changes in the interstitial space of rat testes, determine the sources of nitric oxide production, and evaluate the intensity of oxidative stress in the rat testes during long-term experimental central blocking of the synthesis of luteinising hormone by tryptorelin, with the addition of quercetin to the diet. The experimental group of ten animals received a subcutaneous injection of tryptorelin at a dose of 0.3 mg/kg of active ingredient for 270 days, with simultaneous addition of quercetin to the diet, to model central blocking of luteinising hormone synthesis. Morphologically, the number of macrophages on day 270 was increased at the expense of perivascular macrophages. During the biochemical study of the testicular interstitium, we found that the main production of superoxide anion radical on the 270th day of observation was slightly reduced. The addition of quercetin to the diet reduces changes in the structure of the interstitial space of the testes and impaired nitric oxide production by constitutive isoforms of NO synthase induced by tryptorelin on the 270th day of observation.

Key words: testes, interstitial endocrinocytes, macrophages, NO synthase, iNOS, L-arginine, tryptorelin, quercetin, fibrosis.

Connection of the publication with planned research works.

The study is a fragment of the research project “Experimental and morphological study of the effect of dipherelin, ethanol and 1% methacrylic acid, cryopreserved placental transplants on the morphological and functional state of a number of internal organs”, state registration number 0119U102925.

Introduction.

In 1976, Stanlid reported that some phenolic compounds, including quercetin-3-O-rhamnoside (Q3R, also known as quercetin) and dihydroquercetin, may be involved in polar auxin transport in plants. Quercetin is thought to act as a natural phytoalexin involved in the transport of polar auxin, according to Bruun et al. (1992). On the other hand, wounding during rupture can trigger the production of quercetin-3-D-galactoside (known as hyperoside, Q3DG), which indirectly induces a rooting response (Osterc et al., 2007a). Zohra et al. (2011), [1], working with grape cuttings, observed that quercetin synthesis coincided with rhizogenesis and suggested a direct role of this compound in grapes. Quercetin (rutin aglycone) and rutin are two flavonols that have been extensively studied in several models of intestinal inflammation. There are a large number of clinical trials evaluating the effects of quercetin on diseases, mainly as a product to reduce uric acid levels, improve neuromuscular function and neuroprotection, reduce the effects of inflammatory factors in myocardial infarction, chronic obstructive pulmonary disease, and Covid-19. On the other hand, quercetin has several effects in experimental models of intestinal inflammation and in vitro studies. Quercetin acts as an important antioxidant compound that reduces MPO activity, reduces colonic oxidative stress in acetic acid, trinitrobenzene sulfonic acid models, and counteracts inflammation-induced glutathione depletion. In addition, quercetin acted by modulating the intestinal microbiota, restoring the integrity of the colon epithelium and creating other protective effects [2]. Similar effects have also been observed with rutin in several preclinical studies.

Tryptorelin is a synthetic analogue of the neurohormone gonadoliberin [3]. It suppresses receptor expression in the pituitary gland but does not affect the functioning of the pituitary-testicular complex as a whole. Considerable attention has been given to the development of a new slow-release injectable formulation due to its many advantages, including localised and specific action, extended delivery time, reduced doses, reduced side effects, and improved patient comfort and compliance (Chenite et al., 2000). Sustained-release injectable forms are primarily designed as microparticles, implants, or gel systems. Microparticles are produced using complex methods and are subject to several limitations, such as low drug loads, difficulty in controlling particle size due to aggregation, and difficulty in recovering them to their original size. The efficiency of active ingredient loading methods is limited, and high loading capacity is usually unattainable. The main limitation of implants is the need for surgical intervention to remove the system, which increases the cost and risks. Because of these limitations and disadvantages, many researchers have proposed the use of in situ gel formulations as substitutes (Packhaeuser et al., 2004). Hormonal therapy for cancer involves medical or surgical castration, which leads to a decrease in sex hormone levels to slow or stabilise tumour growth. Hormone therapy is the main treatment for advanced and metastatic prostate and breast cancer.

The aim of the study.

To assess morphological changes in the interstitial space of rat testes, determine sources of nitric oxide production, and evaluate oxidative stress intensity in rats on day 270 of the experiment during central blocking of luteinising hormone synthesis by tryptorelin.

Object and research methods.

The experiments were conducted on 15 sexually mature male white rats. The animals were divided into 2 groups. The first group of 5 animals (control) received a subcutaneous injection of sodium chloride 0.9% throughout the experimental period. The second group of 10 animals (experimental), in which central blocking

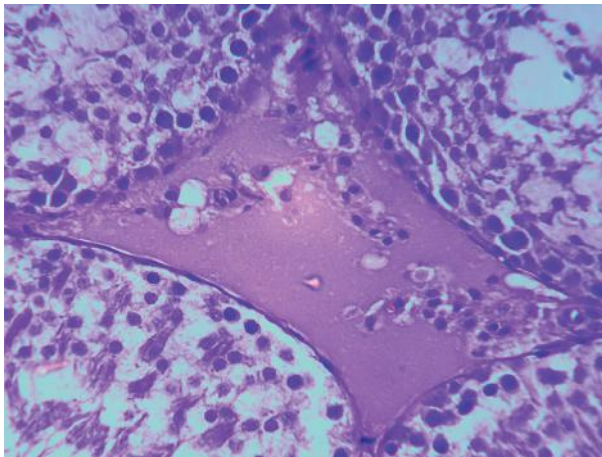


Figure 1 – Fibrosis of the interstitial space of the testes. Convoluted seminiferous tubules on day 270 of the experiment in animals of the experimental group (with central blocking of luteinising hormone synthesis, with the addition of quercetin). Semi-thin section. Hematoxylin-eosin stain. Magnification: 400.

of luteinising hormone synthesis was modelled [4], received a subcutaneous injection of tryptorelin at a dose of 0.3 mg/kg of active ingredient for 270 days, with simultaneous addition of quercetin to the diet, which was administered orally via a gastric tube three times a day. All manipulations involving laboratory animals were carried out in strict accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986). The animals were euthanized using an overdose of ether anesthesia. Small pieces of testes were fixed according to the generally accepted method and placed in paraffin blocks. From these blocks, 4 μ m thick sections were made and stained with hematoxylin and eosin [5],

The study was approved and confirmed by the Bioethics Committee of Poltava State Medical University (Protocol No. 195 – 24.06.2021).

A comprehensive study of histological specimens was performed using a light microscope BIOREX – 3 # 5605. Quantitative cell counting was performed in the fields of view, by visual assessment, using a light microscope with a digital microfilter and software adapted for these studies. Microphotography was conducted with a DCM 900 digital microphotographic attachment, using software specific to these investigations. All biochemical studies were performed in 10% testicular tissue homogenate using an Ulab 101 spectrophotometer. Total nitric oxide production was assessed by total NO synthase (gNOS) activity. The activity of gNOS was judged by the increase in nitrite (NO_2^-) after incubation in Tris-buffered saline (pH 7.4) containing the reaction substrate and electron donor (NADPH-reduced). The nitrite concentration was determined using the Griess reagent at a wavelength of 540 nm. The levels of inducible (iNOS) and constitutive (cNOS) isoforms were determined using the selective iNOS inhibitor aminoguanidine hydrochloride (Sigma). The baseline production of superoxide anion radical ($\text{O}_2^{\cdot-}$) was measured by the increase in the amount of formazan formed in the reaction of $\text{O}_2^{\cdot-}$ with nitroblue tetrazolium after incubation in a buffered solution (pH=7.4) containing sodium hydroxide [6]. The concentration of formazan was determined using spectrophotometry at a wavelength of 540 nm. The study results were statistically processed using

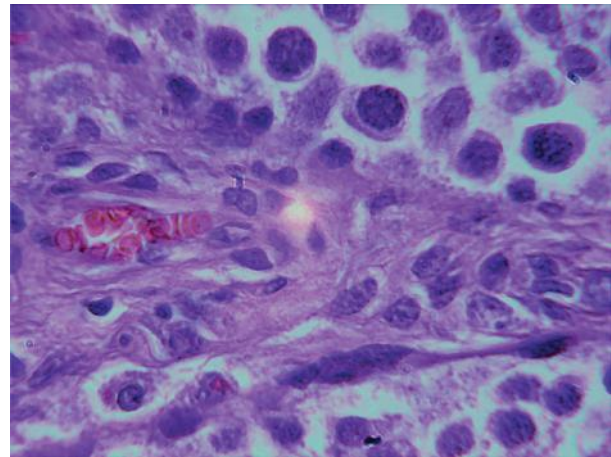


Figure 2 – Interstitial space of the testes on day 270 of the experiment in animals of the experimental group (with central blocking of luteinising hormone synthesis with the addition of quercetin). Semi-thin section. Hematoxylin-eosin stain. Magnification: 1000.

Microsoft Office's Excel program and the Real Statistics 2019 extension.

Research results.

During the 270th day of observation, we observed a reduction in size of the testicular parenchyma, which was represented by visually altered convoluted tubules in the histological preparations of semi-thin sections. We also noted a significant increase in the volume of connective tissue (**fig. 1**) between the convoluted tubules when studying the structure of the interstitial space, compared to previous stages of the experiment. The interstitium has increased due to active collagen production. The total number of blood vessels has increased due to arterioles. Vessel walls are thickened and sometimes swollen. Stasis in the venous vessels is present, and 'sludge' is detected in the capillaries (**fig. 2**).

Upon studying the cellular composition of the interstitial space, an increased number of fibroblastic cells at different stages of differentiation were found, embedded between collagen fibres. Meanwhile, the number of interstitial endocrinocytes was observed to be reduced. The study of these cells revealed a general quantitative decrease in their population compared to the previous terms of the experiment. During the morphological examination of the population of these cells, we identified three groups of cells based on the analysis of their size, nucleus shape, cytoplasmic volume and the number of inclusions in it. The first type is cells with a small nucleus and cytoplasmic volume, single inclusions. The second type is rounded nuclei with increased size and a significant volume of cytoplasm, and a sufficient number of inclusions. The third group of cells has ellipsoidal nuclei, small cytoplasm, and a small number of granules (**fig. 2**). During the visual examination, it was observed that the population consisted mainly of cells of the third type, with no cells of the first type detected.

When studying the quantitative and qualitative composition of macrophages, it was found that their number was increased due to perivascular macrophages, single intravascular macrophages were detected, and peritubular macrophages were absent in the fields of view. The seminiferous tubules displayed abnormalities at various stages of proliferation and differentiation during spermatogenesis.

Table – Markers of oxidative stress in the testes of rats during 270-day central blocking of luteinising hormone synthesis with the addition of quercetin (M±m)

Period	Parameters										
	SOD activity, st. units	Catalase activity, nkat/g	Basic production of O ₂ –, nmol/s	O ₂ production – mitochondrial ETC, nmol/s per g	O ₂ production of microsomal ETC, nmol/s per g	Total NOS 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	MDA concentration, μmol/g	Nitrite concentration, nmol/l
Control	1,87±0,11	182±17,0	0,26±0,01	7,84±0,13	9,55±0,19	0,54±0,04	0,13±0,02	0,41±0,03	2,48±0,05	6,64±1,44	3,83±0,25
9 mon.	2,56±0,02	271±4,0*	0,82±0,01*	8,76±0,05*	8,07±0,07*	0,23±0,01*	0,17±0,04	0,05±0,001*	1,97±0,01*	7,51±0,09*	9,18±0,26*

Notes: * – difference is statistically significant when compared to the control (p<0.05).

In the biochemical study of the testicular interstitium, we found that the main production of superoxide anion radical on the 270th day of observation was slightly reduced compared to the control group of animals (**table**). The production of SAR from mitochondrial and microsomal ETCs was increased and amounted to 8.76±0.05 nmol/s per g. The activity of SOD increased 1.37-fold, and catalase – 1.49-fold. MDA was statistically significantly increased by 1.31 times, which was 7.51±0.09 μmol/g, respectively.

On day 270 of the experiment, a 2.5-fold decrease in gNOS activity was observed (**table**). There were no statistically significant changes in the activity of iNOS in rat testes, which was 0.17±0.04 μmol/min per g protein. The activity of cNOS isoforms decreased 8-fold (0.41±0.03\0.05±0.001). Arginase activity decreased by 28.2%. Nitrite concentration increased more than three-fold to 9.18±0.26 nmol/l.

Discussion of the research results.

Long-term central deprivation of luteinising hormone synthesis leads to the development of oxidative stress in the testes [7]. Nitric oxide production during central deprivation of LH synthesis undergoes complex changes: from an initial decrease in NO synthase-dependent nitric oxide production to hyperproduction of nitric oxide from NO synthases [8]. Reduced cNOS activity may contribute to the endothelial dysfunction observed in the microvasculature on day 270 of the experimental model with tryptorelin and quercetin supplementation. Endothelial dysfunction leads to inadequate blood supply to the testes and the development of hypoxia. Since spermatogenesis requires multiple cell divisions, this process is highly energy dependent. At this stage of the experiment, the absence of nitric oxide derived from cNOS leads to hypoxia, which in turn increases the signalling of the hypoxia-inducible factor [8]. In 2002, at the second international conference of the Society for the Study of Nitric Oxide, it was reported that quercetin restored the activity of flavin-containing monooxygenase, which is normally inhibited by nitric oxide in rats. This study suggests that foods rich in quercetin, such as onions and tomatoes, may be beneficial for people suffering from hepatitis, cirrhosis or liver cancer with increased nitric oxide production.

The immunomodulatory properties of quercetin are also combined with its anti-inflammatory effects, as evidenced by the modulation of several pro-inflammatory and anti-inflammatory cytokines, including IL-1β, IL-33, IL-10, IL-17, TNF-α, IL-6. HIF signalling increases the reactivity of reactive oxygen species formation, which may be the exact mechanism of the increased SAR production observed in our study [9]. Increased HIF signalling may also explain the atypical precapillary spiralling ob-

served in our study, as the HIF pathway also increases the production of vascular endothelial growth factor (VEGF) [9]. Finally, testicular hypoxia leads to fibrosis through the activation of transforming growth factor-β (TGF-β) transcription [8]. Hypoxia-induced testicular fibrosis has also been demonstrated in studies by Abdelhameed RFA et al [10].

In the early stages of the experiment, we observed an acute development of oxidative damage in the testicular tissue and a significant increase in nitric oxide production [11]. At a later stage of the experiment, there was evidence of fibrous changes and a decrease in nitric oxide production [12]. This also suggests that testosterone deficiency may lead to a change in macrophage polarisation [2, 13] towards the prevalence of the M1 (pro-inflammatory) phenotype, as the iNOS/arginase ratio was high [14, 15]. On day 270 of central deprivation of testosterone synthesis, the iNOS/arginase ratio was low, indicating the predominance of M2 macrophage polarisation.

The development of degenerative changes in the 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 [16]. This, in turn, leads to changes in their metabolic processes, permeability of the haematotesticular barrier, and to a persistent impairment of the quality of spermatogenesis. In our opinion, this system includes macrophages, sustentocytes and interstitial endocrinocytes. The disruption of hypothalamic stimulation for testosterone synthesis leads to a change in macrophage polarisation to the M1 phenotype, with subsequent damage to testicular tissue and its replacement by fibrous tissue. Further studies are needed to evaluate the exact changes in the interaction between testicular macrophages, sustentocytes and interstitial endocrinocytes.

Conclusions.

Quercetin supplementation reduces morphological changes in the structure of the interstitial space of the testis and reduces the impairment of nitric oxide production by constitutive isoforms of NO synthase induced by tryptorelin on day 270.

Prospects for further research.

In our opinion, it is promising to identify the pathways of polarisation of M1 and M2 macrophages and their further influence on the tissue stroma. As the change in polarisation can potentially lead to fibrosis, the possibility of a corrective effect of quercetin should be evaluated in parallel.

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ВПЛИВ КВЕРЦЕТИНУ НА СТРУКТУРНУ ОРГАНІЗАЦІЮ ІНТЕРСТИЦІЙНОГО ПРОСТОРУ СІМ'ЯНИКІВ ПРИ ДИЗ-ГОРМОНАЛЬНОМУ СТАНІ ВИКЛИКАНОМУ ТРИПТОРЕЛІНОМ НА 270-ТУ ДОБУ В ЕКСПЕРИМЕНТІ

Стецук Є. В., Шепітько В. І., Запорожець Т. М., Проніна О. М., Борута Н. В.

Резюме. Кверцетин – біофлавоноїд, який міститься в багатьох рослинах. Це гіркий блідо-жовтий кристалічний глікозид, який при гідролізі утворює кверцетин і рамнозу. Метою даного дослідження було оцінити морфологічні зміни інтерстиційного простору сім'яників щурів, визначити джерела продукції оксиду азоту та інтенсивність оксидативного стресу в сім'яниках щурів під час довготривалого експериментального центрального блокування синтезу лютеїнізуючого гормону триптореліном, з додаванням у раціон кверцетину. Досліди були проведені на 15 статевозрілих білих щурах – самцях. Тварини були поділені на 2 групи. Перша група з 5 тварин (контрольна) отримувала підшкірно ін'єкцію натрію хлориду 0,9% протягом усього експериментального періоду. Друга група з 10 тварин (експериментальна), в якій моделювалось центральне блокування синтезу лютеїнізуючого гормону, одержувала підшкірно ін'єкцію триптореліну в дозі 0,3 мг/кг діючої речовини протягом 270 діб з одночасним додаванням у раціон харчування кверцетину. Морфологічне кількісне макрофагів на 270-ту добу збільшена за рахунок периваскулярних, визначалися поодинокі інтраваскулярні клітини, перитубулярні – відсутні в полях зору. Звивисті сім'яні каналці перебували на різних стадіях сперматогенезу, у більшості випадків відмічалися порушення на різних етапах проліферації та диференціації. При біохімічному дослідженні інтерстицію сім'яників нами встановлено, що основна продукція супероксидного аніон-радикала на 270-ту добу спостереження становила $1,1 \pm 0,05$ нмоль/с на г. Була незначно знижена, порівняно з контрольною групою тварин. Продукція SAR із мітохондріальних і мікросомальних ЕТС була підвищена і становила $8,76 \pm 0,05$ нмоль/с на г. Активність СОД збільшилась в 1,37 рази, а каталази – у 1,49 рази. МДА статистично достовірно збільшена в 1,31, що становило $7,51 \pm 0,09$ мкмоль/г, відповідно. Виявлено зниження активності gNOS на в 2,5 рази. Не було визначено статистично значущих змін активності iNOS в яєчках щурів, яка становила $0,17 \pm 0,04$ мкмоль/хв на г білка. Активність ізоформ cNOS знизилась у 8 разів – $(0,41 \pm 0,03) \setminus (0,05 \pm 0,001)$. Активність аргінази зменшилась на 28,2%. Концентрація нітритів збільшилась більше ніж утричі – $9,18 \pm 0,26$ нмоль/л. Додавання кверцетину до раціону зменшує зміни в структурі інтерстиційного простору сім'яників та порушення продукції оксиду азоту конститутивними ізоформами NO-синтази викликаного триптореліном на 270-ту добу спостереження.

Ключові слова: сім'яники, інтерстиційні ендокриноцити, макрофаги, NO-синтаза, iNOS, L-аргінін, трипторелін, кверцетин, фіброз.

THE EFFECT OF QUERCETIN ON THE STRUCTURAL ORGANISATION OF THE TESTICULAR INTERSTITIAL SPACE IN THE DYSHORMONAL STATE INDUCED BY TRYPTORELIN AT DAY 270 IN THE EXPERIMENT

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Abstract. Quercetin is a bioflavonoid that is commonly found in various plants. Quercetin-3-1-rhamnoside is a bitter, pale yellow crystalline glycoside. Upon hydrolysis, it is split into quercetin and rhamnose. The objective of this study was to assess the morphological changes in the interstitial space of rat testes, determine the sources of nitric oxide production, and evaluate the intensity of oxidative stress in the rat testes during long-term experimental central blocking of the synthesis of luteinising hormone by tryptorelin, with the addition of quercetin to the diet. Fifteen sexually mature male white rats were used in the experiments, which were divided into two groups. The first group, consisting of five animals (control), received a subcutaneous injection of sodium chloride 0.9% throughout the experimental period. The experimental group of ten animals received a subcutaneous injection of tryptorelin at a dose of 0.3 mg/kg of active ingredient for 270 days, with simultaneous addition of quercetin to the diet, to model central blocking of luteinising hormone synthesis. Morphologically, the number of macrophages on day 270 was increased at the expense of perivascular macrophages, single intravascular cells were detected, and peritubular cells were absent in the fields of view. The convoluted seminiferous tubules were at different stages of spermatogenesis, in most cases there were abnormalities at different stages of proliferation and differentiation. During the biochemical study of the testicular interstitium, we found that the main production of superoxide anion radical on the 270th day of observation was slightly reduced compared to the control group of animals. The production of SAR from mitochondrial and microsomal ETCs was increased and amounted to 8.76 ± 0.05 nmol/s per g. The activity of SOD increased 1.37 times, and catalase – 1.49 times. MDA was statistically significantly increased by 1.31, which was 7.51 ± 0.09 μ mol/g, respectively. A 2.5-fold decrease in gNOS activity was detected. There were no statistically significant changes in the activity of iNOS in the testes of rats, which was 0.17 ± 0.04 μ mol/min per g of protein. The activity of cNOS isoforms decreased 8-fold (0.41 ± 0.03 \ 0.05 ± 0.001). Arginase activity decreased by 28.2 %. The concentration of nitrite increased more than threefold to 9.18 ± 0.26 nmol/l. The addition of quercetin to the diet reduces changes in the structure of the interstitial space of the testes and impaired nitric oxide production by constitutive isoforms of NO synthase induced by tryptorelin on the 270th day of observation.

Key words: testes, interstitial endocrinocytes, macrophages, NO synthase, iNOS, L-arginine, tryptorelin, quercetin, fibrosis.

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