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DYNAMICS OF CD68 RECEPTOR EXPRESSION IN MACROPHAGES OF THE INTERSTITIAL SPACE OF THE RAT TESTIS UNDER TRIPTORELIN PROLONGED ADMINISTRATION

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Testosterone, the production of which is stimulated by the release of luteinizing hormone (LH) has a remarkable anti-inflammatory and immunomodulatory effect, and in conditions of testosterone deficiency tissue damage can occur due to excessive differentiation of macrophages into a proinflammatory M1 phenotype. The aim of this study was to determine the spread of CD68 receptors as the marker of inflammation on the cells in the interstitial space and testicular vessels under LH synthesis blockade with tryptorelin. Sexually mature white male rats were randomly divided into control (10 animals) and experimental (25 animals) groups. Animals in the experimental group were injected with a tryptorelin acetate solution (0.3 mg/kg). Immunochemical analysis of CD68+ expression was estimated at Olympus FV10i-LIV laser scanning confocal microscope using fluorescent labeling dye. It was shown that luteinizing hormone deprivation led to an increase in the distribution of the CD68 receptor in the interstitial space and in the testicular vessels from day 30 to 180 of the experiment, associated with the increase of inducible NO synthase activity in testis tissue.

K e y w o r d s: CD68, testosterone, luteinizing hormone, tryptorelin, testis, macrophage M1 phenotype.

Luteinizing hormone (LH) is a gonadotropic peptide hormone of the anterior pituitary gland that stimulates the secretion of sex hormones in both women and men. In turn, LH is a central regulator that controls the production of the male sex hormone testosterone through the pituitary-testicular system by stimulating Leydig cells and impacts the growth and development of testicular cells and tissues [1].

In conditions of deficiency or complete deprivation of LH due to the administration of chemotherapy in oncological pathologies, the death of seminiferous tubule cells occurs as a result of apoptosis activation and stress of the spermatogenic cells endoplasmic reticulum as well as supporting sustentocytes [2, 3].

The rise in testosterone concentration under the influence of LH is caused by LH-stimulated proliferation of interstitial endocrinocytes (Leydig cells) [4]. Testosterone has a considerable impact on the functional state of macrophages in the body. It inhibits the macrophages polarization according to the proinflammatory phenotype (M1), which is manifested in a decrease in the production of such cytokines as interleukin 1β (IL-1 β) and interleukin 6 (IL-6) [5, 6].

On the other hand, testosterone promotes a change in macrophage polarization towards an anti-inflammatory (M2) phenotype, even when stimulated with bacterial lipopolysaccharide [7].

Thus, testosterone has a pronounced anti-inflammatory and immunomodulatory effect, and in conditions of its deficiency, tissue damage may develop because of excessive polarization of macrophages by M1 phenotype [8, 9].

The expression of the CD68 receptor on the surface of a macrophage indicates the polarization of this cell by the M1 phenotype [10, 11]. In our ear-

lier work, we have revealed that the expression of the CD68 receptor on testicular macrophages, under conditions of central deprivation of LH synthesis by tryptorelin for 180 days, resulted in hyperproduction of reactive oxygen and nitrogen species [12, 13]. Currently, the scientific literature provides limited data on the interrelation between the expression of the CD68 receptor on testicular macrophages and the activity of macrophage polarization marker enzymes in the case of a prolonged deprivation of luteinizing hormone synthesis in the experiment.

The aim of this study was to determine the spread of CD68 receptor expression on cells in the interstitial space and testicular vessels under conditions of central blockade of LH synthesis by tryptorelin acetate solution.

Materials and Methods

The study was performed on 35 sexually mature white male rats. The animals were randomly divided into 2 groups: control (10 animals) and experimental (25 animals). Animals in the experimental group were injected with a solution of tryptorelin acetate at the dosage of 0.3 mg of active ingredient per kg of animal weight to modulate the central deprivation of luteinizing hormone synthesis [14]. Animals from the experimental group were taken out of the observation on days 30, 90, 180, 270, and 365 by an overdose of ether anaesthesia.

Rats in the control group were administered a saline injection. Animals were held under 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 ether anaesthesia, the animals were decapitated and the prepared small pieces of testes were fixed in a 2.5% glutaraldehyde solution (pH=7.2-7.4). The material was postfixed in a 1% solution of osmium (IV) oxide, dehydrated in propylene oxide and embedded in epoxy resin. Ultrathin sections were prepared on an ultramicrotome, contrasted with a 1% aqueous solution of uranyl acetate and lead citrate according to the Reynolds method, and examined by electron microscopy [15].

Using standard methods, the material was embedded in paraffin blocks, sectioned at 4 μm thickness and stained with hematoxylin and eosin. Histological preparations were examined using a Biorex 3 light microscope with digital microfilter and software adapted for these studies (serial no. 5604).

To provide an immunohistochemical examination of testicular macrophages for CD68 receptors, we deparaffinized the sections after making paraffin blocks and then demasked the antigens. This procedure is designed to restore the original structure of the protein, which can be reconstructed using enzymes (trypsin) or in a microwave oven.

Protocol for processing in a microwave oven. 1. Demasking in PBS (phosphate buffer), pH 6.0, then for 7 min at 700W in a microwave oven, add the buffer solution. Next, 20 min at 350W in a microwave oven. 2. Leave in PBS, pH 6.0 for 15 min. 3. Remove the PBS, pH 6.0, add PBS, pH 7.4, and incubate 2 times for 5 min. 4. Then add the first antibody 1:150, conjugated to MAb 1435. Anti-Macrophages/Antibody, clone ED-1 (Chemicon) for 1 h. 5. Wash with PBS, pH 7.4, 2 times for 5 min. 6. Addition of the second antibody 1:100 Goat anti-Mouse (Murine) IgG (Heave & Light Chain), (Whole Molecule) Hilyte Flour 488 – 30 min in the dark. 7. Wash with PBS, pH 7.4, 2 times for 5 min. Embedding in glycerol with PBS 1:1 under glass.

Prior to the confocal examination, we stained the cell nuclei using a nuclear DNA complex with DRAQ5 (Chemicon). To each sample, 50 μl of DRAQ5 solution (5 μM, Abcam, ab108410) was added and incubated for 15 minutes in the dark. After incubation, the sections were washed twice with Hanks' solution.

For a more detailed study of CD68+ expression, an Olympus FV10i-LIV laser scanning confocal microscope, Olympus cell Sens Dimension software, Olympus 10x NA 0.4 objective, 2.0 confocal aperture, phase contrast and fluorescence detection were used.

Testes tissues were homogenized with tris-buffered solution (pH 7.4) to produce a 10% homogenate, which was used for further biochemical studies on an Ulab 101 spectrophotometer.

The activity of inducible NO synthase (iNOS) and arginase (Arg) was determined in 10% of the testicular homogenate [16]. The ratio of iNOS and Arg activities was also determined by the formula: iNOS activity (μmol/min per g of protein)/Arg activity (μmol/min per g of protein)×100%.

The quantitative index of CD68 receptor distribution in the interstitial space of the testes was calculated as follows: the number of cells with CD68 receptor detected and localized in the interstitial tissue was counted in 10 fields of view in each rat.

Statistical processing of the study results was performed using Microsoft Office Excel and its extension Real Statistics 2019. The non-parametric Mann-Whitney test was used to determine the statistical significance of between-group differences, which was considered statistically significant at $P < 0.05$.

Results

Our study of testicular microscopic specimens on the 30th day of observation revealed a fairly uniform distribution of macrophage CD68 receptor expression both in the interstitial space and within the intravascular space (Fig. 1).

The 90th day of observation was marked by corresponding changes in both the vascular and interstitial components of the testes with a predominance of intravascular macrophages. Interstitial macrophages were located singly or in groups of 1-3 in the field of view near the blood vessel, they were active, had large electron-dense nuclei with a prevalence of heterochromatin. The nuclei are mainly rounded, the karyoplasm is transparent, and the nucleolus is mostly single. The nuclear envelope is dense, two-layered, with pores, without stratification. In most cells, the nuclei are light. Inflammasomes were clearly visualised near the nucleus (Fig. 2).

The cytoplasm of the cells was small in size with a well-developed endoplasmic reticulum, which was formed by numerous branching tubes filled with fine-grained substance. The membranes were dense with a large number of ribosomes. Mitochondria were small, oval, sometimes round, with an osmophilic matrix and a low number of cristae (Fig. 2). In addition, a large number of lysosomes of different sizes and electron density were determined in the cytoplasm of the cells. Lysosomes had clear contours and were compact in size. Lipid content was detected in phagosomes. Externally, a small amount of pseudopodia and invaginations were observed on the cell surface.

On the 180th day of observation, an increase in the distribution of CD 68+ macrophage expression outside the vascular system was detected. The cells were mostly located in clusters of 3-4 in the field of view. Cell nuclei were enlarged, dark, polymorphic, with a large number of invaginations. The karyolemma in some cases did not have a clear contour, with elements of stratification. The cytoplasm was enlarged due to various inclusions that differed in size and density. A large number of lipid granules

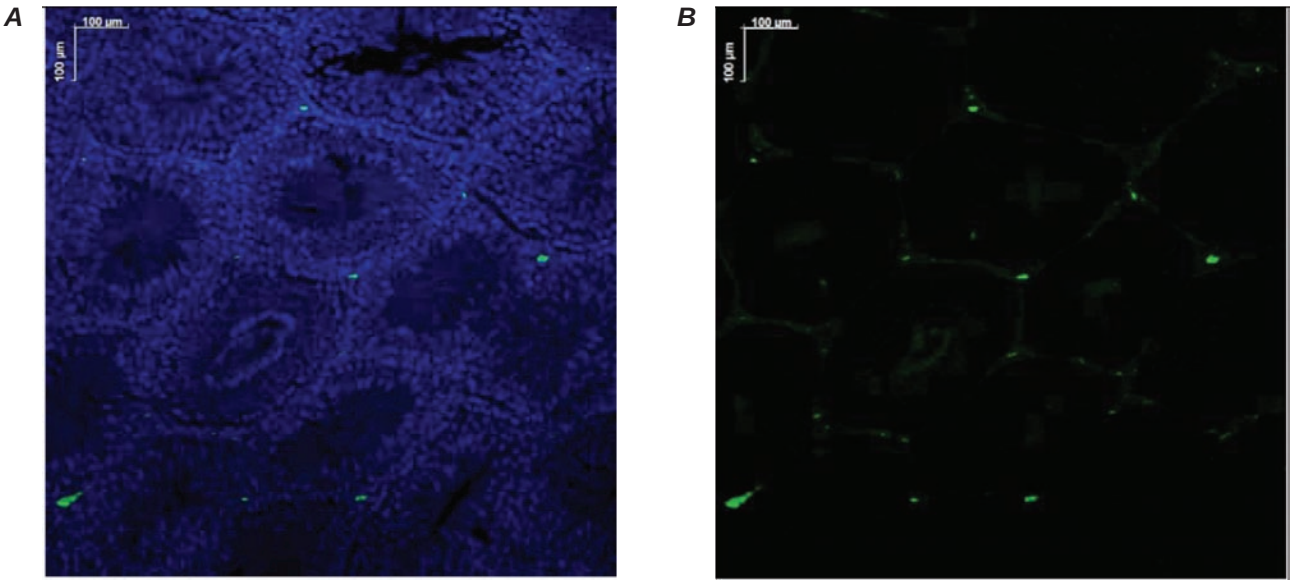

Fig. 1. Day 30 of observation. Expression of CD68 on cells in the interstitial space and in the testicular vessels in different stains. ***A*** *– Cell nuclei staining using Chemicon nuclear DNA complex with DRAQ5.* ***B*** *– No staining of cell nuclei with nuclear DNA complexes with DRAQ5 by Chemicon*

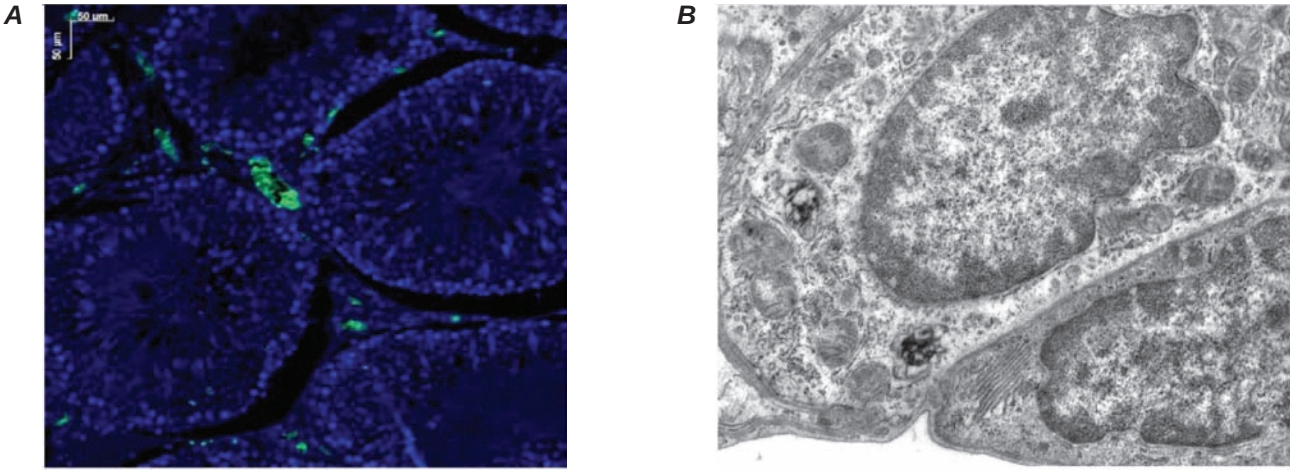

Fig. 2. 90th day of observation. ***A*** *– Expression of CD68 on cells in the interstitial space and testicular vessels. Green – CD68; Blue – DRAQ5. The scale is 50 microns.* ***B*** *– Electron micrograph of an interstitial macrophage*

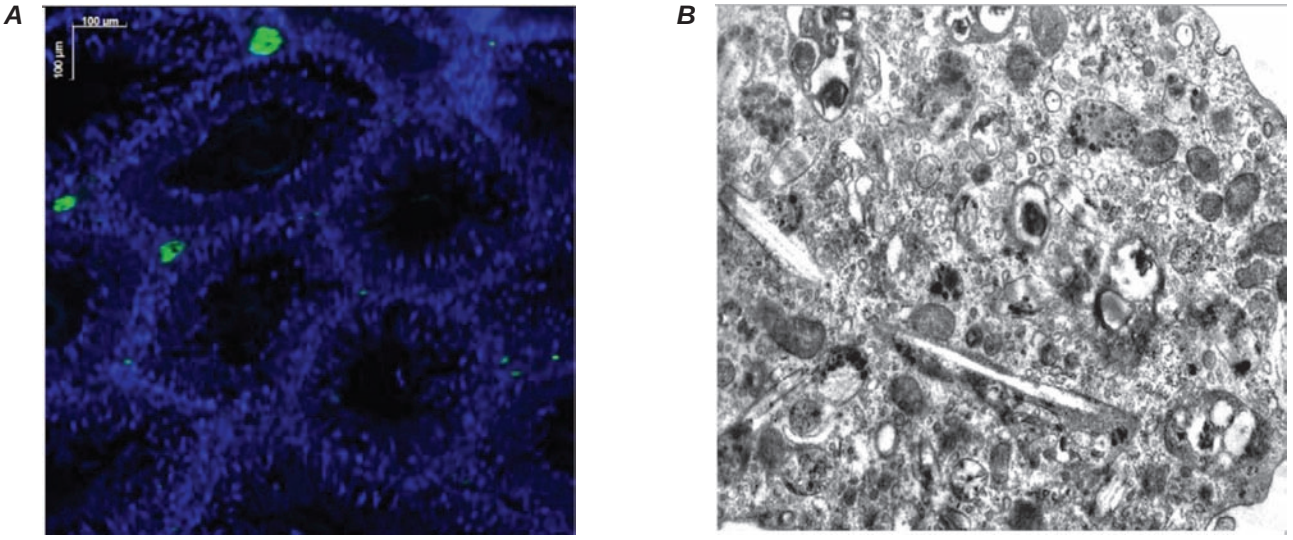

Fig. 3. 180 days of observation. ***A*** *– Expression of CD68 on cells in the interstitial space and testicular vessels. Green – CD68; Blue – DRAQ5. The scale is 100 microns.* ***B –*** *Electron micrograph of an interstitial macrophage*

were present. Phagosomes are observed, varying in size, shape and density. Mitochondria were enlarged in size, rounded, light (Fig. 3).

The 270th day of observation was described by a decrease in the population of macrophages both in the vessels and in the extravascular space. The cells were enlarged in size by the nucleus. The nucleus was polymorphic, with a large number of invaginations, the karyolemma and its structural components (pores) were not clearly visualized. The karyoplasm is mostly homogeneous, sometimes sparse. The cytoplasm contains a large number of small inclusions of varying density. There are a large number of pseudopodia (Fig. 4).

When we analyzed histological specimens on the 365th day of observation, the findings were characterized by a single expression of CD68 on macrophages in the extravascular space, sometimes with a complete absence of such cells in the interstitial space. Interstitial macrophages were clearly visualized on electron micrographs. The cell nuclei were mostly rounded, the karyolemma was clear,

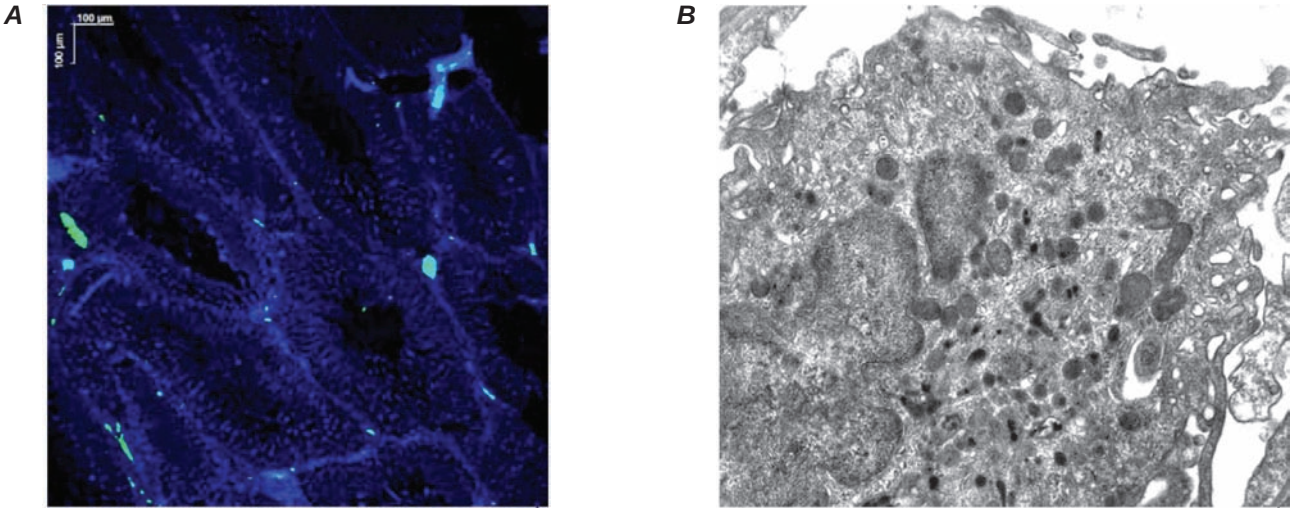

Fig. 4. Day 270 of observation. ***A*** *– Expression of CD68 on cells in the interstitial space and testicular vessels. Green – CD68+; Blue – DRAQ5. The scale is 100 microns.* ***B*** *– Electron micrograph of an interstitial macrophage*

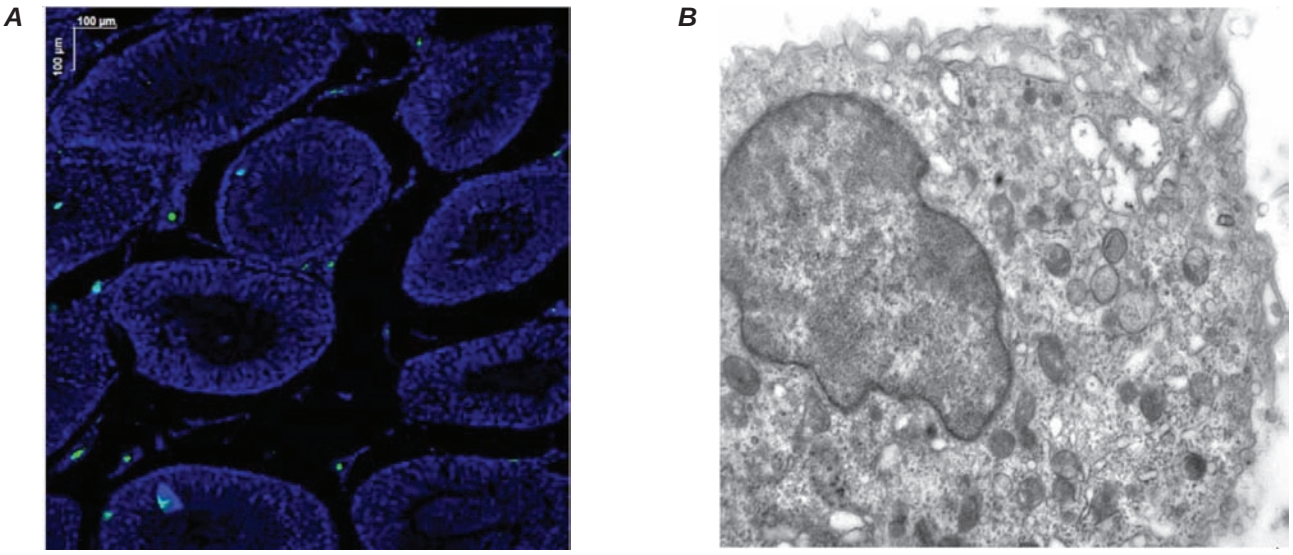

Fig. 5. Day 365 of observation. ***A*** *– Expression of CD68 on cells in the interstitial space and in the testicular vessels. Green - CD68+; Blue – DRAQ5. The scale is 100 microns.* ***B*** *– Electron micrograph of an interstitial macrophage*

and the pores were visible. The cytoplasm was light, transparent, with a moderate number of inclusions. Mitochondria were mostly rounded. There were single pseudopodia (Fig. 5).

On day 30 of central deprivation of LH synthesis by tryptorelin, the iNOS/Arg ratio increased significantly compared with the control group (Fig. 6). On day 90, the growth of the iNOS/Arg ratio continued and reached its peak. On day 180 of the experiment, the iNOS/Arg ratio decreased compared to day 90, but remained higher than the iNOS/Arg ratio in the control group of animals. On day 270 of the experiment, the iNOS/Arg ratio decreased to the level of control animals, and on day 365, a new elevation of the iNOS/Arg ratio was observed.

An increase in the iNOS/Arg ratio against the background of increased CD68 expression on cells in the interstitial space and in the testicular vessels may indicate a change in the prevailing polarization of macrophages in the testes from an anti-inflammatory (M2) phenotype to a proinflammatory (M1) phenotype.

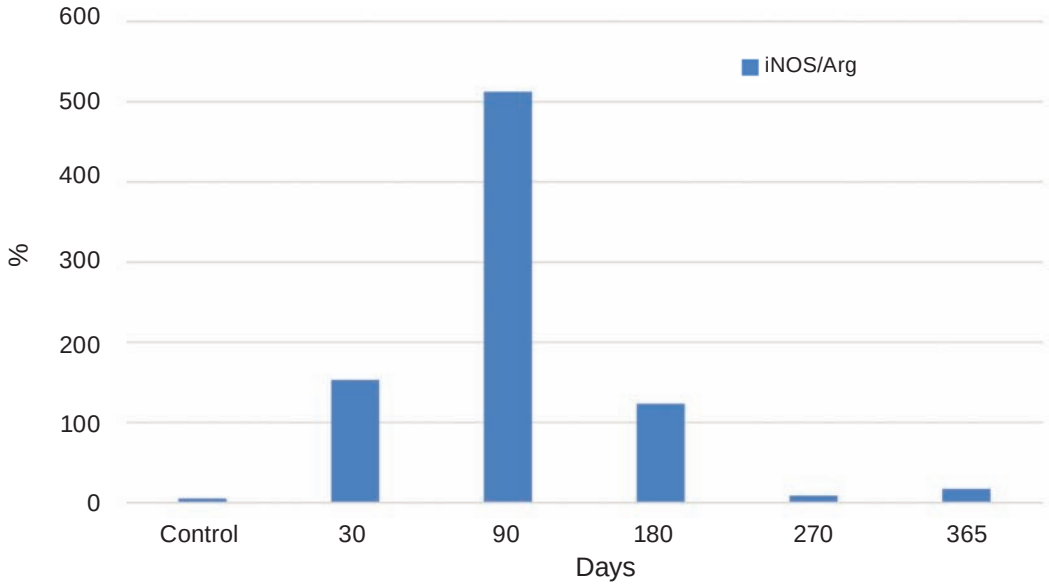

*Fig. 6. Variations in the iNOS/Arg ratio at different time points of the experiment. *The difference is statistically significant when compared with the control group ($P < 0.05$); **The difference is statistically significant when compared with the previous term of the experiment ($P < 0.05$).*

Discussion

The reason for the polarization change of testicular macrophages towards the predominance of the proinflammatory phenotype may be a decrease in the inhibitory effect of testosterone on the macrophages of the testes [17]. Another explanation for the shift in the polarization of testicular macrophages toward the predominance of the M1 phenotype may be the demasking of sperm antigens due to the development of oxidative tissue damage, as described in our previous work [18].

An elevated prevalence of CD68 in the testes was observed on days 30, 90, and 180 of central deprivation of LH synthesis by tryptorelin (Table). This corresponds chronologically with the increase in the iNOS/Arg index observed at the same time. This may indicate the predominance of both morphological and functional polarization of macrophages according to the M1 phenotype during this period. On day 30 of the experiment, interstitial CD68 expression increased by 4.14 times and intravascular expression by 3.22 times compared to the control group. On day 90, interstitial expression of CD68 rose by 3.0 times and intravascular expression by 4.0 times when compared to the control group. On day 180, interstitial CD68 expression is 2.86 times higher and intravascular expression is 3.33 times higher compared to the control group.

T a b l e. Distribution of interstitial and intravascular expression of CD68 receptors in rat testicular macrophages under long-term administration of tryptorelin

Days	Interstitium	Vessels
Control	0.70 ± 0.15	0.90 ± 0.18
30	2.90 ± 0.28†	2.90 ± 0.31†
90	2.10 ± 0.28†	3.6 ± 0,31$^{\dagger/\#}$
180	2.00 ± 0.33†	3.00 ± 0.26$^{\dagger/\#}$
270	1.30 ± 0.21	0.90 ± 0.18*
365	0.09 ± 0.28	2.20 ± 0.29$^{*/\dagger}$

Note: *The difference is statistically significant when compared with the previous term of the experiment ($P < 0.05$); #The difference is statistically significant when compared between the amount of CD68 on interstitial cells and intravascular cells at the same time point of the experiment; †The difference is statistically significant when compared with the control group, ($M \pm m$, $n = 5$)

It is interesting to note that on days 90 and 180, there is a significant predominance of intravascular expression of CD68 over interstitial expression, which may indicate the active involvement of macrophages of bone marrow origin in the process in the testes. This may be due to an elevated level of proinflammatory cytokines (IL-1, TNF-α, etc.) in the blood with a reduced level of testosterone production

by Leydig cells [19]. Proinflammatory cytokines, in turn, have a stimulating effect on the production of macrophages in the bone marrow and promote their migration to the site of cytokine production.

Conclusions. Central deprivation of the luteinizing hormone synthesis by tryptorelin leads to an increase in the distribution of the CD68 receptor in the interstitial space and in the testicular vessels from day 30 to 180 of the experiment, associated with a shift in the functional activity of L-arginine-dependent enzymes towards the predominance of inducible NO synthase activity.

Conflct of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ДИНАМІКА ЕКСПРЕСІЇ РЕЦЕПТОРА CD68 У МАКРОФАГАХ ІНТЕРСТИЦІЙНОГО ПРОСТОРУ СІМ'ЯНИКІВ ЩУРІВ ЗА УМОВ ТРИВАЛОГО ВВЕДЕННЯ ТРИПТОРЕЛІНУ

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Тестостерон, вироблення якого стимулюється вивільненням лютеїнізуючого гормону (ЛГ), має значну протизапальну та імуномодулюючу дію, а в його умовах дефіциту може виникнути пошкодження тканин через надмірну диференціацію макрофагів у прозапальний фенотип М1. Метою роботи було визначення поширення експресії рецептора CD68 як маркера запалення на клітини інтерстиціального простору та судин яєчка у разі блокади синтезу ЛГ трипторелiном. Статевозрілих білих щурів-самців випадковим чином розподіляли на 2 групи: контрольну (10 тварин) і дослідну (25 тварин). Тваринам дослідної групи вводили розчин трипторелiну ацетату (0,3 мг/кг). Імунохімічний аналіз експресії CD68+ оцінювали на лазерному конфокальному мікроскопі Olympus FV10i-LIV із використанням флуоресцентного міченого барвника. Показано, що депривація лютеїнізуючого гормону призводить до збільшення розподілу рецептора CD68 в інтерстиціальному просторі та в судинах яєчок з 30-ї по 180-у добу експерименту і це пов'язано зі збільшенням активності індуцибельної NO синтази в тканині яєчка.

Ключові слова: CD68, тестостерон, лютеїнізуючий гормон, трипторелін, яєчко, фенотип макрофага М1.

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