

UDC 611.018.46:612.419:612.08

DOI: <https://doi.org/10.22141/2224-0721.19.6.2023.1308>R.V. Martynenko¹, V.I. Shepitko¹, Ye.V. Stetsuk¹, N.V. Boruta¹,
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Expression of Ki67 and CD68+ cells of red bone marrow monocyte sprout under triptorelin administration in the hypothalamic- pituitary-testis regulatory system: the experimental study

Abstract. Background. Red bone marrow (RBM) is the main organ of human haemopoiesis. Monocytopoiesis plays an important role in the formation of transitional states: from normal to pathology and in the transformation of pathological processes from one stage to another. In modern urological practice, the substance triptorelin is widely used for androgen deprivation therapy, according to the recommendations of the European Association of Urology. Ki67, a commercially available monoclonal antibody that reacts with a nuclear antigen detected only in proliferating cells, is used to assess immunohistochemical changes. CD68 is a valuable cytochemical marker for immunostaining of monocytes/macrophages during histochemical analysis of tissues in inflammation, cancer and other immunohistopathological purposes. The purpose of the study is to evaluate the proliferative activity and differentiation of progenitor cells through the expression of Ki67 and CD68+ monocyte sprouting of RBM under chemical castration of central origin in male rats caused by the administration of triptorelin solution with quercetin addition to the diet for one year. **Materials and methods.** The study was conducted on 60 adult male white rats. They were divided into 3 groups: group I — control (n = 10), group II (n = 25) — subcutaneous injection of triptorelin, group III (n = 25) — subcutaneous injection of triptorelin acetate and quercetin. Immunohistochemical analysis of biopsy specimens was conducted following a standard protocol at the Department of Pathological Anatomy in Sumy State University, under the supervision of the Head of the Department, Prof. Romaniuk A.M. **Results.** The study evaluated Ki67 expression on microsections of rat red bone marrow through immunohistochemistry, which exclusively reacted with nuclear antigen in the monocyte sprout's proliferating cells. Irregular changes were revealed depending on the experimental groups and time periods. Immunohistochemical analysis of RBM tissue using anti-CD68 antibodies in the experimental groups revealed a strong positive cytoplasmic response in monocytes and resident macrophages located in the monocyte sprout and surrounding environment. The data of the two experimental groups of RBM had a noticeable proliferating compartment, as evidenced by the high content of mitotically active DNA in them. These data correspond to the results obtained in the experiment with triptorelin, where we found a marked positivity of Ki67, depending on the timing of the experiment and the addition of the flavonoid quercetin. This discrepancy suggests that bone marrow cells that grow and proliferate under normal conditions are guided by natural control mechanisms and may lose their Ki67 expression after leaving the progenitor compartment and entering the differentiation compartment. **Conclusions.** Triptorelin administration induces hormonal imbalance in the hypothalamus-pituitary-testis-RBM system, resulting in quantitative and qualitative alterations in the cells of the RBM monocytic lineage. The level of cell proliferation, as measured by Ki67, is highest during the third month of observation. Cytoplasmic expression of CD68 is evident in two experimental groups from the third to the sixth month, suggesting activation of immunoreactive cells as they migrate from the progenitor compartment to the differentiation compartment.

Keywords: hypothalamus-pituitary-testis system; red bone marrow; monocytopoiesis; Ki67; CD68; triptorelin

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Introduction

The red bone marrow (RBM) is a highly innervated and vascularised organ responsible for haematopoiesis, as well as defending against foreign interference and maintaining homeostasis. An uncommitted pluripotent stem cell undergoes ordered proliferation and differentiation to form immature committed progenitor cells. These progenitors eventually become mature cells that are released into the haemocirculation [1].

Cytokines (interleukins, colony-stimulating factor, growth factors), hormones and other humoral factors, such as haemopoietins, which include erythropoietin, leukopoietin, thrombopoietin, monopoietin, are involved in the regulation of haemopoiesis. Monocytopoiesis plays an important role in the formation of transitional states: from normal to pathology and in the transformation of pathological processes from one stage to another [2].

Despite the variety of these processes, they include typical components associated with the development of tissue stress, which is characteristic not only of canonical but also of non-classical inflammation (parainflammation), which develops in response to low-intensity damage without the development of typical local signs of inflammation [3–5].

In modern urological practice and in accordance with suggestions by the European Association of Urology [6], triptorelin is widely used as a synthetic analogue of gonadotropin-releasing hormone for androgen deprivation therapy. The substance, which is a polypeptide in its chemical composition [7], was developed by the laboratory of the French company Ipsen in the 1980s.

Quercetin, a flavonoid found in fruits and vegetables, has distinct biological properties. It has a broad range of effects such as its ability to reduce lipid peroxidation, platelet aggregation, capillary permeability and stimulation of mitochondrial biogenesis, as well as its anti-cancer, anti-inflammatory and antiviral properties [8–10].

Ki67 is a monoclonal antibody that is available commercially and reacts with a nuclear antigen which is only present in proliferating cells [11]. In addition to proliferation markers, Ki67 is also an indirect indicator of cell division rate [12, 13]. CD68 is a valuable cytochemical marker for immunostaining of monocytes/macrophages in histochemical analysis of tissues in inflammation, cancer and other immunohistopathological purposes. CD68 cell-specific expression and differential expression levels are determined by a complex interaction between transcription factors, transcriptional regulatory elements and epigenetic factors [14].

Therefore, the assessment of the spreading and differentiation of monocyte progenitor cells of the RBM in the control group and after administration of triptorelin with quercetin is a current issue that has not been sufficiently discussed in the scientific literature.

The purpose of the study was to evaluate the proliferative activity and differentiation of progenitor cells through the expression of Ki67 and CD68+ monocyte sprouting of RBM under chemical castration of central origin in male rats caused by the administration of triptorelin solution with quercetin addition to the diet for one year.

Materials and methods

The study was conducted on 60 adult male white rats. The rats were divided into 3 groups: group I — controls (n = 10), which were injected with saline solution [15], group II animals (n = 25) received subcutaneous injections of triptorelin acetate at a dose of 0.3 mg/kg body weight, animals of group III (n = 25) were subcutaneously injected with triptorelin acetate and quercetin at 100 mg/kg body weight three times weekly [16].

Experimental animals were kept in standard accommodation in the vivarium of the Poltava State Medical University.

Any euthanasia procedures were executed in strict accordance with the regulations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and the General Ethical Principles for Experiments on Animals as established by the First National Congress on Bioethics (Kyiv, 2001). The animals were euthanised (n = 60) by an overdose of ether anaesthesia in accordance with the relevant regulations.

Using standard methods, the material was embedded in paraffin blocks, and 4 µm thick sections were prepared and then stained with haematoxylin and eosin [17].

Biopsy material was studied using rat red bone marrow tissue pieces, which were fixed in 10% buffered formalin at pH 7.4 for 24 hours and embedded in paraffin. Consequently, 5 µm thick sections were stained with hematoxylin and eosin using automatic Microm HMS 740 Thermo Scientific apparatus and mounted onto Bio-Mount (Bio Optica Milano S.A., Italy).

Immunohistochemical analysis of biopsy material was conducted based on a standard protocol at the Department of Pathological Anatomy, Sumy State University (Head of Department, Prof. Romaniuk A.M.). Serial sections of 5 µm thick were mounted on poly-L-lysine-coated glass slides. Deparaffinisation was carried out, and high-temperature demasking of antibodies was performed using a PT module at 98 °C for 20 minutes. Subsequent procedures were conducted automatically on an Autostainer 360 Thermo Scientific, utilizing the Quanto imaging system. Autostainer protocol included 10 min H₂O₂, 10 min protein block, 30 min primary antibody, 10 min secondary antibody, 5 min DAB. Washing in Tris buffer pH 6 Tween 20. Tissue antigens were detected using a mouse monoclonal antibody to Ki67 (MM1, Diagnostic BioSystems, USA) and CD68 (Chemi-Con, USA).

The proliferative activity of Ki67 was determined using the visual method to count immunoreactive nuclei of RBM monocyte sprout cells in a conventional manner. Three populations were identified based on the degree of activity — high mitotic activity, low mitotic activity, and absence of mitotic activity — in cells expressing Ki67. The resulting data is presented in both percentage and absolute numbers.

CD68 receptor expression was evaluated on the external membrane of monocytes in red bone marrow cells using a standard method of visual counting.

Histological sections were analysed with an Olympus C 3040-ADU light microscope, equipped with a digital microfilter and software adapted specifically for this study (Olympus DP-Soft, license numbers VJ285302, VT310403, 1AV4U13B26802), and Viorex 3 (serial number 5604).

Statistical analysis of the data was conducted using Microsoft Office Excel and the Real Statistics 2019 extension. The non-parametric Mann-Whitney criteria was used to determine statistical significance.

Results

According to the immunohistochemical study of Ki67 expression, which reacts with nuclear antigen, on micro-sections of rat red bone marrow, it was determined only in proliferating cells of the monocyte sprout. The changes were uneven depending on the experimental groups and time periods (Tables 1, 2, Fig. 1, 2, 4B).

Ki67 expression activity increased from the first month of observation in all experimental groups. The maximum total proliferative activity was observed at the third month, with statistical significance at $p < 0.001$. There was a gradual decline from the 6th to the 12th month in the experimental groups. The most significant decrease in the number of cells

expressing Ki67 was observed at month 9 in all observation groups. At month 12, the quantitative measures of mitotic activity in MS RBM did not significantly deviate from the control group in any of the experimental groups. A more precise study of Ki67 expression, depending on the degree of mitotic activity, identified three groups of cells: high level, low level and without activity. Table 3 presents the percentage distribution of these groups.

Immunohistochemical analysis of RBM tissue using CD68 antibodies in the experimental groups demonstrated a highly positive cytoplasmic response in the monocytes and resident macrophages within the monocyte sprout and its environment (Table 4, Fig. 3, 4B). Monocytes were the predominant cell type, comprising 1 : 7 of the total cells. We occasionally observed a faint positive reaction on the endothelial cells and reticular cells of the RBM in addition to macrophages; however, we did not include these cells in our analysis. The highest CD68 cytoplasmic expression

Table 1. Immunohistochemical study of Ki67 expression in MS RBM under triptorelin administration

Parameters	High mitotic activity	Low mitotic activity	Absence of mitotic activity
Controls	3.120 ± 0.125	24.330 ± 1.368	27.230 ± 1.528
1 month	14.030 ± 0.985***	18.860 ± 1.053**	23.350 ± 1.322*
3 months	28.980 ± 1.455***/###	42.310 ± 2.698***/###	40.520 ± 2.512***/###
6 months	9.880 ± 0.789***/###	19.630 ± 1.265**/#	36.010 ± 2.359#
9 months	6.750 ± 0.587*/#	13.720 ± 0.918**/###	25.120 ± 1.554**
12 months	8.550 ± 0.821*/#	20.160 ± 1.326**/#	28.810 ± 1.685

Notes (here and in Table 2): reliability of the difference between the previous research period: * — $p < 0.05$, ** — $p < 0.01$, * — $p < 0.001$; reliability of the difference between the control group and the different study periods: # — $p < 0.05$, ## — $p < 0.01$, ### $p < 0.001$.**

Table 2. Immunohistochemical study of Ki67 expression in MS RBM under triptorelin and quercetin administration

Parameters	High mitotic activity	Low mitotic activity	Absence of mitotic activity
Controls	3.120 ± 0.125	24.330 ± 1.368	27.230 ± 1.528
1 month	13.440 ± 0.912***	16.940 ± 1.003**	24.420 ± 1.389
3 months	23.010 ± 1.187***/###	32.520 ± 2.181***/##	37.170 ± 2.305***/##
6 months	8.980 ± 0.405***/##	18.330 ± 1.147***/#	33.560 ± 2.209*/#
9 months	4.750 ± 0.265**/#	16.410 ± 1.008#	25.850 ± 1.563*
12 months	8.320 ± 0.798**/##	19.930 ± 1.258*/#	28.470 ± 1.693

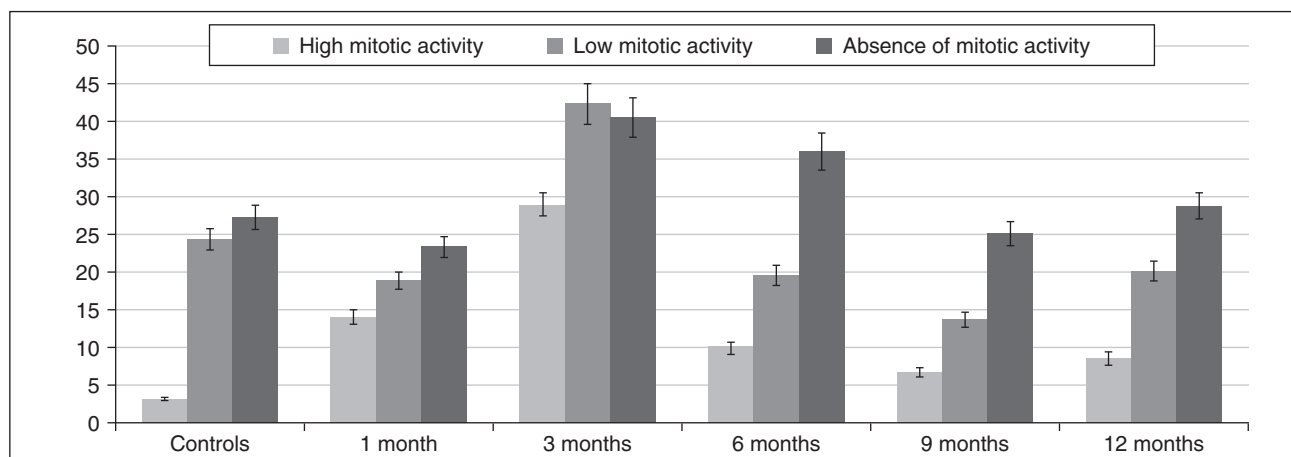


Figure 1. Proliferative activity by Ki67 expression under triptorelin administration

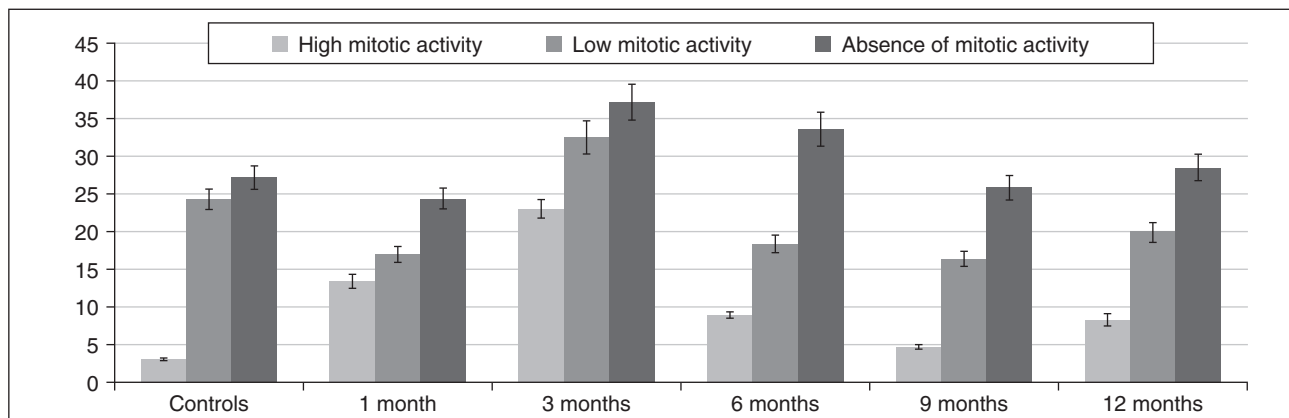


Figure 2. Proliferative activity by Ki67 expression under triptorelin and quercetin administration

was assessed after the 3rd and 6th months of observation in both experimental groups. The values were 14.690 ± 1.136 (3 months in the experimental group with triptorelin) and 13.920 ± 1.181 (3 months in the experimental group with triptorelin with quercetin), respectively, which are 53 and 49 % higher than in the control group (7.020 ± 0.285).

Discussion

Ki67 targets a nuclear antigen expressed by dividing cells in all stages of the cell cycle except G0 and early G1 [18]. It has been used in combination with a monoclonal antibody, BrdU, which reacts selectively with cells in S phase, to determine the percentage of proliferating cells in bone marrow samples [19]. A proliferating compartment was observed in both experimental groups based on the high amount of

mitotically active DNA, as revealed by the data obtained. These findings are consistent with information obtained from studying the effects of triptorelin on stressed bone marrow and on red bone marrow cultured with haematopoietic growth factors. Evidence of increased Ki67 activity was observed depending on the timing of the experiment and the addition of the flavonoid quercetin. This finding suggests that bone marrow cells, which grow and proliferate under normal conditions, are guided by natural control mechanisms and may experience a decline in Ki67 expression upon transitioning from the progenitor compartment to the differentiation compartment.

The migration of monocytes and their transformation into macrophages at sites of inflammation is a critical factor in influencing the outcome of the inflammatory response and

Table 3. Percentage ratio of the mitotic activity level in the immunohistochemical study of Ki67 expression in MS RBM in the experiment with triptorelin and triptorelin in combination with quercetin

Parameters	Triptorelin	Triptorelin and quercetin
Controls	5.7–44.5–49.8	5.7–44.5–49.8
1 month	24.9–33.5–41.6	24.5–30.9–44.6
3 months	25.9–37.8–36.3	24.8–35.1–40.1
6 months	15.2–29.9–54.9	14.8–30.1–55.1
9 months	14.8–30.3–54.9	10.1–34.9–55
12 months	14.8–35.1–50.1	14.7–35.1–50.2

Note: the first number in the ratio is the percentage of cells with high mitotic activity, the second one — with low mitotic activity, and the third number is the percentage of cells with no mitotic activity.

Table 4. Cytoplasmic activity of RBM based on CD68 expression under administration of triptorelin and triptorelin combined with quercetin

Parameters	Triptorelin	Triptorelin and quercetin
Controls	7.020 ± 0.285	7.020 ± 0.285
1 month	7.750 ± 0.308	7.940 ± 0.338
3 months	$14.690 \pm 1.136^{***}/\#$	$13.920 \pm 1.181^{***}/\#$
6 months	$12.920 \pm 0.938^{\#}$	$12.130 \pm 0.907^{\#}$
9 months	$8.930 \pm 0.836^{**}/\#$	$8.410 \pm 0.818^{**}/\#$
12 months	$7.950 \pm 0.886^*$	$7.630 \pm 0.581^*$

Notes: reliability of the difference between the previous research period: * — $p < 0.05$, ** — $p < 0.01$, *** — $p < 0.001$; reliability of the difference between the control group and the different study periods: # — $p < 0.05$, ## — $p < 0.001$.

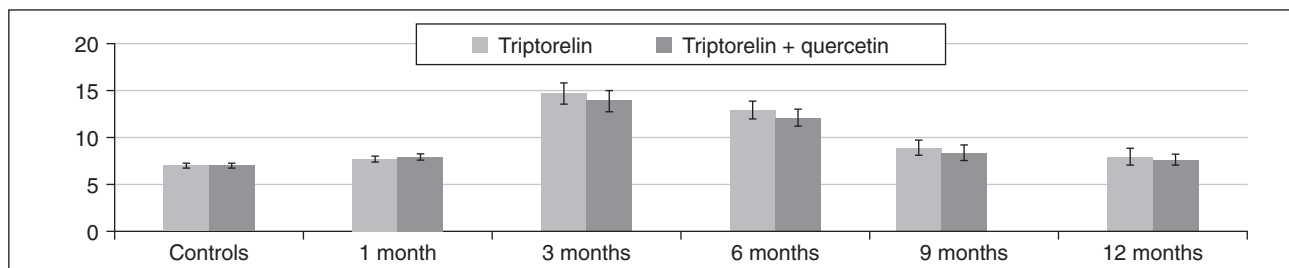


Figure 3. Cytoplasmic activity based on CD68 expression under administration of triptorelin and triptorelin combined with quercetin

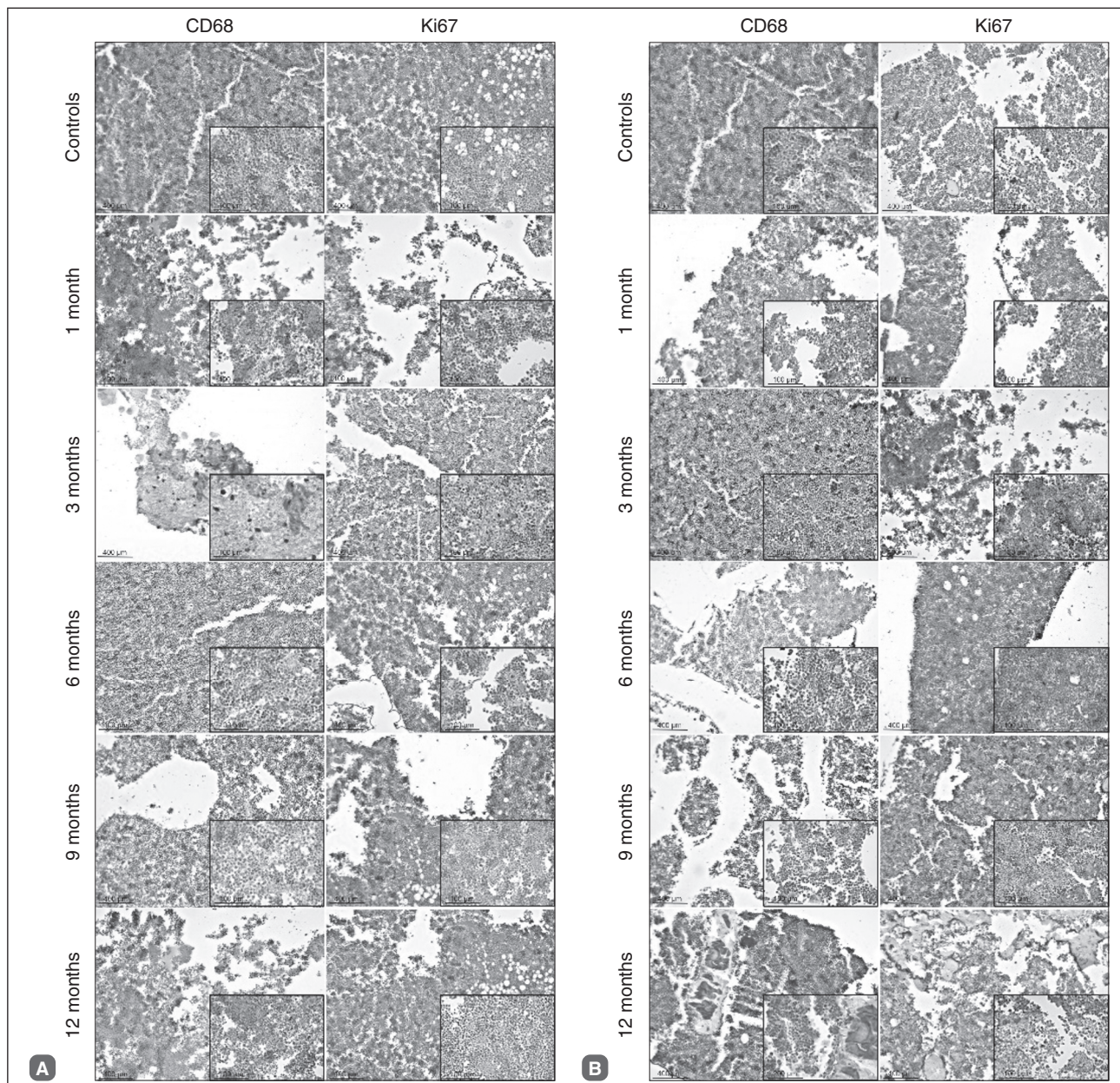


Figure 4. Immunohistochemistry on RBM tissue using antibodies against CD68 and Ki67, with nuclear stain by Mayer's haematoxylin and eosin; the magnification is shown in the lower left corner of each image: A) the experimental group with triptorelin administration; B) the experimental group with triptorelin administration combined with quercetin

initiating tissue homeostasis. CD68 is a glycosylated glycoprotein that is expressed in macrophages and other mononuclear phagocytes [4]. CD68 is a valuable cytochemical marker for immunostaining of monocytes/macrophages in histochemical analysis of tissues in inflammation, cancer and other immunohistopathological purposes [12]. CD68, either alone or combined with other cellular markers of tumour-associated macrophages, has proven to be a valuable prognostic indicator for cancer patient survival [20]. Monocytopenia has been examined in patients who experienced severe acute inflammation from surgical interventions, as well as in those with mild chronic inflammation resulting from gastric or duodenal ulcers [21–25]. It is believed that the state of acute inflammation is associated with a high and rapidly increasing demand for monocytes, in contrast to the constant and re-

latively small number of monocytes in chronic inflammation [26]. The administration of triptorelin causes changes in the human body similar to chronic inflammation [27]. In chronic mild inflammatory reactions, the activity of promonocyte DNA synthesis increased approximately twofold; the promonocyte pool was normal [26]. In patients who underwent surgery, changes in the following parameters were observed within the first 15 hours after the start of treatment: 1) an average increase in the 3H-TDR labelling index by 38 %; 2) an average increase in the promonocyte pool by 34 %; 3) and the release of immature cells from the bone marrow into the blood [28].

Increased DNA synthesis activity, identified through Ki67 marker, and an expanded pool of highly mitotic cells result in a rise in monocytopenia. Changes in cells with

CD68+ activity reflect a reduction in the time of transformation of stem cells into highly differentiated blood cells, namely monocytes, which allows them to adjust to the various needs of the body's homeostasis.

Conclusions

Triptorelin administration induces hormonal imbalance in the hypothalamus-pituitary-testis-RBM system, resulting in quantitative and qualitative alterations in the cells of the RBM monocytic lineage. The highest level of cell proliferation, as measured by Ki67, occurs during the third month of observation. Cytoplasmic expression of CD68 is evident in two experimental groups from the third to the sixth month, suggesting activation of immunoreactive cells as they migrate from the progenitor compartment to the differentiation compartment. An increase in DNA synthesis activity (Ki67 as a marker of proliferative activity) as well as an expansion of the pool of cells with a high level of mitotic activity results in an intensification of monocytopenia. Alterations in cells exhibiting CD68+ activity indicate a decrease in the duration of stem cell differentiation into specialised blood cells, namely monocytes, allowing them to adapt to the different needs of the body's homeostasis.

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Received 29.06.2023

Revised 05.09.2023

Accepted 11.09.2023 ■

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Conflicts of interests. Authors declare the absence of any conflicts of interests and own financial interest that might be construed to influence the results or interpretation of the manuscript.

Authors' contribution. Martynenko R.V. — work concept and design, data collection and analysis, responsibility for statistical analysis, writing the article; Shepitzko V.I. — final approval of the article; Stetsuk Ye.V., Martynenko V.B. — critical review; Boruta N.V. — responsibility for statistical analysis; Rud M.V. — writing the article; Vilkhova O.V. — work concept and design; Moskalenko R.A. — data collection and analysis.

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Експресія клітин Кі67 та CD68+ моноцитарного паростка червоного кісткового мозку при введенні триптореліну в систему регуляції «гіпоталамус — гіпофіз — яєчко»: експериментальне дослідження

Резюме. *Актуальність.* Червоний кістковий мозок (ЧКМ) є основним органом кровотворення людини. Моноцитопоез відіграє важливу роль у формуванні перехідних станів від норми до патології і в трансформації патологічних процесів з однієї стадії в іншу. У сучасній урологічній практиці трипторелін широко використовується для андрогендеприваційної терапії згідно з рекомендаціями Європейської асоціації урологів. Кі67, комерційно доступне моноклональне антитіло, що реагує з ядерним антигеном, виявленим лише в проліферуючих клітинах, використовується для оцінки імуногістохімічних змін. CD68 є цитохімічним маркером імунного забарвлення моноцитів/макрофагів у гістохімічному аналізі тканин при запаленні, раку та інших імуногістопатологічних процесах. **Мета дослідження:** оцінити проліферативну активність і диференціювання клітин-попередників через експресію Кі67 і CD68+ моноцитарного проростання ЧКМ за хімічної кастрації центрального походження у самців шурів, спричиненої введенням розчину триптореліну з додаванням кверцетину до раціону протягом одного року. **Матеріали та методи.** Дослідження проводилося на 60 дорослих самцях білих шурів. Тварин було поділено на 3 групи: I — контрольна (n = 10), II (n = 25) — підшкірне введення триптореліну, III (n = 25) — підшкірне введення триптореліну ацетату і кверцетину. Імуногістохімічний аналіз біопатів проводили за стандартним протоколом на кафедрі патологічної анатомії Сумського державного університету під керівництвом завідувача кафедри професора А.М. Романюка. **Результати.** Експресію Кі67 на мікрорізах ЧКМ шурів оцінювали за допомогою імуногістохімічного аналізу, при якому відмічено реакцію виключно з ядерним

антигеном у проліферуючих клітинах паростка моноцитів. Виявлено нерівномірні зміни залежно від експериментальних груп та періодів часу. Імуногістохімічний аналіз тканини ЧКМ з використанням антитіл проти CD68 в експериментальних групах показав сильну позитивну цитоплазматичну відповідь у моноцитах і резидентних макрофагах, розташованих у паростку моноцитів. Дані двох дослідних груп ЧКМ мали помітний проліферуючий компартмент, про що свідчить високий вміст у них мітотично-активної ДНК. Ці дані відповідають результатам, отриманим в експерименті з триптореліном, у якому виявлено помітну позитивність Кі67 залежно від часу експерименту та додавання флавоноїду кверцетину. Ця розбіжність свідчить про те, що клітини кісткового мозку, які ростуть і проліферують у нормальних умовах, керуються природними механізмами контролю і можуть втратити свою експресію Кі67 після того, як покинуть компартмент попередників і увійдуть у компартмент диференціювання. **Висновки.** Введення триптореліну викликає гормональний дисбаланс у системі «гіпоталамус — гіпофіз — яєчко — ЧКМ», що призводить до кількісних і якісних змін у клітинах моноцитарної лінії ЧКМ. Рівень клітинної проліферації за вимірюванням Кі67 був найвищим протягом третього місяця спостереження. Цитоплазматична експресія CD68 очевидна у двох експериментальних групах з третього по шостий місяць, що свідчить про активацію імунореактивних клітин, коли вони мігрують із компартменту попередника до компартменту диференціювання.

Ключові слова: система «гіпоталамус — гіпофіз — яєчко»; червоний кістковий мозок; моноцитопоез; Кі67; CD68; трипторелін