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Expression features of T-lymphocytes, B-lymphocytes and macrophages in the post-traumatic regenerate of the mandible rats under conditions of filling a bone defect with hydroxyapatite-containing osteotropic material and thymalin injecting the surrounding soft tissues

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ABSTRACT

Aim: The purpose of the study was to determine the features of the expression of T-lymphocytes, B-lymphocytes, macrophages in the post-traumatic regenerate of the mandible rats under conditions of filling a bone defect with hydroxyapatite-containing osteotropic material and thymalin injecting the surrounding soft tissues.

Materials and Methods: An experiment was conducted on 48 mature rats of the WAG population weighing 160-180 grams. Four groups were formed. Group 1 included 12 rats with a simulated holey defect in the lower jaw. Group 2 included 12 rats with a simulated holey defect in the lower jaw followed by its closure with hydroxyapatite-containing osteotropic material (bone graft "Biomin GT"). Group 3 included 12 rats with a simulated holey defect in the lower jaw with injecting the surrounding soft tissues with thymalin. Group 4 included 12 rats with a simulated holey defect in the lower jaw followed by its closure with hydroxyapatite-containing osteotropic material (bone graft "Biomin GT") and injecting the surrounding soft tissues with thymalin. The material for the morphological study was a fragment of the lower jaw from the area of the simulated holey defect. An immunohistochemical study was performed using monoclonal antibodies to CD68, CD20, CD163, CD86, CD3.

Results: A comprehensive experimental and morphological study conducted by the authors revealed that thymalin injection of the soft tissues surrounding the bone defect of the lower jaw, filled with hydroxyapatite-containing osteotropic material "Biomin GT", stimulates local immune reactions in the post-traumatic regenerate, which is manifested, firstly, by an increase in the number T-lymphocytes on the 3rd day of the experiment and their increase up to the 28th day; secondly, by increasing the number of B-lymphocytes on the 14th day of the experiment with their further increase up to the 28th day; thirdly, by increasing the number of macrophages on the 3rd day of the experiment and their growth up to the 28th day; fourth, changes in macrophages phenotypes (decrease in the number of M1-macrophages and increase in the number of M2-macrophages).

Conclusions: Stimulation of local immune reactions in the post-traumatic regenerate can be one of the mechanisms that activate reparative osteogenesis in the lower jaw of rats under the conditions of filling bone defects with hydroxyapatite-containing osteotropic material "Biomin GT" and thymalin injecting the surrounding soft tissues.

KEY WORDS: T-lymphocytes, B-lymphocytes, macrophages, post-traumatic regenerate, hydroxyapatite-containing osteotropic material

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INTRODUCTION

In recent years a rapidly increasing number of publications are focusing on the topic of relationship between bone and the immune system, and this research field has been termed "osteoimmunology" to emphasize the close and complex communication between bone and the immune system [1, 2]. The immune and skeletal systems are found to be closely related, sharing a number of cytokines, chemokines, hormones, receptors, signaling molecules and transcription factors [3]. Bone cells interact with immune cells under physiological and pathological conditions [4].

The immune cells play an important role in reparative osteogenesis [5, 6]. Immune cells serve as the initial responders at the site of injury, mending vasculature, and initiating cascades of signals to recruit cells to carry out the repair processes [7]. Bone tissue damage initial immune response is mainly composed of the innate immune system which includes neutrophils, macrophages, dendritic cells. The later immune response is mainly composed of an adaptive immune system which includes T- and B-lymphocytes [5, 8]. Experimental studies have shown that decrease in morphofunctional activity of T- and B-lymphocytes is associated with impairment in bone mineralization and maturation of osteoblasts with delayed repair and remodeling phases [9]. HIV-positive patients display slower bone fracture healing [7].

Regulating the immune microenvironment is a promising therapeutic target to promote bone tissue regeneration [5]. Our previous morphological study showed stimulation of reparative osteogenesis in the lower jaw of rats under conditions of filling a bone defect with hydroxyapatite-containing osteotropic material and injecting the surrounding soft tissues with thymalin [10]. The features of local immune reactions in the regenerate filling the mandible bone defect under conditions of the above-mentioned therapeutic measures remain unknown, which requires further research.

AIM

The purpose of the study was to determine the features of the expression of T-lymphocytes, B-lymphocytes, macrophages in the post-traumatic regenerate of the mandible rats under conditions of filling a bone defect with hydroxyapatite-containing osteotropic material and thymalin injecting the surrounding soft tissues.

MATERIALS AND METHODS

An experiment was conducted on 48 mature rats of the WAG population, which were divided into four groups (12 animals in each group).

Group 1 included rats that underwent an incision of the skin, subcutaneous tissue and superficial fascia in the left submandibular area with a length of 1-1.2 cm and skeletonized a fragment of the outer surface of the branch and body of the lower jaw under ketamine intraperitoneal anesthesia and ultracaine infiltration anesthesia. A ball-shaped drill bit for a straight tip with a diameter of 3 mm with a rotation frequency up to 1000 revolutions per minute was used to form a transcortical hole defect of the body of the lower jaw in the form of a channel departing from the lower edge of the lower jaw upwards by 2 mm (until the feeling of the bur falling through). The wound was sutured layer by layer with polyamide after the formation of a holey defect.

Group 2 included rats that were modeled with a lower jaw defect similar to group 1. The formed defect was filled with synthetic bone graft "Biomin GT" (RAPID, Ukraine) which included hydroxyapatite and β-tricalcium phosphate. The wound was sutured layer by layer with polyamide.

Group 3 included rats that were modeled with a lower jaw defect similar to groups 1 and 2. The wound was sutured layer by layer with polyamide. Thymalin (LLC PP BIOPHARMA, Ukraine) was injected into the soft tissues around the defect for 10 days (0.01 mg/ml per 100 grams of animal weight).

Group 4 included rats that were modeled with a lower jaw defect similar to groups 1-3, which was filled with synthetic bone graft "Biomin GT" (RAPID, Ukraine). The wound was sutured layer by layer with polyamide. Thymalin (0.01 mg/ml per 100 grams of animal weight) was injected into the soft tissues around the defect for 10 days.

In each groups the animals were removed from the experiment on 3, 7, 14 and 28 days (3 animals for each experimental period).

The material for the study was a fragment of the lower jaw from the area of the simulated holey defect. It was fixed in a 10% solution of neutral formalin (pH 7.4) for 24-48 hours, decalcified and carried out according to the generally accepted method and embedded in paraffin. From paraffin blocks serial sections with a thickness of 4-5 μm were made, which were stained with hematoxylin and eosin.

Immunohistochemical study was performed on Super Frost Plus adhesive slides (Menzel, Germany). The Master Polymer Plus Detection System (Peroxidase, DAB chromogen) (Master Diagnostica, Spain) was used. The citrate buffer (pH 6.0), EDTA buffer (pH 8.0) were used for high-temperature processing of antigen epitopes. An immunohistochemical study was performed using mouse monoclonal antibodies to CD68 (clone KP-1, Master Diagnostica, Spain) (marker of cells of macrophage lineage), CD20 (clone L26, Thermo Fisher Scientific, Great Britain) (marker of B-lymphocytes), rabbit monoclonal antibodies to CD163 (clone EP324, Master Diagnostica, Spain) (marker of M2-macrophages), CD86 (clone B7-2, Thermo Fisher Scientific, Great Britain) (marker of M1-macrophages), CD3 (clone EP41, Master Diagnostica, Spain) (marker of T-lymphocytes). Immunohistochemical reactions with antibodies to CD68, CD20 and CD3 were evaluated by counting the absolute number of immunopositive cells in the field of view of a microscope ×400, and reactions with antibodies to CD86, CD163 – by counting the relative number (%) of immunopositive cells in the field of view of a microscope ×400. Examination of the microslides was carried out using a laboratory microscope ZEISS Primostar 3 (Carl Zeiss, Germany) with a built-in color digital camera.

The indicators in the groups were processed statistically using the PAST program (version 4.15, Natural History Museum, University of Oslo, Norway). Mean values of indicators in groups were compared using the Student's t-test and Mann-Whitney U-test. Differences were considered significant at $p<0.05$.

RESULTS

During an immunohistochemical study in groups 1-4 at all experiment days, $CD20^+$-, $CD3^+$-, $CD68^+$-, $CD86^+$-, $CD163^+$-cells were visualized among the previously described polymorphic cellular infiltration in the regenerate filling the cavity of the bone defect of the rats lower jaw (Fig. 1-3).

The analysis of the mean value of the absolute number of $CD20^+$-cells is shown in Table 1. As indicated in this table, the absolute number of $CD20^+$-cells did not differ ($p>0.05$) in group 2 compared to group 1 at all experiment terms. The absolute number of $CD20^+$-cells in groups 3 and 4 compared to the corresponding indicators in groups 1 and 2 did not differ ($p>0.05$) on days 3 and 7, and on days 14 and 28 had a greater ($p<0.05$) value. At all experiment days, the indicators of groups 3 and 4 did not differ ($p>0.05$).

During the period from the 3rd to the 28th day of the experiment, the absolute number of $CD20^+$-cells in the regenerate did not change ($p>0.05$) in groups 1 and 2. In groups 3 and 4, this indicator also did not change ($p>0.05$) from the 3rd to the 7th day and increased ($p<0.05$) from the 7th to the 28th day.

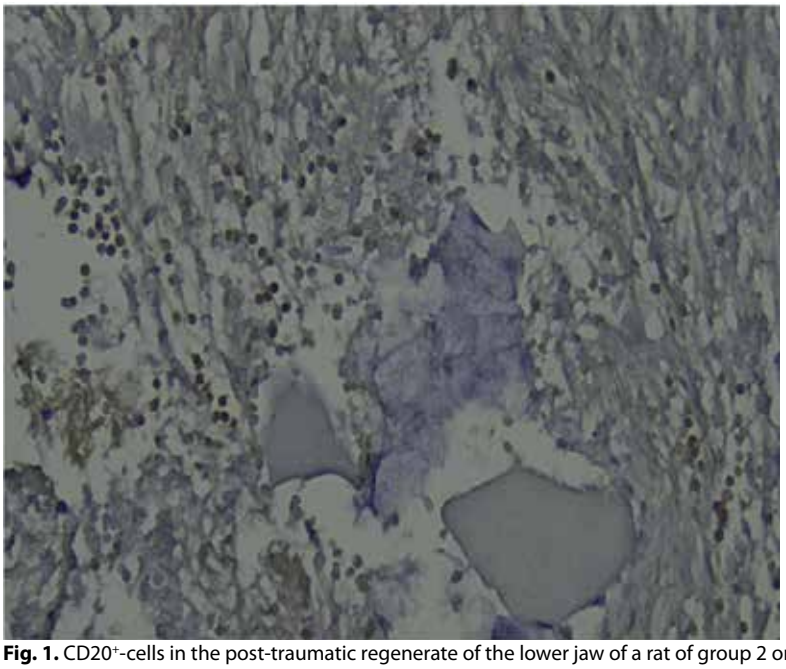

Fig. 1. $CD20^+$-cells in the post-traumatic regenerate of the lower jaw of a rat of group 2 on the 3rd day of the experiment. Immunohistochemical study with a monoclonal antibody to CD20, ×400.

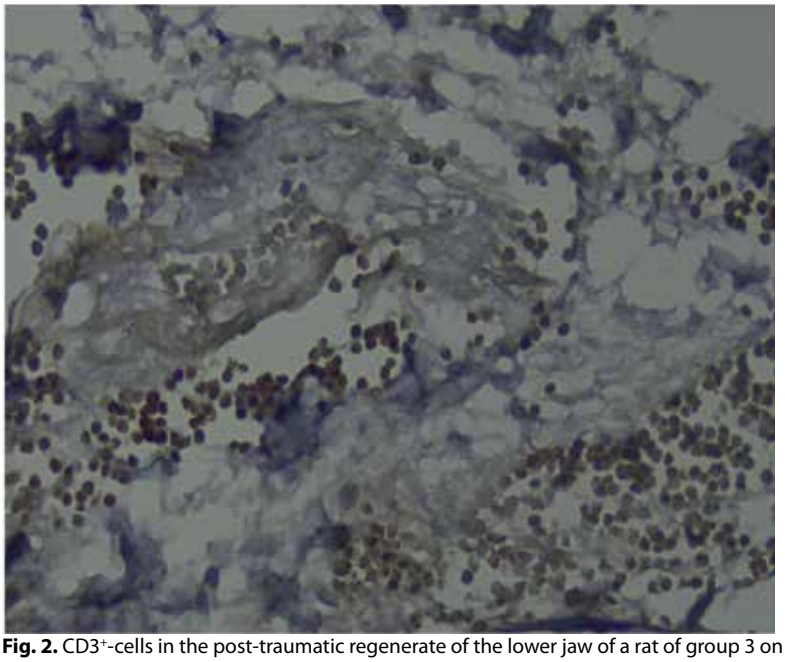

Fig. 2. $CD3^+$-cells in the post-traumatic regenerate of the lower jaw of a rat of group 3 on the 3rd day of the experiment. Immunohistochemical study with a monoclonal antibody to CD3, ×400.

The analysis of the average value of the absolute number of $CD3^+$-cells in the regenerate in groups 1-4 is shown in Table 2. Compared to group 1, in group 2 this indicator had a greater value ($p<0.05$) at all experimental days. From the 3rd to the 28th day of the experiment, the number of $CD3^+$-cells in groups 3 and 4 was higher ($p<0.05$) compared to the indicators of groups 1 and 2. At all days of the experiment, the indicator of group 4 did not differ ($p>0.05$) from the corresponding indicator of group 3. From the 3rd to the 28th day of the experiment, an increase ($p<0.05$) in the number of $CD3^+$-cells was registered in all groups.

The results of counting the number of $CD68^+$-, $CD86^+$-, $CD163^+$-cells in groups 1-4 are shown in Table 3. In group 2, compared to group 1, the absolute number of $CD68^+$-cells did not differ ($p>0.05$) on day 3 and had more ($p<0.05$) values from the 7th to the 28th day. At all days of the experiment, the absolute number of $CD68^+$-cells did not differ ($p>0.05$) in groups 3 and 4, however, in the latter, this indicator had a greater value ($p<0.05$) compared to the indicators of groups 1 and 2.

Analyzing the absolute number of $CD68^+$-cells in groups 1-4 in dynamics (from the 3rd to the 28th day), it was noted

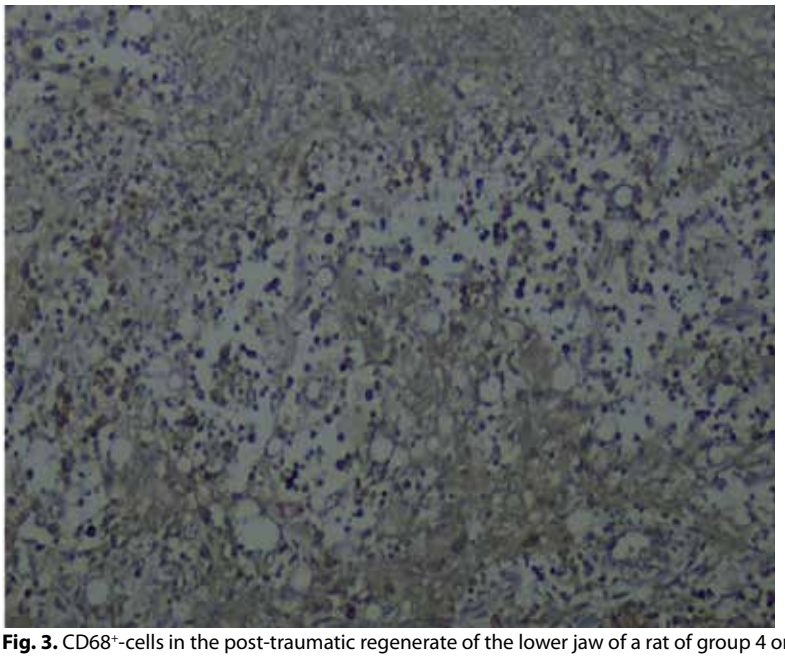

Fig. 3. CD68^{+}-cells in the post-traumatic regenerate of the lower jaw of a rat of group 4 on the 3rd day of the experiment. Immunohistochemical study with a monoclonal antibody to CD68, ×100.

Table 1. Mean values of the absolute number of CD20^{+}-cells

Group number	Day of experiment			
	3	7	14	28
Group 1 (holey defect)	77.3±2.38	81.2±1.96	83.2±1.87	82.2±1.35
Group 2 (holey defect+bone graft «Biomin GT»)	80.2±1.92	82.2±1.33	83.7±1.02	84.3±0.88
Group 3 (holey defect+thymalin)	79.3±1.84	80.7±0.71	100.8±2.24[1, 2, 5]	127.8±1.58[1, 2, 6]
Group 4 (holey defect+bone graft «Biomin GT»+ thymalin)	82.2±0.98	83.2±1.08	108.2±2.41[1, 2, 5]	133.3±1.65[1, 2, 6]

Note: [1] – significance of the differences compared to the indicator of group 1;
[2] – significance of the differences compared to the indicator of group 2;
[3] – significance of the differences compared to the indicator of group 3;
[4] – significance of the differences compared to the indicator on the 3rd day of the experiment;
[5] – significance of the differences compared to the indicator on the 7th day of the experiment;
[6] – significance of the differences compared to the indicator on the 14th day of the experiment.

Table 2. Mean values of the absolute number of CD3^{+}-cells

Group number	Day of experiment			
	3	7	14	28
Group 1 (holey defect)	190.7±4.42	241.2±4.48[4]	300.5±4.04[5]	334.8±3.23[6]
Group 2 (holey defect+bone graft «Biomin GT»)	239.3±3.07[1]	285.2±3.00[1, 4]	336.5±8.12[1, 5]	374.2±6.57[1, 6]
Group 3 (holey defect+thymalin)	339.3±10.66[1, 2]	385.7±10.73[1, 2, 4]	428.2±6.61[1, 2, 5]	470.3±4.15[1, 2, 6]
Group 4 (holey defect+bone graft «Biomin GT»+ thymalin)	344.2±2.43[1, 2]	393.8±5.17[1, 2, 4]	435.2±6.39[1, 2, 5]	473.7±7.33[1, 2, 6]

Note: [1] – significance of the differences compared to the indicator of group 1;
[2] – significance of the differences compared to the indicator of group 2;
[3] – significance of the differences compared to the indicator of group 3;
[4] – significance of the differences compared to the indicator on the 3rd day of the experiment;
[5] – significance of the differences compared to the indicator on the 7th day of the experiment;
[6] – significance of the differences compared to the indicator on the 14th day of the experiment.

Table 3. Mean values of the absolute number of CD68$^+$-cells and the relative number of CD86$^+$-, CD163$^+$-cells

Group number	Cell name	Day of experiment			
		3	7	14	28
Group 1 (holey defect)	CD68$^+$-cells	185.7±12.7	200.7±4.97	240.5±14.43[2]	268.3±26.44[3]
	CD86$^+$-cells	82.8±9.02	83.0±9.90	67.5±12.14[2]	56.0±8.60[3]
	CD163$^+$-cells	17.2±9.02[4]	17.0±9.90[4]	32.5±12.14[2,4]	44.0±8.60[3,4]
Group 2 (holey defect+bone graft «Biomin GT»)	CD68$^+$-cells	201.7±39.37	227.8±19.57[5]	279.8±15.60[2,5]	311.8±13.04[3,5]
	CD86$^+$-cells	81.5±8.46	82.3±7.66	70.8±11.58	56.3±2.94[3]
	CD163$^+$-cells	18.5±8.46[4]	17.7±7.66[4]	29.2±11.58[4]	43.7±2.94[3,4]
Group 3 (holey defect+thymalin)	CD68$^+$-cells	295.2±25.34[5,6]	334.5±9.79[1,5,6]	357.2±17.38[2,5,6]	387.3±12.75[3,5,6]
	CD86$^+$-cells	57.8±6.49[5,6]	49.67±5.82[1,5,6]	33.3±6.06[2,5,6]	18.3±5.16[3,5,6]
	CD163$^+$-cells	42.2±6.49[4,5,6]	50.3±5.82[1,5,6]	66.7±6.06[2,4,5,6]	81.7±5.16[3,4,5,6]
Group 4 (holey defect+bone graft «Biomin GT»+ thymalin)	CD68$^+$-cells	288.7±41.41[5,6]	338.5±10.82[1,5,6]	359.7±8.50[2,5,6]	383.7±17.14[3,5,6]
	CD86$^+$-cells	58.3±6.25[5,6]	49.8±5.49[1,5,6]	34.5±11.81[2,5,6]	16.8±5.31[3,5,6]
	CD163$^+$-cells	41.7±6.25[4,5,6]	50.2±5.49[1,5,6]	65.5±11.81[2,4,5,6]	83.2±5.31[3,4,5,6]

Note: [1] – significance of the differences compared to the indicator on day 3;
[2] – significance of the differences compared to the indicator on the 7th day;
[3] – significance of the differences compared to the indicator on the 14th day;
[4] – significance of the differences compared to the relative number of CD86$^+$-cells;
[5] – significance of the differences compared to the indicator of group 1;
[6] – significance of the differences compared to the indicator of group 2;
[7] – significance of the differences compared to the indicator of group 3.

that in groups 1 and 2 this indicator did not change ($p>0.05$) from the 3rd to the 7th day and increased ($p<0.05$) from the 7th to the 28th day. In groups 3 and 4, the absolute number of these immunopositive cells increased ($p<0.05$) from the 3rd to the 28th day.

When analyzing the average value of the relative number of macrophage phenotypes (M1-macrophages (CD86$^+$-cells) and M2-macrophages (CD163$^+$-cells)) in groups 1 and 2, the relative number of CD86$^+$-cells prevailed ($p<0.05$) at all days of the experiment. In groups 3 and 4, on the 3rd day, there was a predominance ($p<0.05$) of the relative number of CD86$^+$-cells, on the 7th day, no difference was found ($p>0.05$) in the relative numbers of CD86$^+$-cells and CD163$^+$-cells, on the 14th and 28th day, the relative number of CD163$^+$-cells prevailed ($p<0.05$).

During the period from the 3rd to the 28th day in group 1, the relative number of M1- and M2-macrophages did not change ($p>0.05$) from the 3rd to the 7th day, however, from the 7th to 28th day the relative number of M1-macrophages decreased ($p<0.05$), and the relative number of M2-macrophages increased ($p<0.05$) (table 3). In group 2, the relative number of CD86$^+$- and CD163+ cells did not change ($p>0.05$) from the 3rd to the 7th day, the relative number of CD86$^+$- and CD163$^+$-cells tended ($p>0.05$) to decrease and increase, respectively on the 14th day compared to the 7th day, and on the 28th day compared to the 14th day, the relative number of M1-macrophages decreased ($p<0.05$) against the background of an increase ($p<0.05$) in the relative number of M2-macrophages. In groups 3 and 4, from the 3rd to the 28th day, a decrease ($p<0.05$) in the relative number of M1-macrophages and an increase ($p<0.05$) in the relative number of M2-macrophages were recorded.

Intergroup analysis of the relative number of CD86$^+$-, CD163$^+$-cells showed no differences ($p>0.05$) in indicators in group 2 compared to group 1. The relative number of CD86$^+$-, CD163$^+$-cells did not differ ($p>0.05$) in groups 4 and 3, but in the latter, compared to groups 1 and 2, the indicator of the relative number of CD86$^+$-cells had a smaller ($p<0.05$) value, and the indicator of the relative number of CD163$^+$-cells had a greater ($p<0.05$) value.

DISCUSSION

The authors conducted a complex immunohistochemical study, which revealed the features of the expression of markers of the general population of T-lymphocytes (CD3), B-lymphocytes (CD20), macrophages (CD68) and their phenotypes (CD86 and CD163) in the post-traumatic regenerate of the lower jaw of rats under the conditions of bone filling defect with hydroxyapatite-containing osteotropic material "Biomin GT" and encircling the surrounding soft tissues with thymalin.

Thymalin, as is known, is a polypeptide complex isolated from the thymus that regulates the number and ratio of T- and B-lymphocytes, as well as their subpopulations, stimulates cellular immune responses, and enhances phagocytosis [11]. In cases of circumcision with thymalin of the soft tissues surrounding the bone defect of the lower jaw, the authors found an increase in the content of B-lymphocytes in the post-traumatic regenerate on the 14th and 28th day of the experiment.

B-lymphocytes regulate bone formation and have a significant role in the risk of bone metabolism disruption. B-lymphocytes ensure bone homeostasis by producing various cytokines and chemokines (TNFα, TNF-β, IL-6, IL-10)

[1]. Recent studies have disclosed a regulatory effect of B cells, indicating that B cells affect osteoclasts [12]. B cells antagonistically block the effect of RANKL (receptor activator of nuclear factor ligand) by secreting osteoprotegerin and promoting bone tissue regeneration [5].

Thymalin injection of the soft tissues surrounding the bone defect of the lower jaw, filled with hydroxyapatite-containing osteotropic material, led to an increase in the number of T-lymphocytes in the post-traumatic regenerate on the 3rd day of the experiment. Moreover, the absolute number of T-lymphocytes increased from the 3rd to the 28th day.

Activated T cells indirectly or directly regulate bone health and bone remodeling by secreting various cytokines, growth factors [13]. To date, it has been proven that T-lymphocytes affect the activity of bone tissue cells [14]. T cells are divided into αβ T cells and γδ T cells. αβ T cells are further subcategorised into $CD4^+$ and $CD8^+$ T cells, which have dual functions of promoting and inhibiting regeneration. γδ T cells are a small subset of T cells, which are considered to promote regeneration [12]. T cells affect macrophages and fibroblasts, which play important roles in bone tissue regeneration [15].

Deficiency of T- and B-lymphocytes is the cause of suppression of reparative osteogenesis, violations of the processes of mineralization and differentiation of osteoblasts [16].

The complex treatment measures carried out by the authors of the article also led to an increase in the total population of macrophages in the post-traumatic regenerate from the 3rd to the 28th day of the experiment. Against the background of an increase in the number of the total population of macrophages, changes in the content of their phenotypes were recorded: the number of M1-macrophages decreased and the number of M2-macrophages increased.

Macrophages act as phagocytes to prevent pathogens from invading remove necrotic tissue. Macrophages also secrete various cytokines, chemokines, growth factors to initiate the recruitment of fibroblasts, mesenchymal stem cells, and osteoprogenitor cells from their local niches [5]. It's a well-known fact that macrophages may convert into osteoclasts capable of resorbing bone [17].

Macrophages are highly plastic and dynamic cell populations that are capable of changing their phenotype. Today, most scientists distinguish two phenotypes of macrophages: "classically activated" pro-inflammatory M1 phenotype and "alternatively activated" anti-inflammatory M2 phenotype [18]. M1-macrophages secrete pro-inflammatory cytokines, which affect osteoblasts by inhibiting their differentiation and promoting apoptosis. M2-macrophages produce anti-inflammatory cytokines, transforming growth factor-β, vascular endothelial growth factors, bone morphogenetic proteins, which enhance the differentiation and function of bone-healing cells [17].

The correct balance between M1- and M2-macrophages is the key to successful reparative osteogenesis [17].

CONCLUSIONS

A comprehensive experimental and morphological study conducted by the authors revealed that thymalin injection of the soft tissues surrounding the bone defect of the lower jaw, filled with hydroxyapatite-containing osteotropic material "Biomin GT", stimulates local immune reactions in the post-traumatic regenerate, which is manifested, firstly, by an increase in the number T-lymphocytes on the 3rd day of the experiment and their increase up to the 28th day; secondly, by increasing the number of B-lymphocytes on the 14th day of the experiment with their further increase up to the 28th day; thirdly, by increasing the number of macrophages on the 3rd day of the experiment and their growth up to the 28th day; fourth, changes in macrophages phenotypes (decrease in the number of M1-macrophages and increase in the number of M2-macrophages). Stimulation of local immune reactions in the post-traumatic regenerate can be one of the mechanisms that activate reparative osteogenesis in the lower jaw of rats under the conditions of filling bone defects with hydroxyapatite-containing osteotropic material "Biomin GT" and thymalin injecting the surrounding soft tissues.

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CONFLICT OF INTEREST
The Authors declare no conflict of interest

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