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Effect of Bioactive Peptide Complex Isolated from Bovine Serum on Proliferation and Migration of Mesenchymal Stromal Cells *In Vitro* and Reparation of Bone Defects *In Vivo*

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We studied the effect of a bioactive peptide complex isolated from bovine serum on the proliferative potential and migration rate of mesenchymal stromal cells *in vitro*, as well as on the healing of modeled bone defects in rats. This bioregulatory preparation stimulated proliferation of mesenchymal stromal cells from deciduous tooth pulp *in vitro*, but did not affect the rate of their migration in two-dimensional cultures. *In vivo* experiments showed that application of this preparation in combination with hydroxyapatite and chitosan gel accelerated bone tissue regeneration, thus ensuring restoration of morphologically normal bone matrix. Thus, cattle blood serum is an available source for the production of bioregulatory preparations for medical purposes.

Key Words: *bioregulator; blood serum; regenerative medicine; mesenchymal stromal cells; tooth pulp*

In modern regenerative medicine, tissue engineering constructs containing progenitor cells, extracellular matrix components, and growth factors are implanted in the area of damage to restore extensive bone defects. Growth factors can modulate the course of the reparation process via additional activation of the regenerative potential of the recipient, in particular, attraction of host multipotent cells and stimulation of their proliferative activity.

We consider the possibility of using membranotropic homeostatic tissue-specific bioregulator (MHTB) derived from the bovine serum in osteoregenerative therapy. MHTB is a complex of peptides with molecular weights of 1448, 1338, and 1151 Da and a modulator protein, a representative of the serum albumin multifamily [11,13]. These proteins have been previously described in detail. For a peptide with a molecular weight of 1338 Da, N-terminal amino acid sequence (DTPKLEIAAAFK) was determined; it has a 50% homology with the precursor of bovine preproadrenomedulline [10]. For a peptide with a molecular weight of 1448 Da, partial C-terminal amino acid sequence (GGPLASLLLR) was determined by MALDI-TOF mass spectrometry; it has a 100% homology with the N-terminal fragment of P-cadherin identified in the endothelium of the bovine aorta [12]. Partial C-terminal amino acid sequence of a 1151 Da peptide (AYVSP) is identical to bovine granular cell pseudokinase TRB-2 fragment.

It was found [11] that these peptides and the modulator protein in the presence of calcium ions form a complex exhibiting biological activity in ultra-low doses, which prompted a hypothesis about possible osteoinductive properties of MHTB.

The aim of this work was primary assessment of the possibility of medical application of MHTB for bone tissue engineering. To this end, we studied *in*

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vitro effect of the preparation on cultured human mesenchymal stromal cells (MSC) and its *in vivo* effect on healing of modeled bone defects.

MATERIALS AND METHODS

Cell cultures. Cryopreserved cultures of MSC from human deciduous tooth pulp were obtained from the Cryobank of the V. N. Orekhovich Research Institute of Biomedical Chemistry. After defrosting, the cells were cultured in complete growth medium (DMEM/F-12 (1:1) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin; all reagents were from Gibco) in a CO_2-incubator under standard conditions (5% CO_2, 37°C, 80% humidity) until 80-90% confluence; the medium was replaced 2 times a week. Cells of passages 4-6 were used in the experiments.

Evaluation of the effect of MHTB on the proliferative potential of MSC. The number of viable cells was assessed using CytoTox 96 kit. This method is based on quantitative colorimetric assessment of lactate dehydrogenase activity in lysed cell cultures that is directly proportional to the content of viable cells. Cell cultures were transferred to a 24-well plate. To this end, the cells were incubated with 0.25% trypsin and Versene (1:1; PanEco) for 5 min at 37°C, the cell suspensions were centrifuged, and resuspended in a medium containing MHTB in concentrations of 10^{-13}, 10^{-12}, and 10^{-11} mg/ml) (experimental groups) or in a growth medium without MHTB (control group); 2-ml aliquots (12,500 cells/ml) were transferred to plate wells. In 1, 3, 5, 7, and 10 days of standard culturing, the number of viable cells in wells was assessed using CytoToxAssay kit (Promega) according to manufacturer's protocol with some modifications. The growth media was removed from the wells, the cells were washed 3 times with PBS (2 ml) and then 300 μl PBS was added to each well and the plates were subjected to 3 freezing/thawing cycles for destruction of cell membranes. Then, 50-μl aliquots of cell lysate were transferred to wells of a 96-well plate, mixed with 50 μl substrate reagent, and incubated for 60 min in the dark at room temperature. The reaction was stopped by adding 50 μl stop reagent. Optical density was measured on a Tecan Infinite 200 Pro microplate reader (Tecan).

Evaluation of the effect of MHTB on the rate of MSC migration. The study was performed using an IncuCyte Zoom automated interactive microscope (Essen Biosciences). The cells were cultured in a medium containing a protein preparation in a working concentration for 48 h, then suspended as described above. Aliquots of the resulting suspension (100 μl; cell concentration 125,000/ml) were placed in a 96-well ImageLock plate (Essen Biosciences), incubated for 24 h under standard conditions, and then, standard scratches were made in the cell monolayer with a Wound Maker (Essen Biosciences). The wells were washed 3 times with medium to remove detached cells and 200 μl medium containing the studied preparation was added. The plate was placed in an IncuCyte Zoom device in a CO_2 incubator and incubated under standard conditions for 72 h. Cell migration was photographed every 30 min using IncuCyte Zoom applied software. The cells cultured in the growth medium without the test preparation throughout the experiment served as the control.

***In vivo* experiments.** The experiments were performed on male Wistar rats weighing 180-220 g (n=72) kept under standard conditions in a vivarium of N. K. Koltsov Institute of Developmental Biology. The animals were anesthetized with ether and defects in the distal epiphysis of the femur (2×2 mm) was modeled, after which they were divided into 4 groups (18 rats per group). The bone defects were either left unfilled (group 1) or filled with Collost preparation (Biofarmholding) (group 2), a mixture containing 70% chitosan gel (Bioprogress) and 30% hydroxyapatite (GAP; hydroxyapol gap-99g-0.5; Polistom) (group 3), Matribon composition (30% GAP and 70% chitosan gel supplemented with 10-12 mg/ml MHTB). The animals were sacrificed on day 8, 15, and 31 of the experiment. Fragments of the femoral bones containing the defects and surrounding healthy tissues at a distance of 2-3 mm from the defect were fixed in 4% paraformaldehyde, washed in running water, and dried in air. The samples were dusted with gold using a Sputter Coater SCD-005 device (BAL-TEC), and the preparations were examined under a LEO 1430VP scanning electron microscope (Carl Zeiss).

Statistical analysis. Statistical analysis was performed using the free-access R project (www.r-project.org). The differences between the groups were evaluated using non-parametric Mann—Whitney test. The differences were considered statistically significant at $p \leq 0.05$.

RESULTS

In our previous studies, we have shown that MHTB stimulated adhesion, migration, and proliferation of connective tissue cells, thus providing its repair with the preservation of all morphological units [3-5,8]. The effectiveness of the preparation for healing of skin wound and burns [5,8] and cartilage defects [6,7] has been demonstrated. Here, the possibility of MHTB application in bone tissue engineering was evaluated.

Mesenchymal cells from human deciduous tooth pulp were used as a model cell culture for evaluation

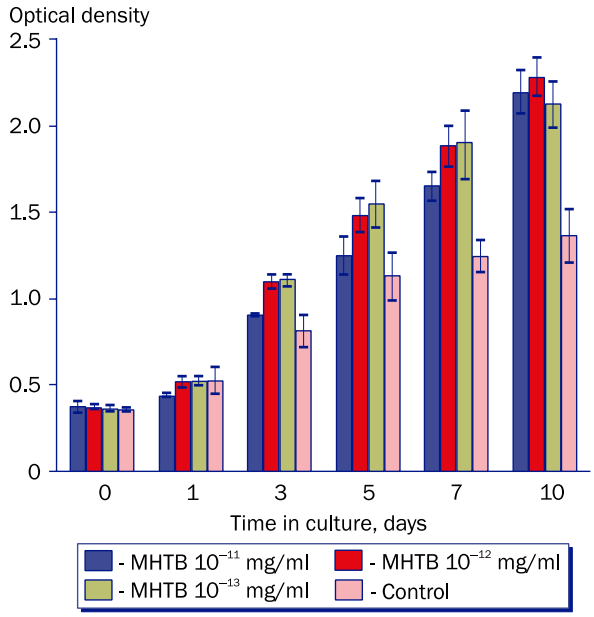

Fig. 1. Effect of MHTB on proliferation of MSC from deciduous tooth pulp.

of the biological effect of the studied bioregulatory peptide complex. The cells derived from this source possess the main characteristics of MSC: they have typical profile of the expression of surface markers and are capable to multilineage differentiation, in particular, towards bone tissue cells [2]. Due to their availability, MSC from deciduous tooth pulp are a promising cell material for regenerative medicine [1]. The effect of MHTB on the proliferative potential of cultured cells was studied over 10 days.

Starting from day 3 of culturing with the test preparation, stimulation of proliferative activity of MSC was observed (Fig. 1). The effect of MHTB persisted throughout the experiment, due to which the number of cells cultured with the preparation was significantly higher than in the control culture. There were no significant differences in the efficacy of the three MHTB concentrations used.

It was hypothesized that MHTB can modulate migration activity of MSC. The rate of cell migration was assessed using an IncuCyte Zoom automated microscope (Essen Biosciences). The study represented an adapted scratch-test. In brief, after the scratches were created in the monolayer cell cultures (90-100% confluence), photographic registration of their filling by migrated cells was performed. The rate of scratch healing was assessed as the percentage of surface area inside the scratch covered with cells to the initial scratch area. To this end, the corresponding parameters for analysis of the images were selected in the IncuCyte software (Fig. 2).

It was shown that healing of the scratches in all wells was completed by day 3 after infliction. There were no differences in the dynamics of this process in the presence of MHTB in the working concentration and in the control culture (Fig. 3).

These findings indicate that the preparation stimulates the proliferation of MSCS from the deciduous tooth pulp, but does not affect the rate of cell migration in the scratch test. It should be noted that this method allowed assessing only the speed of cell movement, but not chemoattractant potency of MHTB in stimulating directed cell migration.

To assess the effect of the studied peptide complex on *in vivo* bone tissue reparation, 2-mm defects were modeled in the femoral bones and MHTB in combina-

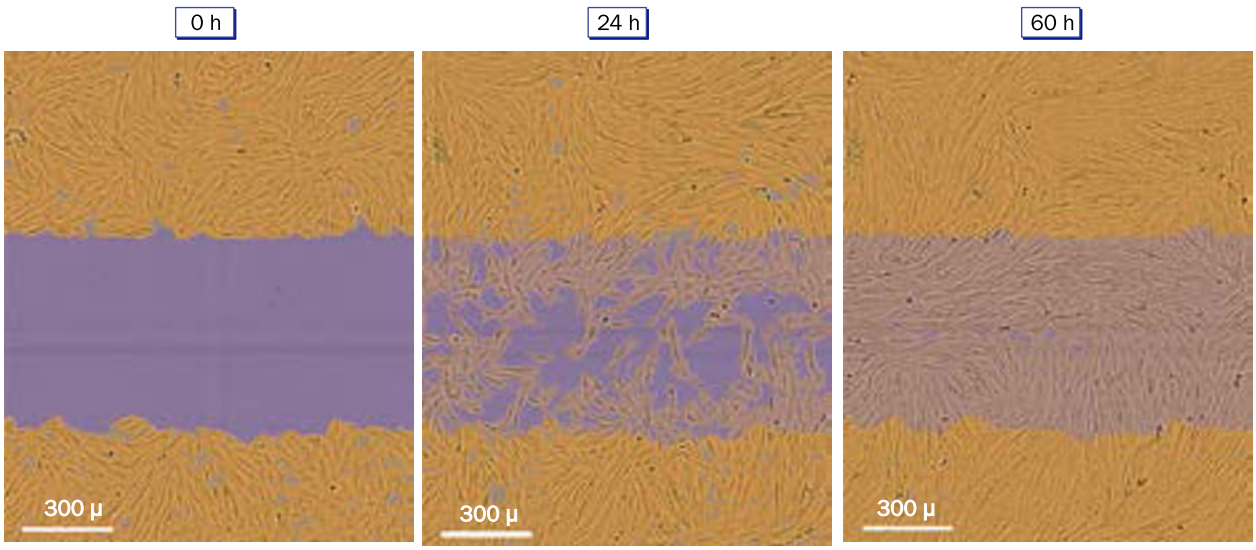

Fig. 2. Evaluation of the migration rate of MSC from deciduous tooth pulp immediately (0 h) and in 4 and 60 h after scratching. Scratch area is highlighted in blue, the area covered by cells is highlighted in yellow.

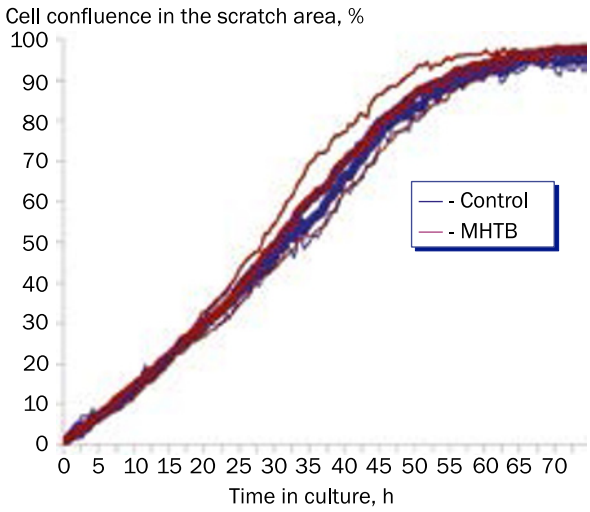

Fig. 3. The effect of MHTB on the migration rate of deciduous tooth pulp MSC in the scratch test.

tion with chitosan gel and GAP (Matribon composition) was introduced into the defect area. In the control groups, the defects remained unfilled or filled with components of Matribon composition without MHTB, or Collost preparation.

In control animals (no therapy), visible bulky wound defects with pronounced signs of inflammation were detected on day 8 after the beginning of the experiment. In 15 days, the formation of the bone matrix was noted along the borders of the defects. By day 31, the defects were partially filled with amorphous bone tissue with a large number of cavities of different sizes (Fig. 4).

When the defects were filled with Collost, wounds closure was visually detected in 8 days after injury, but the defect area looked depressed with residual signs of the inflammation process. Scanning electron microscopy revealed empty spaces between the boundaries of

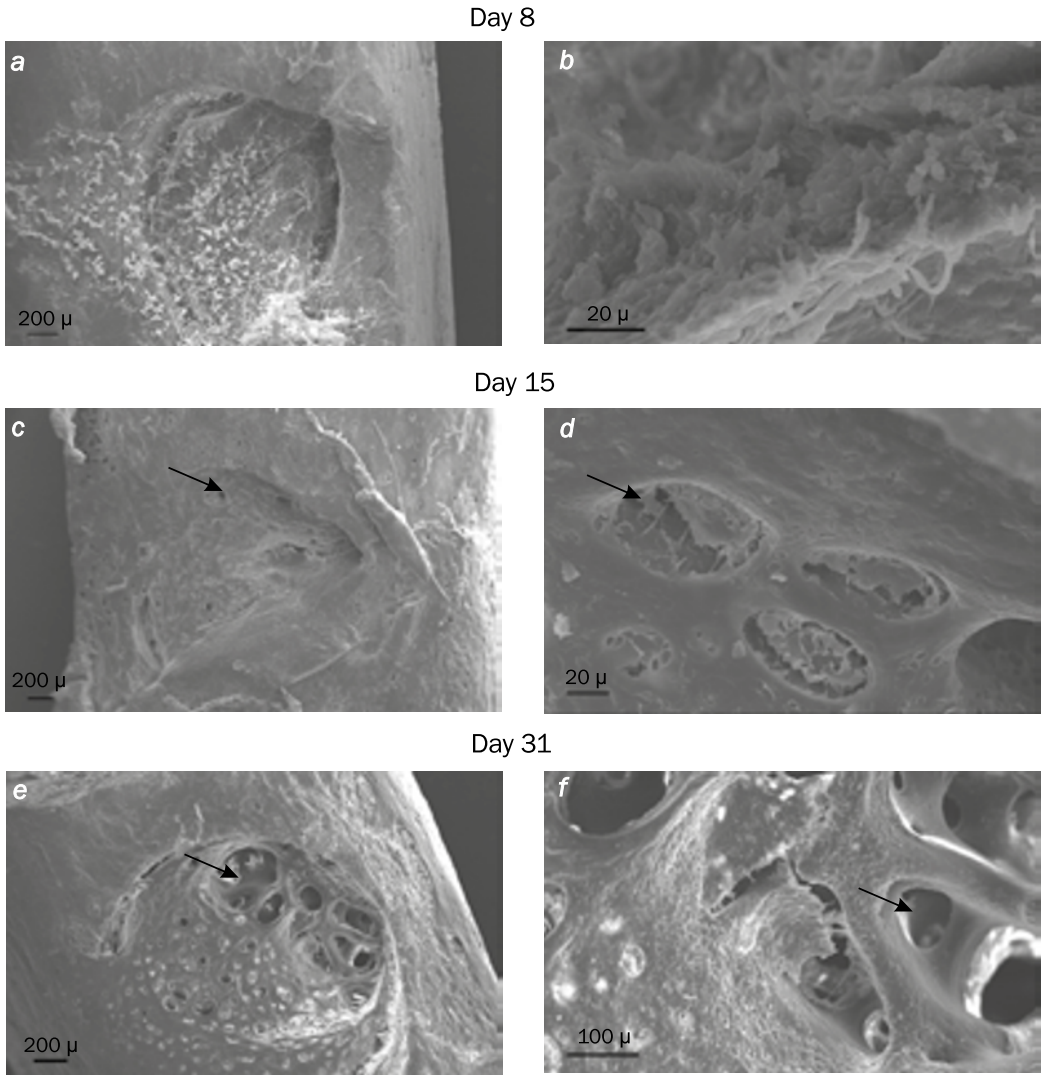

Fig. 4. Healing of femoral bone defects in rats of the control group: without filling of defects. Scanning electron microscopy, low (*a*, *c*, *e*) and high (*b*, *d*, *f*) magnification. Arrows show cavities in newly formed loose bone tissue.

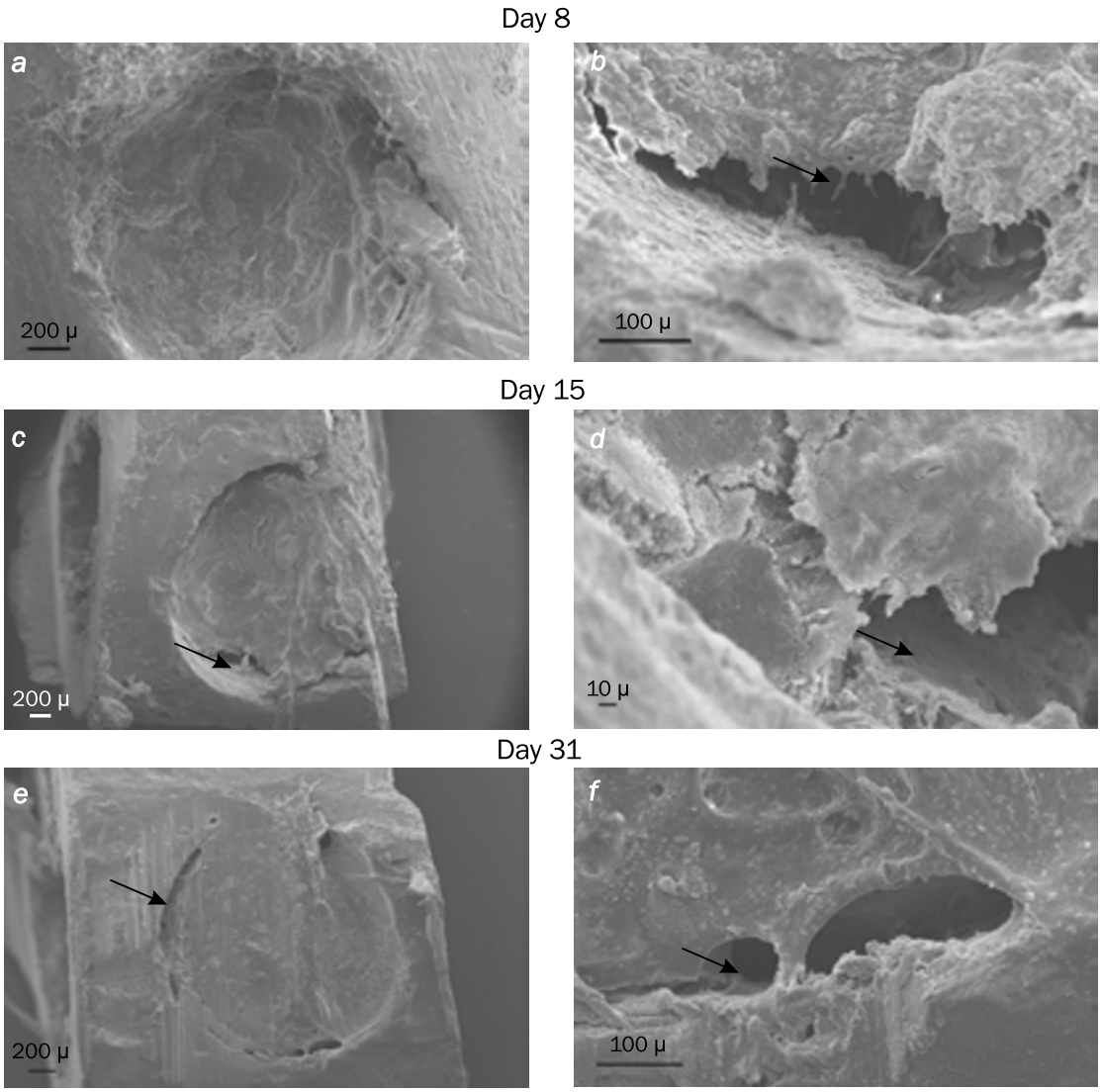

Fig. 5. Healing of femoral bone defects in rats after implantation of Collost preparation. Scanning electron microscopy, low (*a*, *c*, *e*) and high (*b*, *d*, *f*) magnification. Arrows show cavities in newly formed loose bone tissue.

the wound cavity and preparation granules. By day 15, the defects were partially filled with the bone tissue with the formation of numerous cavities; their number somewhat decreased by day 31 (Fig. 5). By the end of the experiment, the space between the edges of the defect and the introduced preparation remained unfilled.

Implantation of GAP with chitosan gel (without MHTB) also led to only partial repair of the defects. By day 15, osteogenesis was weakly expressed: GAP particles occupying the defect cavities were only slightly coated with the bone matrix. In 31 days, the space between the edges of the defects and GAP particles was filled with the bone tissue, but similar to the variant with Collost transplantation, the formation of cavities was observed (Fig. 6).

After application of Matribon composition (MHTB in combination with GAP and chitosan gel), pronounced signs of neoosteogenesis in the area of damage were observed as soon as on day 8. In 15 days after surgery, free space in the defect cavity was filled with regenerating bone tissue. By that term, GAP particles were completely coated with the newly formed bone matrix. In 31 days after implantation of the composition, complete filling of the defect cavity was observed, GAP crystals were completely incorporated into the bone matrix (Fig. 7). No cavities were seen.

The results of *in vivo* experiments showed that complete recovery of the model defects occurred only in animals treated with Matribon composition. Only in this case, the area of damage contained no cavities by day 31 after the beginning of the experiment. Implantation of Collost widely used in reconstructive surgery led to only partial filling of the defect space with the bone tissue containing a large number of cavi-

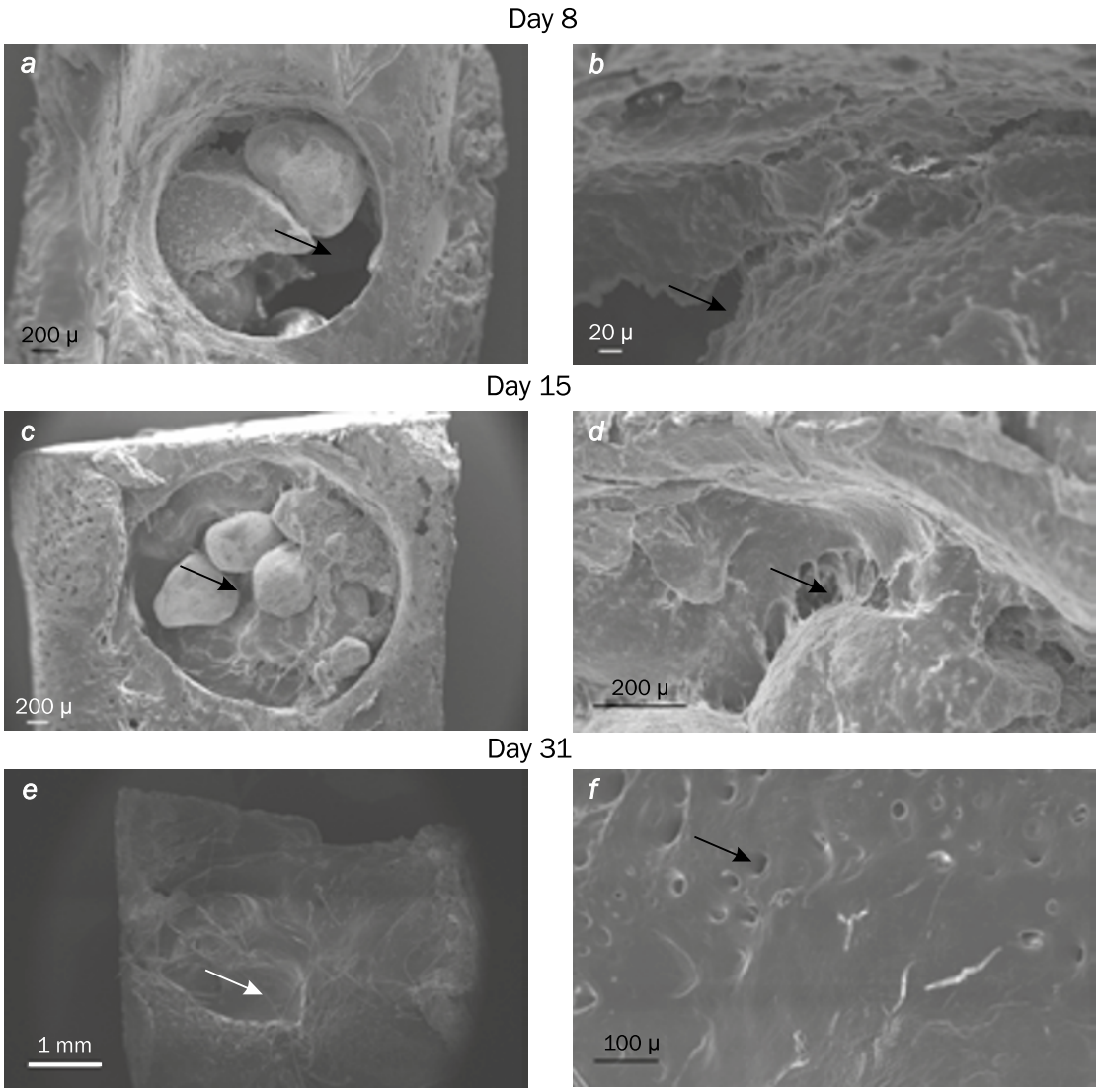

Fig. 6. Healing of femoral bone defects in rats after implantation of GAP with chitosan gel. Scanning electron microscopy, low (*a*, *c*, *e*) and high (*b*, *d*, *f*) magnification. Arrows show cavities in newly formed loose bone tissue.

ties. Similar results were obtained after implantation of individual components of Matribon composition without MHTB, which indicated that the presence of this preparation ensures high efficiency of the preparation.

Our findings allow us to conclude that the bioregulatory preparation developed by us can be used as a component of tissue-engineered bone analogues for induction of cell proliferation and stimulation of neoosteogenesis. The obtained data are consistent with the results of the evaluation of the effect of Matribon composition on healing of model bone defects in rats [9]. Further study of the biological properties of the bioregulator will include analysis of its effect on cell differentiation and stimulation of the migration of recipient's progenitor cells to the area of injury.

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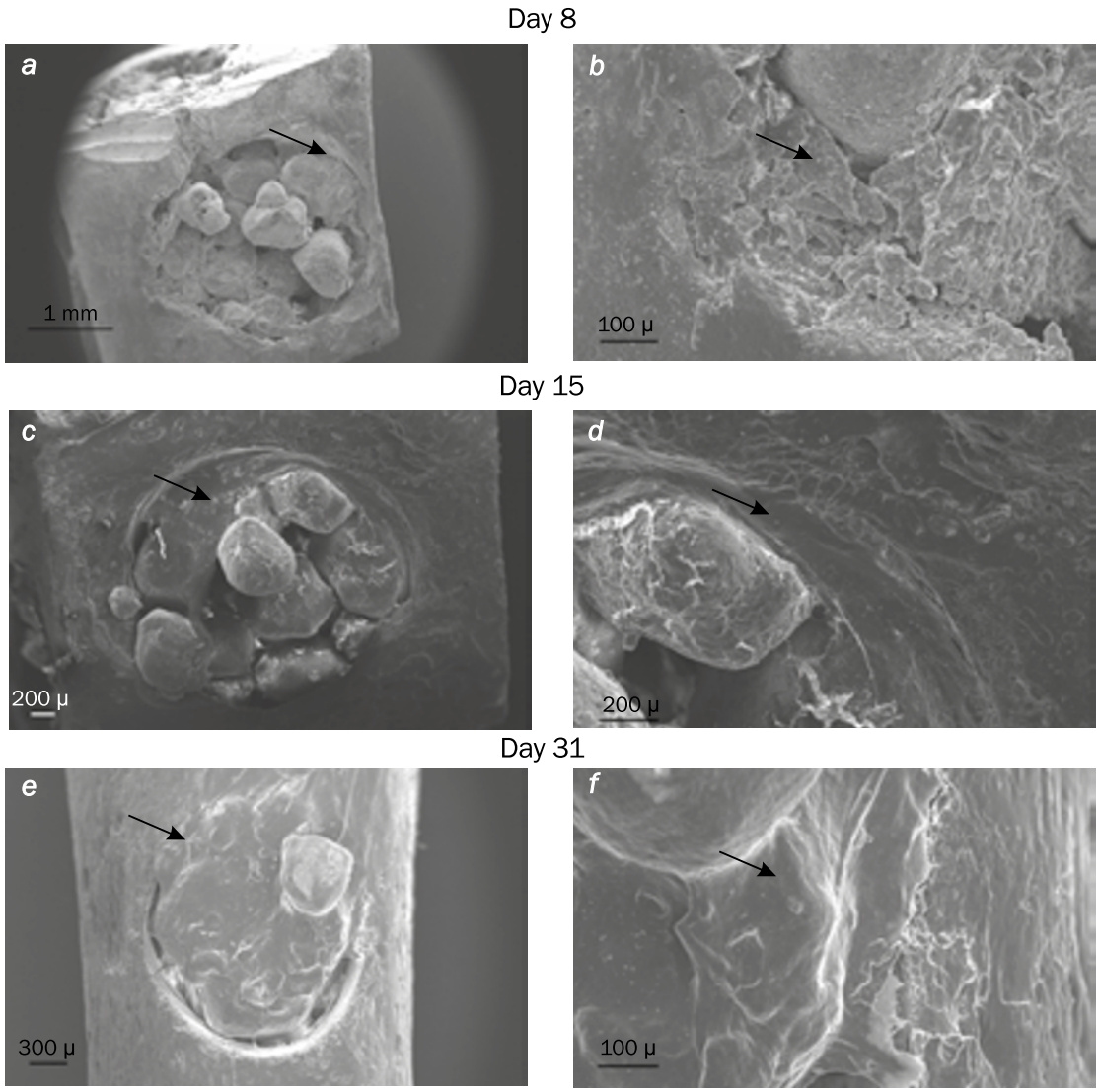

Fig. 7. Healing of femoral bone defects in rats after implantation of Matribon composition. Scanning electron microscopy, low (*a*, *c*, *e*) and high (*b*, *d*, *f*) magnification. Arrows show marked signs of neoosteogenesis and newly formed dense bone tissue that did not differ from the adjacent normal bone tissue.

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