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Stimulation of osteoreparation in the zone of modeled femoral pseudarthrosis in rats using autologous bone marrow

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Purpose To study the morphological features of bone regeneration by using a local injection of autologous red bone marrow in the conditions of an experimentally produced pseudarthrosis. **Materials and methods** The study was conducted on thirty (30) white laboratory rats that were bred at the Sitenko Institute for Spine and Joint Pathology of the Ukraine National Academy of Medical Sciences (6 months old, weight of 250-270 g). The experiment was conducted according to the international rules of experimental animals protection. The protocol of the experimental studies was approved by the ethics board of the Sitenko Institute for Spine and Joint Pathology of the National Academy of Medical Sciences of Ukraine. **Results** Culturing of mesenchymal stromal cells requires high-tech laboratory equipment and takes a long period of time. Therefore, their widespread clinical use is limited. Native bone marrow that contains mesenchymal stromal cells is easily available. The process of its preparation and transplantation is not technologically difficult. Therefore, it has advantages for being used for stimulation of reparative osteogenesis in bone fractures or in disorders of reparative osteogenesis. **Conclusion** The study found that autologous red bone marrow stimulated bone tissue development in the zone of pseudarthrosis. The area of bone tissue was 22.8 % larger as compared with the bone area in the untreated animals. There were no pseudarthrosis-related cavities in the regenerate structure in the cases of red bone marrow injection into the zone of traumatic injury. The features of reparative process were the following: angiogenesis activation, enchondral ossification accompanied by cancellous bone tissue formation with a network of bone trabeculae, and decrease in the destructive manifestations in the maternal bone parts located above and below the injury area.

Keywords Experiment, bone marrow, stromal cells, osteogenesis, rats

INTRODUCTION

Treatment of patients with posttraumatic bone regeneration disorders is one of the important issues in traumatology and orthopaedics. As reported by several authors, the incidence of the disorders in reparative osteogenesis following injuries is rather high, from 2.5 to 25.0 % [5, 10]. Such a situation forces researchers to look for new approaches to the problem of treating patients with non-uniting fractures and pseudarthrosis.

Recently, a new direction, a regenerative medicine, has been actively developing in practical medicine. Various studies in the field of bone regeneration were performed with the application of cell technologies that used stromal cells from red bone marrow or fat tissue, thrombocytes or thrombocytic growth factor, bone morphogenetic proteins, insulin-like growth factor, transforming growth factor, fibroblast growth factor, and cytokines [17, 18, 20]. Their use seems particularly important for the disorders of reparative osteogenesis such as a delay in bone consolidation or pseudarthrosis. There were experimental works that studied the effect of native bone marrow on osteoreparation when it was introduced into bone defects [3, 15], or used in distraction osteogenesis [12]. However, there are only a few experimental and clinical studies that investigated the processes that take place in the regenerate following the transplantation of native red bone marrow or cultured bone marrow stromal cells into the pseudarthrosis area [1, 7, 15].

Purpose To study the morphological features of bone regeneration after a local injection of autologous red bone marrow in the conditions of an experimentally produced pseudarthrosis.

MATERIALS AND METHODS

The experiment was performed on 30 white laboratory 6-months old rats (weight between 250 g and 270 g) that were bred at the *Sitenko Institute for Spine and Joint Pathology* of the National Academy of Medical Sciences of Ukraine. The work was conducted according to the international rules of humane attitude to experimental animals [14] and the protocol of experimental studies was approved by bioethics committee (protocol No 120 from 09.09.2013).

Surgical technique Surgical intervention was performed under general anesthesia (intramuscular injections of aminazinum in the dosage of 10 mg/kg of live weight and ketamin in the dosage of 50 mg/kg of live weight. Transverse osteotomy in the middle third of the femur was produced using a Gigli saw. The wound was closed with full-thickness sutures.

Additional fixation of bone fragments was performed for 14 days using PVC (polychlorovinyl) tubes, 2 or 3 cm long, that are used in the system for intravenous injections and were strengthened with a copper wire. Immobilization of this type allowed the animals to move around the cage and bear weight on the limb. Immobilization was withdrawn on day 14, at the stage of tissue-specific regeneration (regenerated soft tissues). The animals loaded a functionally imperfect limb.

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The formation of pseudarthrosis in the osteotomy area was checked with X-rays at several time-points (days 21, 35 and 49). Our previous study showed that pseudarthrosis in the rats happened by day 49 following osteotomy [9]. At that time point, the animals were divided into two groups:

1) a control group (animals without treatment, injection of 0.3-ml of the physiologic saline into the area of pseudarthrosis;

2) an experimental group (injection of autogenous red bone marrow into the area of pseudarthrosis.

On day 49, autogenous red bone marrow cells were injected into the area of the pseudarthrosis formed (into its central part and in the proximity to bone fragments) of the animals of the experimental group. Bone marrow was obtained from the femur of the non-operated limb under general anesthesia immediately before the injection. For this purpose, a transcortical perforated defect was produced in the left femur laterally in the area of the distal metaphysis using a dental drill (1.3-1.5 mm in diameter) under aseptic conditions, and an amount of 0.4 to 0.5 ml of red bone marrow was aspirated using a syringe needle. The aspirate of red bone marrow in the volume of 0.3 ml was injected into the pseudarthrosis area of the right femur. It had been found by previous experiments that 0.3 ml of bone marrow was enough to be introduced into the zone of modeled pseudarthrosis in the rat's femur. The limbs of the animals from the experimental and control groups were splintered in a physiological position.

After surgery, the animals were under a constant observation. They were withdrawn from the experiment on days 7, 14, 28 after pseudarthrosis had been formed (day 49+) by intramuscular injection of a lethal dose of 20 % thiopental sodium solution. The time-points of withdrawal were chosen according to the phases of reparative osteogenesis development.

Methods of the study Histological and histomorphometrical methods were used to evaluate the condition of regeneration in the zone of femoral osteotomy. The femurs were processed according to a standard technique [11]. The material was fixed in a 10 % solution of neutral formalin, decalcified in a 5 % solution of nitric acid, dehydrated in the ascending solutions of ethyl alcohol (from 60° to 96°) and of ethyl alcohol with diethyl ether (1:1), and compacted in thick celloidin with chloroform vapor. The material was embedded in celloidin. The sections of the femur with a fracture zone (7-10-μm thick) were obtained using Reichert sledge-type microtome and then stained with ferric Veigert hematoxylin and eosin. The investigation and photos of histological sections were performed using an *Axio Star Plus* light microscope (eyepiece 10×, objectives – 4×, 10×, 40×).

Morphometrical methods were used to evaluate the area of the total regeneration and the areas of its tissue types (bone, chondroid and fibrous connective ones; percentage from the total regenerate area (100 %). The tissue areas were measured in mm with an *Olympus BX-60* microscope in all the observation periods. The numerical values were processes by the variation statistics technique using the Student's t-test.

RESULTS AND DISCUSSION

The animals of the control and experimental groups were active throughout the study period, used their operated limb for weight-bearing but limped. The animals' behavior and food consumption was normal. The thigh in the fracture area was thickened.

Microscopic investigation. In the experimental group on day 7 *(49+7)* after red bone marrow injection, the study of the femur preparations that included the regenerated zone established that the regenerate that connected the bone fragments was represented by the bands of dense fibrous connective tissue and intermittent chondroid fields. High density of low differentiated cells and cells of osteoblast lineage was noted in the connective tissue of the regenerate marginal and central parts that is in the assumed zones of red bone marrow injections. The foci of osteoblast proliferation were also revealed (**Fig. 1a**). They had large nuclei and abundant basophilic cytoplasm. Clusters of cells formed osteoids.

Replacement of chondroid parts by both cancellous bone tissue of a small loop-like structure (**Fig. 1b**), and foci of the forming osteoid were noted. The tape-shaped proliferates from osteoblasts were arranged on the surface of the newly formed bone trabeculae (**Fig. 1b**).

Extensive chondroid areas and small areas of newly formed bone tissue in the marginal parts of the fragments prevailed in the regenerate of the control group animals.

Fields of dense fibrous connective tissue (**Fig. 2**) as well a chondroid area with chondrocytes that were of different size were present in the zone of the regenerate between the femoral fragments on day 14 *(49+14)* after injection of bone marrow cells (similar to 7 days).

Unlike in the regenerate on day 7, random fissure-like blood vessels were observed in its connective tissue and chondroid parts (**Fig. 3a**) that indicated the start of the regenerate reorganization process. Blood vessels of the capillary type that sprouted into the regenerate from the intertrabecular spaces of the adjacent bone tissue were revealed in some parts of the regenerate. Bone tissue was formed around some of them (**Fig. 3b**).

In some places, presumably in the area of bone marrow injection, clusters of large clearly basophilic cells were determined of the phenotype that was similar to osteoblast lineage cells (**Fig. 4**).

Sporadic newly formed bone trabeculae with brightly colored osteocytes in high density on the surface were located in the medullary canal, proximal and distal to the formed regenerate, along the cortex on the endosteal surface. Some of them grew out into the regenerate thickness. At a distance from the fracture, the medullary canal contained red bone marrow.

The signs of destructive changes were revealed in the area of the cortices of the femoral fragments located proximal and distal to the fracture; these signs were a non-uniform density of osteocytes, foci of bone matrix without osteocytes, as well as microcracks. Areas of bone matrix rarefaction represented by acellular zones were revealed in some parts. Some vascular bone canals were widened.

Compact bone tissue along the femoral sides was well-marked on the X-rays in the experimental group animals on day 28 *(49+28)* (**Fig. 5b**). The shaft was somewhat thickened in the area of the femoral osteotomy. Formation of the regenerated bone continued. The zone of traumatic injury in the control group was radiolucent (**Fig. 5a**).

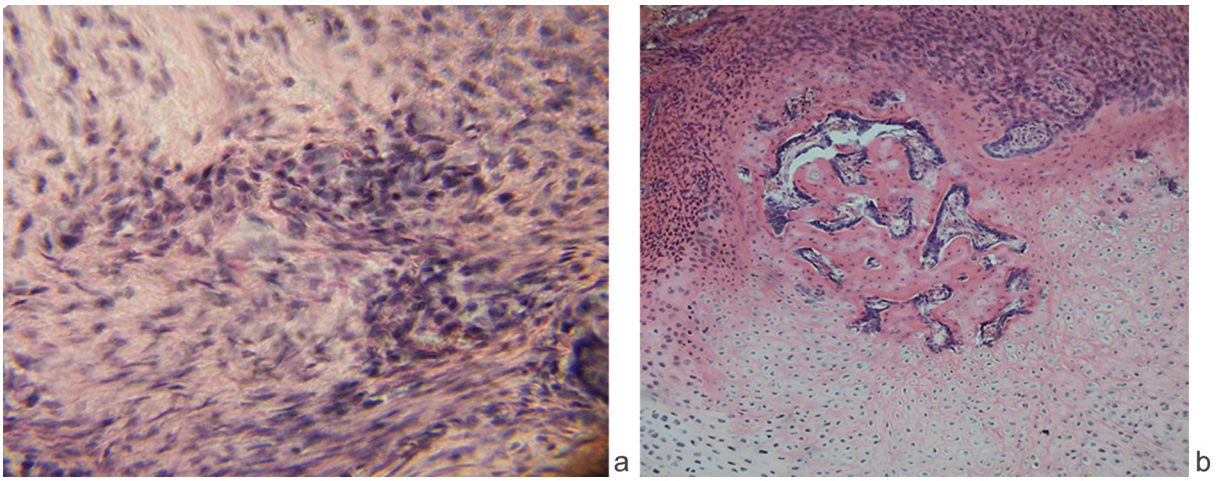

Fig. 1 Regenerate parts: foci of osteoblast proliferation in the regenerate connective tissue (**a**). Newly formed bone tissue in the central part of a chondroid (**b**). 7 days after red bone marrow injection. Hematoxylin and eosin staining: Magnification ×400 (**a**). Magnification ×200 (**b**)

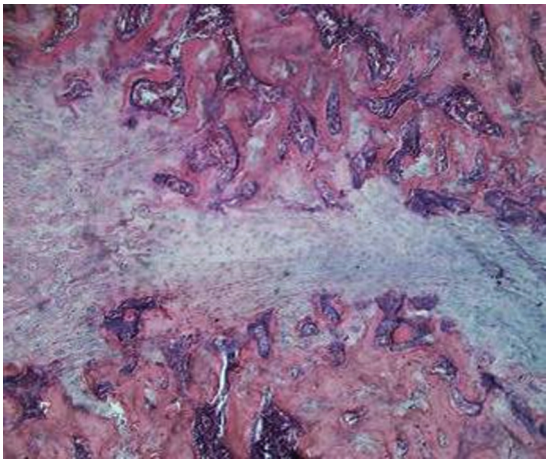

Fig. 2 Zone of pseudarthrosis. Dense fibrous connective tissue in the regenerated area that connects the femoral fragments after osteotomy. Day 14 after injection of autologous red bone marrow. Hematoxylin and eosin. Magnification ×40

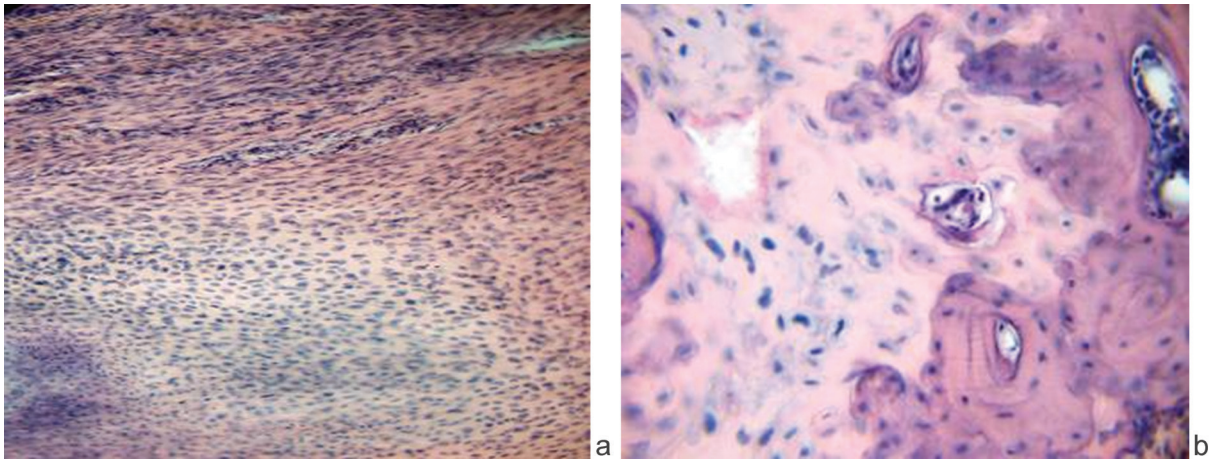

Fig. 3 Parts of the regenerate: foci of chondroid and dense fibrous tissue (**a**). Vessels of capillary type. The regenerate part presented by a chondroid with blood vessels and formed bone tissue around them (**b**). Day 14 after injection of autologous bone marrow. Hematoxylin and eosin staining: Magnification ×200 (**a**). Magnification ×400 (**b**)

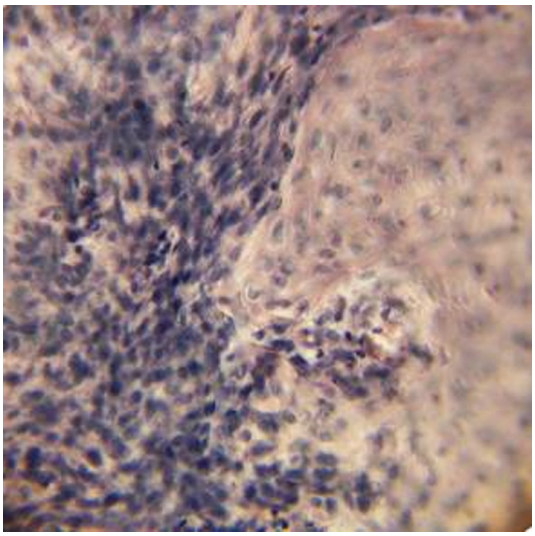

Fig. 4 Area of a supposed injection of bone marrow cells. Clusters of osteoblast lineage in the regenerate. Day 14 after autologous red bone marrow injection. Hematoxylin and eosin. Magnification ×400

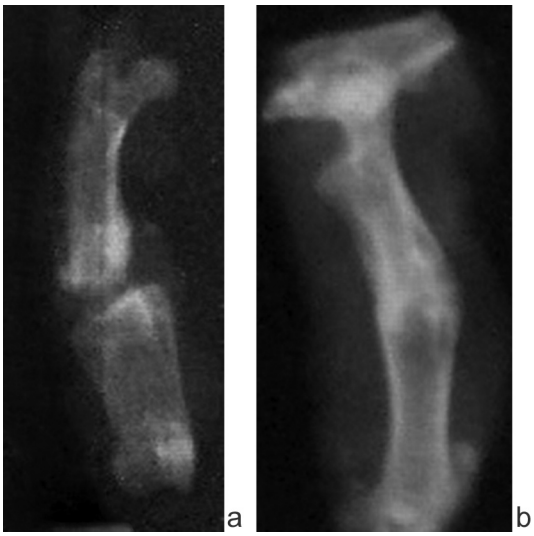

Fig. 5 Femoral X-rays of the control (**a**) and experimental (**b**) group on 49 + 28 day

Histologically, the regenerate in the zone of femoral fracture in the experimental group animals on day 28 was represented not only by the portions of dense fibrous connective tissue and chondroid but also by extensive fields of newly formed bone tissue. In the chondroid area of practically all the preparations, the sprouting blood vessels with the surrounding bone tissue that was represented by a network of bone trabeculae were observed. Intertrabecular spaces were filled with red bone marrow. Unlike the regenerate by day 14, the newly formed bone tissue by day 28 was characterized by high osteocyte density. Bone tissue was also determined in the periphery parts – on the endosteal surface of the cortex, proximal and distal to the fracture area.

Extensive areas of the newly formed bone tissue which replaced chondroid were found (**Fig. 6a**).

We believe that the formation of such bone tissue zones is associated with the injection of red bone marrow cells. A distinctive feature of the histological picture on day 28 was the presence of the zones of practically a completely reorganized regenerate, represented by bone tissue (**Fig. 6b**).

The areas of connective tissue and chondroid prevailed in the pseudarthrosis zone of the control group animals (**Fig. 7**)

Weakly expressed changes were revealed in the cortex structure of the distal and proximal femoral fragments: increase in vascular density, uneven staining of the intercellular substance, and different density of osteocytes in the areas. They showed reactive reorganizations of the cortex in response to a traumatic injury. Low density osteocyte areas prevailed in the control series along with the above mentioned changes.

The area of the tissues (chondroid, bone and connective ones) was determined morphometrically at each time-point in order to objectively reveal the morphological differences in the regenerate structure of the control and experimental groups (**Table 1**).

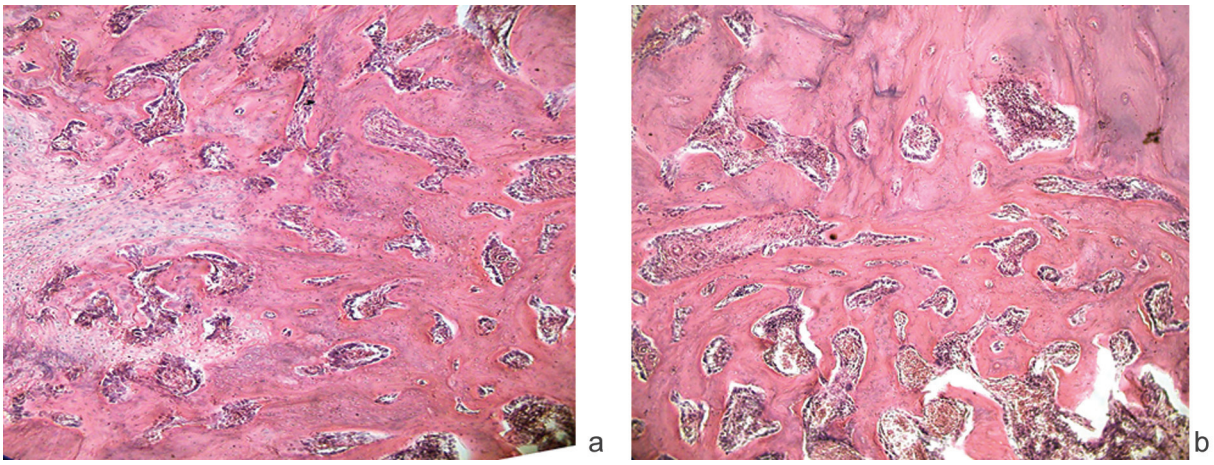

Fig. 6 Areas of the regenerate formed: fields of newly formed bone tissue replace the chondroid in the regenerate (**a**). Extensive fields of bone tissue that have replaced the chondroid in the regenerate (**b**). Day 28 after autologous red bone marrow injection. Hematoxylin and eosin. Magnification ×100

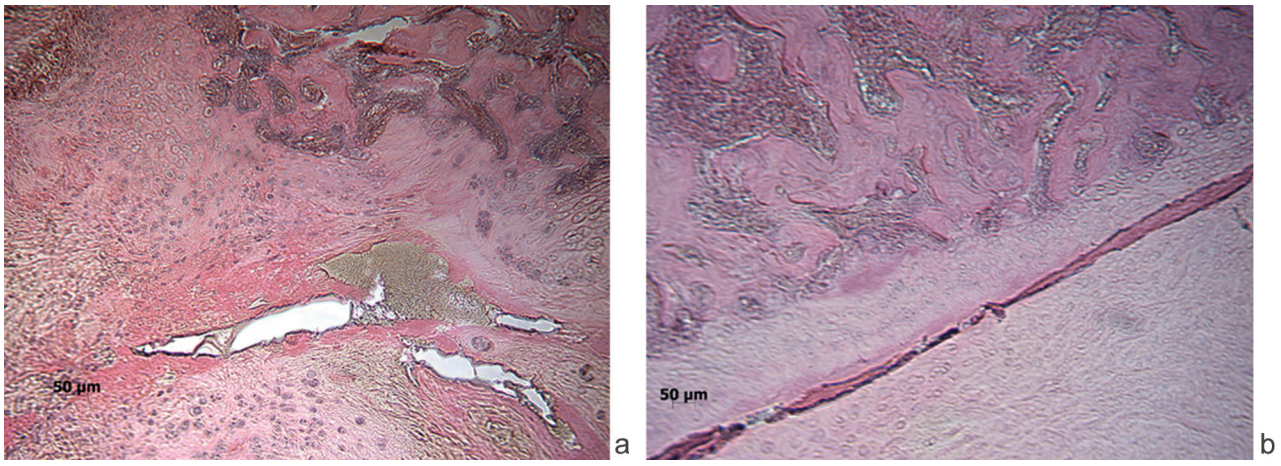

Fig. 7 Area of pseudarthrosis: connective tissue and chondroid (**a**). Chondroid prevalence. Hematoxylin and eosin (**b**). Magnification ×100

Table 1

Tissue areas of the regenerate (M ± m, mm^2 and % from the whole regenerate area) in the control and experimental groups (n = 14)

Periods of study	Regenerate area	Chondroid area	Bone tissue area	Connective tissue area
Experimental group				
Experiment group, Day 49+7	13.929 ± 0.355	8.143 ± 0.390 58.4 %	3.429 ± 0.228 24.6 %	2.714 ± 0.221 19.4 %
Experiment group, Day 49+14	14.071 ± 0.385 P > 0.05	7.214 ± 0.447 51.2 % P < 0.05	4.571 ± 0.309 32.5 % P < 0.05	2.429 ± 0.228 17.3 % P > 0.05
Experiment group, Day 49+28	13.571 ± 0.272 P1 > 0.05 P2 > 0.05	5.857 ± 0.312 43.3 % P1 > 0.05 P2 < 0.05	5.286 ± 0.370 39 % P1 > 0.05 P2 < 0.01	2.143 ± 0.231 15.8 % P1 > 0.05 P2 > 0.05
Control group				
Control group, Day 49+7	13.786 ± 0.485 P5 > 0.05	9.786 ± 0.367 71 % P5 < 0.01	1.714 ± 0.174 12.4 % P5 < 0.001	2.929 ± 0.352 21.2 % P5 > 0.05
Control group, Day 49+14	14.286 ± 0.284 P3 > 0.05	9.571 ± 0.293 66.9 % P3 < 0.01	2.214 ± 0.182 15.5 % P3 < 0.001	2.640 ± 0.210 18.5 % P3 > 0.05
Control group, Day 49+28	14.021 ± 0.284 P4 > 0.05	9.479 ± 0.293 67.6 % P4 < 0.001	2.271 ± 0.182 16.2 % P4 < 0.001	2.343 ± 0.210 16.7 % P4 > 0.05

Note: P – comparison of the values of group Experiment 49 + 14 days with group Experiment 49+7 days; P1 – comparison of the values of group Experiment 49 + 28 days with group Experiment 49 + 14 days; P2 – comparison of the values of group Experiment 49 + 28 days with group Experiment 49 + 7 days; P3 – comparison of the values of group Control 49 + 14 days with group Experiment 49 + 14 days; P4 – comparison of the values of group Model 49 + 28 days with group Experiment 49 + 28 days; P5 – comparison of the values of group Model 49 +7 days with group Experiment 49 + 7 days

The area of bone tissue in the regenerates was by 12.2 % larger *on day 7* and the area of chondroid by 12.6 % smaller as compared with the values of the control animals. The area of connective tissue in the studied periods did not differ significantly.

On day 14 after red bone marrow injection, the area of bone tissue grew by 7.9 % to the values of day 7, and that of the chondroid was 7.2 % lower. As compared with the values of the control group, the area of bone tissue in the regenerate of the experimental group was greater by 17 % and the chondroid area was 15.7 % smaller. The decrease in the area of the chondroid was observed *on day 28* as well. Thus, the area of the chondroid decreased by 15.1 % as compared with day 7 and by 7.9 % relative to day 14. It was revealed that the area of bone tissue increased by 14.4 % and 6.5 % as compared with day 7 and day 14, respectively. The area of connective tissue remained practically unchanged.

The comparison of the areas of the tissue types in the regenerate of the experimental and control animals *on day 28* demonstrated that the chondroid area in the experimental group was 24.3 % smaller in this period and the bone tissue area was larger by 22.8 % as compared with the control animals. The fact that the total area of bone tissue in the regenerate was 39 % by the end of the experiment proved a positive effect of autologous red bone marrow. The tendency towards the decrease in the area of the entire regenerate due to the regenerate reorganization and its reduction in the bone canal was also observed. The bone marrow effect on reparative osteogenesis may be explained by the fact that the transplanted bone marrow

is not only rich in biologically active substances but also contains mesenchymal stromal cells (MSCs) that are capable to supplement the pool of bone-forming osteoblast cells as they are able to differentiate into osteoblasts [13, 19, 21]. As it has been proven that bone marrow MSCs are the basis of bone tissue cell differentiation in reparative osteogenesis disorders [4], contribute to osteogenesis and restore the integrity of bone tissue in cases of its defects [6, 7, 8]. However, high-tech laboratory equipment is required for obtaining MSCs while culturing takes a prolonged time that restrain their widespread clinical use. Native red bone marrow that contains MSCs is easily available. The technologies of its harvesting and transplantation are simple. Therefore, it seems beneficial to use it to promote reparative osteogenesis in bone fractures and pseudoarthrosis.

CONCLUSION

An experimental study on the investigation of bone tissue regeneration following a fracture with a developed pseudarthrosis and on the effect of an autologous red bone marrow injection to optimize reparative process was performed on 30 white laboratory rats. The study revealed that autologous red bone marrow stimulated bone tissue development in the pseudarthrosis zone as the bone tissue area in it was by 22.8 % larger than that in untreated animals. There were no pseudarthrosis-related cavities in the cases of red bone marrow injection into the zone of injury. The following features of reparative process were observed: angiogenesis activation, enchondral ossification accompanied by formation of cancellous bone tissue with bone trabeculae of small loop-like organization, and reduction of destructive manifestations in the maternal bone parts located above and below the injury zone.

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