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Histological evaluation of reparative osteogenesis in critical size femoral bone defects in rats of different ages after introduction of allografts saturated with blood plasma growth factors

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The increase in injuries and gunshot wounds because of the war in Ukraine makes it imperative to find methods for optimizing bone regeneration and filling large-size bone defects. Aim. Study morphological features of reparative osteogenesis when critical size femoral bone defects in rats in the early reproductive and mature stages are filled with allografts saturated with blood plasma growth factors (GF). Methods. Defects (3 × 3 mm) were created in the distal femoral metaphysis of 60 white laboratory rats, 3-months-old (n = 30) and 12-months-old (n = 30). The defects were filled with bone allografts saturated with GF in the two experimental groups (AlloG+GF), and unsaturated bone allografts in the two control groups (AlloG). All groups contained 15 rats of each age. At 14, 28 and 90 days after the surgery, 5 rats from each group were sacrificed, and histological analyses were performed. Results. In the AlloG groups, excessive formation of connective tissue was observed 14 and 28 days after the surgery, most evident in the 3-month-old rats. In the AlloG+GF groups, bone formation was delayed at 14 days independent of age, while at 28 and 90 days, the area of bone trabeculae did not differ from the values in the AlloG groups. Throughout the experiment, decreases in allograft area (almost all of it was replaced by bone after 90 days) and connective tissue (completely absent in 3-month-old rats after 90 days) were observed in both AlloG+GF groups. The area of bone trabeculae increased in the period from 14 to 28 days. Conclusion. Saturating allografts with blood plasma growth factors facilitates an increase in the rate at which allografts are replaced by bone tissue, independent of the recipient's age. However, excessive formation of connective tissues in the defect 14 and 28 days after the surgery, especially in 3-month-old rats, may negatively affect the mechanical properties of the bone, which should be considered in clinical practice.

Збільшення кількості травм і вогнепальних поранень внаслідок війни в Україні обумовлює пошук шляхів оптимізації регенерації кістки та необхідність пластики великих за розмірами дефектів кісток. Мета. Вивчити морфологічні особливості репаративного остеогенезу в критичних дефектах стегнових кісток щурів репродуктивного раннього та зрілого пізнього вікових періодів за умов пластики алогенними кістковими імплантатами, насиченими факторами росту плазми крові (ФР). Методи. Використано 60 білих лабораторних щурів 3-місячного (n = 30) та 12-місячного (n = 30) віку, яким відтворювали дефект у дистальному метафізі стегнової кістки (3 × 3 мм). Дефект заповнювали: у дослідних групах (АлоІ+ФР) — кістковими алоімплантатами, насиченими ФР; у контрольних (АлоІ) — алоімплантатами. У кожній групі було по 15 тварин кожного віку. Через 14, 28 і 90 діб після втручання щурів виводили з експерименту, проводили гістологічний аналіз. Результати. У групах АлоІ виявлено надлишкове утворення сполучної тканини в ділянці дефекту через 14 і 28 діб після операції з більшим проявом у молодших щурів. У дослідних групах визначено затримку кісткоутворення на 14-ту добу не залежно від віку, а на 28-му і 90-ту — показники відносної площі кісткових трабекул не відрізнялися від показників груп АлоІ. Упродовж експерименту у щурів обох вікових груп АлоІ+ФР зменшувалися відносні площі алоімплантата (процес його перебудови майже завершився на 90-ту добу) та сполучної тканини (до повної відсутності в 3-місячних щурів на 90-ту добу), а кісткових трабекул — збільшувалися (із 14-ї до 28-ї). Висновки. Насичення кісткових алоімплантатів факторами росту плазми крові сприяє швидшій їхній перебудові зі заміщенням на 90-ту добу кістковою тканиною пластинчастої структури незалежно від віку реципієнта. Проте надлишкове утворення сполучної тканини в ділянці дефекту через 14 і 28 діб, особливо в молодших тварин, може негативно позначитися на механічних властивостях кістки, що слід ураховувати в клінічних умовах. Ключові слова. Регенерація кістки, алоімплантат, фактори росту, моделювання на тваринах, гістологія.

Key words. Bone regeneration, allograft, growth factors, modeling on animals, histology

Introduction

The search for ways to optimize bone regeneration is due to the need to repair large bone defects formed as a result of injuries, gunshot wounds, removal of bone tumors, in the case of revision endoprosthetic repair, surgical interventions on the spine. According to the latest publications, during the war between Ukraine and Russia, due to the use of high-energy weapons (ballistic trauma), the number of patients (both military and civilians) with long bone fractures, complicated by the formation of significant defects, increased [1]. According to the UN, as of 15 August 2022, the number of wounded civilians was 7,698, but the real figure is probably higher [2].

To optimize the process of bone regeneration, bone material of auto- and allogeneic origin is used, which has osteoinductive and osteoconductive properties. Since the use of bone autografts is limited due to their small volume, shape that does not correspond to the size of the defect, and the need for additional surgical interventions, it is a common practice to use synthetic or allobone implants to fill bone defects [3]. Unfortunately, the latter partially lose their osteoconductive and osteoinductive qualities in the process of manufacturing, sterilization, and storage [4]. However, they provide the mechanical strength of the bone in the area of use, which ensures early loading of the operated area of the skeleton, and is a matrix for the formation of bone tissue. It is possible to increase the osteoinductive properties of alloimplants by saturating them with biological agents, in particular, growth factors.

One of the promising approaches is the use of platelet-enriched plasma or fibrin due to their content of growth factors (platelet-derived (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor (TGF)), which contribute to the acceleration of angiogenesis [5], cell migration and proliferation [6] and, accordingly, bone regeneration [7]. The liquid component of the blood centrifuge, in addition to growth factors, contains adhesive proteins (fibronectin and vitronectin), which increase the biocompatibility of the material [8]. In addition, it is easier to permeate bio-implants with it than with fibrin.

Purpose: to study the morphological features of reparative osteogenesis in critical femoral defects of rats of reproductive early and mature late age periods under the conditions of repair with allogeneic bone implants saturated with blood plasma growth factors.

Material and methods

The study was approved by the Bioethics Committee at the State Institution Professor M. I. Sytenko Institute of Spine and Joint Pathology of the National Academy of Medical Sciences of Ukraine (Protocol No. 191 dated 22.04.2019) in accordance with the requirements of humane treatment of experimental animals regulated by the Law of Ukraine «On Protection of Animals from Cruelty» (No. 3447-IV dated 21.02.2006) and European Convention on the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes (Strasbourg, 18.03.1986).

Animals

The study involved 60 white laboratory rats of 3-month (n = 30) and 12-month (n = 30) age, which were reproduced with a defect in the metaphysis of the femur. The animals were divided into four groups depending on age and the material used to fill the defects:

– study (Alloimplant+GF groups) — bone alloimplant saturated with growth factors of blood plasma (GF) (15 animals of each age),

– control (Alloimplant group) — bone alloimplant (15 animals of each age), histological studies of this group were published by us earlier [9].

In addition, 15 6-month-old rats were used as donors for the production of allo-implants, 6 — for obtaining blood plasma growth factors.

Surgical interventions

Operations were performed in aseptic and antiseptic conditions under general anesthesia (ketamine, 50 mg/kg of body weight, intramuscularly). After trimming the fur on the left knee and treating the area with Betadin® antiseptic, the distal metaphysis of the femur was opened with an anterior-lateral approach, and with the help of a dental bur, a hole defect of critical size was modeled — a minimal defect that does not heal on its own during the life of the animal or during the experiment [10]. Earlier, we substantiated that the size of such a defect for the distal metaphysis of the rat femur is: the diameter of the defect is 3 mm, the depth is 3 mm [9]. Cylindrical cortical-cancellous alloimplants of the appropriate size were placed in the area of the defect in rats of the control groups. In the experimental groups, before installation, alloimplants were kept for 20 minutes in 0.2 ml of blood plasma with growth factors. Next, after the introduction of the alloimplant defect into the cavity, the remaining volume of blood plasma was injected into the implant and the surgical site with a syringe. After local treatment with an antibio-

tic, the muscles and skin wound were sutured, and the surgical site was treated with an antiseptic.

14, 28, and 90 days after surgery, 5 animals from each group were removed from the experiment by decapitation under ether anesthesia.

Manufacturing of bone alloimplants

Alloimplants were made from the metaphyses of donor femurs or tibias of 6-month-old rats (n = 15), removed after the administration of a lethal dose of anesthetic (sodium thiopental 90 mg/kg intramuscularly), in accordance with the «Methodology for obtaining bone alloimplants» (Osteomatrix Sytenko allogeny (OMS-A), certificate of conformity No. UA. TR. 101-21-2016). Sterilization of alloimplants was performed using radiation at a dose of 15 to 25 kGy [9, 11].

Obtaining blood plasma growth factors

8 ml of venous blood was taken into an 8.5 ml vacuum tube with an anticoagulant. A vacuum tube with blood was centrifuged in a laboratory clinical centrifuge OPn-3.02 DASTAN (ILGK.061214.009, GOST 12.2.025-76) at 1500 rpm for 10 minutes. After the procedure the supernatant fraction was removed with Pasteur pipettes, and the plasma left at the bottom of the test tube was used for further research.

Histological studies

After removing the animals from the experiment, the selected operated femurs were cleaned of soft tissues and fixed for 4 days in 10 % neutral formalin. Decalcification was performed in a 5 % solution of trichloroacetic acid, washed in ethyl alcohol, metaphyses with the defect area were cut out, dehydrated in isopropyl alcohol, soaked in a mixture of isopropyl alcohol and paraffin, then a series of paraffins, poured into paraffin. The prepared histological sections were stained with hematoxylin and eosin.

The structure of cells and intercellular substance in the area of the simulated defect was analyzed in an Olympus BX63 light microscope (Japan). A digital camera DP73 (Olympus) and Cell Sens Dimension 1.8.1 software (Olympus, 2013) were used for photography.

Histomorphometry

In the zone of the defect, the areas of newly formed bone and connective tissues and implants were measured (on 7 central sections of each animal) using the Cell Sens Dimension 1.8.1 software (Olympus, 2013), then their relative content (%) from the total area was calculated defect

Statistical methods

Measurement results are given as mean and standard deviation. The normality of the distribution was checked using the Kolmogorov-Smirnov method. Results were compared using the Student's t-test method. Data analysis was performed using the IBM SPSS Statistics 20 software. The difference was considered statistically significant in $p < 0.05$.

Results

14 days after implantation

In the 3-month-old rats of the Alloimplant+GF group, a bone implant in the process of reconstruction, connective tissue located between its trabeculae and in some places along its perimeter, and newly formed bone tissue of a coarse-fiber structure were found in the area of the defect (Fig. 1, a).

In the connective tissue, osteoblastic and fibroblastic differon cells, capillary-type vessels moderately filled with erythrocytes were observed. Cell density was high. Functional activity of fibroblasts was reflected by the presence of hypochromic nuclei with nucleoli, developed cytoplasm. In the newly formed bone trabeculae, the density of osteocytes was significant. Lacunae were not detected around some of them, making it possible to assert their participation in reparative osteogenesis. Functionally active osteoblasts were located on the outer surface of newly formed bone trabeculae, as well as in places on the surface of the alloimplant (both outer and inner). Besides, multinucleated cells of foreign bodies of the osteoclast type, which participate in its reconstruction, were observed on the surface of the alloimplant.

According to the results of histomorphometry, the relative area of connective tissue in the defect zone was (28.51 ± 8.83) % and was 2.9 times greater ($p < 0.001$), and bone trabeculae ((8.86 ± 3.17) %) and bone marrow ((43.33 ± 9.80) %) smaller by 2.1 times ($p < 0.001$) and 1.2 times ($p = 0.022$), respectively, compared to the group of the same age with alloimplant without GF (group Alloimplant). At the same time, the area of the alloimplant did not differ (Fig. 2).

In *12-month-old rats of the Alloimplant+GF group*, the content of the defect zone did not differ from the rats of the younger group: they were found to have bone alloimplant in the process of reconstruction, connective and newly formed bone tissue (Fig. 1, b). Compared with 3-month-old rats, the relative area of the alloimplant was equal to (24.36 ± 4.83) % and was 1.3 times larger ($p = 0.003$), bone marrow ((53.18 ± 9.34)%) — 1.2 times ($p = 0.002$), connective tissue ((14.83 ± 10.18) %) — 1.9 times less ($p < 0.001$). Connective tissue was located in the middle of the alloimplant, between its trabeculae. The formation of bone trabeculae, the relative area of which did not differ from that of 3-month-old animals, was

observed on the side of the defect both in the cancellous bone and in the cortical layer. As in younger rats, osteoclasts were found on individual trabeculae of the alloimplant.

As in 3-month-old rats, in 12-month-old rats, the relative area of connective tissue was 1.8 times larger ($p = 0.014$), bone trabeculae and bone marrow were 1.9 times smaller ($p < 0.001$) and 1.2 times ($p < 0.001$), respectively, comparable to the indicators of the Alloimplant group of the same age (Fig. 2).

28 days after implantation

At this observation period, in the *3-month-old rats of the experimental group*, alloimplant fragments were located in the central part of the defect in the form of thin trabeculae, between which connective tissue of varying degrees of maturity was formed. The rest of the defect, including in the cortical layer, was filled with newly formed small looped bone trabeculae with reticulo-fibrous tissue and red bone marrow between them (Fig. 1, c). Compared to

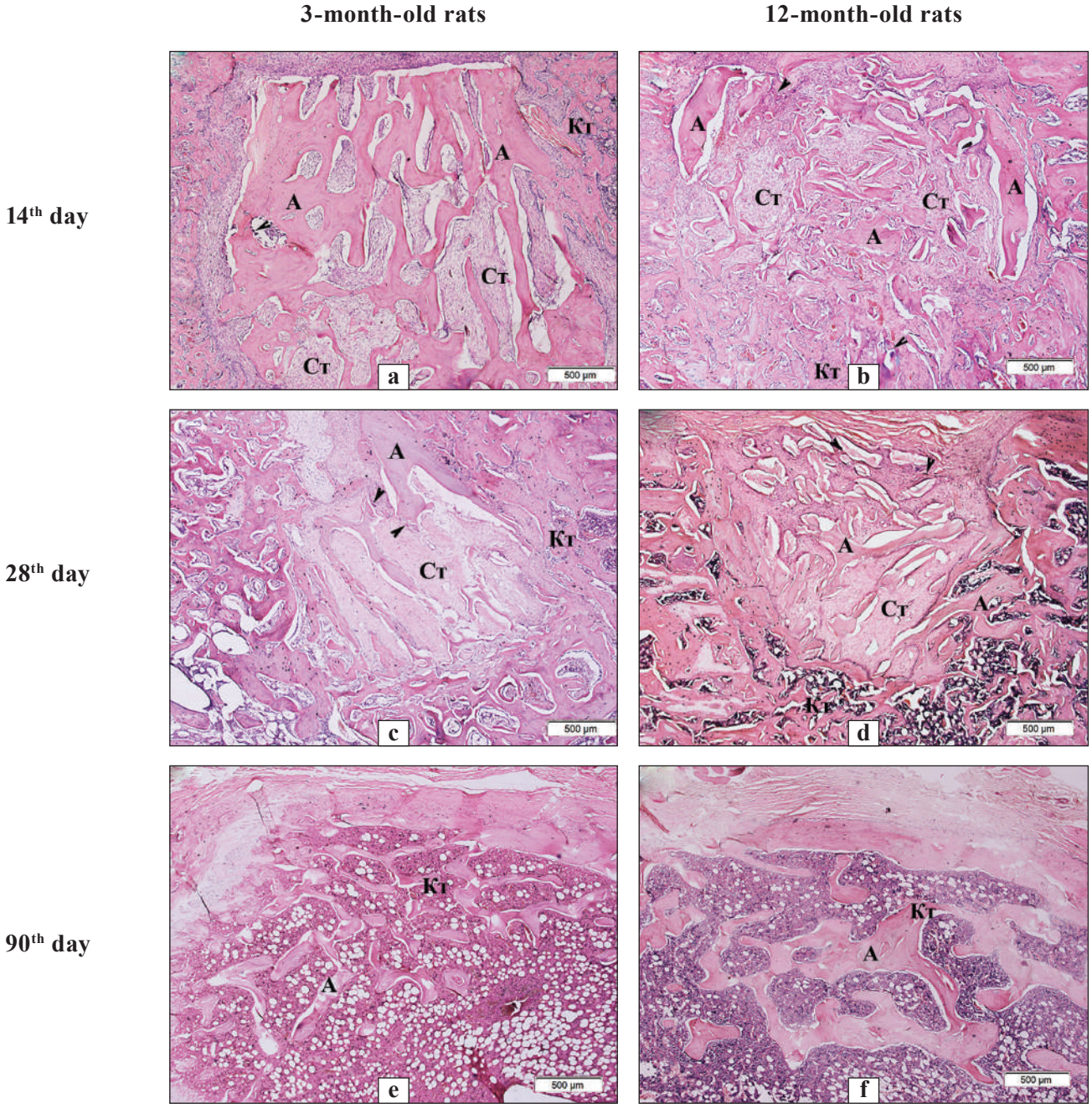

Fig. 1. Histological pattern of the defect site in the metaphysis of the femur of 3-month-old (a, c, e) and 12-month-old (d, e, g) rats on the 14th, 28th, and 90th days after the introduction of the alloimplant (A) saturated with factors blood plasma growth. Fragments of the alloimplant, areas of connective tissue (St), newly formed bone tissue (Kt), multinucleated cells of the osteoclast type (arrows) on the surface of the allogeneic material are shown. H&E stain

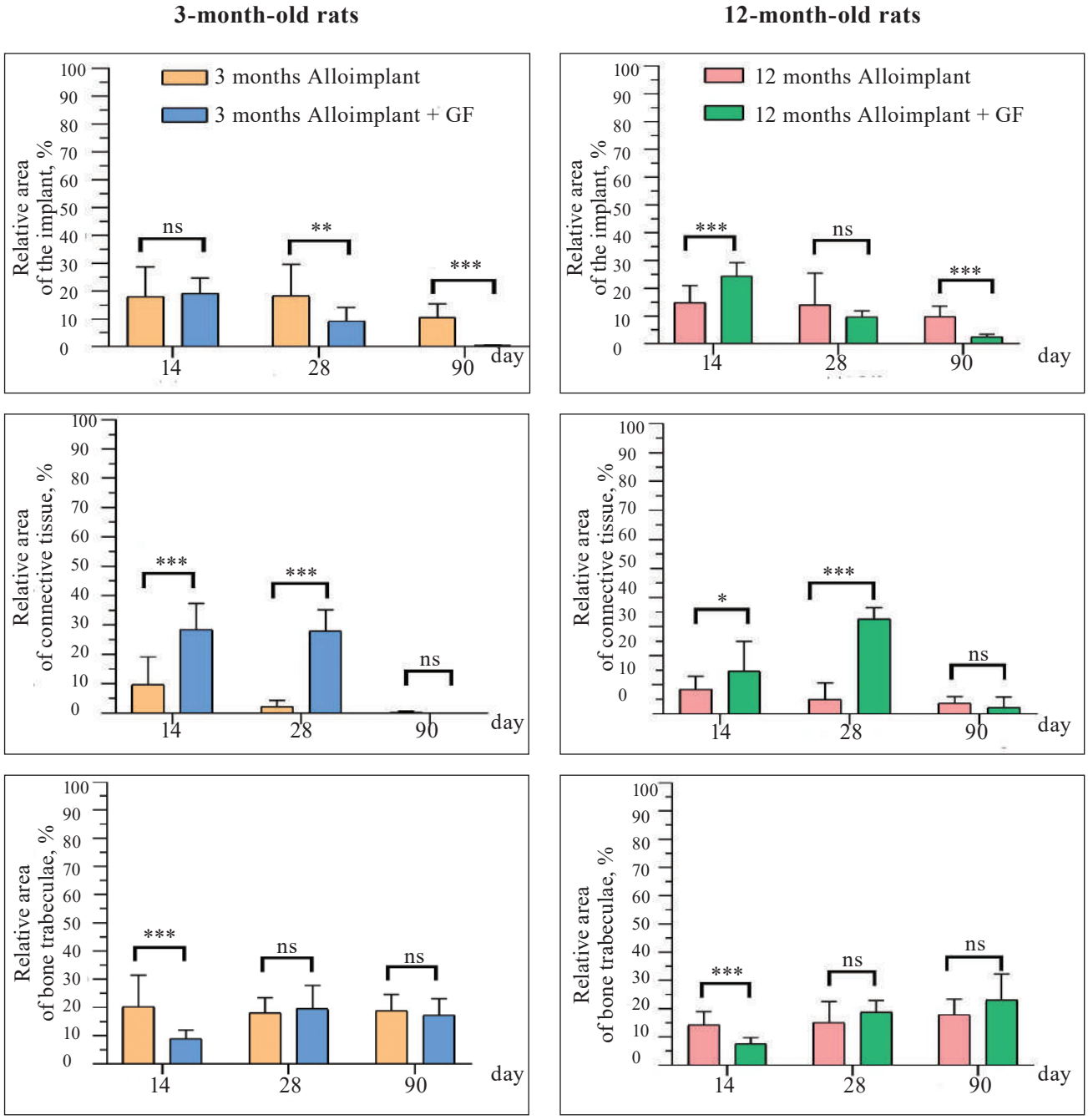

Fig. 2. Relative areas of the alloimplant and newly formed tissues in the area of the critical defect of the femur of 3- and 12-month-old rats, depending on the saturation of blood plasma growth factors (GF) on the 14th, 28th, and 90th days after the introduction of the alloimplant; ns — no statistically significant difference; * — $p < 0.05$; ** — $p < 0.01$; *** — $p < 0.001$. Figures are given as mean and standard deviation. Comparison of groups for each observation period was performed using the Student's t-test method

the 14th day of observation, the relative area of the alloimplant decreased by 2.1 times ($p < 0.001$) and amounted to (9.31 ± 4.91) %, and the bone trabeculae increased by 2.2 times ($p < 0.001$) and was equal to (19.50 ± 8.30) %, while the areas of connective tissue and bone marrow did not change significantly.

Compared to the Alloimplant group of the same age, the relative area of the alloimplant was 1.9 times smaller ($p = 0.003$), bone marrow — 1.4 times ($p < 0.001$) and was (43.21 ± 3.31) %, connective tissue — 12.7 times greater ($p < 0.001$) and equal to (27.98 ± 7.23) %, the relative area of bone trabeculae did not differ (Fig. 2).

In *12-month-old rats of the experimental group*, the histological pattern of the defect zone was similar to that described in 3-month-old animals at this time (Fig. 1, d). Fragments of the alloimplant were found in it, some of its trabeculae contained osteoblasts. Bone

tissue was formed from the bone marrow and cortex. Compared to the previous period of the study, the relative area of the alloimplant decreased by 2.5 times ($p < 0.001$) and was equal to (9.67 ± 2.23) %, bone marrow ((38.87 ± 4.04) %) by 1.4 times ($p < 0.001$), connective tissue ((32.70 ± 3.84) %), on the contrary, increased by 2.2 times ($p < 0.001$), bone trabeculae ((18.76 ± 4.08) %) by 2.5 times ($p < 0.001$). Compared with 3-month-old rats, the area of connective tissue was 1.2 times larger ($p = 0.013$), bone marrow was 1.1 times larger ($p < 0.001$), other parameters did not differ (Fig. 2).

Compared with 12-month-old rats of the Alloimplant group, the relative area of the connective tissue was 6.6 times larger ($p < 0.001$), and the bone marrow was 1.7 times smaller ($p < 0.001$), but the area of the alloimplant, and bone trabeculae did not differ (Fig. 2).

90 days after implantation

In rats of both experimental age groups, the bone alloimplant was almost completely replaced by bone tissue of lamellar structure. The remains of the alloimplant were found among the rebuilt bone trabeculae (Fig. 1, e, g). Its relative area did not exceed 1–3 % (3-month-old rats — (0.45 ± 0.21) %, 12-month-old rats — (2.56 ± 0.88) %) of the total area of the defect and decreased compared to 14 day 42.9 times ($p < 0.001$) and 9.6 times ($p < 0.001$) in younger and older animals, respectively. At the same time, the indicator of the alloimplant area in 12-month-old rats was 5.7 times greater than the indicator in 3-month-old rats ($p < 0.001$). The injured cortical layer was restored to its original structure in 3-month-old rats, and small cells of connective tissue were noted in it in 12-month-old rats.

Compared to the 14th day of observation, the relative area of bone trabeculae and bone marrow in 3-month-old rats increased by 1.9 times ($p < 0.001$) and amounted to (17.21 ± 5.90) % and (82.35 ± 5.04) %, respectively, in 12 months — 3.0 times ($p < 0.001$) and 1.4 times ($p < 0.001$) and was equal to (23.01 ± 9.32) % and (72.17 ± 11.07) % respectively. Connective tissue was not detected in the defect zone of 3-month-old rats at this time, and in 12-month-old rats, although its area decreased by 6.6 times compared to the 14th day ($p < 0.001$), and from the 28th to 14.5 times ($p < 0.001$), still accounted for (2.26 ± 3.62) % of the total area of the defect.

Compared with age-matched groups using an alloimplant without GF for plastic repair of the defect in 3-month-old rats, the relative area of the alloimplant in the defect area on the 90th day of observation was 23.6 times smaller ($p < 0.001$), and of the bone marrow was 1.2 times larger ($p < 0.001$), other indicators did not differ. In 12-month-old rats, the relative area of the alloimplant was 3.9 times smaller ($p < 0.001$), other indicators did not differ (Fig. 2).

Discussion

In the presented study, performed using laboratory rats of different ages (3 and 12 months at the beginning of the experiment), the possibility of improving the osteoinductive and osteoconductive properties of structural allogeneic bone implants sterilized by γ-radiation was studied by saturation with blood plasma growth factors. Because of the increase in incidence due to military conflicts (in particular, the full-scale war between Russia and Ukraine [1]) of large bone defects that do not heal on their own, we chose critical size defects (3 mm depth, 3 mm diameter) in the distal metaphysis of the femur as a model [9].

Bone alloimplants are used for bioreconstruction of large bone defects after injuries, removal of tumors, during operations on the spine and endoprosthesis of large joints, etc. They have advantages over autografts, as their acquisition does not require additional surgical intervention and prevents problems with the donor site [12, 13]. The main disadvantage of bone alloimplants is low osteoinductive and strength qualities due to processing after receiving from the donor, namely freezing, drying or processing with a glycerin-based solution [14, 15]. It negatively affects the strength of the alloimplant and γ-radiation [16], which is associated with the destruction of the molecular structure of collagen [17], also affecting the osteoinductive properties.

Therefore, it is of interest not only to develop methods of preservation, processing and sterilization of alloimplants, but also to find ways to improve their osteoconductive and osteoinductive qualities. For this purpose, we used blood plasma growth factors obtained by centrifugation of blood at 1500 rpm, which were used to saturate the alloimplant before installation in the cavity of the defect and then additionally injected into the area of the traumatic injury. It is believed that the use of the liquid component of blood centrifuge (obtained by centrifuging blood without an anticoagulant in a non-glass tube at approximately 1300 to 2400 rpm [18, 19]) makes it possible to infiltrate bio-implants. In addition, it contains, in addition to growth factors, proteins fibronectin and vitronectin, which increase the biocompatibility of the material [8]. It was determined that the growth factors obtained in this way influence the proliferation and differentiation of gingival fibroblasts and osteoblasts [20, 21], and their combination with allogeneic ma-

terial *in vitro* increases the viability, migration and proliferation of human osteoblasts [22].

In our study, in rats of both age in the experimental groups (the defect was filled with an alloimplant saturated with growth factors of blood plasma) compared to a series of experiments where an alloimplant without growth factors was used as an osteoplastic material, excessive formation of connective tissue in the area of the defect was determined 14 and 28 days after the operation with greater manifestation in 3-month-old rats. Presumably, this is due to the high content of interleukins (IL): IL-4, IL-6 and IL-10 in the used blood centrifuge, in particular IL-4, which affects the differentiation and migration of fibroblasts [23]. At the same time, a delay in bone formation was detected in rats of both age groups only on the 14th day, and on the 28th and 90th, the indicators of the relative area of bone trabeculae did not differ from the indicators of the Alloimplant group [9]. During the experiment, in rats of both age groups, the relative area of the alloimplant decreased and the process of its reconstruction, in contrast to the Alloimplant groups [9], was almost completed by the 90th day. Also, with the passage of time (from the 28th to the 90th day), the relative area of connective tissue decreased (to its complete absence in 3-month-old rats on the 90th day), and bone trabeculae (from the 14th to the 28th) and bone marrow (from the 28th to the 90th) — increased. The anatomical structure of the femurs was normal in all cases.

Therefore, on the basis of the conducted histological study, it was determined that saturation of the alloimplant with growth factors obtained by centrifugation of blood at 1500 rpm contributes to its faster reconstruction with replacement by bone tissue of the lamellar structure on the 90th day, regardless of the age of the recipient. However, in clinical conditions it is necessary to take into account the excessive formation of connective tissue in the area of the defect after 14 and 28 days (especially in younger recipients), which can negatively affect the mechanical properties of the bone and limit the load on it.

Conclusions

Saturation of bone alloimplants with growth factors of blood plasma contributes to their faster reconstruction (compared to a series of experiments where only alloimplant was used as osteoplastic material) with replacement on the 90th day by bone tissue of the lamellar structure, regardless of the age of the recipient. However, excessive formation of connective tissue in the area of the defect after 14 and 28 days (with the greatest manifestation in younger animals) can negatively affect the mechanical properties of the bone, which should be taken into account in clinical conditions.

Conflict of interest. The authors declare no conflict of interest.

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