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Morphology of the repair of critical size bone defects which filling allogeneic bone implants in combination with mesenchymal stem cells depending on the recipient age in the experiment

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Mesenchymal stem cells (MSC) can be used to facilitate reparative osteogenesis. In the case of critical-size defects, MSC can attach to allogenic bone implants (AlloI) that serve as a matrix. Objective. Analyze the morphological features of reparative osteogenesis in critical-size defects in femurs of rats (3 and 12 months old) when the defects are filled with MSC along with AlloI. Methods. 60 white lab rats, 3 months (n=30) and 12 months (n=30) old were used. Defects (3mm in depth, 3mm in diameter) were created in the femoral metaphysis of each rat, and filled with AlloI in the control groups and with AlloI and adipose-derived MSC in the experimental groups. Each group contained 15 rats of a particular age. 14, 28, and 90 days after the surgery, histological studies were conducted. Results. The area of AlloI decreased with time. 14 days after the surgery, in the experimental group, the area of AlloI was 1.6 times greater in 3-month-old (3mo) rats than in 12-month-old (12mo) rats. In comparison to the control, the area of AlloI was greater 14 days after surgery in 3mo rats and 28 days after surgery in 12mo rats. 14 and 28 days after the operation, the area of connective tissue was greater in rats of both experimental groups than in the control. For the 3mo rats, the same was true 90 days after the operation. The area of newly formed bone was 1.6 times lower in 3mo rats than in 12mo rats 14 days after the operation. 90 days after the operation, the area was 2.3 greater in 3mo rats. For 12mo rats, the highest area of bone tissue occurred 14 days after the surgery, and subsequently did not significantly change or differ from the control. For 3mo rats, the area of bone tissue was lower than control 14 and 28 days after the surgery, but greater than control 90 days after the surgery. Conclusions. The use of MSC along with AlloI to fill traumatic bone defects causes slower bone formation and excessive formation of connective tissue, independent of the age of the recipient.

Мезенхімальні стромальні клітини (МСК) використовують для оптимізації репаративного остеогенезу. Каркасом для їхнього прикріплення можуть бути алогенні кісткові імплантати (АлоІ). Мета. Проаналізувати перебіг репаративного остеогенезу в дефектах критичного розміру стегнових кісток щурів (вік 3 і 12 міс.) за умов пластики АлоІ із МСК. Методи. Використано 60 білих лабораторних щурів віком 3 міс. (n = 30) і 12 міс. (n = 30). Дефект (глибина 3 мм, діаметр 3 мм) у метафізі стегнової кістки заповнили в контрольних групах АлоІ (по 15 щурів кожного віку), у дослідних — АлоІ із МСК із жирової тканини (по 15 тварин кожного віку). Через 14, 28 і 90 діб після операції виконано гістологічні дослідження. Результати. Відносна площа АлоІ протягом експерименту зменшувалася. У досліді на 14-ту добу в 3-місячних щурів вона виявилася більшою в 1,6 раза, ніж у 12-місячних, а порівняно з контролем була більшою в молодших щурів на 14-ту добу, у старших — на 28-му. Відносні площі сполучної тканини за умов використання АлоІ та МСК у тварин обох вікових груп були більшими на 14- та 28-му доби, а у 3-місячних щурів — і на 90-ту, ніж у разі застосування АлоІ окремо. Відносна площа новоутворених кісткових трабекул у щурів віком 3 міс. на 14-ту добу була нижчою в 1,6 раза, на 90-ту — більшою у 2,3 раза, ніж у 12-місячних тварин. В останніх площа кісткової тканини досягла вищого показника на 14-ту добу і надалі значуще не змінювалася та не відрізнялася від контролю цього віку. У 3-місячних щурів на 14- та 28-му доби показник був нижчим приблизно в 1,5 раза, а на 90-ту — більшим в 1,9 раза порівняно з контролем цього віку. Висновки. Введення МСК разом із АлоІ у випадках свіжих травматичних ушкоджень кісток спричинює уповільнення кісткоутворення незалежно від віку реципієнта і надлишкове формування сполучної тканини. Ключові слова. Модель на тваринах, дефект кістки критичного розміру, вік, регенерація кістки, алоімплантат, мезенхимальні стромальні клітини.

Key words. Animal model, critical-sized bone defect, age, bone regeneration, alloimplant, mesenchymal stromal cells

Introduction

Bone regeneration is a complex process that depends on many physical and biological factors. Important among the latter is the state of the bone tissue at the time of the injury, namely: the functional activity of its cells — osteoblasts, osteocytes, osteoclasts, the presence of a sufficient number of mesenchymal stromal cells (MSC), poorly differentiated cells of the endosteum and periosteum. With age, the imbalance of bone homeostasis leads to deterioration of bone quality and impairment of its ability to repair. The process of bone regeneration slows down [1–3], which increases the risk of unsatisfactory results after reconstructive interventions on the skeleton (endoprosthesis of hip and knee joints; replacement of bone defects after removal of tumors, gunshot wounds and traumatic injuries; spine surgery, etc.). In clinical settings, secondary displacements after a fracture of the distal radius occurred 1.5 times more often in patients older than 65 years compared to persons aged 18–44 years in the range of up to 8 weeks after applying a plaster cast [2]. The incidence of nonunion of fractures of the diaphysis of the humerus was higher in patients over 55 compared to younger patients [1]. In view of the constant increase in the world population of people over 65 years of age, the total number of which may reach 1.5 billion in 2050 [4], the development of effective methods for optimizing bone regeneration is an urgent problem in orthopedics and traumatology.

MSCs that migrate to the area of bone damage are capable of proliferation and differentiation in the osteogenic direction. In the model of tibial bone fracture in mice, in 8 weeks (85 ± 10) % of osteoblasts in the bone callus were shown to originate from bone marrow MSCs [5], indicating the critical role of these cells in osteoreparation. Accordingly, an insufficient number of MSCs or altered structural and functional features due to age can negatively affect bone regeneration. Experiments on 2- and 24-month-old mice showed an age-related decrease in the number of MSCs and osteoblast precursor cells in intact and injured bones [6], a decrease in their ability to form colonies *in vitro* and differentiate in chondrogenic and osteogenic directions, as well as an increase in old cells of genes that are associated with a decrease in bone formation and an increase in osteoresorption [7]. The transformation of red bone marrow into yellow during aging, that is, the accumulation of adipocytes in it, was found to significantly worsen the healing of fractures in mice due to a decrease in the participation of bone marrow MSCs in the process. In the regenerate, the area of cartilage tissue increases, and the mineralized tissue decreases, accordingly, the mineral density of bone tissue in the fracture area reduces [8]. In addition, a decrease in the migration ability of MSCs (obtained from old donors compared to young ones) in response to signaling factors of tissue damage has been established [9]. Violation of this function of MSCs leads to limitation of their participation in restorative processes and endangers tissue healing. Based on the above, it is logical to introduce additional cultured MSCs, which have a significant reparative potential, into the zone of non-union or delayed union of the fracture, significant bone defects. As evidenced by recently published literature reviews [9, 10], the results of many animal studies and clinical observations confirm the possibility of successful use of MSCs to optimize reparative osteogenesis. The use of various matrices for their cultivation makes it possible to preserve the tissue-specific properties of cells, create a graft of the required shape and size, facilitates the process of transplantation and retention of cells in the area of bone damage.

This is especially important in the case of large bone defects that do not heal on their own, where injections of MSCs alone may not be enough because there is no scaffold to which they can attach. Autologous or allogeneic bone grafts can be used as such a framework. Autografts remain the gold standard in orthopedic surgery. However, the small amount of extracted material, additional surgical intervention and the impossibility of obtaining in patients with severe injuries restrict their use. An alternative is bone alloimplants, which, due to technical processing, are devoid of living cells and are successfully used to optimize reparative osteogenesis [11] as they provide mechanical strength in the area of use and are a matrix for the formation of bone tissue. Bone alloimplants used in orthopedic and traumatological practice can be structural (cortical, cancellous, cortical-cancellous) and unstructured (chip) [12]. Their main drawback is low osteoinductive qualities. It seems possible to solve this issue by simultaneously introducing allo-implants, growth factors or MSCs. The need to use biologically active factors to accelerate reparative osteogenesis arises not only in cases of the formation of bone defects of critical size, but also in the case when the own reserve of osteoblast progenitor cells decreases, and the process of bone remodeling shifts towards osteoresorption, leading to the loss of bone tissue and deterioration of bone quality, observed in elderly and senile patients.

Purpose: to analyze the morphological features of reparative osteogenesis in critical femoral defects of rats

of reproductive early and mature late age periods in plastic repair with allogeneic bone implants with MSCs.

Material and methods

The experimental study was carried out in compliance with the requirements of humane treatment of experimental animals, regulated by the Law of Ukraine «On the Protection of Animals from Cruelty Treatment» (No. 3447-IV of 21.02.2006) and the European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Research goals (Strasbourg, 18.03.1986) [13, 14]. The plan of experimental research 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 of 22.04.2019).

Design of the study

The study involved 60 white laboratory rats of 3-month (n = 30) and 12-month (n = 30) age, receiving a defect in the metaphysis of the femur which was filled with a bone alloimplant in the control groups (15 animals of each age), in experimental animals the defect was filled with bone alloimplant with cultured MSCs (also 15 animals of each age).

The material for the manufacture of implants was obtained from donor femurs or tibias of 6-month-old rats (n = 15), which were removed after administration of a lethal dose of anesthetic (sodium thiopental 90 mg/kg intramuscularly). The obtained implants from the metaphyses of the above-mentioned bones were processed according to the OMC-A method (certificate of conformity No. UA.TR. 101-21-2016), which involves dehydration and release of the organic component.

Surgical interventions were performed under aseptic and antiseptic conditions under general anesthesia (ketamine, 50 mg/kg body weight, intramuscularly). After shaving the fur on the left knee and treating it with an antiseptic Betadin®, the area of the distal metaphysis of the femur was opened using 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 [15]. We chose a defect with a diameter of 3 mm, a depth of 3 mm, which exceeds the minimum size of a critical defect for rats [16] and at the same time there is no need for additional fixation. Cylindrical cortical-spongy alloimplants of the appropriate size were placed in the area of the defect. After local treatment with an antibiotic layer by layer, the muscles and skin wound were sutured, the surgical site was treated with an antiseptic.

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

Obtaining allogeneic MSCs

Adipose tissue was obtained from the omentum of rats, transferred from a sterile 15 ml Falcon-type test tube with DMEM medium (Biowest, Lo102-500, France) in a 1:5 volume ratio of tissue to enzymes. In a sterile Petri dish, the tissue was chopped into pieces about 2 × 2 mm, which were subjected to disaggregation in a mixture of collagenase type I enzyme (Worthington, 49P19751, USA) at a concentration of 0.075 mg/ml and Dulbecco's buffer solution without calcium and magnesium (Biowest, LO615-500, France) (1:10 ratio of volume of tissue and enzymes) and incubated in a thermostat at 37 ºC for 60 min. After that, adipose tissue fragments were centrifuged (Nuve NF800R centrifuge, Turkey) at 1200 g for 5 min and two fractions were obtained. Adipocytes were located in the upper light layer, and cells of the stromal vascular fraction with an admixture of hematopoietic cells were located in the sediment.

The supernatant was collected using a sterile serological pipette, and the pellet was resuspended in 20 ml of DMEM medium (Biowest Lo102-500, France). Next, 3.0×10^5 cells were seeded into 25 cm^2 culture flasks (TPP, 20200482, Switzerland) with the addition of 10 % fetal bovine serum (Biowest, SOOCT100R, France), filtered twice through 0.22 μm Millex®GV syringe filters (Merck Millpore Ltd, SLGP033RB), and 0.01 % gentamicin sulfate. Culture vials with cells were placed in a CO2 incubator EC 160 (Turkey) at a temperature of 37 ºC, a CO_2 content of 5 % in air and 95 % humidity.

In 24 hours, the culture medium with unattached cells was drained, and the flask with attached cells was washed with DMEM medium. After that, fresh DMEM medium with 10 % fetal bovine serum and 0.01 % gentamicin sulfate was added and changed every 3 days.

After the formation of a monolayer of cells on the 9th day, they were removed by incubating for 5 min in a mixture of 0.25 % trypsin (Boiwest, X0915-100, France) heated to 37 ºC with 0.02 % Versen solution (VETLINE AGROSCIENCES LLC, Ukraine) in a ratio of 1:9, resuspended in culture medium and precipitated by centrifugation at 1000 rpm for 10 min. We assessed the concentration of cells in the Horyaev chamber using an MS–100X MICROS microscope and re-scattered 3.0 × 105 cells into each culture vial.

In 7; 8; 9 days of cultivation, cells were removed and transferred to sterile microtubes in the amount

of 1.0×10^6 per 0.5 ml of culture medium (DMEM with the addition of 10 % fetal bovine serum) for infiltration of implants and introduction into defects of rats. Cell viability was assessed at each stage after removal from the culture flask using trypan blue staining.

Histological studies

Operated femurs were isolated from rats, cleaned of soft tissues and fixed for 4 days in 10 % neutral formalin. After washing with tap water, the bones were decalcified in a 5 % solution of trichloroacetic acid [17], washed in ethyl alcohol, the metaphyses with the defect area were cut out, and dehydrated in isopropyl alcohol of increasing concentration. Then they were soaked in a mixture of isopropyl alcohol and paraffin, then a series of paraffin, poured into paraffin. Sections were made on a Reichert sled microtome (Austria), stained with hematoxylin and eosin.

The structure of cells and intercellular substance in the area of the simulated defect and around the implanted bone material 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 of the areas of the regenerate tissues in the implantation zone was performed using the Cell Sens Dimension 1.8.1 software (Olympus, 2013). The areas of newly formed tissues (osseous and connective) and implants were measured (on 7 central sections of each animal) and then their relative content (%) from the total area of the defect was calculated.

Statistical methods

The evaluation of the obtained results was performed using the IBM SPSS Statistics 20 software. Measurement results are given as mean and standard deviation. The normality of the distribution was checked using the Kolmogorov-Smirnov analysis method. Comparison of indicators of groups of different ages or groups with/without the use of MSCs was performed using the Student's t-test method (comparison of two groups). The difference was considered statistically significant at $p < 0.05$.

Results and their discussion

Morphology of culture of mesenchymal stromal cells of rat adipose tissue

In 12 days of cultivation (the second passage after subculturing), the formation of a monolayer of fibroblasts was detected on the entire surface of the culture flask. They formed nodules of various sizes — from small (8–10 cells) to large (20–40) (Fig. 1, a). Smaller elongated fibroblasts were located in the nodules; larger polygonal fibroblasts were located between the nodules. Their nuclei were large, contained condensed chromatin, 2–3 nucleoli. Granular content was found in the cytoplasm of most fibroblasts. Cells with two nuclei and figures of mitosis were observed (Fig. 1, b). The identified structural features of the cells testify to their viability and proliferative activity.

Morphology of the distal metaphysis of the rat femur

14 days after implantation

At this time of the study, in 3-month-old rats, almost the entire area of the critical defect was occupied by an allogeneic bone implant (Fig. 2, a). Its relative area was 1.7 times larger ($p < 0.001$) compared to the group of animals of the same age without the use of MSCs (Fig. 3).

Coarse fibrous bone tissue was located around the alloimplant, mainly from the side of the bone-marrow cavity and the opposite cortical layer. Newly formed bone trabeculae were characterized by a high density of osteocytes, isolated osteoclasts were found on their surface, which indicates remodeling of newly formed bone tissue (Fig. 2, d).

In addition, multinucleated cells of the osteoclast type were found on the allogeneic bone material, reflecting its remodeling. Most rats at this time, in addition to the newly formed bone tissue, were found to have significant areas of loose connective tissue. Chondroid formation was determined on the surface of the maternal cortex.

According to the results of histomorphometry, the relative area of connective tissue was significantly 2.8 times larger ($p < 0.001$), and the newly formed bone trabeculae and bone marrow were 1.6 times ($p = 0.014$) and 1.9 times smaller ($p < 0.001$), respectively compared to the group of this age without MSCs (Fig. 3).

In *12-month-old rats*, remnants of allogeneic material, surrounded mainly by connective tissue, were observed in the implantation area. Multinucleated cells of the osteoclast type were located on their surface. Newly formed bone tissue, which did not differ in structure from that described in 3-month-old animals, was also found at the border with the mother bone (Fig. 2, g, j).

The relative area of the allogeneic bone implant in the defect zone was 1.6 times smaller ($p = 0.004$) compared to 3-month-old rats (Fig. 4) and did not differ from the indicators of animals of this age without the use of MSCs (Fig. 3). The relative areas of connective tissue and bone marrow were smaller by 1.7 ($p < 0.001$) and 1.6 times ($p < 0.001$), respectively, and bone trabeculae were 1.5 times larger ($p = 0.007$) compared to the 3-month rats (Fig. 4). Compared

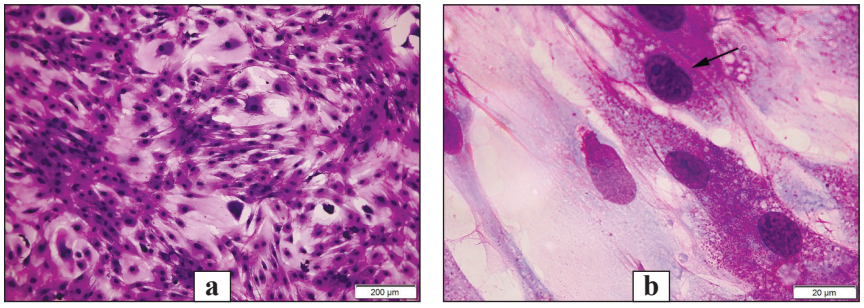

Fig. 1. MSCs culture from rat adipose tissue: a) formation of nodules of different sizes; b) condensed chromatin in MSCs nuclei, a cell at the telophase stage of mitosis (arrow). Romanovsky-Giemsa staining

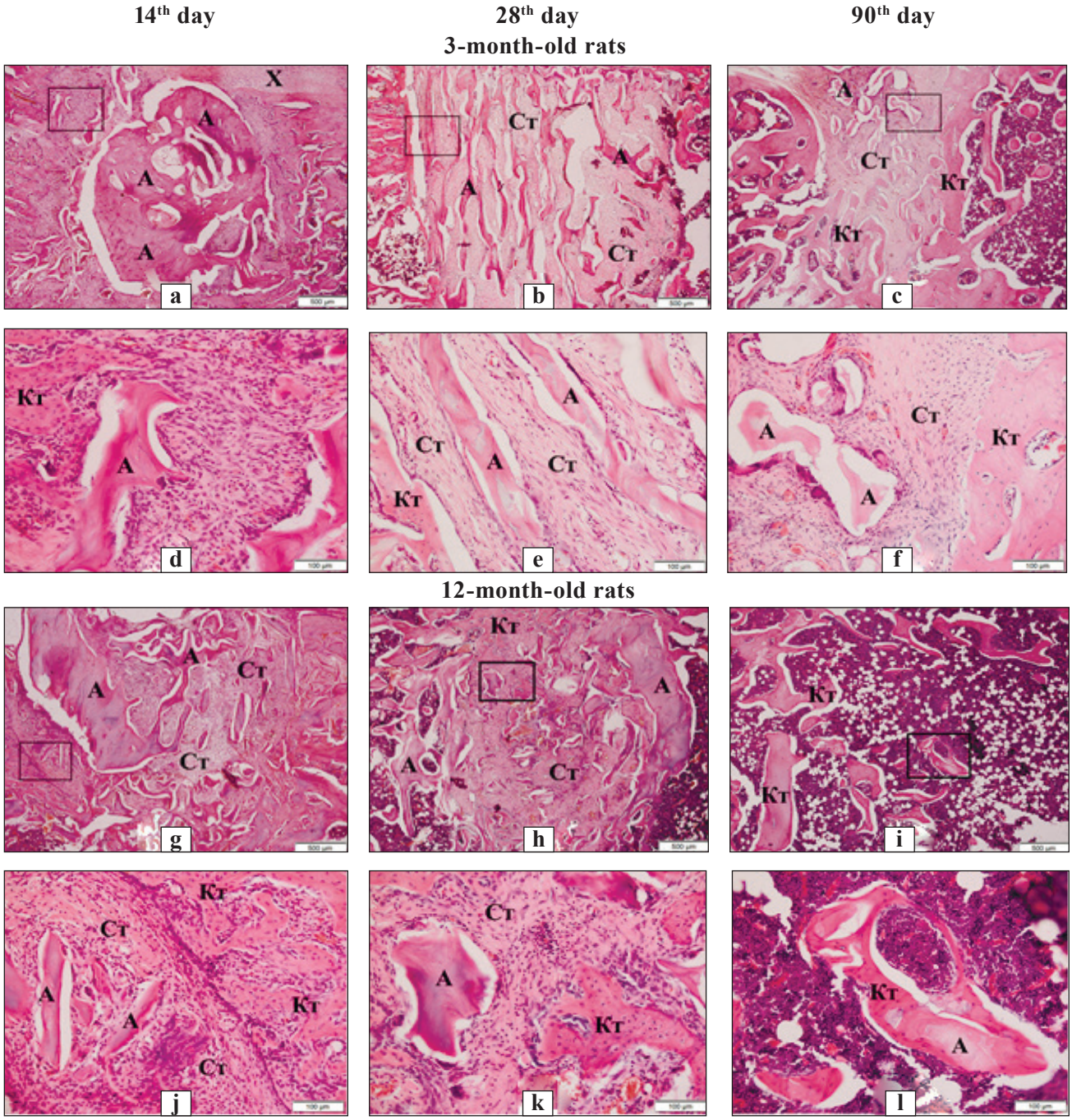

Fig. 2. Histological picture of the defect site in the femur of 3-month-old (a–f) and 12-month-old (g–m) rats on the 14th, 28th and 90th day after the introduction of an alloimplant (A) with MSCs. Fragments of the allograft, the areas of connective tissue (Ct), chondroid (Ch), newly formed bone tissue (Bt) are shown. Figures d, e, f are fragments of Figures a, b, c, respectively, and Figures k, l, m are fragments of Figures g, i, j, respectively. H&E staining

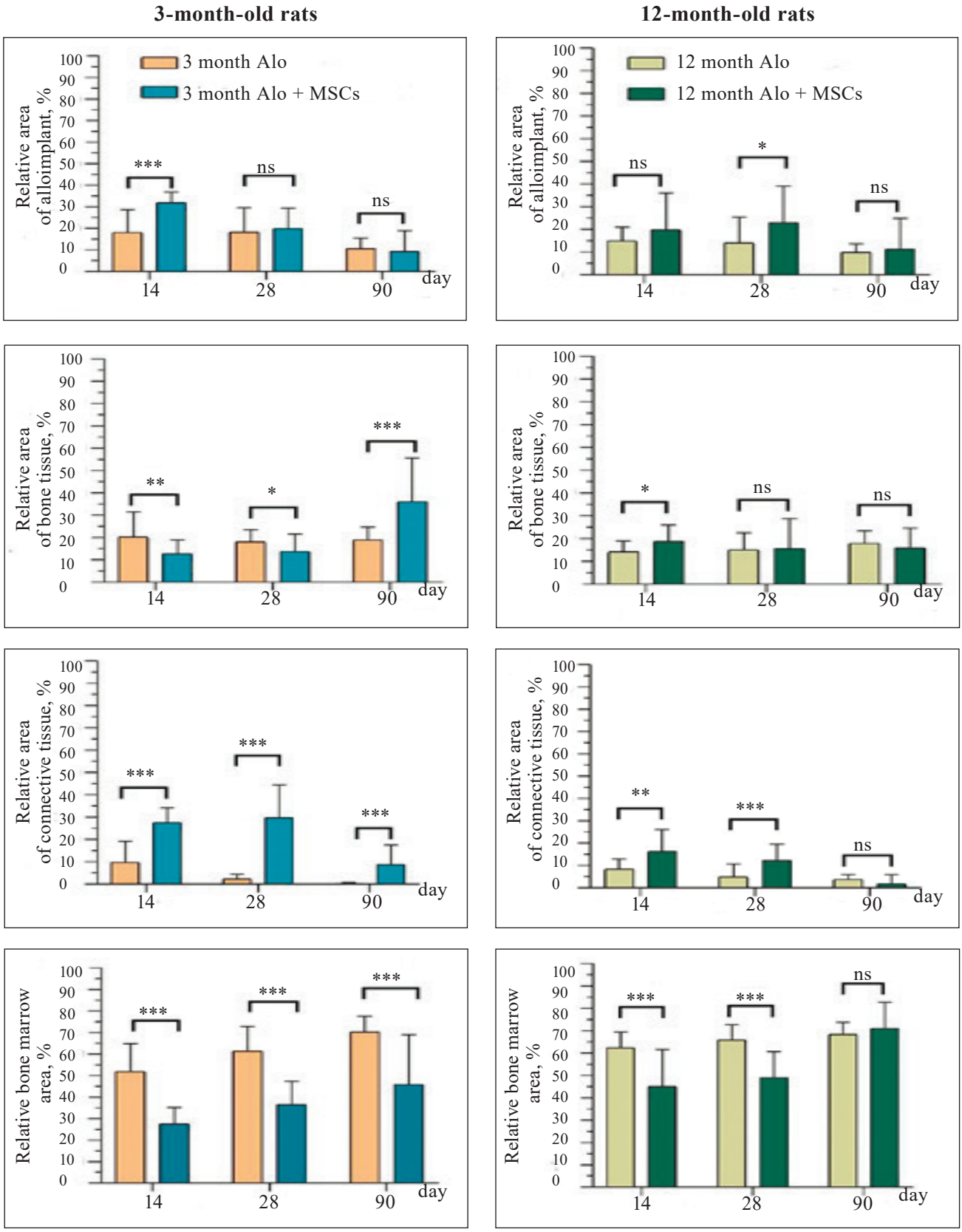

Fig. 3. Relative areas of the alloimplant (Alo) and newly formed tissues in the area of the critical defect of the femur of 3- (3 months) and 12-month-old (12 months) rats depending on the use of mesenchymal stromal cells (MSCs) on the 14th, 28th, and 90th day after the introduction of the alloimplant. ns — no statistically significant difference; * — $p < 0.05$; ** — $p < 0.01$; *** — $p < 0.001$. Indicators are given as mean and standard deviation. Comparison of groups with/without the use of MSCs for each observation period was performed using the Student's t-test method.

to the group of 12-month-old rats without the use of MSCs, the relative areas of connective tissue and bone trabeculae were 1.7 ($p = 0.003$) and 1.3 times ($p = 0.026$) larger, respectively, and the area of bone marrow was 1.5 times smaller ($p < 0.001$) (Fig. 3).

28 days after implantation

In *3-month-old rats*, fragments of allogeneic bone material, surrounded mainly by connective tissue of varying degrees of maturity, were identified in the area of the defect. It contained capillary-type blood vessels, fibroblasts with hypochromic nuclei, and fibrocytes, the long axis of which was directed along the bone trabeculae of the alloimplant. A layer of osteoblasts was found on the surface of its fragments, indicating osteoinductive properties. The newly formed bone was located along the perimeter of the defect at the border with the parent bone and formed a cortical layer represented by spongy bone tissue (Fig. 2, b, e).

According to the results of histomorphometry, the relative area of the allogeneic bone implant in the defect zone did not differ from the indicator of animals of this age without the use of MSCs. As on the 14th day of observation, compared to the group without MSCs of this age, the relative area of connective tissue was 13.5 times ($p < 0.001$), and the relative areas of bone trabeculae and bone marrow were 1.3 times smaller ($p = 0.049$) and 1.7 times ($p < 0.001$), respectively. Compared to the 14th day of observation, the relative area of the implant decreased by 1.6 times ($p < 0.001$), and that of the bone marrow increased by 1.3 ($p = 0.004$). The indicators of connective tissue and bone trabeculae did not change significantly (Fig. 3).

In *12-month-old rats*, alloimplant fragments were located between fields of osteogenic connective tissue in the area of the defect. It mainly contained osteoblastic and fibroblastic differon cells, capillary-type blood vessels of various diameters, and lymphocytic infiltration was determined around some of them. Newly formed bone trabeculae with a high density of osteocytes and a layer of active osteoblasts on the surface were located mainly along the perimeter of the defect, but also occurred in its central areas. The cortex was formed by spongy bone tissue (Fig. 2, i, l).

The relative area of the alloimplant did not differ from that of 3-month-old rats, but was 1.6 times greater ($p = 0.049$) compared to animals of the same age without the use of MSCs. The relative area of connective tissue was 2.4 times smaller ($p < 0.001$), bone marrow was 1.3 times larger ($p = 0.001$), and bone trabeculae did not differ significantly from the indicators of 3-month-old rats (Fig. 4). Compared to a group of animals of the same age without the use of MSCs, the relative area of connective tissue was 2.5 times larger ($p = 0.001$), bone marrow was 1.3 times smaller ($p < 0.001$), and bone trabeculae did not differ. All evaluated tissue and implant areas on the 28th day did not differ statistically significantly from the indicators on the 14th day (Fig. 3).

90 days after implantation

In *3-month-old rats*, connective tissue and fragments of allogeneic bone alloimplant were preserved in the area of the defect (Fig. 2, c, f), the relative area of which, as on the 28th day, did not differ from that of animals of the same age without the use of MSCs. The relative areas of connective tissue and newly formed bone trabeculae were larger 43.4 ($p < 0.001$) and 1.9 times ($p = 0.001$), respectively, and that of the bone marrow 1.5 times smaller ($p < 0.001$) compared to the group without MSC of this age. Compared to the 14th day after implantation, the relative area of the implant decreased by 3.4 ($p < 0.001$), that of connective tissue by 3.2 times ($p < 0.001$), and that of bone trabeculae and bone marrow increased by 2.8 ($p < 0.001$) and 1.6 times ($p = 0.003$), respectively. Compared to the 28th day, the area of the implant decreased by 2.2 times ($p = 0.001$), the area of connective tissue by 3.4 times ($p < 0.001$), and that of bone trabeculae increased by 2.6 times ($p < 0.001$), the area of bone marrow was not found to have statistically significant changes (Fig. 3).

In *12-month-old rats*, almost no connective tissue was found in the area of the defect. Small fragments of bone alloimplant were surrounded by newly formed bone (Fig. 2, j, m). The relative area of the allogeneic bone implant did not differ significantly from the group of animals of the same age without the use of MSCs and from the group of 3-month-old rats with the use of MSCs. The relative areas of connective tissue and bone trabeculae were 4.8 ($p = 0.003$) and 2.3 times ($p < 0.001$) smaller, respectively, and bone marrow was 1.5 times ($p < 0.001$) larger compared to 3-month-olds rats and did not differ significantly from the indicators of 12-month-old animals without the use of MSCs. Compared to the 14th day, the relative area of connective tissue decreased by 8.9 times ($p < 0.001$), and that of the bone marrow increased by 1.6 times ($p < 0.000$), that of bone trabeculae and implant did not change statistically significantly. Compared to the 28th day, the relative area of the implant decreased by 2.1 times ($p = 0.017$), that of the connective tissue by 6.8 ($p < 0.001$), that of the bone marrow increased by 1.4 times, the relative area of the bone trabeculae did not change significantly (Fig. 3, 4).

Discussion

In the conducted study on rats, the dependence of the incorporation and remodeling of structural

bone alloimplants on age (3 and 12 months at the beginning of the experiment) and the use of mesenchymal stromal cells of adipose tissue was analyzed. Defects of critical size (depth 3 mm, diameter 3 mm) in the distal metaphysis of the femur were chosen as the model. Defects in the metaphyses of long bones of rats of a similar size are used to evaluate the effectiveness of osteoplastic materials [15, 16, 18, 19].

For the distal metaphysis of the femur of rats, the minimum size of the critical defect is a diameter and depth of 2.5 mm [16], and in the case of a diameter of 3.5 mm and a depth of 4 mm, the authors [20] suggest performing plate stabilization. That is, the size of the defect chosen by us, on the one hand, exceeds the minimum critical one; on the other hand, it does not require the use of additional means of fixation, which also affect the course of reparative osteogenesis.

During the morphological study, we paid attention to the speed of reconstruction of the implant and the tissues formed in the area of the defect. A gradual decrease in the relative area of the alloimplant during the experiment (which indicates its restructuring) with the formation of connective and bone tissue in different proportions on the 14th, 30th, and 90th days was determined. This is consistent with the results of histological studies of patients' tissues, which show that in the process of incorporation of bone alloimplants, their incomplete reconstruction occurs with the formation of connective tissue [21–23]. At the same time, we established a significant difference between age groups on the 14th day: in 3-month-old rats, the relative area of the alloimplant was 1.6 times larger than in 12-month-old rats. A distinctive feature was also a larger area of the implant in younger rats on the 14th day and in older rats on the 28th day, compared to age-matched groups of rats using alloimplants without MSCs for plastic repair of defects.

The relative areas of connective tissue under the conditions of using allogeneic bone implants with MSCs in animals of both age groups were greater than in the case of using allogeneic bone implants alone. This may be related to the immune response to the introduction of allogeneic MSCs, mediated by the functioning of T- and B-lymphocytes, resulting in inhibition of bone formation [24]. In addition, administration of MSCs immediately after acute injury causes is known to suppress or alter local and

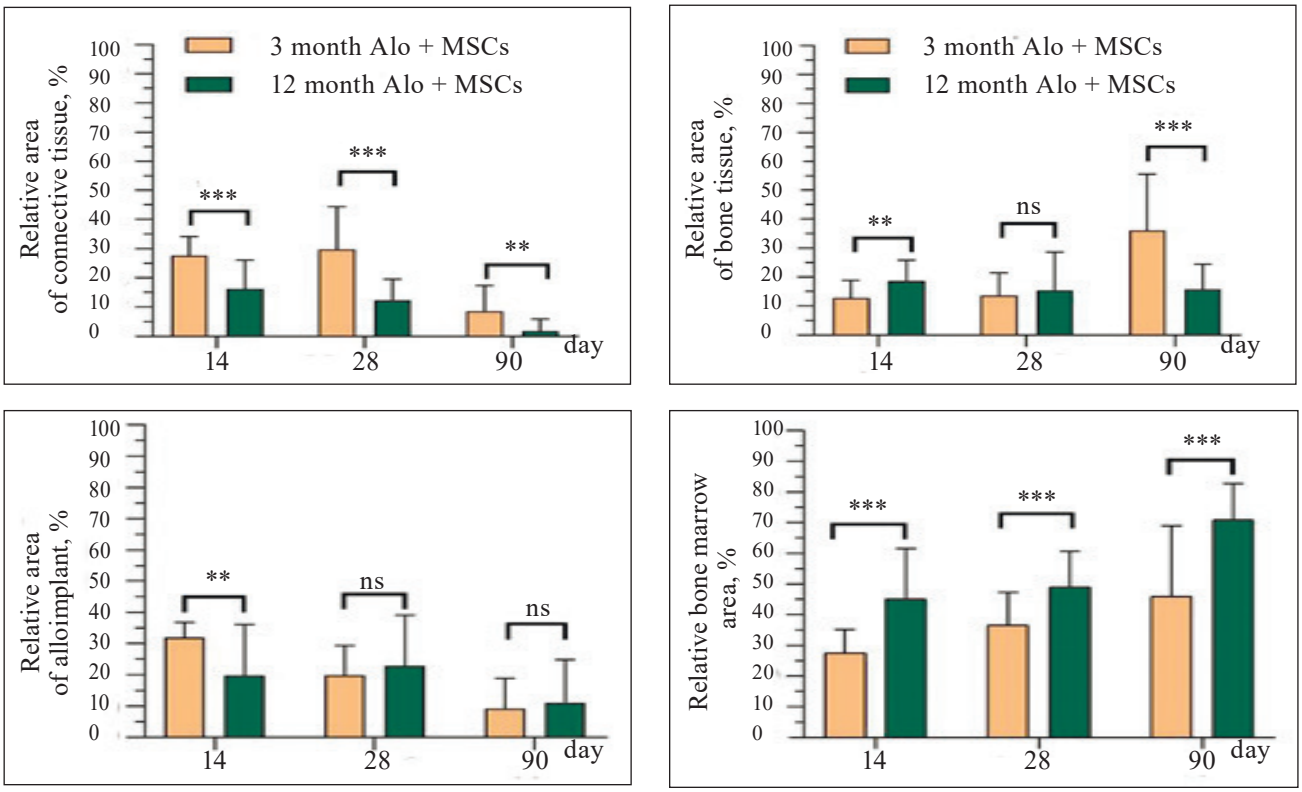

Fig. 4. Changes in the relative area of the alloimplant (Alo), in the case of its use with mesenchymal stromal cells (MSCs), and newly formed tissues in the area of the critical defect of the femur of 3- (3 months) and 12-month-old (12 months) rats on the 14th, 28th and 90th day after the introduction of the alloimplant. ns — no statistically significant difference; ** — $p < 0.01$; *** — $p < 0.001$. Indicators are given as mean and standard deviation. Comparison of 3- and 12-month-old groups for each observation period was performed using the Student's t-test method

systemic responses (inflammatory responses) via the release of immunosuppressive factors such as transforming growth factor β, prostaglandin E2, and indoleamine-2,3-dioxygenase 1 [25] . MSCs also secrete the protein SDF-1 (stromal cell-derived factor), which affects neoangiogenesis [26] and, together with other cytokines, changes the types of immune cells recruited to the site of injury [25]. This can lead to stimulation of bone formation and connective tissue itself. In our study, after the introduction of alloimplants together with MSCs, the relative area of connective tissue in the defects of 3-month-old rats for all experimental periods was on average 3 times greater compared to 12-month-old rats and significantly exceeded the indicators of 3-month-old animals with defects filled only with alloimplants . In 12-month-old rats, the values of the relative area of connective tissue in defects on the 14th and 28th day after surgery exceeded the values obtained in animals of the corresponding age without using MSCs. A common feature of the age groups was a sharp decrease in the area of connective tissue on the 90th day compared to other periods, the most significant in 12-month-old rats — by 8.9 times compared to the 14th day. There is an opinion that the formation of connective tissue around the implant prevents its complete reconstruction [27], and in fact, we determined the presence of fragments of allogeneic material in the defects of rats of both age groups at the final observation period.

The relative area of newly formed bone trabeculae in 3-month-old rats on the 14th day was 1.6 times lower, and on the 90th day, it was 2.3 times larger than in 12-month-old animals. In the latter, the area of bone tissue reached a higher value on the 14th day and subsequently did not significantly change and did not differ from the group of this age without the introduction of MSCs. In 3-month-old rats, on the 14th and 28th days, the indicator was approximately 1.5 times lower, and on the 90th day, it was 1.9 times higher compared to the group of the same age without MSCs administration.

Despite the fact that MSCs have been studied for a long time and even used to improve bone regeneration, the specifics of their influence on this process have not yet been fully elucidated. Namely, whether MSCs participate in morphogenesis during regeneration or whether they are only a source of factors that regulate the behavior and functions of other cells. Encouraging results were obtained in clinical observations. It was shown that the introduction of autologous bone marrow MSCs together with platelet-rich plasma and alloimplant into the tibial fracture site allowed to shorten the healing time of the fracture by two times compared to the group of patients without the use of MSCs (1.5 months vs. 3) [28]. The ability of MSCs obtained from adipose tissue to form bone tissue in mice in the case of their use in a critical skull defect no less than under the conditions of the introduction of MSCs induced in the osteogenic direction has been experimentally proven [29]. In rats, better bone formation in a critical defect of rats was demonstrated after the use of MSCs together with ceramics compared to the group without MSCs [30]. Our results are opposite and probably related to the fact that the microenvironment in the area of injury stimulates the differentiation of MSCs in the direction required for fracture regeneration [31], but much depends on the immune response of the recipient's body, which can lead to their death [32] or excessive inflammation reactions.

Based on our results, the introduction of allogeneic MSCs together with an allogeneic bone implant causes the formation of significant areas of connective tissue on the 14th and 28th days after implantation, regardless of the age of the rats. Along with this, in 12-month-old rats, the stimulation of bone tissue formation was determined at an early term (14th day) compared to the group without the use of MSCs. In younger rats, a significant increase in bone tissue was established only on the 90th day, but the area of connective tissue remained significantly higher compared to the group of this age without the use of MSCs. All of the above can negatively affect the mechanical properties of the bone and cause repeated fractures. Therefore, we believe that the combination of allogeneic MSCs with an allogeneic bone implant is inappropriate to use in the early stages of reparative osteogenesis and without the use of osteoinduction of cells during cultivation or immunosuppressants, especially in young recipients.

Conclusions

The study showed a gradual decrease in the relative area of the bone alloimplant during the experiment (indicating its restructuring) with the formation of connective and bone tissue in different proportions on the 14th, 28th, and 90th day.

The relative areas of connective tissue under the conditions of the use of allogeneic bone implants and MSCs in animals of both age groups were greater on the 14th and 28th day, and on the 90th day in 3-month-old rats, than in the case of the use of allogeneic bone implants alone.

The introduction of MSCs together with an allograft in cases of fresh traumatic bone injuries caused a slowdown in bone formation, especially in younger recipients: on the 14th and 28th days, the rate was lower by about 1.5 times compared to the age group without the introduction of MSCs.

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

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MORPHOLOGY OF THE REPAIR OF CRITICAL SIZE BONE DEFECTS WHICH FILLING ALLOGENEIC BONE IMPLANTS IN COMBINATION WITH MESENCHYMAL STEM CELLS DEPENDING ON THE RECIPIENT AGE IN THE EXPERIMENT

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