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Histological structure of the rat femurs after filling of defects in the distal metaphysis with 3D-printed implants based on polylactide and tricalcium phosphate in combination with mesenchymal stromal cells

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Polylactide (PLA) frameworks printed on a 3D printer are used for filling the bone defects. The osteotropic properties of 3D-PLA can be improved by combining with tricalcium phosphate (TCP) and mesenchymal stromal cells (MSCs). Objective. Study the reconstruction in the rat femurs after implanting 3D-printed implants based on PLA and TCP (3D-I) in combination with cultured allogeneic MSCs into defects in the distal metaphysis. Methods. 48 white laboratory rats (age 5–6 months) were used, which were randomly divided into groups: Control — 3D-I; Experiment-I — 3D-I, saturated MSCs; Experiment II — 3D-I, with injection of 0.1–0.2 ml of medium with MSCs into the area of surgical intervention 7 days after implantation. 15, 30 and 90 days after the operation, histological (with histomorphometry) studies were conducted. Results. The area of 3D-I decreased with time in all groups and connective and bone tissues formed in different ratios. 15 days after the surgery, in the Experiment-I group, the area of the connective tissue was 1.9 and 1.6 times greater (p<0.001) in comparison to the Control and Experiment II; 30 days it was greater 1.6 times (p < 0.001) and 1.4 times (p=0.001), respectively. 30 days after the surgery, the area of newly formed bone in the Experiment-I group was 2.2 times (p < 0.001) less than in the Control. On the contrary, in the Experiment-II, the area of newly formed bone was 1.5 and 3.3 times greater (p < 0.001) compared to Experiment-I and Control, respectively. Conclusions. The studied 3D-I with time after their implantation into the metaphyseal defects of the rats' femurs are replaced by connective and bone tissues. The use of 3D-I, saturated MSCs, 15 and 30 days after the surgery, caused excessive formation of connective tissue and slower bone formation. Local injection of MSCs 7 days after the implantation of 3D-I caused to the formation of a larger area of newly bone 30th day after surgery compared to 3D-I alone and 3D-I with MSCs.

Для заміщення дефектів кісток використовують надруковані на 3D-принтері каркаси з полілактиду (polylactide, PLA). Їхні остеотропні властивості можна покращити шляхом комбінації з трикальційфосфатом (ТКФ) та мезенхімальними стромальними клітинами (МСК). Мета. Дослідити перебудову стегнової кістки щурів після імплантації в дірчастий метафізарний дефект 3D-друкованих імплантатів на основі PLA та ТКФ (3D-I) в поєднанні з культивованими алогенними МСК. Методи. Використано 48 білих лабораторних щурів (вік 5–6 міс.), яких у випадковий спосіб розподілили на групи: Контроль — 3D-I; Дослід I — 3D-I, насичений МСК; Дослід II — 3D-I з ін'єкційним введенням 0,1–0,2 мл середовища з МСК у ділянку хірургічного втручання через 7 діб після імплантації. Через 15, 30 і 90 діб після операції виконано гістологічні (з гістоморфометрією) дослідження. Результати. Відносна площа 3D-I протягом експерименту зменшувалася в усіх групах з утворенням сполучної та кісткової тканин у різних співвідношеннях. У групі Дослід-I через 15 діб після операції відносна площа сполучної тканини виявилася більшою в 1,9 і 1,6 разу (p < 0,001) порівняно з величинами в групах Контроль і Дослід II відповідно; через 30 діб — в 1,6 разу (p < 0,001) і в 1,4 разу (p = 0,001) відповідно. На 30-ту добу в групі Дослід-I площа кісткової тканини була в 2,2 разу (p < 0,001) меншою порівняно з Контролем, а в групі Дослід-II, навпаки, — більшою в 1,5 і 3,3 разу (p < 0,001) порівняно з Дослід-I і Контроль відповідно. Висновки. Досліджені 3D-I з плином часу після їхнього встановлення в метафізарних дефектах стегнових кісток щурів заміщуються сполучною та кістковою тканинами. Використання 3D-I, насиченого МСК, призводить до утворення більших обсягів сполучної тканини на 15 та 30-ту доби й уповільнення кісткоутворення. Локальне введення МСК через 7 діб після встановлення 3D-I сприяло утворенню більшого обсягу кісткової тканини на 30-ту добу після операції порівняно з 3D-I самостійно та 3D-I одночасно з МСК. Ключові слова. Моделювання на щурах, дефект кістки, регенерація кістки, адитивні технології, полілактид, трикальційфосфат, мезенхімальні стромальні клітини.

Key words. Rat model, bone defect, bone regeneration, additive technologies, polylactide, tricalcium phosphate, mesenchymal stromal cells

Introduction

The development of osteoplastic materials for orthopedic surgery is related to the need to treat bone defects that do not heal on their own due to their large size and are the result of trauma, gunshot wounds, removal of neoplasms, infection, etc. The problem became more acute in Ukraine due to active military operations, when the use of high-energy weapons among the victims increased the number of people with fractures of long bones, complicated by the formation of significant defects [1]. Precisely segmental diaphyseal defects of long bones of critical size, by definition, are incapable of spontaneous healing and, therefore, are an indication for surgical intervention [2].

Bone autografts remain the «gold standard» for replacing bone tissue. However, their use is limited by the amount of material, the need for additional surgical intervention, the tenderness of the donor site, and possible complications [3, 4]. A promising alternative to auto- and allografts are artificial bioceramic frames based on phosphate and calcium sulfate [5], the use of which is constantly increasing due to achievements in the field of bone tissue engineering [6–8]. However, the properties of the new products are far from optimal. It is associated with the low rate of growth of bone tissue in them and undesirable effects [7]. The use of mesenchymal stem cells (MSCs) in combination with biomaterials to increase the osteogenic qualities of the latter became a new stage in the development of a bioengineering approach to optimize the restoration of bone tissue [9]. The number and functional activity of MSCs is one of the important components of the bone regeneration process, which was confirmed in experimental studies on mice. The authors showed that 8 weeks after tibial fracture reconstruction (85 ± 10) % of osteoblasts in the regenerate are derived from bone marrow MSCs [10]. In addition, MSCs are able not only to migrate to the site of injury and directly differentiate into bone cells, but also can locally change the environment by synthesizing growth factors, which contributes to the recruitment of other cells and improvement of reparative osteogenesis [9, 11].

Since the 1980s, the technology of three-dimensional (3D) printing for the creation of medical implants has been developing quite rapidly [12]. It provides a possibility to produce individual samples, which in terms of shape and volume correspond to the defect in the injured part of the skeleton, with sufficient mechanical strength and a favorable macro- and microstructure [13]. For 3D printing, highly porous titanium (acetabular components for total hip arthroplasty) [14, 15], ceramic materials [16], various polymers, but more often — polylactide (PLA) and polyglycolides, as well as composites based on them are used for 3D-printing [17, 18]. The advantages of implants printed on a 3D-printer from biodegradable materials include the absence of repeated surgery for their removal, osteoconductive and osteoinductive properties, the appropriate defect shape, simplicity and speed of manufacture. However, changing the physico-chemical properties of materials (chemical composition, biomechanics, added biological agents, etc.) in order to improve their quality requires complex preclinical studies to determine their biocompatibility, strength, ability to biodegrade, and osteotropy.

Purpose: to investigate the remodeling of the rat femur after implantation into a holey metaphyseal defect of 3D-printed implants based on polylactide and tricalcium phosphate in combination with mesenchymal stem cells.

Material and methods

The research plan was approved at a meeting of the Bioethics Committee at the State Institution Professor M. I. Sytenko Institute of Spine and Joint Pathology of the National Academy of Sciences of Ukraine (Protocol No. 205 of 13 July 2020) in accordance with the Law of Ukraine No. 3447-IV of 21.02.2006 «On the Protection of Animals from Cruelty» (Articles 26, 31), the European Convention on the Protection of Animals vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986) and Directive 2010/63/EU [19, 20].

Animals

The study involved 48 white laboratory rats from the population of the experimental biological clinic of the State Institution Professor M. I. Sytenko Institute of Spine and Joint Pathology of the National Academy of Sciences of Ukraine, which underwent making a hole defect in the distal metaphysis of the femur. The age of the animals at the beginning of the experiment was 5–6 months, the body weight was (365.8 ± 6.4) g. The rats were randomly divided into groups depending on the implanted material:

– Control — 3D-printed implant (3D-I);

– Trial I — 3D-I in combination with cultured allogeneic MSCs;

– Trial II — 3D-I with injection of 0.1–0.2 ml of cultured allogeneic MSCs into the surgical site 7 days after implantation.

Additionally, 3 rats were used to obtain mesenchymal stem cells according to the method described in detail [21].

5 animals from each group were removed from the experiment on days 15; 30; 90 after surgery, they were decapitated under open inhalation anesthesia with diethyl ether (for blood sampling for research [22]) and the operated femurs were removed from them.

Implants

For printing implants on the 3D-printer «Easy3D-Print» with an extruder (Studio 3D-print Easy 3D-Print, Company, Kharkiv, Ukraine) a composite filament of PLA and tricalcium phosphate (TCP) was used, made by mixing 60 % of PLA granules and 40 % of a mineral compound (20 % PLA and 80 % TCP), heating and extrusion. The thickness of the thread was equal to 1.75 mm, the diameter of PLA (L-PLA) and TCP (TCP medical, China) granules was 10 μm. The cylindrical implants obtained (length 30 mm, diameter 2.5 mm, pore size 300 μm, porosity 45 %) were divided into fragments 3 mm long. Before implantation in the bone of the rats of the Trial I group, the 3D-printed implant was soaked for 20 minutes in 0.5 ml of culture medium with 106 cells, the rest of the cells were injected into the defect cavity.

Surgical interventions

Operations were performed under general anesthesia (ketamine 50 mg/kg, intramuscularly) under aseptic and antiseptic conditions. Using the anterolateral approach, a hole defect with a diameter of 2.6 mm and a depth of 3 mm was modeled using a dental drill, according to the size of the manufactured implants. The wound was washed with a dexasan solution, dried, and the test samples were placed in the bone defect using the press-fit technique. Soft tissues were sewn up in layers, treated with an antiseptic.

Histological studies

For histological analysis, the selected femurs with the implantation area were cleaned of soft tissues, fixed for 4 days in a 10 % neutral formalin solution, decalcified in a 10 % formic acid solution. After that, the severed distal metaphyses with the defect zone were dehydrated in alcohols of increasing concentration and embedded in paraffin. The prepared frontal histological sections of 5–6 μm thickness were stained with hematoxylin and eosin (H & E) and van Gieson's picofuchsin, and analyzed under a BX63 light microscope (Olympus, Japan). Digital images were obtained using a DP73 camera (Olympus).

Histomorphometry

The defect area of the implant, connective and bone tissues was measured (on 4 central sections of each animal), then their relative content (%) to the total area of the defect was calculated. The software «Cell Sens Dimension 1.8.1» (Olympus, 2013) was used.

Statistical studies

Data analysis was performed using «IBM Statistics SPSS 23 software» and «Microsoft Office Excel 2007». Measurement results are given as mean ± standard deviation in case of normal distribution. The influence of different types of materials after their implantation on the formation of connective and bone tissue was evaluated according to the Student–Fisher method. The difference was considered statistically significant in $p < 0.05$ [23].

Results and their discussion

15 days after surgery

3D-I was clearly visualized on histological samples in the area of the metaphyseal defect of the femur of rats of all studied groups, on the surface of which zones of formation of connective and bone tissues sprouted into its external pores (Fig. 1, a–c).

Connective tissue was characterized by different degrees of maturity, consisted of bundles of collagen fibers, between which blood capillaries, cells of fibroblastic and osteoblastic diferons were distributed. Some of them had elongated nuclei and narrow cytoplasm, others had rounded nuclei with 1–2 nucleoli and developed basophilic cytoplasm, indicating their high functional activity aimed at the biosynthesis of matrix components.

Newly formed bone trabeculae of coarse fibrous structure contained densely located osteocytes with hypochromic nuclei and developed cytoplasm, and functionally active osteoblasts with hypochromic eccentric nuclei on the outer surface. No signs of inflammation were found in any animal.

Histomorphometric findings (Fig. 2) showed that the area of the implant in the defect zone of the rats of the Trial I group was equal to (45.28 ± 13.90) % and was smaller than the indicator of the Control ((69.31 ± 17.33) %) in 1.5 times ($p < 0.001$), Trial II ((54.19 ± 5.11) % — 1.3 times ($p = 0.009$). The relative area of connective tissue, on the contrary, was 1.9 times greater ($p < 0.001$) and 1.6 times ($p < 0.001$) compared to the values in the Control (Trial I — (52.25 ± 13.56) %, Trial II — (43.06 ± 4.82) %, Control — (27.75 ± 16.70) %), respectively; and bone tissue did not differ. The comparison between the experimental groups showed significant differences only in terms of the relative area of the implant — it was 1.2 times larger ($p = 0.024$) in the Trial II group .

30 days after surgery

The histological structure of the defect area did not change compared to the previous period of the study: 3D-I was located in it, dense connective tissue and newly formed bone trabeculae were located around

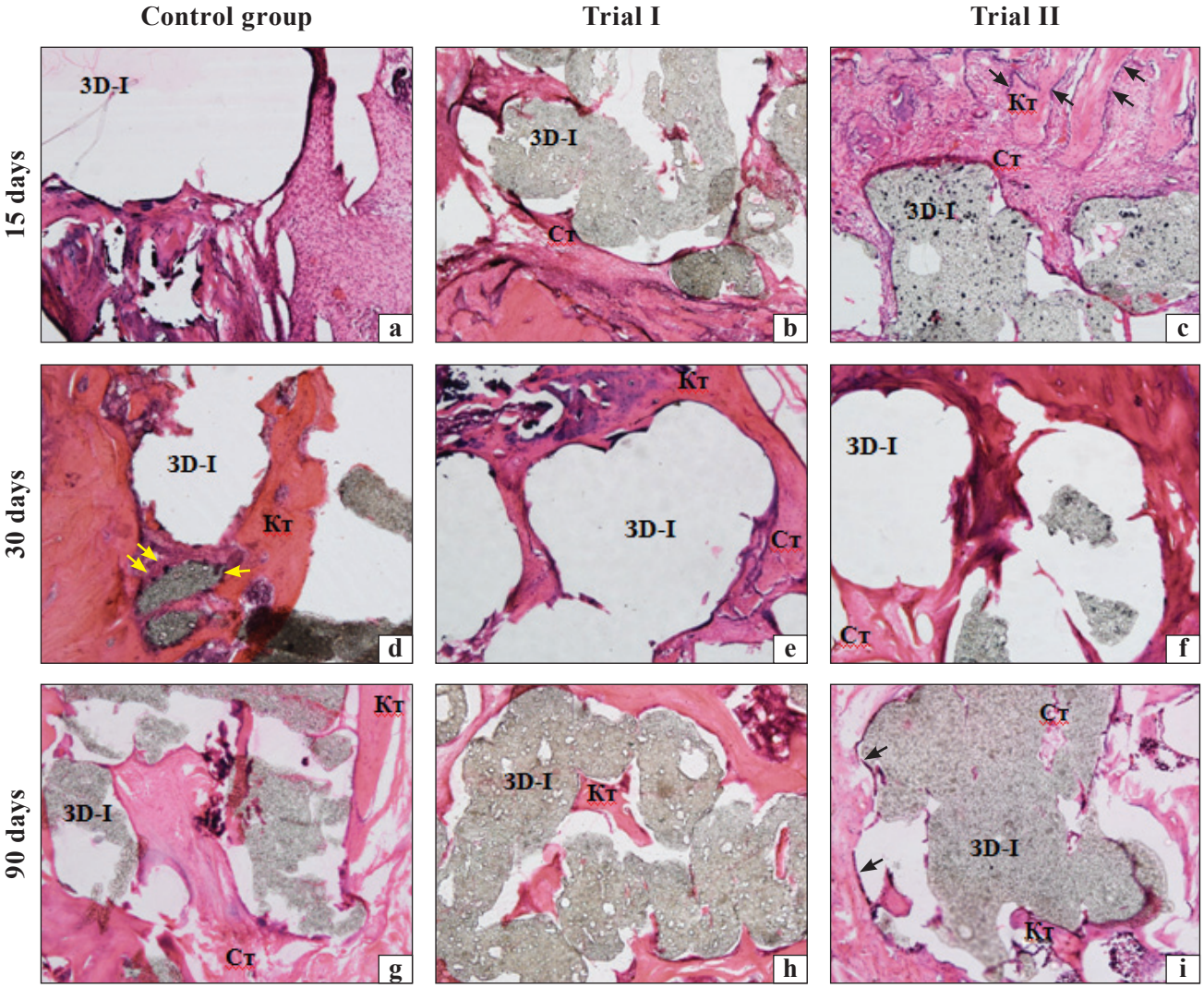

Fig. 1. Fragments of the distal metaphyses of rat femurs 15, 30, and 90 days after implantation of a 3D-printed implant (3D-I) (Control group), 3D-I in combination with allogeneic cultured MSCs (Trial I), 3D-I with the introduction allogeneic cultured MSCs 7 days after surgery (Trial II). Connective (Ct) and bone (Bt) tissues around and in the pores of the implant are shown; black arrows, c — functionally active osteoblasts on the surface of newly formed bone trabeculae; yellow arrows, d — multinucleated cells of foreign bodies on the surface of fragments 3D-I. ×10. Hematoxylin and eosin

it and in its pores (Fig. 1, d–e). Bundles of collagen fibers in the connective tissue were directed parallel to the surface of the implant and penetrated into its middle. Between them were blood vessels and elongated blast cells with hyperchromic nuclei with fine-grained chromatin. Newly formed bone trabeculae, which also sprouted into the middle of the implant, were characterized by a significant density of osteocytes located in lacunae, and a layer of functionally active osteoblasts was found on their outer surface. Multinucleated cells of foreign bodies were also observed on the surface of 3D-I (Fig. 1, d), which indicated the course of cellular resorption.

Histomorphometric findings (Fig. 2), compared to the indicators on the 15th day of observation, showed a decrease in the relative area of 3D-I by 1.2 times ($p = 0.022$) in the Control groups ((56.04 ± 12.68) %) and Trial II ((46.19 ± 8.86) %); an increase in the relative area of bone tissue by 4.1 ($p < 0.001$), 2.2 ($p = 0.005$) and 6.5 times ($p < 0.001$) — respectively, the Control group ((11.95 ± 4.02) % versus (2.94 ± 1.18) % on the 15th day), Trial I ((5.51 ± 3.48) % vs. (2.47 ± 1.92) %), Trial II ((18.01 ± 9.49) % vs. ((2.75 ± 1.17) %. Comparison of the measured indicators between groups for the same study period (30th day) showed smaller relative areas of 3D-I by 1.2 times ($p = 0.021$ and $p = 0.013$ — Trial I ((44.47 ± 14.94) %) and Trial II, respectively) relative to values in the Control, without differences between experimental groups. The relative area of connective tissue was greater in the Trial I group ((50.02 ± 13.97) %) by 1.6 times ($p < 0.001$)

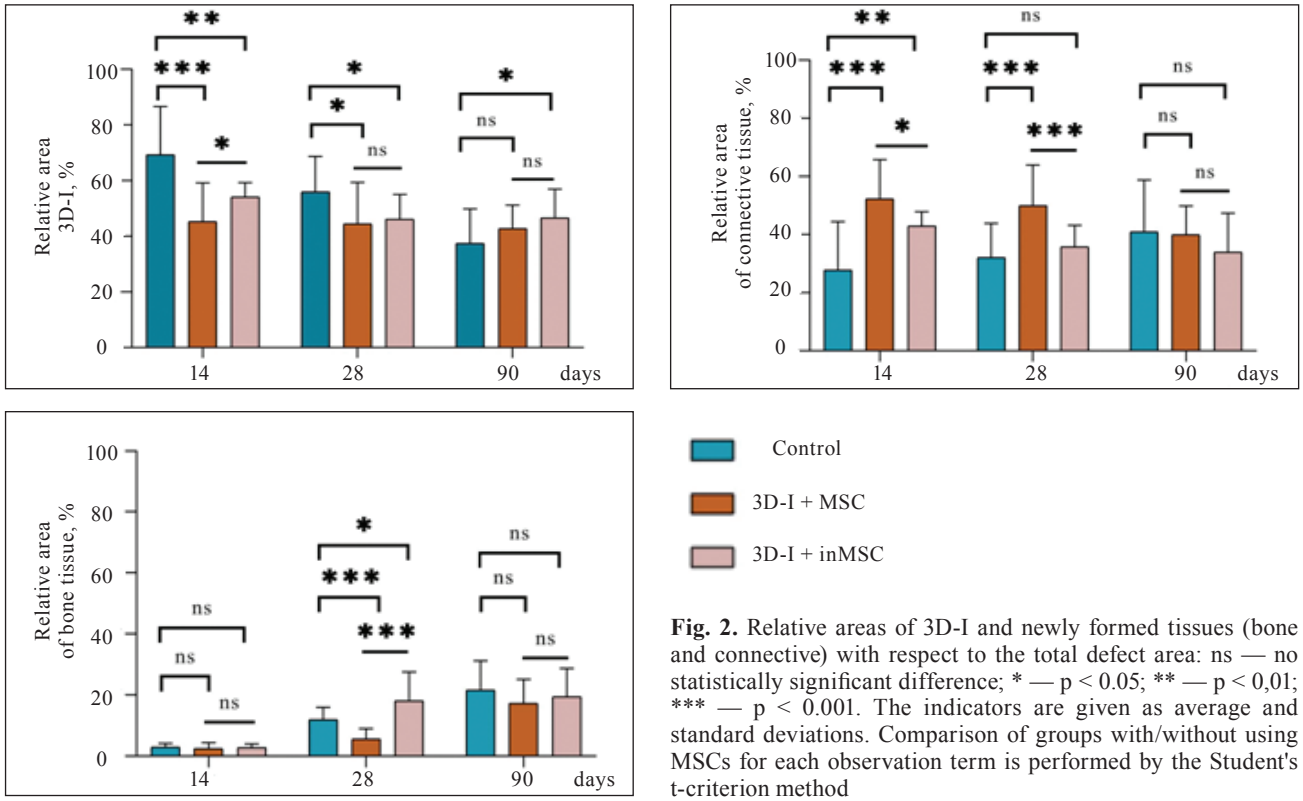

Fig. 2. Relative areas of 3D-I and newly formed tissues (bone and connective) with respect to the total defect area: ns — no statistically significant difference; * — $p < 0.05$; ** — $p < 0,01$; *** — $p < 0.001$. The indicators are given as average and standard deviations. Comparison of groups with/without using MSCs for each observation term is performed by the Student's t-criterion method

compared to the Control ((32.01 ± 11.86) %), by 1.4 times ($p = 0.001$) — with the Trial II group ((35.80 ± 7.43) %).

90 days after surgery

Fragments of 3D-I, the shape of which was changed due to its biodegradation, were identified on histological sections in the area of the defect. Bone and connective tissue were located along the perimeter and in the 3D-I pores. Blood vessels and cells of fibroblastic and osteoblastic diferons penetrated into small-diameter pores (Fig. 1, g–j).

Histomorphometric findings (Fig. 2) showed that the relative area of 3D-I decreased: in the Control ((37.48 ± 12.38) %) by 1.8 times ($p < 0.001$) compared to the indicator on the 15th day, by 1.5 ($p = 0.001$) on the 30th day; Trail II ((46.65 ± 10.31) %) by 1.2 times ($p = 0.011$) compared to the indicator of the same group only on the 15th day; in Trial I ((42.80 ± 8.42) %) it did not change significantly. At the same time, there were no significant differences in 3D-I areas on the 90th day of observation between the Control and Trial I, Trial I and Trial II groups, as well as bone tissue between all groups (Control — ((21.64 ± 9.54) %), Trial I — (17.30 ± 7.80) %, Trial II — (19.42 ± 9.32) %). It should be noted that compared to the values obtained on the 15th day after surgery, the area of bone tissue was increased by 7.4 times ($p < 0.001$) in the Control group and by 7.1 times ($p < 0.001$) in Trial II, and in group Trial I it did not change significantly.

The relative area of connective tissue, which was detected mainly in the internal pores of the implant, decreased by 1.3 times ($p = 0.008$) compared to the 15th day in the group of Trial II ((33.93 ± 13.47) %), but did not differ from the indicators of the Control ((40.88 ± 17.92) %) and Trial I ((39.90 ± 10.00) %) groups on the 90th day after surgery.

Discussion

The article presents the results of histological analysis of bone tissue reconstruction of implants based on polylactide and tricalcium phosphate, manufactured using 3D printing technology and installed in hole defects in the distal metaphyses of rat femurs. The animals were randomly divided into 3 groups: Control (3D-I was installed independently), Trial I (3D-I was saturated with cultured allogeneic MSCs before installation) and Trial II (3D-I alone, and 7 days later, a suspension was injected locally into the injury zone cultured allogeneic MSCs). The size of the defect was chosen to be 3 mm deep and 2.6 mm in diameter, since defects in the metaphyses of the long bones of rats of a similar size are used to evaluate the effectiveness of osteoplastic bone substitute materials [5, 21, 24, 25].

To create three-dimensional implants, PLA is taken as one of the most commonly used biosoluble polymers for 3D frameworks in tissue engineering due to its proven plasticity, bioinertness, ability to biodegrade, and the ability to remove decay products naturally [17, 18, 26]. The osteotropic properties of PLA in 3D structures can be improved by combining it with TCP [27], which is characterized by osteoinduction, that is, the ability to stimulate cell differentiation in the osteogenic direction [5, 28, 29]. In addition, we used a biological factor that can potentially contribute to the formation of bone tissue [30] — MSCs obtained from adipose tissue of rats.

In this study, using the histomorphometry method, we evaluated the rate of implant reconstruction with bone and connective tissue replacement. It was determined that after the use of 3D-I composites with PLA and TCP alone or in combination with allogeneic cultured MSCs for plasticity of bone defects of a critical size, the relative area of 3D-I decreases over time (which indicates their restructuring) with the formation of connective and bone tissues in different ratios. In the experimental and control groups, during the experiment, a decrease in the relative area of connective tissue and 3D-I, and an increase in the area of bone was established. However, on the 15th day after implantation in the area of the defect of groups Trial I (introduction of MSCs together with 3D-I) and Trial II (introduction of MSCs 7 days after installation of 3D-I), the relative area of connective tissue was significantly increased compared to the Control, and in the Trial I group — on the 30th day. In addition, in this same group (3D-I, saturated MSC) a slowing of bone formation was recorded, reflecting a 2.2 times smaller ($p < 0.001$) relative area of bone tissue on the 30th day compared to the use of 3D-I alone. Unfavorable conditions and slowing down of the healing of the defect by bone tissue creates the development of pronounced inflammation with a significant increase in the content of glycoproteins, interleukin-6 and total protein in the serum of rats of Trial I group, which we reported earlier [21]. In turn, this response of the body may be related to the effect of allogeneic MSCs on the functioning of T- and B-lymphocytes [31]; local response to their administration immediately after an acute injury with the release of such cytokines by the recipient's body as transforming growth factor β, prostaglandin E2 and indoleamine-2,3-dioxygenase 1 [32]; as well as the synthesis of MSC protein SDF-1 (stromal cell-derived factor), which affects neoangiogenesis [33]. It is not known how the combination of these biological factors will affect regenerative processes: stimulation of both osteogenesis and the formation of connective tissue (loose or dense) can be expected. Taking into account the results obtained by us and findings of other authors regarding the excess formation of connective tissue on the 15th and 30th days in the metaphyseal defects of the femurs of rats filled with bone alloimplants in combination with allogeneic MSCs [21], we consider it unjustified to simultaneously introduce into the area of the defect a manufactured implant by 3D printing technology from PLA and TCP, with allogeneic MSCs in cases of fresh traumatic bone injuries.

Delayed (7 days after the installation of 3D-I based on PLA and TCP) injection of allogeneic MSCs led to faster bone formation in the defect area on the 30th day compared to the introduction of MSCs at the time of implantation and with the control, confirming greater indicators of its relative area in 3.3 and 1.5 times ($p < 0.001$), respectively.

Conclusions

The study showed a decrease over time in the relative area of all investigated composites implants made by 3D-printing technology from PLA and TCP (alone or in combination with MSCs), with the formation of connective and bone tissue after their installation in metaphyseal defects of rat femurs.

The use of 3D-I, saturated with cultured allogeneic MSCs before implantation, leads to the formation of larger volumes of connective tissue on the 15th and 30th days after surgery by 1.9 and 1.6 times ($p < 0.001$), respectively, and to the slowing down of bone formation, which shows a 2.2 times smaller ($p < 0.001$) relative area of bone tissue on the 30th day compared to the Control group (3D-I alone).

Administration of MSCs 7 days after the installation of 3D-I in the area of the defect contributed to the formation of a larger volume of bone tissue on the 30th day after surgery by 1.5 times ($p < 0.001$) compared to the Control (3D-I alone) and by 3.3 times ($p < 0.001$) compared to the Trial I group (3D-I simultaneously with MSCs).

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

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HISTOLOGICAL STRUCTURE OF THE RAT FEMURS AFTER FILLING OF DEFECTS IN THE DISTAL METAPHYSIS WITH 3D-PRINTED IMPLANTS BASED ON POLYLACTIDE AND TRICALCIUM PHOSPHATE IN COMBINATION WITH MESENCHYMAL STROMAL CELLS

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